JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 102, No. 6, 485–496. 2006
DOI: 10.1263/jbb.102.485

REVIEW

Applications of Lipopolysaccharide Derived from *Pantoea agglomerans* (IP-PA1) for Health Care Based on Macrophage Network Theory

Chie Kohchi,[1,2] Hiroyuki Inagawa,[1,2,3] Takashi Nishizawa,[1] Takatoshi Yamaguchi,[4] Shiro Nagai,[4] and Gen-Ichiro Soma[1,2]*

Institute for Health and Sciences, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima, Tokushima 770-8514, Japan,[1] Institute for Drug Delivery Systems, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan,[2] Department of Applied Aquabiology, National Fisheries University, Nagatahon-machi, Shimonoseki, Yamaguchi 759-6595, Japan,[3] and Yaegaki Bio-industry Corporation, 681 Mukudani, Hayashida, Hyogo 679-4298, Japan[4]

Received 9 May 2006/Accepted 3 July 2006

Innate immunity is a universal prophylactic system which all multi-cellular animals possess. Macrophages are the cells that play the central role in the innate immune system. In 1991, we discovered a substance in a water extract of wheat flour that activated macrophages after oral or intradermal administration. The active substance was lipopolysaccharide (LPS), which is derived from the cell walls of *Pantoea agglomerans*, gram-negative bacteria that grows symbiotically with wheat. We named the substance IP-PA1 (immuno potentiator from *P. agglomerans*, former name: LPSp). The IP-PA1 is considered to be useful in various fields such as health food (to prevent and improve metabolic syndromes), skincare products (to maintain healthy skin, to improve atopic dermatitis, and to resist aging), and as active ingredients in feeds for stockbreeding and aquaculture (to act as a defense against infection). In this manuscript, we discuss the significance of activation of macrophages through oral or intradermal administration, the discovery of IP-PA1 as a macrophage-activating substance, the chemical structure of IP-PA1, the use of IP-PA1 to improve various disorders, the mechanism of action, and the possibility of application of IP-PA1 to various fields.

[**Key words:** innate immunity, prophylaxis, macrophage network, TLR4]

MACROPHAGES

Macrophages in animal evolution The immune system can be classified into two groups. One is an innate immune system without immune memory, and the other is an acquired immune system with immune memory. Although these two systems are closely related and collaborate in higher vertebrates, the innate immune system is a more primitive prophylactic system which is possessed by all multicellular animals, while currently the acquired immune system exists only in vertebrates. The acquired immune system is characterized by the possession of an expression system in which there is a rearrangement of genes, as occurs in antibody molecules or antibody receptor molecules and lymphoid cells, especially B cells and T cells. By contrast, the main characteristic of the innate immune system is that it does not employ the rearrangement of genes. Instead, it functions by utilizing innate gene expression and myeloid cells, especially macrophages.

The major function of an immune system is the defense of the body against the invasion of foreign substances. Hence, the immune system can technically be referred to as a prophylactic system. Burnet proposed the clonal-selection theory (1) and stated that the role of the immune system is to assure integration of the individual organism (2). Mizuno *et al*. proposed the concept of ontogenic inflammation and hypothesized that the starting point of the immune system lies in its function of assuring ontogeny (3). Furthermore, Tada proposed the existence of a super system in individual organisms, that was a system organized to be self referring with reference to a changing self. He considered the immune system as a typical super system (4).

It is reasonable to assume that the immune system and the macrophages play an essential role in the maintenance of homeostasis in all multi-cellular animals. In fact, various abnormalities are observed in osteopetrotic (op/op) mice that are deficient in macrophages because of a deficiency of

* Corresponding author. e-mail: sma5628@tokushima.bunri-u.ac.jp
phone: +81-(0)88-622-9611 fax: +81-(0)88-622-3217

colony-stimulation factors (5–8).

Role of macrophages The existence and importance of macrophages was first recognized in starfish larva by Metchnikov in 1882 (after larvae were pricked with rose thorns, macrophages engulfed and detoxified the pricks). After this discovery, Metchnikov performed an extensive systematological phylogenetic study and proposed a theory that "macrophages play an important role in prophylaxis throughout the animal kingdom, from simple multi-cellular organisms to man". The most fundamental function of macrophages is phagocytosis. Macrophages consume foreign substances (including invaded pathogens), waste materials, and the organism's own dead cells and confine the substances in phagosomes. Phagosomes containing the foreign bodies are next fused with lysosomes. Then, the foreign bodies are destroyed by the action of lysosomal enzymes, and finally, small fragments are expelled. Thus, macrophages are scavenger cells that function early in the process of preventing infection. However, the roles of macrophages are not limited to scavenging and preventing infection by phagocytosis. Part of the destroyed fragments in macrophages are presented on cell surfaces as antigens. As antigen presentation is a first step for antibody production with histocompatibility complexes, the antigen presentation leads to the activation of the acquired immune system. Moreover, macrophages stimulated with pathogens produce nitric oxide to kill the pathogens, and they secrete various effector molecules (specific cytokines etc.), which regulate other macrophages or immune cells. All of these functions contribute to the maintenance of homeostasis (9).

Existence of tissue macrophages Cells produced in bone marrow differentiate into two major groups: myeloid progenitor cells and lymphoid progenitor cells. Macrophages are differentiated from monocytes of myeloid lineage. Monocytes, after leaving the bone marrow, enter the blood circulation system where they circulate for several days, and migrate into various tissues. In the tissues, monocytes differentiate into tissue-specific macrophages in response to the environment of the tissues. Thus, macrophages exist in every tissue. They are not necessarily referred to as macrophages. For example, macrophages in the brain are called microglia, and in liver, kupffer cells. There are many macrophages in mucosal tissues such as in the lungs or intestines. Macrophage-like cells exist also in skin and are called Langerhans cells. These tissue macrophages are known to play important roles, at the individual level, not only for defense against infection but also in metabolic control and in wound healing (9). It is important that macrophages function normally.

Macrophage network theory Since macrophages exist in every tissue including mucosal tissues, some are always in contact with the external environment and external stimuli. Thus, they can be the earliest cells to recognize changes. Macrophages express on their cell surface and/or secrete various receptors and ligands in response to foreign bodies. These protein expressions are believed to function as tools for inter-cellular communication. Thus, information related to the external environment that was obtained by local macrophages could be transferred in a paracrine, juxtacrine and autocrine manner to other tissue macrophages. We hypothesized the existence of a network formed by tissue macrophages and termed this putative communication network a macrophage network (Fig. 1) (10). To maintain body homeostasis it is required that there be a flexible system at the locus which quickly recognizes changes in the external environment and reacts to this change while transmitting information to the whole body. We believe that the macrophage network functions as a system that permits plasticity within an individual organism.

Significance of macrophage activation Assuming that the macrophage network plays a role in maintenance of homeostasis, then a decline in macrophage functioning may create a situation where there is a danger of a failure in homeostasis. Conversely, if we can prevent the decline in macrophage functions in response to environmental stimuli, there is a possibility of preventing the failure in homeostasis

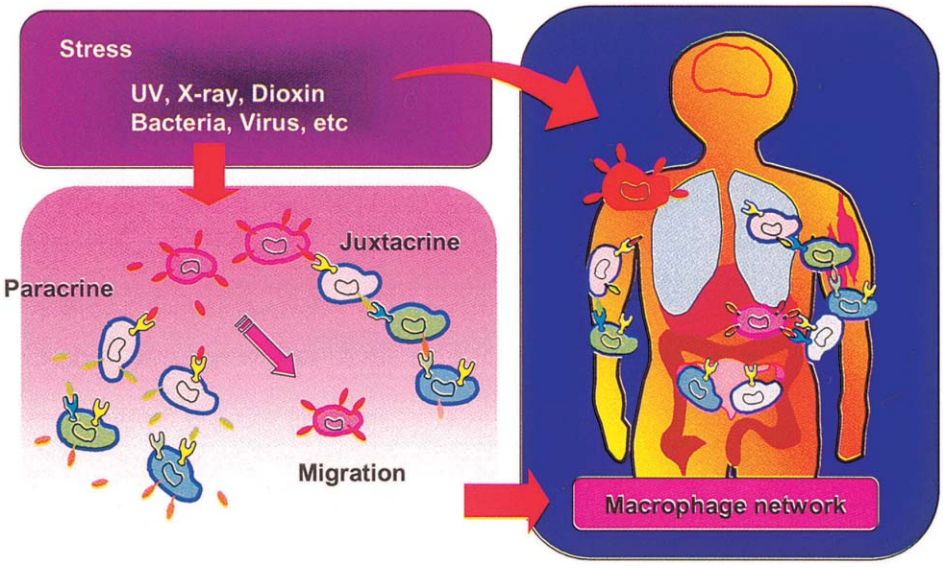


FIG. 1. Macrophage network theory.

