Preventative and Therapeutic Potential of Lipopolysaccharide Derived from Edible Gram-Negative Bacteria to Various Diseases

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Abstract: Gram-negative bacteria contain lipopolysaccharides (LPS), which are generally considered to be an endotoxin that has negative impacts on humans. There have been very few therapeutic drugs developed that contain LPS. Recently, it has been reported that hygienic improvements have decreased exposure to LPS, and this is correlated with an increase in allergenic diseases. Lack of exposure to LPS may adversely affect the immune balance in the body.

LPS is the substance that has the greatest known effect in activating macrophages which play a central role in the innate immune system. We have hypothesized the existence of a network formed by tissue macrophages and have termed this putative communications network a macrophage network. We studied certain edible Gram-negative bacteria that have a long history of use in traditional food production, in order to discover ways to improve and/or maintain health.

In 1991 we discovered that the LPS of *Pantoea agglomerans* (named IP-PA1 by us) was a macrophage-activating substance that could be obtained from water extracts of wheat flour. LPS is also part of the make-up of cells of other well known Gram-negative bacteria used in food processing such as *Acetobacter* (vinegar, yogurt) and *Xanthomonas* (xanthan gum). This demonstrates that humans have a long history of consuming Gram-negative bacteria and LPS.

In this manuscript, we discuss the potential for utilizing IP-PA1 and other LPS from edible Gram-negative bacteria. Forms of LPS can be used in various fields, such as in health food, to prevent and improve metabolic syndromes and allergies. They can also be used in feedstuffs for stockbreeding and in aquatic culture as defenses against infection where they can replace antibiotics or chemical substances.

Key Words: Innate immunity, LPS, macrophage network, TLR4, hygienic hypothesis, IP-PA1.

INRODUCTION

It is experientially known that health can be maintained or improved by environmental stimulation with moderate exercise, acupuncture, diet, etc. These stimulations increase resistance to infectious and lifestyle-related diseases, reduce chronic fatigue, and have other beneficial effects. Animals have biological mechanisms that maintain homeostasis of the body. This mechanism receives environmental information and transmits this information throughout the body. Though a number of mechanisms may be responsible for the control of this phenomenon, we have focused on the role of phagocytes (mainly macrophages in mammals) that distinguish between self and non-self including foreign substances and apoptotic cells [1]. Macrophages play an indispensable role in maintaining health and preventing disease by eliminating non-self substances or cells [1].

The macrophages are phylogenetically conserved cells that play the central role in providing innate immunity. They exist in the front line of the internal and external interface receiving environmental information. Peripheral blood monocytes migrate to every tissue and differentiate into tissue-specific macrophages. Macrophage lineages have high plasticity and flexibility corresponding to environmental information [2-4]. According to Tomio Tada, a Japanese immunologist, organisms have an active system, a "super system," that organizes itself by referring to changes in the self [5]. In this context, macrophages appear to be the obvious leaders of this super system. With this line of thinking, it is reasonable to propose a new strategy in which it is the macrophages that maintain the control of homeostasis.

We found that macrophages that had been activated by some types of environmental information expressed and transmitted this information through membrane-bound tumor necrosis factor (proTNF) on their surfaces to neighbor macrophages [6-9]. This juxtacrine, macrophage-macrophage interaction, included bidirectional regulation. Because macrophages exist throughout the body, we conceived the concept that macrophages formed an information network. This network integrates environmental information that is received locally to the rest of the body. We tentatively referred to this system as a "macrophage network" [10, 11]. This review discusses issues related to problems of health and immunity

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regulation that are difficult to analyze by reductionism. A holistic approach to understanding this regulation requires an analysis of the tissue macrophages (the macrophages which have become differentiated by adapting to specific tissues). We believe that this information will help in the understanding of organism integrity.

