コイの実験的細菌感染症に対するβ-1,3-グルカンの防御効果

誌名	日本水産學會誌
ISSN	00215392
著者	矢野, 友紀 松山, 博子 Mangindaan, R.E.P.
巻/号	55巻10号
掲載ページ	p. 1815-1819
発行年月	1989年10月

Nippon Suisan Gakkaishi 55(10), 1815–1819 (1989)

Enhancement of the Resistance of Carp *Cyprinus carpio* to Experimental *Edwardsiella tarda* Infection, by Some β-1, 3-Glucans

Tomoki Yano*, Remy E. P. Mangindaan*, and Hiroko Matsuyama*

(Received August 15, 1989)

Schizophyllan, scleroglucan and lentinan, which are β-1,3-glucans derived from *Schizophyllum commune*, *Sclerotium glucanicum* and *Lentinus edodes*, respectively, were evaluated for their ability to enhance protection to *Edwardsiella tarda* infection in carp *Cyprinus carpio*. Intraperitoneal injections of these polysaccharides (2–10 mg/kg) to carp 6 and 3 days prior to intraperitoneal challenge with *E. tarda* (5×10^7 CFU/100 g) resulted in a significantly increased survival rate. In all polysaccharide-treated groups, the rapid elimination of the challenge bacteria from the blood was observed and phagocytic activity of pronephros cells against baker's yeast was significantly elevated. Furthermore, it was shown that these polysaccharides activated the alternative complement pathway of carp. These results indicate that schizophyllan, scleroglucan and lentinan enhance the resistance of carp to bacterial infection through the activation of the non-specific immune system.

The rapid expansion of fish farming in recent years and the corresponding increase in fish diseases have increased the demand for effective prophylactics, and thus far several vaccines have been developed.[1–5] However the advantage of using immunopotentiators to induce non-specific immunity in cultured fish has received little attention.

Recently, Olivier *et al.*[6] found that strong anti-furunculosis immunity could be induced in coho salmon by an adjuvant (modified Freund's complete adjuvant, MFCA) alone, and that the active factor in the adjuvant was a killed *Mycobacterium butyricum* preparation. Furthermore, they demonstrated that the induced immunity was non-specific and probably due to the reticuloendothelial system (macrophages). More recently, Kitao and Yoshida[7] showed that intraperitoneally injected FK-565 (a lactoyl tetrapeptide) increased phagocytic activity and resistance to *Aeromonas salmonicida* infection in rainbow trout.

Schizophyllan,[8] scleroglucan[9] and lentinan[10] derived from *Schizophyllum commune*, *Sclerotium glucanicum* and *Lentinus edodes*, respectively, are β-1,3-glucans which have some β-1,6-glucosidic side chains. They are known as antitumour polysaccharides and as stimulators of the non-specific immune system of mammals.[11–14] In this study we investigated whether these polysaccharides have a stimulatory effect on the non-specific immune response of carp, and if they induce protection against bacterial infection in carp.

Materials and Methods

Fish

Carp *Cyprinus carpio*, weighing 30 to 38 g were purchased from a fish farm in Fukuoka Prefecture, Japan. The fish were acclimated to laboratory conditions for more than 1 week in 60 *l* aquaria. The water temperature was kept at 25°C and the fish were fed commercial pellets during the experiment.

Injection of Polysaccharide

Schizophyllan and scleroglucan were obtained by courtesy of Taito Co., Tokyo. Lentinan was isolated from fruit bodies of *Lentinus edodes* (shiitake, an edible mushroom in Japan) and purified as described by Chihara *et al.*[10]

These polysaccharides were dissolved or suspended in sterile saline and injected intraperitoneally to carp at doses of 2 to 10 mg/kg twice at an interval of 2 days. Control fish received saline.

* Laboratory of Fisheries Chemistry, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan (矢野友紀, R. E. P. Mangindaan, 松山博子: 九州大学農学部水産化学教室).