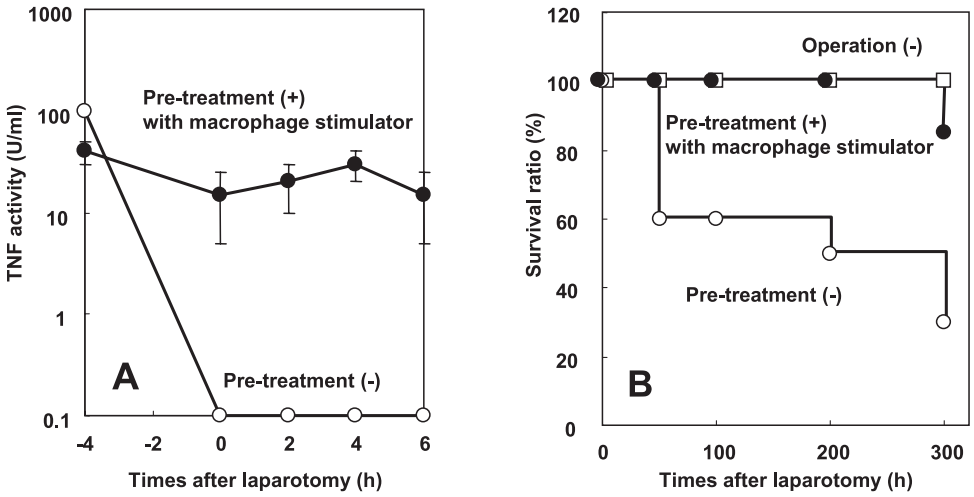


FIG. 2. Effect of laparotomy stress on macrophage activity in serum and resistance to bacterial infection. (A) BALB/c mice were given laparotomy stress. After the stress, mice were intravenously administrated with OK-432 (a macrophage stimulator) and the serum TNF levels were examined as an indicator of macrophage activity. Macrophage activity was dramatically decreased after the operation stress. However, when mice were stimulated with OK-432 before the operation stress, the serum TNF levels were not affected. (B) BALB/c mice were given laparotomy stress. After the stress, mice were infected with 10^7 of *Staphylococcus aureus*. Although, mice which were not given operation stress were resistant to the bacterial infection, mice given operation stress were highly susceptible to the infection. However, when OK-432 (a macrophage stimulator) was administrated to mice before the operation stress, the mice were relatively resistant to the bacterial infection.

after an individual organism has been exposed to unfavorable environmental stimuli.

Various stresses such as environmental factors are known to significantly decrease macrophage function. For example, the mortality rate of mice infected with *Staphylococcus* increased significantly after laparotomy (operational stress) compared to mice without the operation (Fig. 2). In other words, when an operational stress is given to an individual, a significant decline in macrophage functions occurs. As a result, resistance to bacterial infection decreases dramatically. If this decline of macrophage function after exposure to stress could be prevented so that resistance to bacterial infection does not decline under stress. When OK432, a macrophage activator, was injected intravenously before laparotomy stress, the functional decline in the macrophages after the operation stress was observed to be small, and the mortality rate caused by the infection was about the same as for mice without the operation (11). A similar phenomenon was also observed in the suppression of lung metastasis in an experiment with operation stress. The important factors in preventing a lowering of macrophage function and suppressing a decline in the resistance to infection (or suppressing lung metastases) were the amount and the timing of the administration of a macrophage-activating agent before the operation.

The results mentioned above indicate that appropriate pre-activation of macrophages could effectively prevent homeostasis failure in response to various stresses. Furthermore, this conceptual modality may lead to the establishment of new techniques for preventing disease, and these techniques that have a completely different action mechanism than conventional drugs, *i.e.*, antibiotics that kill microorganisms.

SCREENING OF MATERIALS WHICH ACTIVATE MACROPHAGES AFTER ORAL OR INTRADERMAL ADMINISTRATION AND DISCOVERY OF IP-PA1

Screening Based on the macrophage network theory, we hypothesized that a substance which can activate macrophages would be effective in disease prevention and/or recovery from diseases. It is known that there is a stage called priming in macrophage activation. Macrophages in the priming stage do not secrete effector molecules such as cytokines and nitric oxide, but after stimulation, larger amount of effector molecules are secreted compared to macrophages that are in the normal stage (12). For example, radiation or IFN-γ is known to initiate priming activity (13, 14). Although, the mechanism of induction of priming is not fully clarified, up-regulation of receptors for recognition of foreign substances, such as TLR4, has been reported (15). Also, augmentation of membrane-bound TNF, but not free TNF, on the cell surface of priming macrophages seems to be implicated in this biological state (16). The induction of a priming state in macrophages is advantageous, as it does not cause unnecessary loading when a counter attack is not required, but allows a more effective response in times of emergency (such as during an infection). For this reason, we screened for substances (preferably natural substances) that would initiate the process leading to the priming state in macrophages. We also preferred a substance that could be administered in a non-invasive way, *i.e.*, with oral or intradermal administration. In our tests, mice were administrated primer candidates orally or intradermally and were then injected with OK432 as the trigger for macrophage activation. After several hours, the serum level of TNF was measured as an indicator of macrophage activation. As a result of this screening process, we found that administration of a water

extract of wheat flour augmented the serum TNF level after OK432 injection (17). This substance alone did not augment the TNF level without the additional triggering by OK432.

Discovery of IP-PA1 Initially, we believed that the active substance was derived from wheat. However, further analysis revealed that it was a lipopolysaccharide (LPS) of the gram-negative bacteria *Pantoea agglomerans*, which was symbiotically attached to the wheat (18).

P. agglomerans is a bacterial strain which attaches or lives symbiotically not only with wheat, but also with other food plants, such as fruits and potatos. Living *P. agglomerans* exists in the flour. Recently, Kariluoto *et al*. demonstrated that *P. agglomerans* was necessary counterpart, during fermentation of rye sourdough, by producing and providing folate to Lactobacillus (19). It is considered to be a non-pathogenic bacteria which humans have been ingesting from ancient times (20). The bacteria were present in the eight kinds of wheat (cultivated in various countries) that we tested. Interestingly, some strains of the bacteria produce antibiotics, and the living bacteria are used as bio-control agents for prevention of corruption of fruits by fungus (21–23). The *P. agglomerans* strain we isolated does not produce antibiotics.

The active substance for macrophage priming was LPS, which is a cell-wall component characteristic of gram-negative bacteria. We named the substance LPSp, and lately renamed it as IP-PA1, meaning immuno potentiator from *P. agglomerans*. It was already well known that LPS is a strong trigger for macrophage activation. When macrophages are directly stimulated with LPS, large amount of cytokines are secreted. As LPS can cause lethal shock when it enters the blood stream, LPS is also known as an endotoxin. However, it is very interesting that the same LPS is not always toxic; it has priming activity when administrated orally or intradermally. This fact suggests that the biological effect is modified depending on whether absorption is from the mouth, gastrointestinal mucosa, or skin.