Macrophage-activating agents such as β-1,3-glucan and peptidoglycan of lactic-acid bacteria are already used as ingredients in health foods. Though lipopolysaccharide (LPS) of Gram-negative bacteria is best known as an endotoxin, we have proposed oral or percutaneous administration of LPS as an effective and safe way to maintain homeostasis [12]. Even though LPS is medically considered to be a bacterial toxin, it also has the strongest known macrophage-activating activity. Recent information shows that humans are frequently exposed to low doses of LPS over long periods of time without harmful effect or without impairing their immunological condition [13]. The LPS of Gram-negative bacteria can be a useful health food additive for the prevention of allergies or infectious diseases. In this paper, we introduce a wheat fermentation extract of *Pantoea agglomerans* for this purpose.

THE ROLE OF MACROPHAGES IN THE MAINTENANCE OF HOMEOSTASIS

The macrophage is a type of cell named by Elie Mechnikov about 100 years ago [14]. These migratory cells can recognize and distinguish foreign bodies and can eliminate them by phagocytosis. Macrophages are phylogenetically conserved in all animals and provide protection from invasive pathogens (bacteria, virus, fungus, and protozoa). Recently, there has been a large amount of information accumulated for vertebrates and invertebrates on the mechanism for distinguishing foreign bodies using gene cloning of Toll like receptors (TLRs) [15]. Macrophages recognize invading pathogens as well as self-cells which have become useless (ex. apoptotic cells or tumor cells). Apoptotic cells express phosphatidylserine (PS), which usually exists inside of the cell membrane; such cells are phagocytosed by macrophages after PS receptor recognition [16]. It was reported that suppression of macrophage phagocytic function by inhibition of PS recognition induces autoimmune disease symptoms (production of antinuclear antibodies that causes nephritis) [17]. In unicellular animals the phagocytic functions of macrophages (phagocytes) are necessary for taking in food; this ability has been further adapted in multicellular animals for the essential function of maintaining body integrity.

Here, we focus on the environmental factors that affect macrophage functions. It is known that low dose exposure to radiation activates macrophages (this is known as the hormesis effect on health) [18]. We found that certain stressors such as sleep deprivation or water deprivation induced macrophage activation [19]. These facts suggest that macrophages directly or indirectly receive and rapidly respond to environmental factors. Though a macrophage-defective animal model does not exist, it is possible to temporarily eliminate some macrophages by using liposome-encapsulated dichloromethylene-diphosphonate [20]. Mice treated in this way lost the integrity of intestinal homeostasis and became highly sensitive to methicillin-resistant *Staphylococcus aureus* (MRSA) infection [21]. We previously found that surgical stress (30 minutes laparotomy) significantly suppressed macrophage functions and remarkably increased sensitivity to MRSA infection and lung metastases, but administration of macrophage activating agents prior to surgery reduced these detrimental immunosuppressive effects [22] (Tables **1** and **2**). Based on the above information, we believe that macrophages receive environmental information from both inside and outside of the body, and use this information to maintain homeostasis.

LIPOPOLYSACCHARIDE (LPS) IS THE STRONGEST KNOWN MACROPHAGE ACTIVATING SUBSTANCE

β-1,3-glucan, peptidoglycan, and LPS are known as macrophage-activating substances. They are polymers consisting polysaccharides and are not absorbed in the intestine. When LPS was administered orally, very little was absorbed in the intestine, and the amount sampled in the portal vein was trivial [23, 24]. LPS was rapidly absorbed by the liver which kept it from being transferred throughout the body. When LPS does enter the vascular flow, it very efficiently binds to LPS binding protein (LBP) in the plasma and is transported to the complex with CD14, toll like receptor-4 (TLR4) and MD-2 on the immune cells [15]. The signal from TLR4 through intracellular MyD88 induces the activation of transcription factors such as NF-κB or AP-1. This highly sensitive signal system provokes inflammation after an intravenous injection of only 10 ng/kg of LPS in humans or 40 ng/kg of LPS in mice [25]. In contrast, β-1,3-glucan (known as a health food) requires 0.1 mg/body weight (4 mg/kg) in mice to obtain the same level of macrophage activation as is obtained with LPS [26]. This means that 100,000 times more β-1,3-glucan is needed to cause the same level of signal transduction as occurs with LPS. The molecular structure of both LPS and β-1,3-glucan consists only of polysaccharides with some lipids; thus, they do not induce any toxicity after oral ingestion. Animals have developed a supersensitive system against LPS when it is in the bloodstream that involves the production of cytokines. Therefore, it seems more useful to study LPS in the role of a powerful substance that transmits environmental information than as an endotoxin.

