

The Stimulation of Cytokine-like Proteins in Tilapia (*Oreochromis niloticus*) Orally Treated with β -1, 3-glucan

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Abstract

The stimulation of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-10 (IL-10) or interleukin-12 (IL-12)-like proteins using the enzyme-linked immunosorbent assay (ELISA) was investigated in tilapia (*Oreochromis niloticus*) orally treated with β -1,3-glucan (β G) for five days. The stimulation of each cytokine were determined in the plasma of tilapia orally treated with β G. The TNF- α reactive protein was significantly stimulated in the plasma of tilapia treated with β G. The levels of IL-1 β , IL-10 and IL-12 antibody-reactive proteins also increased in the plasma of fish treated with β G. On the other hand, the phagocytic indices of macrophages in tilapia treated with β G significantly increased. These results suggest that the immunostimulation in tilapia orally treated with β G may relate with the production of cytokine-like proteins.

Introduction

Immunostimulants can increase resistance to infectious diseases. Usually, phagocytes of fish treated with immunostimulants show enhanced phagocytic activities (Sakai 1999). Immunostimulants like concanavalin A or lipopolysaccharides (LPS) induce enhanced mitogen responses in lymphocytes (Hardie et al. 1991; Siwicki et al. 1996).

Cytokines are simple polypeptides or glycoproteins that act as signaling molecules within the immune system (Thomson 1994). In fish, the genes for interleukin 1 β (IL-1 β) and transforming growth factor β (TGFB) have been isolated and its expression was analyzed (Secombes et al. 1999). Furthermore, the existence of proteins that function as interleukin 2 (IL-2), macrophage

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activating factors (MAF) and interferon has been suggested for several fish (Manning and Nakanishi 1996). Proteins reacting with antibodies against mammalian interleukin 3 (IL-3), interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α) have also been reported (Manning and Nakanishi 1996). Cytokines such as IL-1 and TGF β are known to play important roles in both specific and non-specific immune responses of fish (Secombes et al. 1996). The effects that immunostimulants have on the TNF- α and MAF activities of fish had only been reported by Robertsen et al. (1994) and by Jang et al. (1995) that were treated with β -glucan and glycyrrhizin, respectively.

In the present study, tilapia were treated orally with β -1,3 glucan in order to investigate the effect of immunostimulants on both the production and activity of cytokine in animals. The IL-1 β , IL-10, IL-12 and TNF- α in the plasma were determined through ELISA using human antibodies.

Materials and Methods

Fish

A total of 100 juvenile tilapia, *Oreochromis niloticus* (mean weight 83 g) were obtained from the Division of Fisheries Sciences in Miyazaki University, Japan. Fish were acclimated in indoor tanks with running fresh water at 24°C for two weeks and fed with a commercial dry pellet diet (Nosan Co., Ltd., Japan) twice daily before administering the experimental diets.

Immunostimulants

β -1,3 glucan (β G) was obtained from the Wako Junyaku Co. Dry feed pellets were coated with β G using food oil (Riken Co., Ltd., Japan) to deliver 0, 2 or 10 g·kg of fish.

Administration of β -1,3 glucan

Each diet was fed at 2% of the average body weight of fish/day in indoor flow-through tanks containing 20 fish. Before taking blood samples and collecting air-bladder macrophages, the fish were fed with the diets at the Miyazaki University Fish Disease Unit/Laboratory for five days. The mean water temperature during the feeding period was 24.5°C. Fish fed with diet without β G were used as control. These experiments were repeated three times and five fish from each fish group were sampled for each procedure.

Blood samples

After five days of feeding, blood samples were collected from the caudal vein of both treated and control fish using heparinized syringes and the plasma was separated by centrifugation at 3000 rpm for 10 min.

Macrophage isolation

Macrophages were isolated from the air-bladder following the method of Matsuyama et al. (1999). The collected macrophages were suspended in Hank's balanced salt solution and adjusted to 1×10^6 cells·ml.

Phagocytosis assay

Phagocytosis was examined as described by Yoshida et al. (1993). The cell number was adjusted to 10^6 cells·ml in Hanks solution. Equal volumes of the macrophage solution and zymosan solution (0.5 mg·ml) were mixed in microtubes and incubated for 60 min at 25°C. After centrifugation at 700 rpm for 5 min, the cells were smeared onto a glass slide and fixed with methyl alcohol (100%) for 5 min and stained with Giemsa. The number of phagocytic cells per 100 cells was counted microscopically while the phagocytic activity was calculated using the following equation:

$$PA = \frac{\text{Number of phagocytizing cells}}{\text{Number of total cells}} \times 100$$

The phagocytic index was determined by multiplying the number of zymosan phagocytized by 20 phagocytic cells in each group.

