Comparative Immunological Analysis of Innate Immunity Activation after Oral Administration of Wheat Fermented Extract to Teleost Fish

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Abstract. *Background: Intradermal or oral administration of lipopolysaccharide derived from Pantoea agglomerans (IP-PA1) has shown prophylactic and antitumor effects without serious side-effects. While it is known that tumor necrosis factor (TNF)-α produced by activated macrophages plays an important role in the expression mechanism following intradermal administration, details of the mechanism after oral administration remain unclear. In this study, the activation of innate immunity using fish as an animal model was investigated. In fish, the innate immunity system is dominant over acquired immunity. Materials and Methods: Carp (Cyprinus carpio L) were fed IP-PA1 for 7 days. Total RNA was extracted from the head kidney (a major immune organ of teleost fish), and interleukin (IL) -1β, IL-6, IL-8, IL-10, IL-12, TNF-α and transforming forming growth factor (TGF)-β mRNAs were quantified by one-step real-time PCR. Phagocytic and bactericidal activity of head kidney leukocytes were estimated using zymosan and Aeromonas hydrophila (a pathogenic bacteria), respectively. Serum lysozyme activity was assayed with Remazol brilliant Blue stained Micrococcus lysodeikticus. Results: Oral administration of IP-PA1 for 7 days augmented the quantity of mRNA expression of IL-1β, IL-8, and TNF-α mRNA and reduced the expression level of IL-6 mRNA in the head kidney. Phagocytic and bactericidal activity of head kidney leukocytes were significantly enhanced. Moreover, serum lysozyme activities were significantly augmented. Conclusion: The results suggest that oral administration of IP-PA1 induced activation of M1 type macrophages in the immune organ of fish, and this enhanced the function of pathogen elimination. Since the functions of macrophages are highly preserved in comparative immunology, there is a high probability that the preventative or curative effect on various diseases that have been observed in mammals is also related to the activation of macrophages to the M1 type.*

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Macrophages are essential cells that both play a central role in innate immunity and function in maintaining homeostasis (such as in the elimination of foreign substances). Macrophages phagocytose unnecessary cells such as apoptotic cells and regulate the immune balance. It is known that activation of macrophages could prevent certain diseases and provide therapeutic effects. Lipopolysaccharide (LPS), a cellular-wall component of Gram-negative bacteria, is known to activate macrophages at the lowest dose of any known immuno-potentiation (1). However, it has been little used clinically because it is known that intravenous administration of LPS induces systemic inflammation and causes endotoxin shock.

However, we have found that the intradermal administration of LPS derived from *Pantoea agglomerans* (IP-PA1) did not cause serious side-effects and also provided an antitumor effect in mouse experimented models and in clinical studies (2, 3). An analysis of the

mechanism indicated that intradermal administration of IP-PA1 caused a sustained release of IP-PA1 from the skin site into the blood and of tumor necrosis factor (TNF)-α from the accumulated inflammatory cells at the site of administration.

Furthermore, we found that oral administration of IP-PA1 improved hyperlipidemia, atopic dermatitis and allergies (4, 5). In addition, it had an analgesic effect and a preventative effect on diabetes, and on bacterial and viral infections (5, 6). However, the molecular and cellular mechanism after oral administration of immunopotentiating substances such as β-glucan, peptidoglycan and LPS have been little studied. One reason is that in mammals it is difficult to analyze the mechanism after the oral administration of immunopotentiators. To solve this problem, it seemed useful to study lower vertebrate animal species in which innate immunity is dominant.

Fish are lower vertebrates that possess an immune system similar to mammals except that it has been reported that innate immunity is dominant over acquired immunity (7). Fish macrophages are believed to play the central role in the innate immune system (8). Because they have various receptors for the recognition of foreign substances, fish macrophages are believed to have the same functions as in mammals (9). Many cytokines control the activation of immune and phagocytic cells which then produce reactive oxygen and nitric oxide (8, 10-12). For these reasons, it was believed that fish could be used as an animal model for an analysis of the effect of immunopotentiators on the innate immune system. We found that there was a clear preventative effect on infection after oral administration of IP-PA1 in fish such as yellowtail, carp and ayu (5). Thus, we believed that these fish species would be useful model animals to analyze the innate immune system.