In the medical field, LPS has been treated as a toxic substance and is referred to as an endotoxin while similar macrophage-activating substances, such as lactic acid bacteria are considered as non-toxic. This does not make sense as lactic acid bacteria will also produce endotoxic shock in mice if it is intravenously administered in sufficient quantities [27]. TNF-α and IL-1β Gram-positive bacteria contain peptidoglycan a major bacterial cell wall component, which activates macrophages mainly through TLR2. Lipoteichoic acid (cell membrane lipids of Gram-positive bacteria) makes intracellular signals by TLR4. TLR4 and TLR2, and have the same intracellular adaptor molecule (MyD88) to induce NF-κB and AP-1 activation [15]. Therefore, lactic acid bacteria cause a biological response similar to the receptor-signal system of Gram-negative bacteria and LPS. This means that they belong to the same general category of macrophage activation. Neither LPS nor lactic acid bacteria cause detrimental side effects when administered orally and/or percutaneously. Nevertheless, LPS is generally treated only as if it were a toxin, and is considered a harmful substance for par-

Table 1. Survival Rate After *Staphylococcus aureus* Infection in Laparotomy Mice

	Laparotomy	Treatment	Survival (%)	Statistics*
Exp. 1	+	Saline	33	
	-	Saline	100	p=0.01
	+	TNF	89	p=0.04
	+	OK-432	89	p=0.03
Exp. 2	+	Saline	44	
	+	IP-PA1	89	p=0.05

BALB/c mice were laparotomized for 30 minutes while anesthetized. After the operation, mice were inoculated with *Staphylococcus aureus* (ATCC 25923, 1 x 10^8 cfu), and the survival periods were determined. Recombinant TNF (0.23 μg/mouse) or OK-432 (0.1 KE/mouse, dead bacterial bodies of *Streptococcus pyogenes*) (experiment 1), and IP-PA1 (10 ng/mouse) (experiment 2) was injected as macrophage-activating substances 3 hours before the operation.

*: Statistical significance between the control group of laparotomy with saline and laparotomy with macrophage activating agents (Kaplan-Meyer method, log-rank test).

enteral usage such as injection, catheters and needles because small amount of LPS can induce significant inflammatory response with severe side effects.

GRAM-NEGATIVE BACTERIA IN FOOD MANUFACTURING AND LPS IN FOOD

Gram-negative bacteria have long been part of human diets, and we believe these bacteria should be evaluated as food additives for immune system activation. The use of Gram-negative bacteria in normal foods is summarized in Table **3**. A number of species of Gram-negative bacteria genera are used to make traditional foods, such as *Acetobactor* (brewing vinegar, making Caspian Sea yoghurt, and nata de coco), *Zymomonas* (tequila), *Xantomonas* (xanthan gum), and *Pantoea* (fermented rye bread) [28]. We determined that *Acetobactor aceti* contained LPS using the *Limulus* reaction and nitric oxide production from a macrophage cell line [29]. Significant amounts of LPS were also confirmed to be in many other foods and in Chinese medicines (Table **4**) [30]. These facts show that certain species of Gram-negative bacteria have long been present in food production.

LPS exists widely in commercially available foods (vegetable powders, health foods, and Chinese medicines) sometimes in quite large amounts (0.16 to 600μg/g) (Table **4**). For example, the amount of LPS in the daily intake of certain health foods such as wheat bran and germ was calculated as 132 and 180μg/g using the *Limulus* reaction. Xanthan gum, which is widely used as a salt/acid resistant thickener, a highly efficient suspension agent, an emulsifier, and a high viscosity filling agent in various foods was approved as a food additive by the FDA. We found significant amounts of LPS in xanthan gum. This demonstrates that modern human diets may contain considerable incidental quantities of LPS.