Structure of IP-PA1 LPS consists of a lipid portion called lipid A, polysaccharide components with inner and outer core, and a highly variable O-antigen portion composed of oligosaccharide subunits (24). The chemical structures (chemotype) of LPS are different depending on the bacteria (25). The O-antigen portion of typical *Escherichia coli* LPS (LPSe) consists of a repetition of oligosaccharide units composed of two colitoses, a gucosamine, a glucose, and a galactose (compared to rhamnose and glucose units in IP-PA1) (Fig. 3A). Moreover, an SDS–PAGE analysis revealed that the number of repetitions of the sugar unit is less in IP-PA1 than in LPSe. The result is that the overall molecular weight of IP-PA1 is much smaller than of LPSe (Fig. 3C) (17). The amphipathic LPS easily forms micelle in solution. LPS with short sugar chains constructs small-diameter micelle, which seems to be suitable for permeation through mucosa and/or skin. We then analyzed the curative effects of IP-PA1 against various diseases after oral or intradermal administration.

EFFECT OF IP-PA1 ON VARIOUS DISEASES AFTER ORAL OR INTRADERMAL ADMINISTRATION

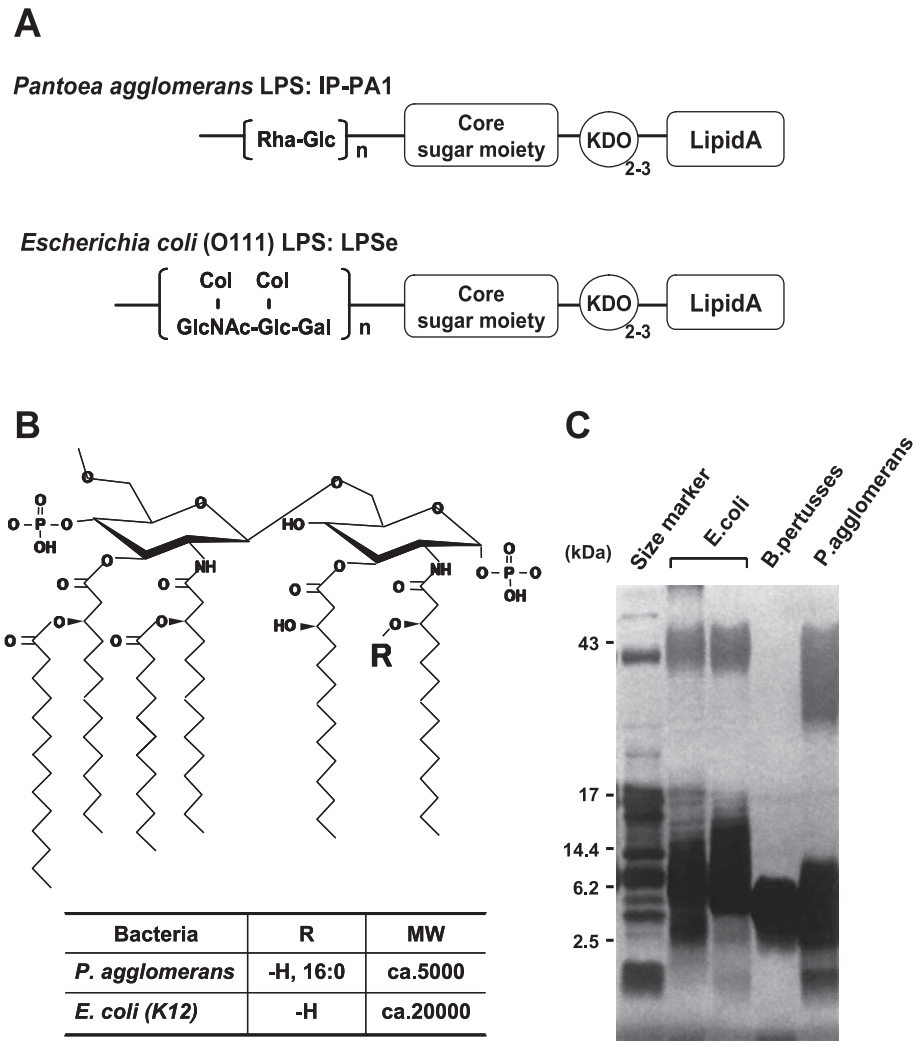
Effect on hyperlipidemia The effect of IP-PA1 on cholesterol catabolism was examined using WHHL (Watanabe heritable hyperlipidemic) rabbits, which is an experimental model for familial hyperlipidemia (26). The serum cholesterol level of the rabbits decreased with uptake of IP-PA1 contained in water (1 μg/ml). Following cessation of uptake of IP-PA1, the cholesterol level gradually elevated. The serum level of apolipoprotein B, a constituent of low-density lipoprotein (LDL), also decreased along with the serum cholesterol at a nearly coincident rate. Conversely, the level of apolipoprotein A–I, a constituent of high-density lipoprotein (HDL), did not change, nor did the HDL-cholesterol. As macrophages are known to phagocytose LDL, these results may have been caused by the activation of phagocytotic functions of the macrophages.

Effect on gastric ulcers The protective effect of LPS from various bacterial sources (*E. coli*, *Serratia ficaria*, *Enterobacter cloacae*, *Bordetella pertussis*, *Alcaligenes faecalis*, and *P. agglomerans*) on gastric ulcers when administered orally was examined in a mice ulcer model (27). Ulcers were induced by subcutaneous injection of indomethacin (60 mg/kg). The results were evaluated by the sum of the lengths of the necrotic lesions of the stomachs. One hour prior to indomethacin administration, LPSs (10 μg/kg) was given to mice by sonde. LPSs showed a protective effect against the occurrence of ulcers. Of the various types of LPS tested, IP-PA1 derived from *P. agglomerans* was shown to provide a significant protective effect.

Effect on infection The protective effect of IP-PA1 against *Toxoplasma gondii* when administered orally was examined in a mice model (28). *Toxoplasma* is an obligate intracellular parasite and is an important pathogen in humans and other animals. An oral administration of IP-PA1 to mice at a concentration of 20 ng/ml in drinking water beginning 1 d after infection significantly decreased mortality and prevented weight loss after an acute infection with *T. gondii*. By comparison, 71% of the mice in a control group that did not receive IP-PA1 died of toxoplasmosis, and only 14% of the mice treated with IP-PA1 died. The administration of LPS derived from *B. pertussis* also significantly decreased the mortality of infected mice. LPS from *E. coli* and synthetic lipid A, however, did not cause a significant decrease in mortality. As the administration of IP-PA1 started after the infection, these results indicate that IP-PA1 administration has an effect not only in preventing infection, but also for clearing the pathogens after an infection has begun.

Effect on diabetes After intradermal administration of IP-PA1, there was a suppressive effect on diabetes progression in type 1 diabetes in non-obese diabetic (NOD) mice (29). In this experiment, NOD mice were given IP-PA1 (10 μg/mouse/week) from 6 to 12 weeks of age. The onset of glycosuria was delayed about 9 weeks when compared with the onset in non-administered control mice, and the survival rate was significantly improved.

Effect on allergy Intradermal administration of IP-PA1 also showed a suppressive effect on IgE-dependent allergy. When mice were injected with anti dinitrophenol IgE mono-



Bacteria	R	MW
P. agglomerans	-H, 16:0	ca.5000
E. coli (K12)	-H	ca.20000

FIG. 3. Comparison of the chemical structure and molecular size of LPS (IP-PA1) from *Pantoea agglomerans*'s with the LPS from other species. (A) Sugar constructions, (B) chemical structures of lipid A, (C) molecular sizes of LPS extracted from *Escherichia coli*, *Bordetella pertussis*, and *Pantoea agglomerans* observed by SDS–PAGE.

clonal antibodies, following application of dinitrofluorbenzene as an antigen on the ear, the ear became swollen. (This is one of the animal models for IgE-dependent allergy.) If IP-PA1 was intradermally administrated before antigen application, the swelling was suppressed to the same degree as the negative control mice which were not given IgE antibodies. A therapeutic effect on atopy (an IgE-dependent allergy) in humans was also obtained.