In order to study the activation of macrophages following oral administration of IP-PA1, the gene expression of M1-type cytokines (which enhance the elimination of foreign substances by macrophages) and M2-type cytokines (which enhance wound healing and suppress inflammation) was estimated using cells from the main immune organ of fish, the head kidney.

Materials and Methods

Animals and experimental design I. C3H/He male mice were purchased from Japan SLC Inc. (Shizuoka, Japan). They were 6, 8-weeks of age and weighed 18-24 g at the start of the experiments. Prior to performing the experiments, the experiments were approved by the Animal Ethics Committee of the Biotechnology Research Center, Teikyo University. Mice were given water containing LPS derived from *Escherichia coli* (LPSe) or LPS derived from *Pantoea agglomerans* (IP-PA1) (10 μg/ml) *ad libitum* for 3 days. Then the mice were orally administered LPSe or IP-PA1 (100 μg/mouse) by gavage.

Detailed procedures for determining TNF-α production are described elsewhere (13-16). Briefly, mice were intravenously administered 1 Klinische Einheit (KE) of OK-432 as a triggering agent 3 h after oral administration of LPSe or IP-PA1. Blood was taken 2 h after OK-432 administration. Serum was collected from blood and kept at –80˚C until used. TNF activity was assayed by a method described by Ruff and Gillford (17).

Animals and experimental design II. Prior to use in experiments, common carp (*Cyprinus carpio* L) were purchased and acclimatized to laboratory conditions. They were fed Carp feed (Hayashikane Sangyo Co., Yamaguchi, Japan). The experiments were carried out in aquariums with a 60-l capacity that contained tap water at 23±2˚C. There was a 12 h dark/12 h light photoperiod. Each aquarium was stocked with 5 fish (average weight 20 g). The water was changed on alternate days. Fish in each aquarium received one of two different diets for 1 week: fish in two aquaria were fed with a commercial diet (HayashikaneSangyo co.) (control group), while the fish of the other two aquaria were fed with the same diet supplemented with substances from water extract of wheat flour (IP-PA1, final concentration: 10 μg/ml).

Expression analysis. Total RNA was extracted from the head kidney using a Fast pure RNA kit (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. Real-time quantitative PCR with primer combinations (Table I) was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Primers for β-actin (Table I) were used as an internal control for real-time PCR. All qRT-PCR reactions were run in duplicate, and the average Ct value was calculated. Amplification and detection were performed in an Opticon3 monitor (Bio-Rad, Tokyo, Japan). PCR conditions were: 1 cycle of 50˚C for 5 min and 95˚C for 10 min, 40 cycles of 95˚C for 20 s, 60˚C for 20 s and 72˚C for 20 s.

Isolation of head kidney cells. The head kidney phagocytic cells of the carp were isolated according to the modified method described by Braun-Nesje *et al.* (18). Head kidneys were removed and pushed through a nylon mesh with RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan) containing 50 μg/ml ampicillin, 40 μg/ml kanamycin, 0.2% heparin (Wako, Osaka, Japan), and 10% carp serum (CS). The head kidney phagocytic cells were isolated by centrifugation on a 40%/50%/60% discontinuous Percoll density gradient (Sigma-Aldrich). The cells were resuspended in RPMI-1640 supplemented with 10% CS.

Phagocytosis assay. A microscopic method was used for the phagocytosis assay as described elsewhere (19). Briefly, head kidney phagocytic cells were collected with a discontinuous Percoll density gradient and prepared to 1×10^6 cells/well in 8-well chamber slides (Iwaki, Tokyo, Japan). The slides were then flooded with 0.1 ml of zymosan (1×10^8 cells/ml) (Sigma-Aldrich). Phagocytosis was allowed to proceed for 1 h before excess zymosan was washed off with RPMI-1640, and the slides were air-dried. After formalin fixation, the cells were stained sequentially with May-Grünwald (Sigma-Aldrich) and Giemsa stains (Merck, Darmstadt, Germany).