Bacterium

A virulent strain of *Edwardsiella tarda* NG 8104, isolated from a diseased flounder, was kindly donated by Dr. Kitao (Miyazaki University, Japan). The virulence of the isolate was maximized by passing cultures three times through carp. An inoculum was prepared by growing the isolate on Heart Infusion (HI) agar (Eiken Chemical Co., Tokyo) at 37°C for 24 h and suspended in saline at a concentration of 5×10^7 CFU/m*l*.

Challenge

Carp were inoculated intraperitoneally with *E. tarda* (5×10^7 CFU/100 g) and monitored for 5 days. Kidney samples of all resulting mortalities were examined by culture on HI agar at 37°C to verify that the challenge organism was responsible for the death.

Counting of Bacteria in the Blood

Fish were anesthetized with MS-222 (Sankyo Co., Tokyo) and about 100 μl of blood was withdrawn from the caudal vein using a heparinized syringe. The blood was serially diluted with HI broth (Eiken Chemical Co., Tokyo) supplemented with 0.6% agar at 40°C, and 0.1 m*l* droplets of appropriate dilutions were put into petri dishes, covered with sterile 0.6% agar, and incubated at 37°C for 24 h. Microcolonies were counted under a dissecting microscope.[15]

Collection of Pronephros Cells

Fish were anesthetized with MS-222 and bled from the caudal vein using syringes. Pronephros of each fish was excised and placed in 5 m*l* of ice-cold RPMI-1640 (Nissui Pharmaceutical Co., Tokyo) containing 10% fetal calf serum, 10 mM HEPES (Wako Pure Chemical Industries Co., Osaka), antibiotics (50 units/m*l* of penicillin and 50 μg/m*l* of streptomycin) and 20 units/m*l* of heparin. The tissue was then teased and passed through a stainless steel mesh. The pronephros phagocytic cells thus obtained were washed twice with the above medium by centrifuging at $400\times$g for 5 min, and suspended in the same medium at a concentration of 5×10^7 viable cells/m*l*. Cellular viability was determined by the trypan blue exclusion test.

Assay of Phagocytic Activity

Phagocytic activity of the pronephros cells against baker's yeast (Oriental Yeast Co., Tokyo) was determined as follows: An aliquot (100 μl) of the pronephros phagocytic cell suspension (5×10^7 cells/m*l*) was mixed with 200 μl of heat-inactivated baker's yeast suspension (5×10^7 cells/m*l*) and the mixture was incubated at 20°C for 1 h in a siliconized micro test tube. Then, the mixture was smeared on a slide glass, air dried, and stained with Wright solution. Phagocytic index (PI) was determined by enumerating 500 phagocytic cells under a microscope.

$$\mathrm{PI}=\frac{\text{No. of phagocytic cells ingesting yeast}}{\text{No. of phagocytic cells}}\times\frac{\text{No. of yeast ingested}}{\text{No. of phagocytic cells}}\times100$$

Assay of the Alternative Complement Pathway (ACP) Activity

An aliquot (200 μl) of carp serum was incubated with 50 μl of polysaccharide suspension (0.25–5 mg/m*l*) at 20°C for 30 min. After centrifugation, residual ACP activity (ACH50) was assayed according to the method described in the previous paper.[16]

The carp serum used in this experiment was obtained from a fish weighing about 1.2 kg and stored at −80°C until use.

Statistics

Fisher's exact probability test was used to evaluate the levels of significance of survival in the challenge experiment. T-test was applied to evaluate the means of PI. In both cases, if $P<0.05$ the difference was considered significant.

Results

Survival Rate of Carp Challenged with E. tarda

Carp were injected intraperitoneally with 0, 2, 5 and 10 mg/kg of polysaccharide twice on the 1st and 4th day. On the 7th day, the fish were challenged with *E. tarda*.