Two sets of experiment with five fish in each group were conducted. Data of phagocytic activity and index were expressed as mean \pm SE. Data was analyzed using two- and one-way ANOVA and Student's t-test.

The enzyme immunoassay to detect IL-1 β , IL-10, IL-12 and TNF- α like proteins

The enzyme-linked immunosorbent assay (ELISA) was used for detecting fish IL-1 β , IL-10, IL-12 and TNF- α like molecules. The 96 well microtitreplates (Iwaki Glass, Japan) were coated with 100 μ l of tilapia plasma diluted 10 times in physiological buffered saline (PBS) and incubated in a moist chamber overnight at 4°C. Antihuman IL-1 β , IL-10, IL-12 or TNF- α rabbit polyclonal antibody was used as the primary antibody (Sigma, USA). Vectastain ABC-PO kit (Vector Lab.,USA) was applied for ELISA and peroxidase ABTS substrate kit (Vector Lab.,USA) was used for coloration. The plate was read at 490 nm on a multiscan spectrophotometer (Pharmacia, Sweden). For the blank, physiological saline was used instead of plasma. Fish plasma not treated with β G was used as negative control. Positive controls could not be set up, as none of the fish proteins such as IL-1 β , IL-10, IL-12 or TNF- α have been purified. However, to confirm our ELISA experiments, human IL-1 β , IL-10, IL-12 or TNF- α proteins were used as control.

The ELISA was repeated five times and data was expressed as mean \pm SE. The stimulation of cytokines was determined by the significant difference

between the negative control and the experimental samples using Student's t-test.

Results

Phagocytosis

The phagocytosis in the macrophages of tilapia fed with pellets containing 0.2% or 1% β G is shown in figures 1 and 2. Although the phagocytic activity did not show any significant increase in fish treated with 0.2%, statistical differences were observed in fish fed with 1% β G (Fig. 1). The phagocytic index showed a significant increase in fish treated with β G (Fig. 2).

The stimulation of antihuman TNF- α , IL-1 β , IL-10, IL-12 reactive proteins in plasma of tilapia after β G treatment

The stimulation of antihuman TNF- α reactive proteins in the plasma of tilapia after β G treatment is shown in figure 3a. The TNF- α reactive protein was produced in the plasma of fish treated with pellets containing 0.2% and 1% β G and was significantly higher than untreated fish ($P < 0.05$). The stimulation of TNF- α was higher in fish fed with 0.2% β G than in fish fed with 1% β G.

Figure 3b shows the stimulation of antihuman IL-1 β reactive proteins in the plasma of fish fed with pellets containing 0.2% and 1% β G. The IL-1 β reactive protein also significantly increased in the plasma of fish treated with β G.

The IL-10 and IL-12 reactive proteins were also stimulated in tilapia treated with β G (Figs. 3c and 3d). IL-10 and IL-12 production in the plasma

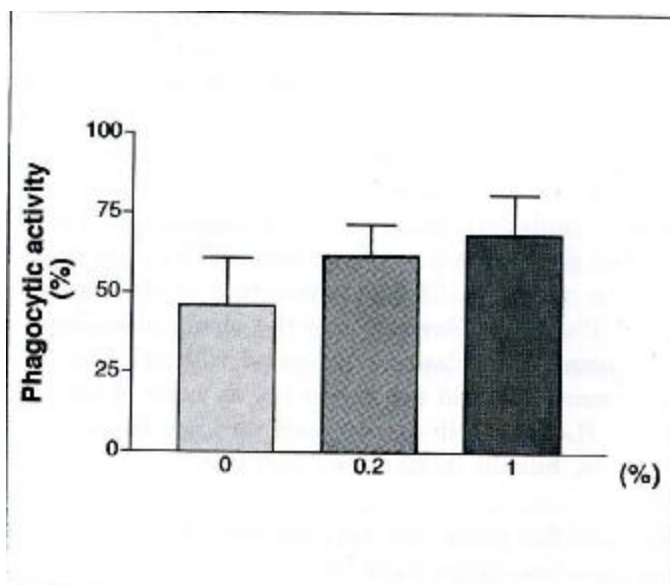


Fig. 1. The phagocytic activity in macrophages of tilapia treated with 0.2 or 1% β -1,3-glucan. Samples were collected five days after the treatment and five individual fish of each group were examined. * $P < 0.05$.

of fish treated with 0.2% and 1% β G significantly increased compared with that of the control fish.

The ELISA systems could not detect concentrations ranging from 10 ng to 100 ng of human TNF- α , IL-1 β , IL-10, and IL-12 in the positive control.