Bacterial killing assay. This was performed according to Chen and Ainsworth with slight modification (20). A bacterial inoculum of *Aeromonas hydrophila* of approximately 1×10^8 cfu/ml was used as

Table I. *Primers used for gene expression.*

Gene	Primer sequence (5'→3')	Size (bp)	Accession number
β-Actin	F: CTATGTGGCTCTTGACTTCG R: CCGTCAGGCAGCTCATAGCT	85	M24113
IL-1β	F: CTGGAGCAATGCAATACAAA R: AGGTAGAGGTTGCTGTTGGAA	206	AJ245635
IL-6	F: GATTGGTACAACGAAGAAGA R: GCATGACCCATATATGACCCA	256	AY102633
IL-8	F: GTCTTAGAGGACTGGGTGTA R: ACAGTGTGAGCTTGGAGGGA	160	DQ453125
IL-10	F: GCTGTCACGTCATGAACGAG R: CCCGCTTGAGATCCTGAAATAT	132	AB110780
IL-12	F: GAGCGCATCAACCTGACCAT R: AGGATCGTGGATATGTGACCTCTAC	151	AJ621425
TGF-β	F: ATCCTGTGGAGGAGGAATAC R: CTGGAAACGTTGTGATGAGC	174	AF056942
TNF-α	F: TGTGTGGTGTCCTGCTGG R: TGGAAAGACACCTGGCTGTA	169	AJ311800

a stock. From this, 0.1 ml was taken and mixed with 0.1 ml of head kidney phagocytic cell suspension (1×10^7 cells/ml) and incubated at 23˚C for 2 h with occasional shaking. After 2 h, 10 μl of the bacteria-phagocyte mixture was diluted with 9.99 ml of sterile distilled nutrient medium (Nissui, Tokyo, Japan) to release the living bacteria from the phagocytes. This was serially diluted, plated on nutrient agar plates, incubated at 37˚C for 24 h, and the number of colonies was counted.

Lysozyme assay. Lysozyme activity was measured with the colorimetric method described by Ito *et al*. (21). Briefly, a solution of 200 mg Remazol brilliant blue (RBB-R) (Sigma-Aldrich) in 20 ml of distilled water was added to a suspension of 300 mg of *Micrococcus lysodeikicus* in 20 ml of distilled water at 50˚C with stirring. During the following 30 min, 4 g of sodium sulfate were added to the mixture in several portions. A solution of 200 mg of trisodium phosphate in 2 ml of distilled water was then added, and the mixture was stirred for an additional 30 min at 50˚C. The reaction mixture was centrifuged (2,600 rpm, 10 min) and the supernatant was again discarded. The pellet of labeled cells was suspended in 20 ml of 0.05 M KH_2PO_4-NaOH buffer (0.05 M phosphate buffer, pH 7.0) with a vortex mixer, centrifuged, and the supernatant was discarded. In the same manner, the cells were washed with the same buffer repeatedly until the supernatant became colorless. Finally, the cells were resuspended in 10 ml of the same buffer to provide RBB-R labeled *M. lysodeikticus* (blue *M. lysodeikticus* solution), which was stored in a refrigerator until use. The blue *M. lysodeikticus* solution was divided into 190 μl portions in 1.5 ml-microtubes. After preincubation at 37˚C, 10 μl of supernatant from head kidney homogenization ware added to each tube and the mixture was briefly mixed with a vortex mixer, and then incubated at 37˚C for 1 h. After incubation, the mixture was centrifuged (15,000 rpm, 5 min), and the absorbance peak (592 nm) of RBB-R was measured. Hen egg-white lysozyme (Sigma-Aldrich) was used as a comparative control in this study.

Statistical analysis. Data were analyzed with Student's *t*-test. Differences between treatment and control groups were considered statistically significant at $p<0.01$ and $p<0.05$.

