カードラン(β-1,3-グルカン)経口投与のヒラメ非特異的免疫機構に与える効果と投与期間の影響について

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Oral Administration of Curdlan (β-1,3-Glucan) Potentiates the Non-specific Immune System of Japanese Flounder, Paralichthys olivaceus, and Its Effect Is Influenced by the Feeding Period

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Abstract: Activation of the non-specific immune system by oral administration of curdlan was studied in Japanese flounder, Paralichthys olivaceus. In the short period feeding test, the phagocytic activities of peritoneal exuded leucocytes, circulating leucocytes and head kidney leucocytes were significantly increased until 2 weeks with everyday feeding of curdlan, and the \( O_2^- \) generation by peritoneal exuded leucocytes in fish fed curdlan everyday for 3 weeks was enhanced from 10 to 20% in the presence (in vitro) of low concentrations of curdlan (＜10 nm). When fish were fed every 2 days for 4 months (long period feeding test) their phagocytic activities increased but they were significantly lower than those in the short period feeding test, whereas the \( O_2^- \) generation by peritoneal exuded leucocytes did not increase. Thus, the effect of curdlan was found to be influenced by the feeding period and the feeding mode.

Key words: Japanese flounder; Curdlan; Phagocytosis; Superoxide

It is known that some β-glucans can increase the host systematic immune response, such as increasing the anti-tumor activity and non-specific defense against pathogenic bacteria, fungi and viruses in mammals\(^1,2\). Such immunostimulatory activities of β-glucans have been demonstrated not only in mammals but also in fish: Carp, \( Cyprinus carpio \)\(^3\), and yellowtail, \( Seriola quinqueradiata \)\(^4\), injected with glucans intraperitoneally (i. p.) showed increased phagocytic activity and alternative complement pathway, and enhanced resistance against bacterial infection. Oral administration is much simpler than i. p. injection and can be applied to mass of fish. However, there have been few reports on the effect of glucan by oral administration on host defense. In this study, we investigated the effects of an orally fed bacterial β-1,3-glucan on the phagocytic activity and superoxide generation of leucocytes.

Materials and Methods

Fish and Diets
Japanese flounder, Paralichthys olivaceus, were purchased from Marusuisan Corporation in Hiroshima prefecture, Japan. The fish were acclimatized to our laboratory conditions before use. Curdlan (bacterial β-1,3-glucan)\(^5\) was donated by Takeda Chemical Industries, Ltd. The experimental diets, \( i. e. \), curdlan-containing diets (1% curdlan) and control diets, were prepared by Marubeni Ltd. The control diets contained 1% cellulose in place of curdlan. The experimental diets were fed at a rate of 1.1% body weight per day (\( i. e. \), 110 mg curdlan/kg body

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Feeding Schedules and Sampling Protocols

Fish (200 ± 9 g) were kept in 200 l tanks with continuous supplies of air (3.6 l/min) and seawater (3.0 l/min). The fish were divided into 3 groups with 15 fish per group. One group was used for a short period feeding test and the other two groups for a long period feeding test. In the short period feeding test, the fish were fed on the 1% curdlan-containing diet everyday for 4 weeks. Two or three samples of fish were taken from the tank on the first day of feeding (0 weeks) and every 1 week after feeding to investigate the effects of the experimental diets. The average rearing water temperature was 24.8°C.

In the long period feeding test, the fish in each group were fed on the 1% curdlan or the control diet every 2 days for 4 months, and three fish were taken from each group after 4 months from the start of feeding. The average rearing water temperature was 23.5°C.

Preparation of Leucocytes

After feeding, the fish were i.p. injected with 2% sodium caseinate (Acros Organics) in phosphate-buffered saline (PBS: 0.14 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.4) to obtain intraperitoneal casein-elicited leucocytes. The peritoneal exuded leucocytes were collected with a syringes at 15 h after injection and centrifuged. The precipitates were washed with PBS and then diluted in RPMI 1640 medium (Bio Whittaker). The resultant cells were peritoneal exuded leucocytes. Circulating leucocytes were isolated by Ficoll-Paque (Pharmacia Biotech) density gradient centrifugation: Blood obtained from the caudal vein was added to the same volume of PBS (containing heparin), and an aliquot (4 ml) of the resultant mixture was overlaid on Ficoll-Paque (3 ml); the overlaid sample was centrifuged at 450 x g for 30 min at 20°C; and the middle white band obtained was collected as the leucocyte fraction, which was subsequently washed with PBS and suspended in RPMI 1640 medium. For head kidney leucocytes, scissored head kidney was incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco Laboratories) for 1 h at room temperature. The suspension was filtered through a 0.5 x 0.5 mm stainless steel mesh. The filtrate was centrifuged with Ficoll-Paque reagent and then leucocytes were obtained in the same manner as in the case of blood samples.