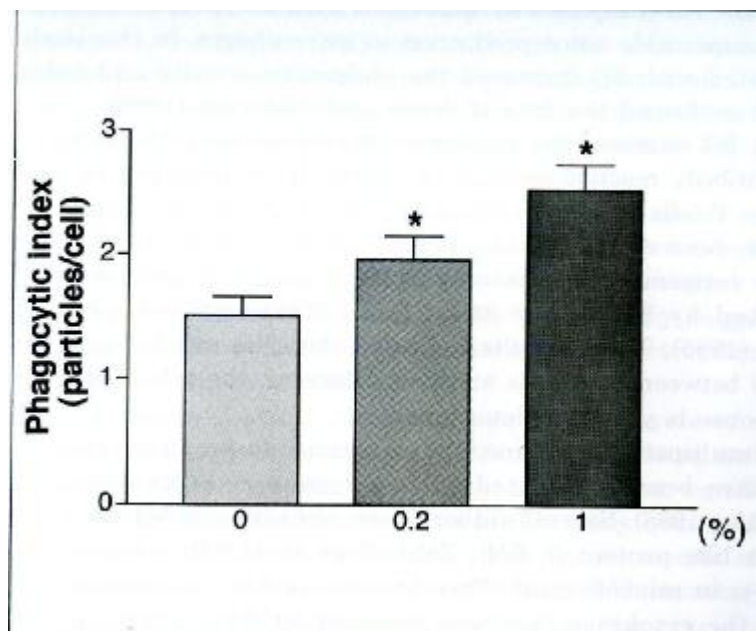


Fig. 2. The phagocytic index in macrophages of tilapia treated with 0.2 or 1% β -1,3-glucan. Samples were collected 5 days after and five individual fish of each group were examined. * $P < 0.05$.

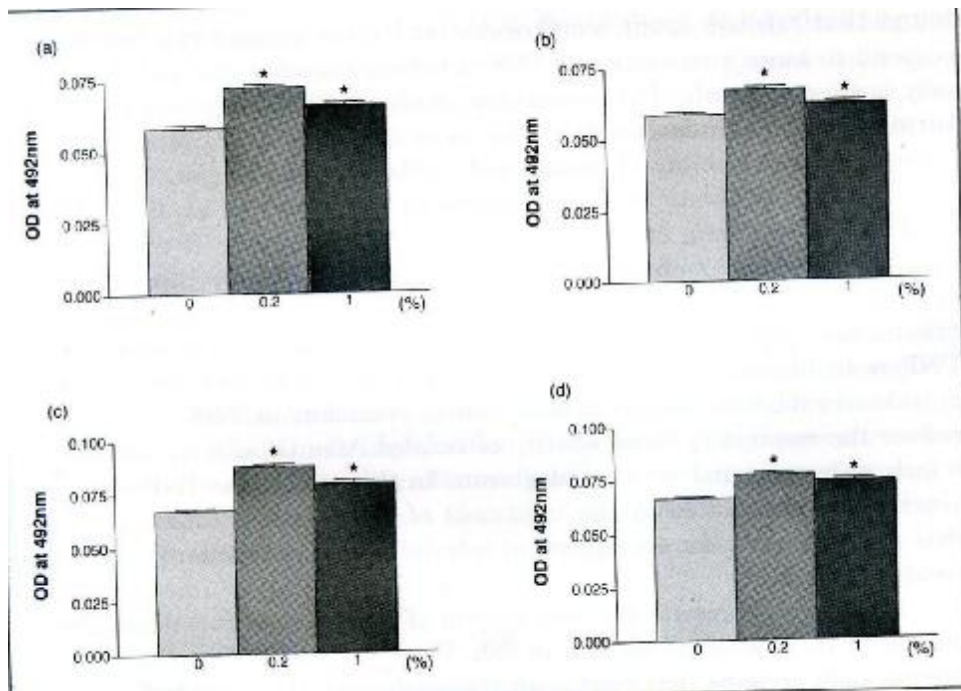


Fig. 3. The production of proteins reactive to antihuman TNF- α (a), IL-1 β (b), IL-10 (c) and IL-12 (d) antibodies in the plasma of tilapia treated with 0.2 or 1% β -1,3-glucan. Samples were collected five days after treatment and five individual fish from each group were examined. * $P < 0.05$.

Discussion

It is well-known that fish treated with immunostimulants increased the phagocytic activity (Sakai 1999). Jeney and Anderson (1993) have already reported that rainbow trout injected or immersed with β G increased phagocytic activity and superoxide anion production in macrophages. In this study, tilapia orally treated with β G increased the phagocytic activity and index. Thus, our results confirmed the data of Jeney and Anderson (1993).