As hypothesize that the mechanism for suppressing the IgE-dependent allergy is a shift of the immune balance (Fig. 4). As described above, the immune system is divided into an innate immune system where macrophages play the main roles and an acquired immune system where T cells and B cells play the main roles. The acquired immune system is further subdivided into humoral immunity and cellular immunity. Humoral immunity is important for defense against parasites, but excessive activity causes allergies. By contrast, cellular immunity is important for protection against intracellular pathogen, but excessive activity causes inflammatory diseases.

Which form of immunity becomes dominant correlates with the direction of differentiation of naive T-cells, (either the Th1 direction or the Th2 direction) (30). Although the direction of differentiation depends on the kind and intensity of antigen stimulation, it also depends on the cytokines that exist in the body. For example, IL-12 or IFN-γ stimulates differentiation in the Th1 direction and IL-4 or IL-10 stimulates differentiation into the Th2 type. Furthermore, differentiated T cells secrete the same group of cytokines and repress the production of cytokines that have the reverse action (31). Thus, once the immune system shifts in one direction, the same trend is apt to continue. In the case of atopy, it is believed that the immune balance has shifted to Th2 type (32).

As activated macrophages are known to produce Th1 type cytokines (33), we hypothesize that IP-PA1 sets the immune balance to the right via activation of macrophages to secrete Th1 type cytokines.

Cancer We also examined the antitumor effect of intradermal administration of IP-PA1 on murine Meth A fibro-

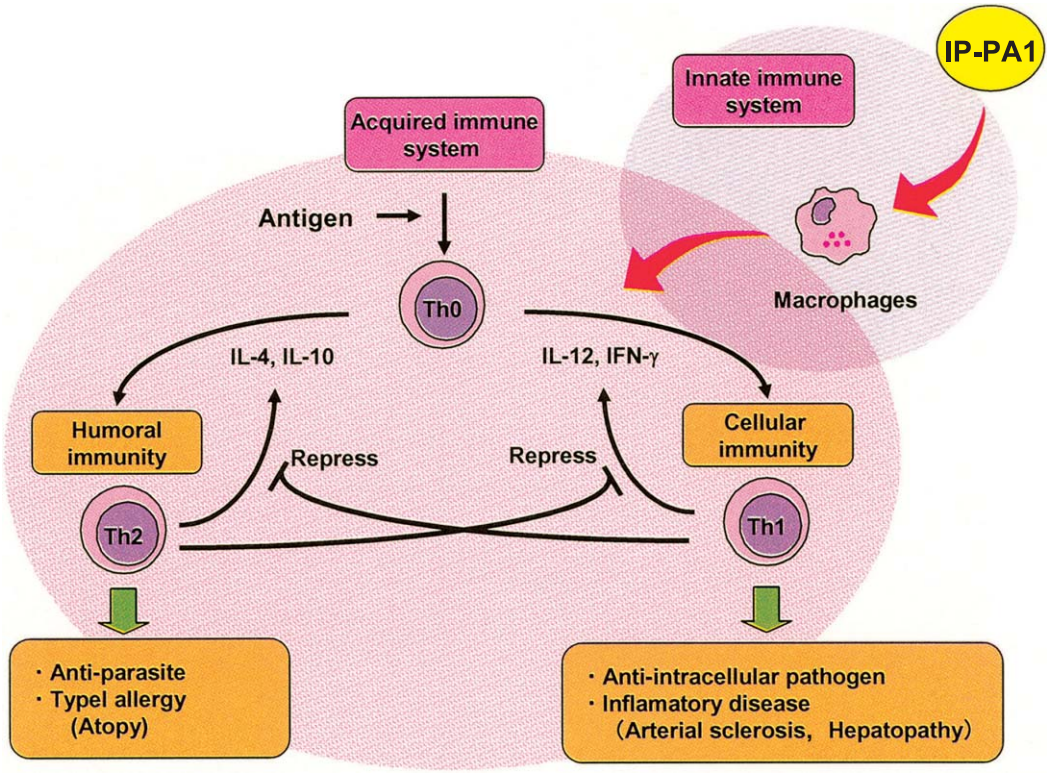


FIG. 4. A hypothetical mechanism for suppression of the IgE-dependent allergy. Shift of immune balance (Th1/Th2) through macrophage activation with IP-PA1.

sarcoma, MH134 hepatoma, and Lewis lung (LL) carcinoma (34). IP-PA1 alone had a significantly suppressive effect on the growth of all tumors. Complete regressions were observed in 75% of the mice bearing Meth A tumors (though, there were no complete regressions for MH134 or LL tumors). Moreover, when given in combination with cyclophosphamide (CY) once prior to the IP-PA1 administration, the antitumor effects of IP-PA1 were significantly augmented, and there was complete regression in all types of tumors.

The effect on tumors in humans was further tested under approval by an ethical committee. In this study, intradermally administrated IP-PA1 was less toxic and elicited a tumor response in combination with CY (35). Incidently, the tumor microenvironment contains many migratory macrophages (36), that are referred to as tumor-associated macrophages (TAMs). Although the TAMs are suspected of promoting tumor progression and metastasis, Ohno *et al*. found that the macrophages that exist in a nest of tumor cells may be correlated with tumor suppression (37).

Other effects Another significant result was that oral administration of LPS induced an analgesic effect to various types of pain (3). In the acetic acid-writhing mouse model, oral administration of IP-PA1 (10 μg) significantly inhibited writhing. Moreover, oral administration of IP-PA1 (30 μg) reduced post-operative pain and increased the serum level of β-endorphine in patients who underwent endoscopic cholecystectomy surgery.

MECHANISM OF ACTION OF IP-PA1

Discovery of TLR and signal transduction When we found IP-PA1 16 years ago, none of the molecular mechanism for macrophage activation by LPS was understood. However, during the last decade, receptors for recognition of foreign molecules, called toll like receptors (TLRs), have been discovered as the homologue for prophylactic molecules of *Drosophila*, and as the mechanism by which bacteria and/or viruses activate immune cells (38).

Macrophages express various TLRs to foreign substances on their cell surfaces. The specific receptor for LPS derived from gram-negative bacteria is a TLR4/MD2 complex (39). Extracellular LPS are first captured by plasma LPS binding proteins (LBP) or CD14, concentrated around the cells, and transferred to the TLR4/MD2 complex expressed on the cellular membrane. Then, this event triggers assembly of adaptor molecules to the LPS/TLR4/MD2 complex in the cytoplasm, followed by signal transduction (40). There are two pathways for LPS signaling. One is a MyD-88 dependent pathway where adaptor molecules, MyD-88 and TIRAP (Mal), assemble with the intracellular domain of TLR4, resulting in activation of NF-κB or MAP kinase through TRAF6. The other is a MyD-88 independent pathway activated when TRIF and TRAM assemble into TLR4, and where the IRF3 is activated after phosphorylation and dimarization. Depending on the activation of these transcription factors, certain types of genes are expressed in the nucleus. Based on these gene expressions, functional activation of macrophages occurs, and as a consequence, there is regula-

tion of the whole body.

Different types of signal transduction depending on differences of sugar moiety of the LPS TLR4 binds to the lipid-A portion of the LPS, and it appears that the signal transduction pathways are similar for all forms of LPS that have a lipid A moiety that is independent of sugar chain construction (41). However, we found that, the response to IP-PA1 and LPSe are different depending on the type of cell. For example, when testing dose dependency on NO production by LPSs, it was found that IP-PA1 induced NO in lower concentrations than LPSe (42). Also, in human peripheral blood monocytes, IP-PA1 induce higher levels of IL-12 than did LPSe. These results suggest that the quality or quantity of LPS signal transduction might be different depending on the LPS chemotype. As the differences in the lipid A portions of IP-PA1 and LPSe are minor (Fig. 3B) (43), it is presumed that in this case, the portion that causes these differences is the sugar-chain portion.

Additional information on this topic was reported by Jiang *et al*. who reported that the TLR4/MD2 complex responded to differences in the construction of sugar moieties, at least with respect to the length of the sugar chain (44). They found that macrophages from $CD14^{-/-}$ mice did not produce TNF when stimulated with smooth LPS (which is LPS with a long sugar chain derived from *Salmonella abortus*) but did produce with rough LPS (which is LPS derived from *Salmonella minnesota* with a short sugar chain). Furthermore, they clarified that the MyD-88 independent pathway did not function even from rough LPS, when CD14 was deficient. In other words, they showed the possibility that quality and quantity of signal transduction may be different depending on the LPS chemotype and the availability of CD14.