Table 3. 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)
Acetobacter xylinum	Nata de coco (Philippines)
Xantomonas	Xanthan gum (worldwide)
Acetobacter orientalis	Caspian Sea yogurt (Caucasus)
Pantoea agglomerans	Fermented rye bread (Northern Europe)

These measurements demonstrate that humans are routinely exposed to food-derived LPS. However, this does not prove that the quality and quantity of LPS is adequate to

Table 2. Lung Metastasis After Meth A Tumor Injection in Laparotomy Mice

Laparotomy	Treatment	Number of metastatic foci	Statistics*
+	Saline	54.3 ± 25.6	
-	Saline	37.4 ± 20.0	NS **
+	TNF(3 h)	10.1 ± 10.7	p=0.008
+	OK-432(3 h)	0 ± 0	p=0.0004

BALB/c mice were laparotomized for 30 minutes while anesthetized. After the operation, mice were intravenously inoculated with Meth A fibrosarcoma (5 x 10^5 cells). The number of metastatic foci on the lungs were counted after 21 days of Meth A fibrosarcoma inoculation. Recombinant TNF (0.23 μg/mouse) or OK-432 (0.1 KE/mouse) was injected as macrophage-activating substances 3 hours before the operation.

*: Statistical analysis between laparotomy control and other treatment groups were performed using Student's *t*-test. **: NS: not significant.

Table 4. LPS Content in Health Food and Chinese Herbs

Health food (Scientific name)*	LPS** (μg/g)	Amout of LPS*** in daily dosage
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 *****	LPS (μg/g)	
Kanboui (*Sinomenium accutum*)	600	
Ginseng (*Panax ginseng*)	50	
Bupleuri radix (*Bupleurum scorzoneraefloiurm*)	40	
Licorice (*Glycyrrhiza glabra*)	30	
Kakkon (*Pueraria lobata*)	30	

*: All materials were commercially available health foods in Japan. Samples were suspended in distilled water and heated for 5 hours at 60ºC. **: Endospecy (LPS specific detection) were used for the LPS content in the samples. ***: The amount of LPS was calculated based on the daily recommend dosage. ****: Food used by ulcerative colitis patients. *****: Chinese herbs that were commercially available in Japan were washed with distilled water and dried. These samples were suspended in distilled water and heated for 5 hours at 60ºC.

provide beneficial functions. Though there is LPS present in certain foods, the kind and amount is generally unknown as these types of measurements are not being made. Each serotype of a Gram-negative bacteria has its own individual LPS structure, so there are a large number of different structures of LPS. Differences in the lipid and polysaccharide portions affect their biological activity [31]. Therefore, the quality (origin and structure of the LPS) and the quantity (optimal dosage for beneficial biological activity) of the LPS need to be evaluated to develop standards for functional foods. We described our processing method for obtaining an immunopotentiator from the edible Gram-negative bacteria, *Pantoea agglomerans* LPS (IP-PA1).

BIOLOGICAL SIGNIFICANCE BY LPS-TLR4 SIGNAL CASCADE IN HOMEOSTASIS MAINTENANCE

Currently, Gram-negative bacteria and/or LPS are not considered to be edible, even though humans have obtained considerable benefits with respect to homeostasis control and immunologic enhancement. Recently, the "hygienic hypothesis [13]" has suggested that the increase in hygienic management has resulted in an increase in allergies because of the decrease of exposure-opportunities and dose of Gram-negative bacteria and LPS. In other words, Gram-negative bacteria and/or LPS which humans have been unknowingly consuming in their food are now thought to be necessary for maintaining health.