As shown in Fig. 1, the fish treated with schizophyllan, scleroglucan and lentinan had a significantly greater survival rate than the control fish. All control fish died within 3 days, while the survival rates of the treated groups, 5 days after challenge, were 73–80% for schizophyllan-treated group and 50–75% for scleroglucan- and lentinan-treated groups. In schizophyllan-treated group, there was no apparent difference in survival rates among doses

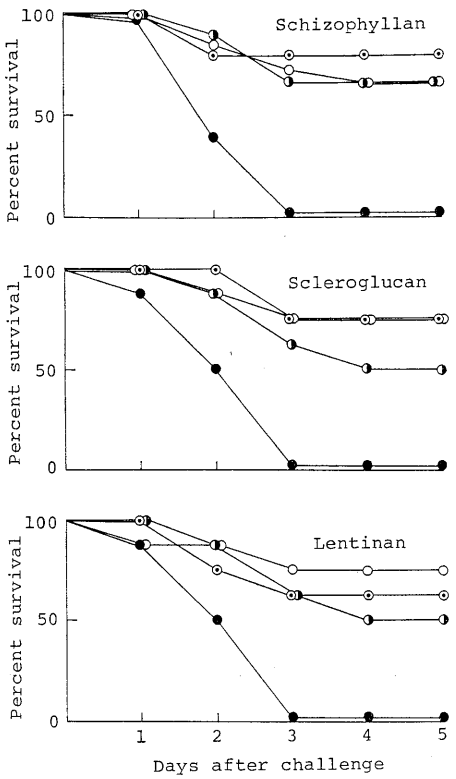

Fig. 1. Effect of polysaccharide administration on survival of carp challenged with *Edwardsiella tarda* (5×10^7 CFU/100 g). Polysaccharide (2–10 mg/kg) was injected intraperitoneally 6 and 3 days prior to the challenge. N=8 per group. ◐, 2 mg/kg; ⊙, 5 mg/kg; ○, 10 mg/kg; ●, control.

of 2, 5 and 10 mg/kg, while in scleroglucan- and lentinan-treated groups, doses of 5 and 10 mg/kg resulted in slightly higher survival rates than a dose of 2 mg/kg. We observed in preliminary experiments that doses below 1 mg/kg resulted in much lower survival rates.

Elimination of Challenge Bacteria from the Blood

Carp were injected intraperitoneally with 5 mg/kg of polysaccharide twice on the 1st and 4th day. On the 7th day, the fish were inoculated intraperitoneally with *E. tarda* (5×10^7 CFU/100 g) and the number of bacteria in the blood was counted at 6, 12 and 24 h intervals.

The changes in the number of bacteria in the blood of each polysaccharide-treated and each non-treated fish are shown in Fig. 2. The number of bacteria in the blood of treated fish was apparently reduced within 24 h. In 3 out of 5 scleroglucan- and lentinan-treated fish, the number of bacteria decreased to below 10^2 CFU/ml by 24 h. On the other hand, in the blood of non-treated fish, the number of bacteria temporarily decreased at 12 h, but had begun to increase at 24 h.

Effect of Polysaccharides on the Phagocytic Activity of Pronephros Cells

Three days after polysaccharide injection (5 mg/kg×2), the phagocytic activities of pronephros cells from treated and non-treated fish were assayed.