The information above explains the differences in bioactivity between IP-PA1 with a short sugar chain and LPSe with a long sugar chain. Moreover, their discovery suggests another important aspect. Although the TLR4/MD2 complex is widely expressed in immune cells, CD14 expression is relatively limited. For this reason, IP-PA1 with a short sugar chain will activate a broader range of cells than will LPSe with a long sugar chain, as LPS with a long sugar chain cannot transmit an LPS signal without CD14.

APPLICATION OF LPS

As described above, IP-PA1 has curative effects on a variety of diseases after oral or intradermal administration. For this reason there is excellent potential for using IP-PA1 in a variety of fields, such as health foods, skin-care cosmetics, or as feed for animals. Several practical examples are cited below.

Application to animal food Currently, antibiotics and chemical antimicrobial agents are being used to control infectious diseases in animal husbandry and aquaculture. But it is now recognized that overuse of antibiotics and chemical antimicrobials in food animals are leading to serious problems (WHO report, 2002), because they put public health at risk and cause environmental pollution. In particular, two major risks to public health are that ingestion and/or accumulation of residual antibiotics in food cause direct health disorders, and they cause the development of antibiotic resistant bacteria. Thus, establishing new alternative strategies for preventing infection that is not dependent on antibiotics is a pressing issue in the fields of animal husbandry and aquaculture.

IP-PA1 activates macrophages, and therefore it activates the innate immune system. All species of animals are subject to the principle of innate immunity. Thus, feeding IP-PA1-containing food to animals, including fish, will serve to strengthen the prophylactic ability that all animals possess. Thus, with IP-PA1 would be possible to maintain productivity without using antibiotics, and to continue to provide products which consumers demand, but in a safe manner. Prevention of infection by IP-PA1 reduces both the risks of the appearance of drug-resistant bacteria and indirectly reduces the uptake/accumulation of drugs in humans, through reduction of usage of antibiotics. Furthermore, since IP-PA1 is effective at trace amounts (on the order of μg per kg bodyweight of animals per day) and is environmentally degradable, environmental pollution will not occur. We have obtained data proving the protective effect of IP-PA1 in feed in test models with broiler chickens, spawning ayu fish, kuruma shrimp, carp, and yellowtail.

Reduction of mortality of broiler chickens Five thousands broiler chickens per group were fed food containing either IP-PA1 (0.14 mg/kg-food) or Calspollin (living bacillus preparation, commercially available) (2 g/kg-food) during the period from 3 weeks to 7 weeks after hatching, and the total mortality of the chickens just before shipment (9 weeks after hatching) was examined. Vaccinations were performed 3 weeks after hatching as usual. Antibiotics were not used. Two independent tests showed that the mortality of the IP-PA1-fed group was lower (1.1–1.2%) than the Calspollin-fed group (2.8–2.9%).

Strengthening eggshells Oral administration of IP-PA1 given at 60 μg/hen/d in drinking water markedly enhanced eggshell strength (45). The monthly percentage of eggs laid with a shell strength of more than 4 kg was 32% in the group given IP-PA1 in drinking water, while it was 12% in the control group given plain water. At the same time, IP-PA1 caused a 30% enhancement in the total monthly number of eggs laid.

Effect on prophylaxis of kuruma shrimp against WSV The effect of oral administration of IP-PA1 for prophylactic efficacy against *Penaeid acute viraemia* (white spot virus, WSV) in kuruma shrimp was examined (46). IP-PA1 feeding (20 μg/kg-body weight/d) prominently increased the survival rate against WSV (80% versus 0% for the control). There was an increase of phagocytotic activity and phenoloxidase (PO) production of shrimp haemocytes.

Effect on prophylaxis of ayu fish against *Pseudomonas* Ayu were fed food containing IP-PA1 for 7 d before injection with *Pseudomonas*. Survival was monitored for 15 d after the injection. The survival rate of the control group was 44%, while the 4 μg-fed group had 56% survival, and the 20 μg-fed group was 72%. In this experiment IP-PA1 feeding was shown to be effective against pseudomonas disease.

Effect on prophylaxis of ayu fish against *Flavobacterium* IP-PA1 treatment was shown to be effective also against

coldwater disease. Ayu were fed food containing IP-PA1 for 7 d. Then the ayu were immersed in IP-PA1-containing water for 2 h every other day. *Flavobacterium* infection was started for 1 week after 7 d pre-feeding. On the 19th day, the survival rate of the control was 11%, while the survival rate of the 4 μg-feeding combination with immersion in IP-PA1 water (4 μg/*l*) was 46%, and the 20 μg-feeding combination with immersion in IP-PA1 water (20 μg/*l*) was 32%.

Effect on prophylaxis of carp against koi herpesvirus Carp were fed food containing IP-PA1 7 d before virus infection. After the infection, survival was monitored for 10 d. All control carp died within the 10 d. By contrast, IP-PA1-fed carp showed significantly higher survival rates. In particular, the 20 μg-fed group had 65% survival (PCT/JP2004/013812).

As can be seen, IP-PA1 feeding provided a prophylactic effect on livestock and various aquatic animals.

Application to skincare products When skin dries because of inadequate moisture, natural oils are not produced as well. Thus allergens or stimulus substances can more easily enter through the skin. Furthermore, dried skin is sensitive to ultraviolet. Crinkling or itching is also caused by dryness. Therefore, many skin problems are caused by dryness. For this reason, some skin problems will be prevented if there is adequate retention of moisture. IP-PA1 is an amphipathic molecule having both an oleophilic lipid portion and a hydrophilic sugar-chain portion. The oleophilic lipid portion of IP-PA1 is speculated to attach to natural oil, while the hydrophilic sugar chain retains moisture. This means that IP-PA1 can function in covering the skin and retaining moisture. With this in mind, IP-PA1 may be a suitable ingredient for basic skin care.

In addition to the moisturizing action, the effect of activation of innate immunity by IP-PA1 is believed to improve skin problems. IP-PA1, with a molecular weight of 5000, forms micelle in solution. The diameter of the micelle is expected to be about 60 nm. It is believed that the maximum diameter of a particle that can be adsorbed into the skin is 250 nm, so some of the micellae of IP-PA1 will enter the skin.

Langerhans cells in the epidermis are analogous to macrophage cells (9). Epidermal Langerhans cells that are activated by IP-PA1 phagocytose and remove cells damaged by heat, ultraviolet light, and other physical forces. Moreover, when there is an infection, they can kill directly by phagocytosis and by producing bactericidal molecules, resulting in the augmentation of prophylactic activity.

Macrophages also exist in dermis along with other immune cells. Activation signals are transmitted through the macrophage network from the epidermal Langerhans cells to dermal macrophages. As activated macrophages secrete Th1 type cytokines, activated dermal macrophages calm the allergy state; the immune balance shifts to the Th2 type, and histamine and/or IgE antibodies are overproduced by mast cells and B cells. Because of these effects, it can be anticipated that skincare products containing IP-PA1 may help prevent infection, may aid wound healing, and may cause the remission of allergies such as atopy.

Based on these hypotheses, we prepared trial substances containing IP-PA1. A water-soluble cream (IP-PA1: 0.1 μg/g) and bath agent (IP-PA1: 0.1 μg/*l*) were applied to the skin of normal healthy people, and also to the skin of people with burns, pressure sores, and atopy. The trial IP-PA1 cream was confirmed to be effective for moisture retention and treatment of rough skin surfaces in normal healthy people, and was also shown to have curative effects for burns, pressure sores, and atopy. A double-blind test was performed using a bath agent containing IP-PA1 (100 participants). When compared to a control bath agent without IP-PA1, all measured parameters had higher point values (heat retention, stiff neck, muscle ache, backache, nerve pain, rough skin, atopy, and athletes foot).