According to the hygienic hypothesis, the molecular mechanism of allergy suppression by LPS is the result of a Th1 shift in theTh1/Th2 balance through the activation of a TLR2 and/or TLR4 signal cascade. For example, synthetic lipopeptide (Pam2CSK4), which is a TLR4 agonist, suppresses allergic bronchitis in a manner depending on IL-12 production [32]. Also, it has been reported that an allergic rhinitis drug which was developed as a TLR4 agonist (CRX-675) induces a Th1 immune reaction [33].

In addition, it has recently been shown that signal transduction from TLR2/TLR4 is important for control of homeostasis in the intestine. The symptoms of dextran sulfate sodium (DSS) induced colitis were worse in knocked out mice of TLR2, TLR4, or MyD88, which is the intracellular signal transduction factor of TLRs [34]. Moreover, colitis in mice was suppressed by the oral administration of *Escherichia coli* or LPS [34]. It was also reported that LPS induced defensin by bringing the TLR4 on Peneth cells, which provides host defenses against microbes in the small intestine [35]. Interestingly, defensin acts as the ligand for TLR4 on the intestinal epithelial cells [36]. These results indicate that Gram-negative bacteria and LPS seem to contribute to the homeostasis maintenance through TLR4 signal cascade of the intestine [37]. These phenomena suggest that humans can maintain a Th1/Th2 immune balance by consuming Gram-negative bacteria, and that they may do this unaware.

The modern hygienic life style appears to supply insufficient Gram-negative bacteria and/or LPS, and this has caused a shift in the Th2 dominant immune balance resulting in the spread of allergic diseases. Therefore, it seems reasonable to prepare functional foods containing edible Gram-negative bacteria and/or LPS to supply the TLR4 agonist which is insufficient in modern life.

NEW IMMUNOPOTENTIATING MATERIAL *PANTOEA AGGLOMERANS* LPS (IP-PA1)

When general foods were screened in mice using oral or percutaneous administration, we found that a macrophage-activating substance existed in wheat flour [12]. The domi-

nant molecule of this substance was LPS derived from *Pantoea agglomerans,* a concomitant bacterium in wheat. We named this substance IP-PA1 (Immunopotentiator of *Pantoea agglomerans* 1) [11]. We found that oral and percutaneous administrations of IP-PA1 were useful for preventing hyperlipidemia (rabbit), diabetes mellitus (mouse and human), various infectious diseases (mouse, shrimp) (Table **1**, Figs. (**1**) and (**2**)), ulcerative colitis (mouse) (Fig. (**3**)), and analgesic effect (mouse, rat and human), etc. [11, 38-47]. Moreover, IP-PA1 showed no toxicity by 2 weeks repeated oral administration and intradermal administration using rat test (data not published).

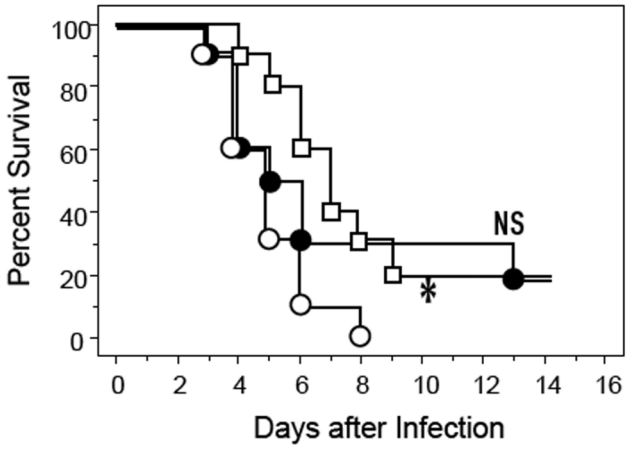

Fig. (1). Preventive effect of oral administration of IP-PA1 *ad libitum* on *Salmonella* infection.