Phagocytic Activity Assay

The phagocytic activities of the three kinds of leucocytes against fluorescent zymosan A (Saccharomyces cerevisiae) bioparticles (Molecular Probes Inc.) were determined as follows. RPMI 1640 medium (1 ml) containing leucocytes (peritoneal exuded leucocytes and circulating leucocytes, 5 x 10⁶ cells/ml; and head kidney leucocytes, 1 x 10⁶ cells/ml) was incubated with opsonized (with Japanese flounder serum) fluorescent zymosan A (20 μg/ml) for 1 h at room temperature, and then the leucocytes were isolated by Ficoll-Paque density gradient centrifugation as described above. The isolated leucocytes were then smeared on a slide, air-dried, and stained with Hemacolor (Merck). Phagocytic activity was determined by counting 600 cells under a fluorescence microscope and calculated with the following formula: Phagocytic activity (%) = (number of phagocytes ingesting zymosan A) ÷ (total number of leucocytes counted) x 100.

Assaying of Superoxide Generation

The superoxide (O₂⁻) generated by peritoneal exuded leucocytes was measured by recording cytochrome c (cyt. c; Wako) reduction as the absorbance change at 550 nm in a total volume of 2 ml at 20°C using a spectrophotometer (Hitachi U-3210). The reaction medium used was 1.95 ml Krebs-Ringer phosphate buffer (KRP buffer: 154 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 10 mM Na₂HPO₄, pH 7.4) containing 50 μM cyt. c, with the addition of the prepared 20 μl peritoneal exuded leucocytes (10⁶ cells/ml), 20 μl glucose (10 mM), 5 μl CaCl₂ (250 μM), solubilized curdlan in 2 μl dimethyl sulfoxide (DMSO) or DMSO (control) and 1 μl phorbol-12-myristate 13-acetate (PMA; Sigma, 50 μM) in that order, as shown in Fig. 1. The changes in absorbance for
Fig. 1. Effect of superoxide dismutase on the cytochrome c reduction by superoxide. The superoxide generation on PMA stimulation was measured by spectrophotometry in the presence of cyt. c. 1 to 3 min were measured after the addition of PMA. The superoxide generating activity were expressed as the increase in absorbance per min, taking that of the control (without curdlan) as 100%. To confirm that the increase in OD$_{550}$ really originated from O$_2^-$ molecules, 35 unit/ml of superoxide dismutase (SOD) was added to the reaction mixture. If OD$_{550}$ is decreased by the addition of SOD, the cyt. c reduction should be caused by O$_2^-$ molecules.

Statistical Analysis
Significance of differences between means was determined using Student’s t-test.

Results

To determine the effect of orally administrated curdlan on the non-specific defense mechanism of Japanese flounder, we compared the phagocytic activities of leucocytes from curdlan-fed fish and control ones. The fish were fed curdlan for 1, 2, 3 and 4 weeks (everyday), and 4 months (every 2 days), from which three kinds of leucocytes were isolated, the phagocytic activities against opsonized zymosan A were assayed. On short period feeding, the phagocytic activities of peritoneal exuded leucocytes, circulating leucocytes, and head kidney leucocytes increased significantly (p < 0.05, 0.001) until 2 weeks, and then decreased to the initial levels (0 weeks) as shown in Fig. 2A (The head kidney sample at 4 weeks was lost.). Moreover, significant enhancement of the activity was observed in leucocytes from the fish fed curdlan for 4 months (p < 0.05, 0.01). However, the activities of peritoneal exuded leucocytes, circulating leucocytes, and head kidney leucocytes at 4 months were significantly (p < 0.01) lower than that at two weeks in the short period feeding test (Fig. 2B).

To determine whether curdlan feeding affects the O$_2^-$ generation by Japanese flounder peritoneal exuded leucocytes or not, the cells were
incubated in the presence of various concentrations (0-100 nM) of curdlan, and then stimulated with PMA. As the O$_2^-$ generation was suppressed on the addition of SOD, as shown in Fig. 1, the increase in OD$_{550}$ caused by addition of curdlan and PMA was found to originate really from O$_2^-$ molecules. The results as to O$_2^-$ generation are presented in Fig. 3. Without added curdlan in vitro, The O$_2^-$ generation of curdlan feeding groups for 1 week, 2 weeks, 3 weeks, 4 weeks, 4 months and control (0 weeks) were 5.7±4.3, 8.3±1.4, 6.4±1.5, 9.7±1.2, 9.1±3.0 and 9.2±3.0 μM/10^6 cells/min, respectively.

Thus, the O$_2^-$ generation of peritoneal exuded leucocytes caused by PMA stimulation was not different in the feeding period. In the short period feeding (everyday feeding) test, curdlan feeding for 1 and 2 weeks led to a small increase in O$_2^-$ generation in the presence of curdlan in vitro, but the feeding for 3 weeks strongly enhanced the O$_2^-$ generation from 10 to 20% by the addition of lower concentrations of curdlan (<10 nM), compare to the control (without curdlan). Especially, when 10 nM curdlan was added in vitro, the O$_2^-$ generation (122.8±7.4) was significantly (p<0.05) higher than the control (103.0±4.2). However higher concentrations (>10 nM) had no effect. In contrast, the fish fed on curdlan for 4 weeks did not exhibit enhanced O$_2^-$ generation from peritoneal exuded leucocytes at any concentrations of curdlan. As shown in Fig. 3(B), such enhancement was not observed in fish fed curdlan every 2 days for 4 months.