As described above, there are multiple lines of evidence for effectiveness of IP-PA1 against a variety of skin problems. However, IP-PA1 is completely different from drug medicines, such as steroids or anti-inflammatory agents, because the fundamental principle is macrophage activation. Thus, skincare products using IP-PA1 could be used daily for prevention and improvement of skin trouble without side effects.

Application to health foods Recently, health-conscious consumers have become increasingly interested in gaining health through diet. Oral administration of IP-PA1 has been shown to be safe in several animal and human experiments, and it is expected that it will be useful in preventing or improving metabolic syndromes.

Under certain circumstances LPS is toxic and is called an endotoxin. It is known to cause severe sepsis and septic shock when large amounts enter the blood stream directly (47). However, oral administration of IP-PA1 was confirmed to be far less toxic relative to GLP in a safety test using rats. We also determined that LPS is contained not only in flour and fruit but also in existing health foods, supplements, and Chinese herbal medicines. Thus, there is considerable human experience with the ingestion of LPS, and LPS has not been found to be toxic when it is taken either orally or through the skin. The existence of LPS in health foods, supplements, and Chinese herbal medicines suggests another important topic; LPS may partly or substantially provide the functionality of these health foods. Although, one could infer from our research that the presence of LPS in health foods or Chinese herbal medicines is important, this has not yet been adequately researched. However, recent epidemiological studies have shown an inverse correlation between the amount of exposure to endotoxins in earlier generations and the presence of atopy, and it has been suggested that LPS administrated orally or through the skin may be useful for remaining healthy (48, 49). Thus, a new concept for health foods would be foods that that contain LPS, and where the uptake of the LPS is controlled in quality and quantity.

We are interested in qualitative differences between various types of LPS, for use as the active ingredient in health foods, cosmetics, and animal feeds. As described above, depending on the species of bacteria LPS has different chemotypes, and the induced signals differ both qualitatively and quantitatively depending on the LPS chemotypes. Because of this, we evaluated the LPS of gram-negative bacteria used in food products, searching for other types of LPS that might be as useful and safe as IP-PA1. For example, acetic acid bacteria are gram-negative bacteria, which have been

traditionally used for production of vinegar in Japan. However, there is little information available on the LPS of acetic acid bacteria. We ascertained that acetic acid bacteria do contain LPS, from the following tests: (i) an extract of acetic acid bacteria was positive in limulus activity, which is a method for examining lipid A; (ii) the extract induced TNF and/or NO from normal macrophages, and the activity was blocked by polymixin B, an inhibitor of LPS; and (iii) the extract did not induce TNF and NO from the macrophages derived from C3H/HeJ mice, which have a defective LPS receptor, TLR4 (50). Although, it has not yet been proven that the LPS from acetic acid bacteria is related to the health-enhancing effects of vinegar, the LPS from acetic acid bacteria is certainly a good candidate for commercial use as the safety of this form of LPS has already been empirically confirmed.

Possibility of application to medical drugs Since the existence of tumor-associated antigens (TAA) were discovered, there have been trials in which TAA has been tested for use in immune therapies for cancer. This is because tumors can theoretically be a target for acquired immunity (51–54). During immune therapies for cancer, the main strategy is the augmentation of cytotoxic T cells specific for TAA. However, now it is recognized that the innate immune system must be activated prior to activation of acquired immunity. As a consequence, it is now beginning to be recognized that it is important to activate the innate immune system in order to be successful with immune therapies for cancer. The medicines that have been in use for activating the innate immune system includes BCG (55, 56), OK-432 (prepared from hemolytic *Streptococcus*), and Bestatin (prepared from *Actinomycetes*) (57–59). Generally, these medicines have only a weak effect on tumor attrition, and so are only used as accessory therapies along with chemotherapy and radiation. However, significant side effects are rare, and sometimes nearly miraculous efficacies have been observed. Recently, trials have been initiated in which different designs for ligands for TLR are being tested as therapies for diseases including cancer. For example, CpG oligodeoxynucleotide (CpG ODN) (a ligand of TLR9) is now in phase III clinical trial for small-cell carcinoma, melanoma, and cutaneous T-cell lymphomani (60–62). It is also presumed that CpG ODN can also be a medicine for atopy (63, 64). Compounds belonging to the imidazoquinoline family, the ligands for TLR7 and TLR8, are also under development as drug medicines (65).

Although LPS, a ligand of TLR4, has never been used as a medication, Coley discovered that bacterial infections diminished malignant tumors. Mixtures of heat-killed gram-negative and gram-positive bacteria (Coley's toxin) were used for cancer therapies (66). However, this therapy decreased quality of life (QOL) of patients, as it induced high fevers and chills, and this therapy was eventually discontinued before there was an understanding of the mechanism. Now, a century after Coley's experiments, we can speculate that the success of these procedures were due to the biological function and mechanism of signal transduction from LPS. The potential of LPS for activating innate immunity holds the promise of being effective against refractory diseases including cancer, research is still necessary to determine the most suitable route and method of administration.

As described above, different types of LPS have different chemotypes depending on the source species of bacteria; in particular, there is significant variety in the sugar portion (25). To develop medicines based on LPS, there needs to be extensive research on the correlation between the function and the sugar portion. Furthermore, uniformity of specifications is required for medical drugs. Unfortunately, the LPS prepared from bacteria is heterotypic, and there is variable repetition in the number of the sugar units. Hence, to develop an LPS-based drug, it will be necessary to synthesize whole molecules with the appropriate sugar portion for a particular therapy.

INDUSTRIAL PRODUCTION OF IP-PA1

It would be prohibitively expensive to produce IP-PA1 from flour extracts. For this reason we developed an inexpensive method for the mass production of IP-PA1 that is both safe and stable (PCT/JP2004/013812). Briefly, *P. agglomerans* is cultured in a medium, free from components of animal origin, that contains flour as the sole carbon substrate. Flour is inexpensive and is a safe substrate. After culture, the culture broth is heated to extricate the IP-PA1 component from the bacteria, and the free IP-PA1 component is concentrated from the soluble fraction of the culture. The final product (a wheat-fermented extract) contains IP-PA1 and a fermented flour component, and has an effect that is equivalent to pure IP-PA1.

This wheat-fermented extract has received authorization for use in animal feeds by the Ministry of Agriculture, Forestry and Fisheries of Japan after safety testing in chickens and carp. Also, a water-soluble skin cream containing flour-fermented extract was examined for allergens, and it was confirmed that the allergens derived from wheat were under detection limits.

FUTURE PERSPECTIVES

IP-PA1 and/or the wheat-fermented extract could be an active ingredient of various health products, as it can be manufactured efficiently, safely, and economically.

During the 20th century, science was driven by reductionism, as represented by the genome project. However, there are still major issues to be resolved, including cancers, zoonotic infections, auto-immune diseases, metabolic syndromes, and others. This fact suggests that there may be a limit to the prevention and therapy of diseases based on reductionism. In the future, we need to develop applied technologies that are based on an understanding of the holisticity of organisms (a clarification of the unknown principles that organisms utilize for self regulation).

The macrophage network that we have proposed appears to be a super system that is the regulatory system that is innate in individual organisms. Therefore, substances that activate the macrophage network (such as LPS from *P. agglomerans* and other gram negative bacteria) would also be substances which quantitatively regulate the macrophage network and also qualitatively control the results. Such substances are prime candidates for use in establishing new,

fundamentally different methods (than conventional drugs or therapies) for prevention and improvement of various diseases.

ACKNOWLEDGMENTS

This research has been supported by an Open Research Center Project, a University-Industry Joint Research Project, and a High-Tech Research Center Project of the Japanese Ministry of Education, Culture, Sports, Science and Technology, and a Regional New Consortium R&D Project and an Industrial Cluster Project (Shikoku Techno Bridge Plan) of the Shikoku Bureau of Economy, Trade and Industry. We thank Sawa, D.V.M. (Livestock Research Institute) and Mr. Yuasa (Fisheries Research Institute) of Tokushima Agriculture, Forestry and Fisheries Technology Support Center, and Professor Takahashi of National Fisheries University for their helpful collaboration.