In this study, β G increased the production of antihuman IL-1 β , IL-10, IL-12 and TNF- α antibody reactive proteins in tilapia. IL-1 β , produced by macrophages, activates T cells and B cells (Janeway and Travers 1994). IL-1 β like activity has already been reported in tilapia and catfish (Secombes et al. 1996). Furthermore, the antigenic cross-reactivity of IL-1 β from fish and mammals has been reported by Ellsaesser and Clem (1994) and Verburg van Kemenade et al. (1995). These results indicated the close similarity in the structure of IL-1 β between mammals and fish. However, the role of IL-1 β in the immune response is yet to be determined.

TNF- α is a multipotent cytokine, that is primarily produced by macrophages that have been stimulated with mitogens or other cytokines (Cerami and Beutler 1988). Several authors have already reported the existence of a TNF- α like protein in fish. Zelikoff et al. (1990) reported the presence of TNF- α in rainbow trout, *Oncorhynchus mykiss*, macrophages. It was also one of the cytokines that was detected by Ahne (1993) in the serum of virus-infected carp. In addition, Hardie et al. (1994) have recently found that rainbow trout lymphocytes and macrophages can functionally respond to human recombinant TNF- α , which suggests that fish leukocytes may possess a specific TNF- α receptor. Such cross-reactivity implies an evolutionary conservation of the TNF- α receptor — a hypothesis that is supported by the ability of monoclonal antibodies raised against the human TNF receptor to ablate the fish response to TNF (Jang et al. 1995). Hardie et al. (1994) and Jang et al. (1995) found that the response of the trout cells to TNF- α requires additional signals from mitogens in the case of lymphocytes or from interferon- γ for macrophages, to enable the TNF- α to elicit its effects. Abel and Czop (1992) reported that yeast glucan elicits production of TNF- α in human monocytes. Robertsen et al. (1994) also showed that a polyclonal rabbit antiserum against human recombinant TNF- α was able to reduce the respiratory burst activity of isolated Atlantic salmon macrophages which were activated with yeast glucan. In this study, the TNF- α reactive protein was stimulated by the treatment of β G. These results suggest that this protein may play an important role in the immunostimulation of fish treated with β G.

IL-10 can potentiate the suppression of macrophage functions. The existence of IL-10 was not studied in fish. Thus, this is the first report on fish having such proteins that react with the antihuman IL-10 antibody. Interestingly, these proteins in plasma increased after treatment with β G and the peak responses of IL-10 manifested five days after β G treatment. IL-10 can produce the stimulation of LPS and act in the suppression of macrophage

functions. We have already observed the production of antihuman IL-10 antibody reactive protein in tilapia kidney leukocytes stimulated by LPS (unpublished data).

This is also the first report that fish have proteins that react with antihuman IL-12 antibody. IL-12 produced from B-cells or macrophages, activates natural killer (NK) cells. Although the activation of NK cells in fish treated with β G has not been investigated, Kajita et al. (1990) reported that when levamisole, an immunostimulant is injected in fish, the NK cell activities are activated. Further studies will examine the stimulation of NK cells in fish treated with β G.

β G is one of the excellent immunostimulants. Jeney and Anderson (1993) reported that the macrophages of rainbow trout injected with β G has increased phagocytic activity. In this study, tilapia treated with β G increased the phagocytic index in leukocytes. Our results may suggest that cytokines, such as IL-1 β or TNF- α , are related to the activation of macrophage functions in tilapia treated with β G.

The present study also investigated the levels of cytokines in fish treated with immunostimulants, determined by using antihuman cytokine antibodies ELISA system. However, it is clear that significant interference by noncytokine molecules can occur in this assay questioning the validity of the results (Ahne 1994; Hausmann 1995). In the results of IL-10 and IL-12, the possibilities indicated by Ahne (1994) and Hausmann (1995) still remain because there are no biological tests for this. However, control fish did not show the stimulation of these proteins. On the other hand, IL-1 β and TNF- α show several biological activities in fish. Especially in IL-1 β , the antigenic similarity between fish and mammals have already been indicated (Ellsaesser and Clem 1994; Verburg van Kemenade et al. 1995). Thus, the stimulation of IL-1 β and TNF- α in tilapia treated with β G should be addressed.

Conclusion

Tilapia orally treated with β G for five days stimulated antihuman IL-1 β , TNF- α , IL-10 and IL-12 rabbit polyclonal antibody-reactive proteins in plasma. The increase of these cytokine-reactive proteins in fish fed with 0.2% β G are higher than that of 1%. The phagocytic index of macrophages showed a significant increase in fish treated with 1% β G. Thus, these results suggest that the immuno-stimulation in tilapia treated with β G may be related to the production of cytokine-like proteins.

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