Results

Priming effect of IP-PA1 after oral administration to mice. To analyze the macrophage-activating mechanism after oral administration of IP-PA1, we initially investigated *TNF-α* production. This is because after the intradermal administration of IP-PA1, the TNF-α that is produced by the macrophages has an important role in exerting the biological effect. Mice were orally administered water that contained IP-PA1 (10 μg/ml) for 3 days *ad libitum*, and then serum was collected. The serum TNF-α activity was estimated by L929 cell cytotoxicity. The result was that the TNF-α activity did not show a difference when compared to the control group (data not shown). Several types of combination protocols were followed for the oral administration of IP-PA1. One protocol succeeded in inducing a primed effect. Mice were orally administered IP-PA1 in water containing IP-PA1 (10 μg/ml) for 7 days *ad libitum* and then 100 μg of IP-PA1 by gavage, then intravenous injection of 1KE of OK432. Administration of IP-PA1 caused the augmentation of TNF-α production by 3.7 times when compared to the control group. LPSe induced 2.8 times more TNF-α production than the control group, but the difference was not significant (Table II).

Augmentation of mRNA expression of M1 type cytokines after oral administration of IP-PA1 in fish. With the immune system of mammals, it may not be possible to easily interpret the biological effects caused by the oral administration of IP-PA1. For this reason, we used teleost fish as an animal model for this analysis, because they are well protected against infectious diseases after oral administration of immunostimulators (5, 22-26). The head kidney is a major immune organ of fish and contains large amounts of

Table II. *Priming effect of LPSs via oral route. Mice were given water containing water (control), LPSe or IP-PA1 (10 μg/ml) ad libitum for 3 days. Then the mice were orally administrated water, LPSe or IP-PA1 (100 μg/mouse) by gavage. Mice were intravenously administered 1 KE of OK-432 as a triggering agent 3 h after oral administration of LPSe or IP-PA1. Blood was taken 2 h after OK-432 administration and then TNF activity in serum was assayed.*

	Control	LPSe	IP-PA1
Log TNF activity	0.60±0.29	1.05±0.32	1.17±0.14*
Relative activity	1.0	2.8	3.7

The data represent means±SD (n=3). Significant differences (*$p<0.05$) were obtained between control and IP-PA1.

Table III. *Gene expression in the head kidney following oral administration of IP-PA1.*

Gene	Treatment	Gene expression in the head kidney	
		At 7 days	±SD
IL-1β	IP-PA1	0.01930*	0.01524
	Control	0.00025	0.00169
IL-6	IP-PA1	0.00024*	0.00043
	Control	0.00706	0.00440
IL-8	IP-PA1	0.00373*	0.00992
	Control	0.00085	0.00039
IL-10	IP-PA1	0.00606	0.01240
	Control	0.00402	0.00901
IL-12	IP-PA1	0.00463	0.00520
	Control	0.00252	0.00697
TNF-α	IP-PA1	0.13898*	0.10984
	Control	0.00386	0.00410
TGF-β	IP-PA1	0.00934	0.01810
	Control	0.00329	0.00302

Fold increase of target gene relative to β-actin. *Significant up- or down-regulation relative to control at $p<0.01$.

phagocytes. It has often been used to analyze leukocyte activation. To study the macrophage-activating mechanism after oral administration of IP-PA1, we quantified the mRNA of four M1 type cytokine genes (*IL-1β, TNF-α, IL-8, IL-12*) and three M2 type (*IL-6, IL-10, TGF-β*). As shown in Table III, the levels of mRNA expression of *IL-1β, TNF-α, IL-8* in the head kidney of the carp after the oral administration of IP-PA1 (10 μg/kg/day) for 7 days were 77-, 36-, and 4.4-fold higher, respectively, than in the control group. Thus significant augmentation in the expressed amount was observed. The expression of *IL-12* mRNA was shown to be 1.8-fold higher than that in the control group, but the difference was not significant. The levels of mRNA expression of M2 type cytokines (*IL-6, IL-10* and *TGF-β*) were 0.03-, 1.5-, and 2.8-fold higher than in the control group, but only the decrease in the expression level of *IL-6* was significant (Table III).