REFERENCES

1. **Burnet, F. M.:** Cellular immunology. Melbourne University Press and Cambridge University Press (1969).
2. **Burnet, F. M.:** The clonal selection theory of acquired immunity. Vanderbilt University Press, Nashiville (1959).
3. **Soma, G.-I. and Mizuno, D.-I.:** Further development of the therapy with lipopolysaccharides of a small molecular size on various intractable diseases, p. 203–220. *In* Fiers, W. and Buurman, W. A. (ed.), Tumor necrosis factor: molecular and cellular biology and clinical relevance. Karger, Basel (1993).
4. **Tada, T.:** The immune system as a supersystem. Annu. Rev. Immunol., **15**, 1–13 (1997).
5. **Wiktor-Jedrzejczak, W. W., Ahmed, A., Szczylik, C., and Skelly, R. R.:** Hematological characterization of congenital osteopetrosis in op/op mouse. Possible mechanism for abnormal macrophage differentiation. J. Exp. Med., **156**, 1516–1527 (1982).
6. **Kodama, H., Nose, M., Niida, S., and Yamasaki, A.:** Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells. J. Exp. Med., **173**, 1291–1294 (1991).
7. **Pollard, J. W., Hunt, J. S., Wiktor-Jedrzejczak, W., and Stanley, E. R.:** A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Dev. Biol., **148**, 273–283 (1991).
8. **Michaelson, M. D., Bieri, P. L., Mehler, M. F., Xu, H., Arezzo, J. C., Pollard, J. W., and Kessler, J. A.:** CSF-1 deficiency in mice results in abnormal brain development. Development, **122**, 2661–2672 (1996).
9. **Burke, B. and Lewis, C. E.:** The macrophage. Oxford University Press, Oxford, New York (2002).
10. **Kohchi, C., Inagawa, H., Hino, M., Oda, M., Nakata, K., Yoshida, A., Hori, H., Terada, H., Makino, K., Takiguchi, K., and Soma, G.-I.:** Utilization of macrophages in anticancer therapy: the macrophage network theory. Anticancer Res., **24**, 3311–3320 (2004).
11. **Nakamoto, T., Yoshimura, H., Honda, T., Nakata, K., Taniguchi, Y., Yoshida, A., Uenobe, M., Yoshioka, N., Yamaguchi, T., Inagawa, H., Kohchi, C., Nishizawa, T., and Soma, G-I.:** Treatments for activating macrophages that reduces surgical stress and post operative mortalities from bacterial infections and tumor metastases. In Vivo (2007). (in press)
12. **Meltzer, M. S.:** Macrophage activation for tumor cytotoxicity: characterization of priming and trigger signals during lymphokine activation. J. Immunol., **127**, 179–183 (1981).
13. **Lambert, L. E. and Paulnock, D. M.:** Modulation of macrophage function by gamma-irradiation. Acquisition of the primed cell intermediate stage of the macrophage tumoricidal activation pathway. J. Immunol., **139**, 2834–2841 (1987).
14. **Suzuki, M., Hisamatsu, T., and Podolsky, D. K.:** Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex. Infect. Immun., **71**, 3503–3511 (2003).
15. **Wolfs, T. G., Buurman, W. A., van Schadewijk, A., de Vries, B., Daemen, M. A., Hiemstra, P. S., and van 't Veer, C.:** *In vivo* expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. J. Immunol., **168**, 1286–1293 (2002).
16. **Tanabe, Y., Kitahara-Tanabe, N., Mizuno, D., and Soma, G.-I.:** Enhanced production of tumour necrosis factor alpha (TNF-alpha) by its precursor on the cell surface of primed THP-1 cells. Cytokine, **6**, 337–348 (1994).
17. **Nishizawa, T., Inagawa, H., Oshima, H., Okutomi, T., Tsukioka, D., Iguchi, M., Soma, G.-I., and Mizuno, D.:** Homeostasis as regulated by activated macrophage. I. Lipopolysaccharide (LPS) from wheat flour: isolation, purification and some biological activities. Chem. Pharm. Bull. (Tokyo), **40**, 479–483 (1992).
18. **Inagawa, H., Nishizawa, T., Tsukioka, D., Suda, T., Chiba, Y., Okutomi, T., Morikawa, A., Soma, G.-I., and Mizuno, D.:** Homeostasis as regulated by activated macrophage. II. LPS of plant origin other than wheat flour and their concomitant bacteria. Chem. Pharm. Bull. (Tokyo), **40**, 994–997 (1992).
19. **Kariluoto, S., Aittamaa, M., Korhola, M., Salovaara, H., Vahteristo, L., and Piironen, V.:** Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. Int. J. Food Microbiol., **106**, 137–143 (2006).
20. **Lindh, E., Kjaeldgaard, P., Frederiksen, W., and Ursing, J.:** Phenotypical properties of *Enterobacter agglomerans* (*Pantoea agglomerans*) from human, animal and plant sources. Apmis, **99**, 347–352 (1991).
21. **Vanneste, J. L., Yu, J., and Beer, S. V.:** Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. J. Bacteriol., **174**, 2785–2796 (1992).
22. **Nunes, C., Usall, J., Teixido, N., Fons, E., and Vinas, I.:** Post-harvest biological control by *Pantoea agglomerans* (CPA-2) on Golden Delicious apples. J. Appl. Microbiol., **92**, 247–255 (2002).
23. **Giddens, S. R., Houliston, G. J., and Mahanty, H. K.:** The influence of antibiotic production and pre-emptive colonization on the population dynamics of *Pantoea agglomerans* (*Erwinia herbicola*) Eh1087 and *Erwinia amylovora* in planta. Environ. Microbiol., **5**, 1016–1021 (2003).
24. **Raetz, C. R.:** Biochemistry of endotoxins. Annu. Rev. Biochem., **59**, 129–170 (1990).
25. **Bergey, D. H., Krieg, N. R., and Holt, J. G.:** Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore (1984).
26. **Okutomi, T., Nishizawa, T., Inagawa, H., Takano, T., Morikawa, A., Soma, G.-I., and Mizuno, D.:** Homeostasis as regulated by activated macrophage. VII. Suppression of serum cholesterol level by LPSw (a lipopolysaccharide from wheat flour) in WHHL (Watanabe heritable hyperlipidemic) rabbit. Chem. Pharm. Bull. (Tokyo), **40**, 1268–1270 (1992).
27. **Inagawa, H., Saitoh, F., Iguchi, M., Nishizawa, T., Okutomi, T., Morikawa, A., Soma, G.-I., and Mizuno, D.:** Homeostasis as regulated by activated macrophage. III. Protective effect of LPSw (lipopolysaccharide (LPS) of wheat flour) on gastric ulcer in mice as compared with those of other LPS from various sources. Chem. Pharm. Bull. (Tokyo), **40**, 998–1000 (1992).