**Discussion**

Our research was focus on investigate an oral administration of curdlan alternate to the conventional i.p. injection to avoid the problem of bacterial infection in fish farming.

In our investigation, we examined the activation of non-specific immune system by continuous oral administration of curdlan to Japanese flounder. Activated host immune systems phagocytes infected microorganisms and produce antimicrobial substances such as active oxygens (O$_2^-$ and H$_2$O$_2$). The most widely used method for O$_2^-$ production by phagocytes is the activation of NADPH oxidase in the plasma membrane with PMA, N-formylmethionylleucylphenylalanine or lipopolysaccharide. Secombes et al. showed that rainbow trout, Oncorhynchus mykiss macrophages produced O$_2^-$ and reduced cyt. c when the cells were stimulated with PMA. We confirmed the reactions in Japanese flounder leucocytes; the cells stimulated with PMA released O$_2^-$, the molecules reduced cyt. c added, and the cyt. c reduction was inhibited by SOD (Fig. 1).

The phagocytic activities of the leucocytes from curdlan-fed fish began to increase in 1
week, became maximum in 2 weeks, and then decreased to the same level as 0 weeks (without curdlan). The phagocytic activity of peritoneal exuded leucocytes was significantly higher than those of other leucocytes. Rabgaoui et al.\textsuperscript{7} reported that casein-derived peptides enhanced the production of oxygen-free radical and 5-hydroxyicosatetraenoic acid (5-HETE). The reason for the high phagocytic activity of peritoneal exuded leucocytes would be that the peritoneal exuded leucocytes were activated on account of such priming effects because the cells were induced with casein. Another reason for that would be difference in the cell composition of peritoneal exuded leucocytes to others (i.e., peritoneal exuded leucocytes were rich in phagocytes).

The enhancement of the O$_2^-$ generation by phagocytes would be cause the resistance against fish diseases\textsuperscript{8}. Our results showed that the O$_2^-$ generation from peritoneal exuded leucocytes by stimulation with PMA was increased after 3 week feeding in the presence of lower concentrations of curdlan in vitro. The suppression of O$_2^-$ generation in higher concentration may be derived from physicochemical property or membrane interaction of curdlan, although we have no evidence. The peritoneal exuded leucocytes from fish fed curdlan for 3 weeks were activated and more susceptible to PMA stimulation, this may cause the increase of the protection ability against bacterial infection. Thus, the non-specific immune system of Japanese flounder was activated highly in 2 or 3 weeks after the start of feeding, and the activation decreased with lengthening the administration periods.

Matsuo and Miyazone\textsuperscript{9}, and Yoshida et al.\textsuperscript{10} reported that the oral feeding of glucan protected bacterial infection in rainbow trout, O. mykiss and African catfish, Clarias gariepinus, but long feeding did not. As the continuous feeding (everyday) of curdlan for a long period seemed to decrease the activation of the non-specific immune system in fish, next, we shifted our attention to the discontinuous feeding. The discontinuous feeding (every 2 days) of curdlan for 4 months enhanced the phagocytic activities of three kinds of leucocytes significantly ($p < 0.05$), but the activation rates were lower than those in the short period feeding test, and the O$_2^-$ generation from peritoneal exuded leucocytes was not increased in this trial (Fig. 2 B, Fig. 3 B).

In human, $\beta$-1,3-glucan enhances the phagocytosis of zymosan and rabbit erythrocytes in at least two ways: activation of the complement via the alternative pathway and stimulation of the pathway from arachidonic acid to eicosanoids\textsuperscript{11-14}. Sveinbjornsson et al.\textsuperscript{15} demonstrated that the aminated $\beta$-1,3-d-polyglucose (prepared from curdlan) fed to salmon are absorbed from the intestine, and distributed in the lymphoid tissues. They suggested the possible use of this immunostimulating polysaccharide as a feed additive in fish. In our studies on curdlan-fed fish, the $\beta$-1,3-glucan would be absorbed and activate the non-specific immune system in fish. However, the cells prepared from fish fed curdlan everyday for a long period (3, 4 weeks) did not show the activation. Figueras et al.\textsuperscript{16} showed that the respiratory burst and phagocytic activities of turbot phagocytes were inhibited by the addition of high concentrations of yeast glucan on in vitro immunostimulation. Similarly in our experiments, the activation of non-specific immune systems may have been suppressed when stimulated in surplus by a long-time feeding of curdlan.

In conclusion the oral administration of curdlan enhanced the phagocytic activity and the O$_2^-$ generation of leucocytes, but the activation rates of long period feeding test were significantly lower than those in the short period feeding test. It seemed that the enhancement depends on the feeding period and the feeding mode.

References

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魚類の細菌感染症に対するカーダラン経口投与の効果について、細胞性非特異的免疫機能にかから評価した。カーダランを単離投与したヒラメの白血球では、投与開始2週間後までは貪食能の、3週間後ではO2産生能の活性化が認められた。しかし、4カ月の長期隔日投与を行ったヒラメの白血球では、貪食能は活性化されず、その程度は短期投与と比較して有意に低く、カーダラン経口投与の効果は、投与期間、投与法に影響された。