Augmentation of the foreign substance elimination function after oral administration of IP-PA1 in fish. Augmentation of mRNA expression of M1 type cytokines was observed in the head kidney of carp after oral administration of IP-PA1. This result suggested that M1 type macrophages were significantly activated and induced in their function of eliminating foreign substances, which is the characteristic action when the M1 type is activated. To analyze this function, bactericidal phagocytic activity and the production of antimicrobial peptides from macrophages after oral administration of IP-PA1 were assayed. After administering IP-PA1 for 7 days, the bactericidal activity of the cells of the head kidney against *A. hydrophila* was 49% in the control group and 65% in the treatment group, indicating a significant increase in bactericidal activity (Figure 1). After oral administration of IP-PA1 for 7 days, phagocytic activity in response to zymosan was 34% in the control group and 43% in the IP-PA1 treatment group, indicating significant elevation when compared to the control group (Figure 2). Lysozyme activity of carp serum after oral administration of IP-PA1 for 7 days was 728 units/ml in the control group and 1282 units/ml in the IP-PA1 administrated group, indicating a significant elevation as compared to the control group (Figure 3).

Discussion

In this study to analyze the mechanism of the effect of oral administration of IP-PA1, we initially measured macrophage activation after oral administration of IP-PA1 in mice. As shown in Table II, in mice the priming effect after oral administration (*ad libitum*) of 10 μg/ml of IP-PA1 followed by forced oral administration of 100 μg of IP-PA1 was only 3.7 times higher than in the control. Because of this result, we decided that it would be difficult to analyze the mechanism of the biological effect after the oral administration of immunostimulators in higher vertebrate animals because they possess a complex immune system in which acquired immunity and innate immunity function mutually. This is why we believed it would be necessary to use an animal model of a lower vertebrate, a species in which innate immunity dominated acquired immunity, to analyze the expression mechanism after oral administration of an immunostimulator.

Fish are animals wherein innate immunity is dominant over acquired immunity, which is why fish appeared to be an suitable group of animals to use for analyzing the innate immunity mechanism. We had already determined that the oral administration of IP-PA1 showed a clear and reproducible preventative effect against viral and bacterial

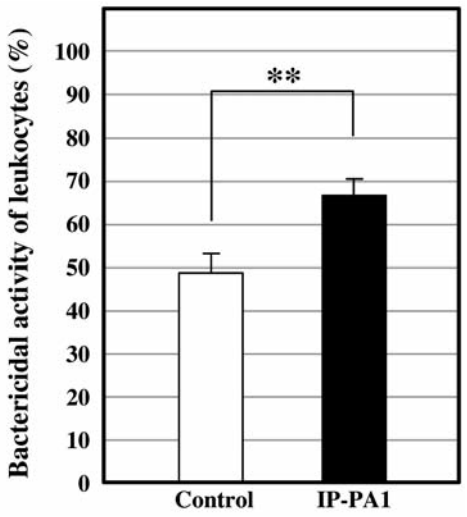

Figure 1. *Bactericidal activity of leukocytes in head kidney of carp fed diets containing IP-PA1 (10 μg/kg/day) for 7 days. Data are presented as the mean±S.D. of five fish. Statistical analysis was performed by Student's t-test (*p<0.01).*

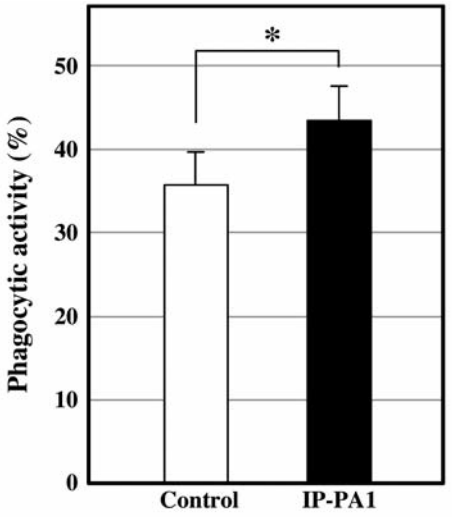

Figure 2. *Phagocytic activity of leukocytes in head kidney of carp fed diets containing IP-PA1 (10 μg/kg/day) for 7 days. Data are presented as the mean±S.D. of five fish. Statistical analysis was performed by Student's t-test (*p<0.05).*