28. **Suzuki, Y., Kobayashi, A., Nishizawa, T., Inagawa, H., Morikawa, A., Soma, G.-I., and Mizuno, D.:** Homeostasis as regulated by activated macrophage. VI. Protective effect of LPSw (a lipopolysaccharide from wheat flour) against acute infection by *Toxoplasma gondii* in mice. Chem. Pharm. Bull. (Tokyo), **40**, 1266–1267 (1992).
29. **Iguchi, M., Inagawa, H., Nishizawa, T., Okutomi, T., Morikawa, A., Soma, G. I., and Mizuno, D.:** Homeostasis as regulated by activated macrophage. V. Suppression of diabetes mellitus in non-obese diabetic mice by LPSw (a lipopolysaccharide from wheat flour). Chem. Pharm. Bull. (Tokyo), **40**, 1004–1006 (1992).
30. **Del Prete, G.:** The concept of type-1 and type-2 helper T cells and their cytokines in humans. Int. Rev. Immunol., **16**, 427–455 (1998).
31. **Mosmann, T. R. and Coffman, R. L.:** TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol., **7**, 145–173 (1989).
32. **Grewe, M., Bruijnzeel-Koomen, C. A., Schopf, E., Thepen, T., Langeveld-Wildschut, A. G., Ruzicka, T., and Krutmann, J.:** A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. Immunol. Today, **19**, 359–361 (1998).
33. **Desmedt, M., Rottiers, P., Dooms, H., Fiers, W., and Grooten, J.:** Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. J. Immunol., **160**, 5300–5308 (1998).
34. **Inagawa, H., Nishizawa, T., Noguchi, K., Minamimura, M., Takagi, K., Goto, S., Soma, G.-I., and Mizuno, D.:** Anti-tumor effect of lipopolysaccharide by intradermal administration as a novel drug delivery system. Anticancer Res., **17**, 2153–2158 (1997).
35. **Goto, S., Sakai, S., Kera, J., Suma, Y., Soma, G. I., and Takeuchi, S.:** Intradermal administration of lipopolysaccharide in treatment of human cancer. Cancer Immunol. Immunother., **42**, 255–261 (1996).
36. **Pollard, J. W.:** Tumour-educated macrophages promote tumour progression and metastasis. Nat. Rev. Cancer, **4**, 71–78 (2004).
37. **Ohno, S., Inagawa, H., Dhar, D. K., Fujii, T., Ueda, S., Tachibana, M., Ohno, Y., Suzuki, N., Inoue, M., Soma, G.-I., and Nagasue, N.:** Role of tumor-associated macrophages (TAM) in advanced gastric carcinoma: the impact on FasL-mediated counterattack. Anticancer Res., **25**, 463–470 (2005).
38. **Romani, L.:** Immunity to fungal infections. Nat. Rev. Immunol., **4**, 1–23 (2004).
39. **Takeda, K. and Akira, S.:** Toll-like receptors in innate immunity. Int. Immunol., **17**, 1–14 (2005).
40. **Akira, S. and Takeda, K.:** Toll-like receptor signalling. Nat. Rev. Immunol., **4**, 499–511 (2004).
41. **Saitoh, S., Akashi, S., Yamada, T., Tanimura, N., Kobayashi, M., Konno, K., Matsumoto, F., Fukase, K., Kusumoto, S., Nagai, Y., Kusumoto, Y., Kosugi, A., and Miyake, K.:** Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int. Immunol., **16**, 961–969 (2004).
42. **Itami, T., Ohno, N., Suzuki, N., Doi, K., Kondo, M., Takahashi, Y., Maeda, M., Soma, G.-I., Inagawa, H., Honda, T., and Yokomizo, Y.:** Shrimp hemocytes can produce superoxide and nitric oxide. ITE Lett., **2**, 70–74 (2001).
43. **Tsukioka, D., Nishizawa, T., Miyase, T., Achiwa, K., Suda, T., Soma, G.-I., and Mizuno, D.:** Structural characterization of lipid A obtained from *Pantoea agglomerans* lipopolysaccharide. FEMS Microbiol. Lett., **149**, 239–244 (1997).
44. **Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., and Beutler, B.:** CD14 is required for MyD88-independent LPS signaling. Nat. Immunol., **6**, 565–570 (2005).
45. **Suzuki, J., Nishizawa, T., Inagawa, H., Okutomi, T., Morikawa, A., Soma, G.-I., and Mizuno, D.:** Homeostasis as regulated by activated macrophage. IX. Enhancement effect of LPSw (a lipopolysaccharide from wheat flour) on hen egg-laying and breaking strength of eggshell. Chem. Pharm. Bull. (Tokyo), **40**, 1274–1276 (1992).
46. **Takahashi, Y., Kondo, M., Itami, T., Honda, T., Inagawa, H., Nishizawa, T., Soma, G.-I., and Yokomizo, Y.:** Enhancement of disease resistance against *penaeid acute viraemia* and induction of virus-inactivating activity in haemolymph of kuruma shrimp, *Penaeus japonicus*, by oral administration of *Pantoea agglomerans* lipopolysaccharide (LPS). Fish Shellfish Immunol., **10**, 555–558 (2000).
47. **Cohen, J.:** The immunopathogenesis of sepsis. Nature, **420**, 885–891 (2002).
48. **Braun-Fahrlander, C., Riedler, J., Herz, U., Eder, W., Waser, M., Grize, L., Maisch, S., Carr, D., Gerlach, F., Bufe, A., Lauener, R. P., Schierl, R., Renz, H., Nowak, D., and von Mutius, E.:** Environmental exposure to endotoxin and its relation to asthma in school-age children. N. Engl. J. Med., **347**, 869–877 (2002).
49. **Lauener, R. P., Birchler, T., Adamski, J., Braun-Fahrlander, C., Bufe, A., Herz, U., von Mutius, E., Nowak, D., Riedler, J., Waser, M., and Sennhauser, F. H.:** Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. Lancet, **360**, 465–466 (2002).
50. **Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B.:** Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. Science, **282**, 2085–2088 (1998).
51. **Mandelboim, O., Bar-Haim, E., Vadai, E., Fridkin, M., and Eisenbach, L.:** Identification of shared tumor-associated antigen peptides between two spontaneous lung carcinomas. J. Immunol., **159**, 6030–6036 (1997).
52. **Hoffman, D. M., Gitlitz, B. J., Belldegrun, A., and Figlin, R. A.:** Adoptive cellular therapy. Semin. Oncol., **27**, 221–233 (2000).
53. **Haupt, K., Roggendorf, M., and Mann, K.:** The potential of DNA vaccination against tumor-associated antigens for antitumor therapy. Exp. Biol. Med. (Maywood), **227**, 227–237 (2002).
54. **Conrad, C. and Nestle, F. O.:** Dendritic cell-based cancer therapy. Curr. Opin. Mol. Ther., **5**, 405–412 (2003).
55. **Dodd, M. J.:** BCG in cancer therapy: theoretical bases of immunotherapy. Am. J. Nurs., **79**, 310–314 (1979).
56. **Sullivan, B. P.:** BCG in cancer therapy: patient responses to BCG therapy for malignant melanoma. Am. J. Nurs., **79**, 320–324 (1979).
57. **Aoyagi, T., Ishizuka, M., Takeuchi, T., and Umezawa, H.:** Enzyme inhibitors in relation to cancer therapy. Jpn. J. Antibiot., **30(suppl.)**, 121–132 (1977).
58. **Talmadge, J. E. and Herberman, R. B.:** The preclinical screening laboratory: evaluation of immunomodulatory and therapeutic properties of biological response modifiers. Cancer Treat Rep., **70**, 171–182 (1986).
59. **Ogawa, Y., Goodman, G. B., Chaplin, D. J., Grulkey, W., and Lam, G. K.:** Combination therapy of pions and SPG (*Sonifilan, Schizophyllan*), a biological response modifier for mouse tumor systems. Int. J. Radiat. Oncol. Biol. Phys., **18**, 1415–1420 (1990).
60. **Jahrsdorfer, B. and Weiner, G. J.:** CpG oligodeoxynucleotides for immune stimulation in cancer immunotherapy. Curr. Opin. Invest. Drugs, **4**, 686–690 (2003).

61. **Klinman, D. M.:** Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol., **4**, 249–258 (2004).
62. **Krieg, A. M.:** Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. Curr. Oncol. Rep., **6**, 88–95 (2004).
63. **Hussain, I. and Kline, J. N.:** CpG oligodeoxynucleotides: a novel therapeutic approach for atopic disorders. Curr. Drug Targets Inflamm. Allergy, **2**, 199–205 (2003).
64. **Hengge, U. R. and Ruzicka, T.:** Topical immunomodulation in dermatology: potential of toll-like receptor agonists. Dermatol. Surg., **30**, 1101–1112 (2004).
65. **Schon, M. P., Wienrich, B. G., Drewniok, C., Bong, A. B., Eberle, J., Geilen, C. C., Gollnick, H., and Schon, M.:** Death receptor-independent apoptosis in malignant melanoma induced by the small-molecule immune response modifier imiquimod. J. Invest. Dermatol., **122**, 1266–1276 (2004).
66. **Starnes, C. O.:** Coley's toxins in perspective. Nature, **357**, 11–12 (1992).