C57BL mice (n=10) administered IP-PA1 at concentrations at 0 ng/ml (○), 20 ng/ml (about 6.4 μg/kg/day) (●) or 200 ng/ml (about 64 μg/kg/day) (□) respectively in drinking water from 3 to 8 weeks of age. A challenge infection was performed by intraperitoneal injection of 4 x 10^5 CFU *Salmonella typhimurium* at 6 week of age. Statistical analysis between control and other treatment groups were performed using Kaplan-Meyer method, log-rank test (*: P=0.012, NS: not significant).

The characteristics of IP-PA1 and *Pantoea agglomerans* are summarized below. The molecular weight of IP-PA1 (main band 5~~000~~kDa) is lower and more uniform when compared to *Escherichia coli* LPS (multi-ladder band 10,000 to 50,000) [25]. Though the lipid A structure of IP-PA1 is almost the same as for *E. coli* and *Salmonella* sp., the composition of the polysaccharide chain is glucose and rhamnose, which is different from *Escherichia coli* [48]. *Pantoea agglomerans* can fix nitrogen and phosphorus, and it adheres to various kinds of plants such as rice, sweet potato, apple and pear worldwide [49-53]. Furthermore, rye bread, which is eaten in Europe and in the U.S.A. may use lactic acid fermentation, and may have a step wherein a Gram-negative bacteria such as *Pantoea agglomerans* is cultured prior to the growing of lactobacilli because this provides a supply of folic acid which is an essential vitamin for growing the lactobacilli [28]. In Europe, viable *Pantoea agglomerans* has been developed as a biological control for post-harvest diseases of pome fruits that are caused by *Penicillium expansum*, *Botrytis cinerea* or *Rhizopus stolonifer*. This information demonstrates that *Pantoea agglomerans*, a Gram-negative bacterium, has a long history of being consumed in foods with apparent safety.

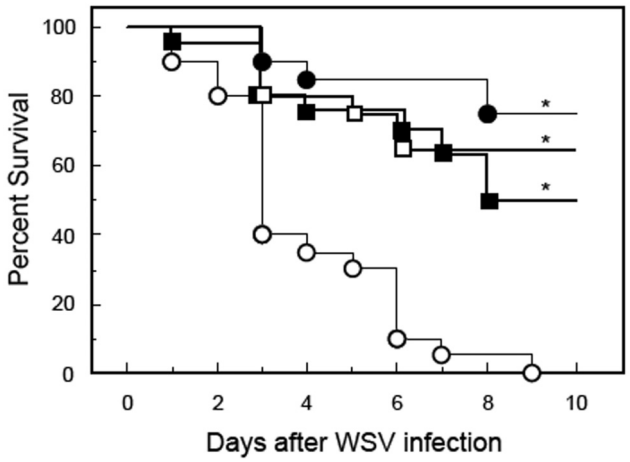

Fig. (2). Shrimp was administered IP-PA1 containing feed (○: Control, ●: 20 μg/kg/day, □: 40 μg/kg/day, ■: 100 μg/kg/day) during 8 days. Then white spot virus (WSV) solution which was prepared from infected kuruma shrimp homogenate was injected into shrimp. Shrimp were continuously fed with IP-PA1 by 10 days after WSV infection. Statistical analysis between control and other treatment groups were performed using Kaplan-Meyer method, log-rank test (*: P<0.0001).

Oral administration of IP-PA1 did not show toxicity in safety evaluations using animals [51]. Research has demonstrated that it is useful for preventing lifestyle-related diseases, allergic diseases, and infectious diseases in both human and animal models [38-47]. In addition, preventive effects have also been demonstrated against infectious diseases in cultured animals such as *Pseudomonas* disease of ayu fish and white-spot disease of the kuruma prawn [11, 46]. The occurrence of drug-resistant bacteria in livestock and aquaculture has become a critical worldwide social problem, and recently, the EU strengthened rules for animal feed and placed restrictions on the use of antibiotics as growth promoters. When livestock and aquaculture organisms must be cultured without drugs, it becomes even more desirable that infectious pathogens can be controlled by application of substances such as IP-PA1.