infections in aquatic animals (5). From these results, we believed that fish would be a good experimental model for an analysis of the activation mechanism of innate immunity after oral administration of IP-PA1. After oral administration of IP-PA1, there was an elevation in the mRNA expression levels of *IL-1β, TNF-α* and *IL-8*, and a decrease in the *IL-6* mRNA level in the head kidney of the carp. Recently, macrophages have been classified roughly into 2 groups: the M1 macrophages (classical activation) and the M2 macrophages (alternative activation) (27, 28). The M1 macrophages augment gene expression of *TNF-α, IL-1β,* and *IL-12*. These induce a Th1 immune response which produces reactive oxygen species and nitric oxide, and is known to have a high capability for eliminating pathogens. Conversely, M2 macrophages augment gene expression of *IL-10* and/or *TGF-β*, which induces the Th2 immune responses and is known to possess curative functions such as wound healing. The significant augmentation in the mRNA expression level of *IL-1β, TNF-α and IL-8* after the oral administration of IP-PA1 suggested that activation of the macrophages in the head kidney is of the M1 type. It is of interest that a significant decrease in *IL-6* mRNA expression was observed after oral administration of IP-PA1. *IL-6* is known to be secreted by various cells such as T-cells, B-cells, hepatocytes, hematopoietic progenitor cells, and neurocytes. Depending on the target cell, it induces cell proliferation or differentiation, suppresses gene expression, and suppresses the production of *IL-1β* or *TNF-α* by monocytes (29). These results strongly suggested that macrophages were activated to the M1 type in the head kidney after oral administration of

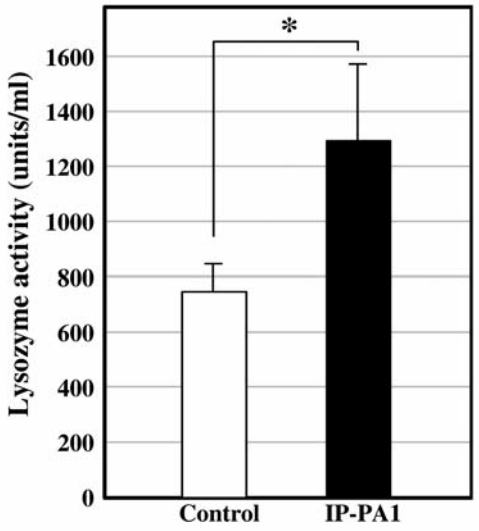

Figure 3. *Lysozyme activity in serum of carp fed diets containing IP-PA1 (10 μg/kg/day) for 7 days. Data are presented as the mean±S.D. of five fish. Statistical analysis was performed by Student's t-test (*p<0.01).*

IP-PA1. This in turn suggests that there would be an augmentation in the functions that eliminate foreign substances, which is characteristic when M1 type macrophages are activated.

To evaluate the function of foreign substance elimination by macrophages after oral administration of IP-PA1, we assayed the bactericidal activity. This was measured by phagocytic

activity in response to zymosan and by the production of antimicrobial peptides. As the results showed a significant augmentation in all assays, it is believed that macrophages secrete cytokines that transform the macrophages to the M1 type after oral administration of IP-PA1. This then augments the foreign substance elimination function. However, it is still not clear whether or not the orally administered IP-PA1 that was absorbed in the intestine reached the head kidney and activated macrophages directly. We plan to clarify this point with further studies that show the distribution of IP-PA1 in the body after oral administration of IP-PA1.

Based on the information that was clarified by this study, it appears that the preventative or therapeutic effects on various diseases after oral administration of IP-PA1 that have been observed in mammals may have been induced by the activation of macrophages to the M1 type. This can be assumed because in comparative immunology, the functions of macrophages are highly preserved. Recently, many researchers reported that macrophages present in malignant tumor tissue (tumor-associated macrophage, TAM) had characteristics similar to M2 macrophages and were known to promote tumor development and metastasis (30, 31). Hagemann *et al*. reported that an antitumor effect was observed in tumor tissue after changing the activated state to the M1 type through re-education of TAMs (32). These reports suggest that changing the TAM phenotype to M1 may be a productive method for establishing new therapies for malignant tumors. The present study suggests that the oral administration of IP-PA1 has potential in preventing tumor development.

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