As shown in Table 1, phagocytic activity of

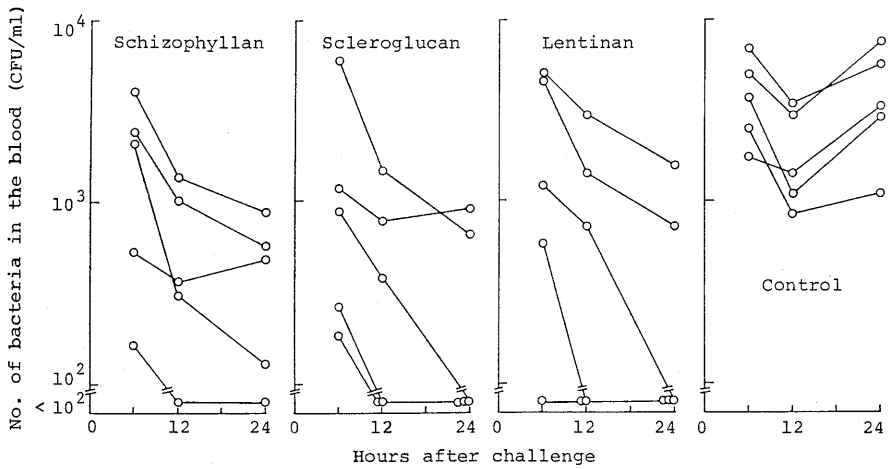

Fig. 2. Changes in the number of bacteria (*Edwardsiella tarda*) in the blood of polysaccharide-treated and non-treated carp. Polysaccharide (5 mg/kg) was injected intraperitoneally 6 and 3 days prior to challenge with *E. tarda* (5×10^7 CFU/100 g). Control fish received saline.

Table 1. Phagocytic activity of pronephros phagocytic cells from carp treated with polysaccharide*[1]

Treatment	Phagocytic Index*[2]
Schizophyllan	3.07±1.59 (n=20)*[3]
Scleroglucan	2.63±0.99 (n=20)*[3]
Lentinan	2.88±1.50 (n=15)*[3]
Control	1.16±0.68 (n=22)

*[1] Pronephros phagocytic cells were obtained from carp 3 days after intraperitoneal injection of polysaccharide (5 mg/kg ×2). Control fish received saline.
*[2] Each value represents the mean±SD.
*[3] Significantly different ($p<0.05$) from control group.

Table 2. Reduction of the alternative complement pathway (ACP) activity of carp serum by polysaccharide treatment*

Polysaccharide	Residual ACP activity (%)				
	Final concentration of polysaccharide (mg/ml)				
	0	0.05	0.1	0.5	1.0
Schizophyllan	100	86	77	68	65
Scleroglucan	100	80	77	54	50
Lentinan	100	83	24	14	13

* Carp serum (200 μl) was incubated with polysaccharide suspension (50 μl) at 20°C for 30 min. After centrifugation at 3000 rpm for 5 min, the residual ACP activity in the supernatant was measured.

pronephros cells from treated fish was significantly higher than that of pronephros cells from non-treated fish. The injection of schizophyllan resulted in 2.6-fold increase in the phagocytic activity, while the administration of scleroglucan and lentinan resulted in 2.3- and 2.5-fold increase, respectively.

Activation of the Alternative Complement Pathway (ACP) by Polysaccharides

Schizophyllan, scleroglucan and lentinan were tested for their potency of complement activation at several doses. In all experiments, carp serum was incubated with polysaccharide suspension at 20°C for 30 min, followed by centrifugation and titration of residual ACP activity in the supernatant. The results were expressed in per cent reduction in hemolytic activity (ACH50) as summarized in Table 2. When carp serum was incubated with increasing concentrations of polysaccharide, the ACH50 activity of the serum decreased though the extent of reduction was different among the polysaccharides. At the highest concentration of polysaccharide (1 mg/ml), the serum hemolytic activity was reduced 35% for schizophyllan, 50% for scleroglucan and 87% for lentinan.

Discussion

In this experiment, it was demonstrated that intraperitoneally injected schizophyllan, scleroglucan and lentinan significantly increased the resistance to *E. tarda* infection in carp. This resistance occurred within a few days earlier than would be expected if the specific immune response was involved. It seems likely, therefore, that the polysaccharides stimulated non-specific resistance of carp.

A rapid reduction of challenge bacteria was observed in the blood of polysaccharide-treated carp. The survival rate of fish was very high if the number of bacteria decreased to below 10^3 CFU/ml (Fig. 2). This is probably due to the elevation of phagocytic activity since we observed that the phagocytic activity of carp pronephros cells was significantly increased by polysaccharide administration. It appears that carp phagocytic cells play an important role at an early stage of bacterial infection.