PERSPECTIVE

In the 20th century, reductionism techniques were remarkably successful in developing analytic technology for proteins and genes, and the functions and structures of a great many proteins and genes were clarified. As the result of these achievements, many molecular target drugs could be developed. The effectiveness of molecular target drugs is ascribed to one-to-one molecule correspondence, and this may lay the foundation for the mainstream of future medicine [54]. For example, gefitinib is the first selective inhibitor of epidermal growth factor receptor's tyrosine kinase domain, but presently it has not been developed so that it provides consistently good outcomes for cancer patients [55]. In contrast, as shown in this paper, just one macrophage activating agent has demonstrated a multitude of advantageous phenomena such as the preventing hyperlipidemia (rabbit),

diabetes mellitus (mouse and human), various infectious diseases (mouse, shrimp) (Table **1**, Figs. (**1**) and (**2**)), ulcerative colitis (mouse) (Fig. (**3**)), analgesic effect (mouse, rat and human), and causes tumor suppression [11, 38-47]. Macrophages are the main participants in body homeostasis and exhibit multifunctional activities. Therefore, macrophage-activating drugs are on the opposite end in a continuum of agents when compared to the very specific nature of molecular target drug. If macrophage-activating drugs can control the homeostatic dynamism to maintain normal conditions, they will be useful for the prevention and treatment of various diseases. In the future, macrophage-activation therapy may be used for more medical conditions than molecular target drugs.

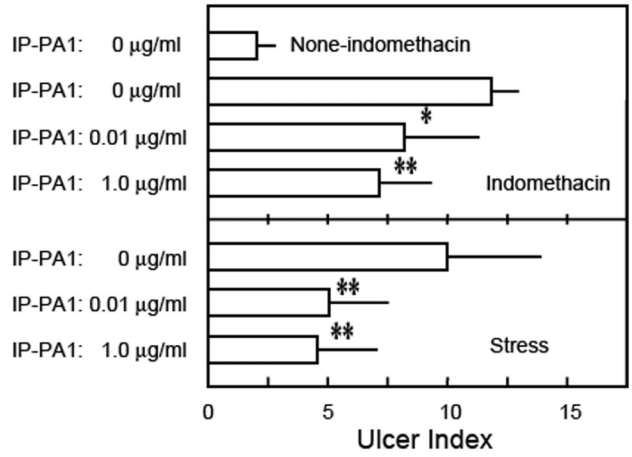

Fig. (3). Protective effect of IP-PA1 with *ad libitum* administration in drinking water on indomethacin or water-stressed ulcer. The BLAB/c mice were administered 0 µg/ml, 1 µg/ml or 0.01 µg/ml of IP-PA1 in drinking water during 4 to 5 days. Ulcers were induced by subcutaneous injection of indomethacin (60 mg/kg) or stress (water immersion). Column shows the average ulcer index (the sum of the hemorrhagic scar length (mm)) and bar their standard deviation. Significant differences between the control and IP-PA1 tested group (*: $P<0.05$, **: $P<0.01$).

The "hygienic hypothesis" states that in the past people lived in an environment that was less hygienic. Thus, they consumed larger amounts of bacteria and LPS. As the modern hygienic life has lead to relatively low consumption of LPS, we propose producing LPS-containing foods that have immune-regulating functions and that suppress allergy generation. We have developed a new method for preparing *Pantoea agglomerans* LPS (IP-PA1) which is similar to the method used for rye-bread fermentation. *Pantoea agglomerans* was cultured on wheat flour, water, and minerals, and then a water extract was prepared containing IP-PA1. Since, IP-PA1 is derived from the edible Gram-negative bacteria, *Pantoea agglomerans*, we believe that this traditional species can be used as an ingredient in new functional foods to prevent lifestyle-related diseases and allergy. We believe this substance can be used in new health preparations, and will help dispel the unwarranted toxic image of LPS.

IP-PA1 was developed as a food supplement with the data of safety as well as the efficacy. To develop it as a therapeutic agent, however, complete toxicology and clinical pharmacological studies are mandatory to be accomplished.

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