We also confirmed that incubation of carp serum with schizophyllan, scleroglucan and lentinan resulted in considerable loss of its ACP activity. This indicates that complement components from C3 and later were activated by these polysaccharides. The role of carp complement in the protection against bacterial infection is still obscure, but when carp complement is activated through the alternative pathway, the biological activities, that is, phagocytosis-promoting activity,[17] chemotactic activity[18,19] and leukocytosis-inducing activity[19] would be generated. These activities may play an important role in the non-specific defense mechanism of carp against bacterial infections.

Finally, the following questions as to whether the polysaccharides exhibit a protective effect when given orally or by immersion, and if they have a stimulatory effect, as adjuvants, on the humoral antibody response of vaccinated fish remain to be settled. In our laboratory, these studies are now in progress.

References

1) J. L. Fryer, J. S. Rohovec, G. L. Tebbit, J. S. McMichael, and K. S. Pilcher: *Fish Pathol.*, **10**, 155-164 (1976).

2) R. W. Gould, P. J. O'Leary, R. L. Garrison, J. S. Rohovec and J. L. Fryer: *Fish Pathol.*, **13,** 63-68 (1978).
3) R. W. Gould, R. Antipa, and D. F. Amend: *J. Fish Res. Board Can.*, **36,** 222-225 (1979).
4) T. Aoki, T. Kitao, M. Fukudome, S. Takahashi, and S. Egusa: *J. Fish Dis.*, **7,** 149-156 (1984).
5) Y. Fukuda and R. Kusuda: *Fish Pathol.*, **20,** 421-425 (1985).
6) G. Olivier, T. P. T. Evelyn, and R. Lallier: *Dev. Comp. Immunol.*, **9,** 419-432 (1985).
7) K. Kitao and Y. Yoshida: *Vet. Immunol. and Immunopathol.*, **12,** 287-296 (1986).
8) S. Kikumoto, T. Shiga: *Taito Kenkyusho Hokoku*, **23,** 77-87 (1965).
9) J. Johnson Jr., S. Kirkwood, A. Misaki, T. E. Nelson, J. V. Scaletti, and F. Smith: *Chem. & Ind.*, **1963,** 820-822 (1963).
10) G. Chihara, J. Hamuro, Y. Maeda, Y. Arai, and F. Fukuoka: *Cancer Research*, **30,** 2776-2781 (1970).
11) N. Komatsu, S. Okubo, S. Kikumoto, K. Kimura, G. Saito, and S. Sakai: *Gann*, **60,** 137-144 (1969).
12) N. Komatsu, N. Nagumo, S. Okubo, K. Koike: *Jap. J. Antibiotics*, **26,** 277-283 (1973).
13) G. Chihara, Y. Maeda, J. Hamuro, T. Sakai, and F. Fukuoka: *Nature*, **222,** 687-688 (1968).
14) T. Okuda, Y. Yoshioka, T. Ikekawa, G. Chihara, and K. Nishioka: *Nature New Biology*, **238,** 59-60 (1972).
15) H. Reichenbach and M. Dworkin: in "The Prokaryotes" (ed. by M. P. Starr, H. Stolp, H. G. Trüper, A. Balows and H. G. Schlegel) Springer-Verlag, New York, 1986, pp. 328-355.
16) H. Matsuyama, K. Tanaka, M. Nakao, and T. Yano: *Dev. Comp. Immunol.*, **12,** 403-408 (1988).
17) K. A. Nash, T. C. Fletcher, and A. W. Thomson: *Comp. Biochem. Physiol.*, **86B,** 31-36 (1987).
18) Y. Suzuki: *Nippon Suisan Gakkaishi*, **52,** 811-816 (1986).
19) T. Iida and H. Wakabayashi: *Fish Pathol.*, **23,** 55-58 (1988).