Vibrio anguillarumリポ多糖に対するアユの免疫応答

誌名	日本水産學會誌
ISSN	00215392
著者	Salati, F. 渡辺, 圭介 川合, 研児 楠田, 理一
巻/号	55巻1号
掲載ページ	p. 45-49
発行年月	1989年1月

Nippon Suisan Gakkaishi 55(1), 45–49 (1989)

Immune Response of Ayu against *Vibrio anguillarum* Lipopolysaccharide

Fulvio Salati,*1,2 Keisuke Watanabe,*1 Kenji Kawai,*1 and Riichi Kusuda*1

(Received August 1, 1988)

In order to investigate immune response of ayu *Plecoglossus altivelis* against *Vibrio anguillarum* lipopolysaccharide (LPS) by intraperitoneal injection, antibody production at various water temperatures and amount of the crude LPS that induce protective immunity were examined. Agglutinating antibody titer against formalin-killed cell and passive hemagglutinating antibody titer against crude LPS and pure LPS increased 1 week after immunization in the fish reared at 25°C and 3 weeks after the immunization in the fish reared at 15–20°C. The fish reared at 10°C showed lower titers at 5 weeks after immunization. Distinctly higher agglutinating antibody titer against formalin-killed cell was observed in the serum and mucus of the fish immunized with 0.05–0.50 mg of crude LPS than that of the control fish. Those immunized fish showed 100% survival rate after challenge by water born infection, while the control fish showed 13.3%. From these results, it is concluded that ayu shows good immune response against *V. anguillarum* LPS at a range of 15–25°C and 0.05 mg of LPS per fish induces an enough immune protection against the infection.

Efficacy of vaccines for preventing vibriosis of cultured ayu *Plecoglossus altivelis* is well documented in some papers.[1–4] A probable protective antigen of the vaccine has been considered to be the lipopolysaccharide (LPS) of the pathogen *Vibrio anguillarum* from the result of oral[5] and immersion[6] administration of the vaccine. Protective antigenicity of LPS has been reported also on *Edwardsiella tarda* infection of eel *Anguilla japonica*[7] and *E. ictaluri* infection of channel catfish *Ictalurus punctatus*[8] in the field of fisheries. As it has been reported that LPS of gram negative bacteria contains a lot of biological and immunological activities,[9] the protective effect of the LPS in the studies mentioned above may have been brought by two or more activities of the LPS, but the explanation has not been clarified.

Though injection method is not practical for vaccination in the field of fish production except some cases, it presents a fundamental information concerning the immune response of the fish more clearly than the immunization by the oral or immersion method. The purpose of this study was to investigate principal immune response of ayu to *V. anguillarum* LPS by the intraperitoneal injection under some conditions and to confirm the protective effect of LPS against subcutaneous infection of the pathogen.

Materials and Methods

Two experiments were performed. Experiment 1 (exp. 1) was conducted to examine the effect of various water temperatures on the antibody production and experiment 2 (exp. 2) was to examine effective amount of LPS to prevent from vibriosis by a challenge test.

Experimental Animal and Aquarium

Experimental ayu were obtained from an ayu farm and kept in 200 *l* tanks with recirculating, aerated and filtered fresh water. The fish were maintained in the tanks for few days before the experiments to acclimate to new conditions. The fish were fed with commercial ayu food during the experiment. In the exp. 1, 4 groups of 20 fish weighing 38–40 g were reared at 10, 15, 20 and 25°C. In the exp. 2, 4 groups of 20 fish weighing 52–54 g were reared at 20°C.

Bacterium

Vibrio anguillarum strain PT-84060, serotype J-O-1,[10] was used to prepare immunogen and reactive antigen. The strain was originally isolat-

*1 Fish Disease Laboratory, Faculty of Agriculture, Kochi University, Nankoku, Kochi 783, Japan (フルビオ・サラティ, 渡辺圭介, 川合研児, 楠田理一: 高知大学農学部水族病理学講座).
*2 Present address: Via Doni 5, 12080 Monastero di Vasco (CN), Italy.

ed from a diseased ayu in 1984 and supplied from Tokushima Prefectural Fisheries Experimental Station.

Preparation of Antigen

Crude LPS was extracted by the method of Westphal and Jann[11] from lysozyme treated whole cells of PT-84060. Purified LPS was obtained by high speed centrifugation at 30,000×g for 5 h of the crude LPS followed by Cetavlon treatment. Polysaccharide and lipid A fractions were obtained by mild acid hydrolysis of the purified LPS as described by Salati *et al.*[7]

Immunization

In exp. 1, fish were intraperitoneally injected with 0.1 m*l* of crude LPS solution corresponding to 0.4 mg of the crude LPS. In exp. 2, 3 groups of fish were injected with 0.1 m*l* of the crude LPS solution corresponding to 0.05, 0.25 and 0.50 mg of the crude LPS. Control fish in exp. 2 were injected with 0.1 m*l* sterile physiological saline.

Titration of Antibody

The serum was collected from five fish in each group and pooled at 1, 3 and 5 weeks after immunization in exp. 1 and 3 weeks after immunization in exp. 2. The body surface mucus was collected after bleeding from five fish by scraping with a small sponge holding 2 m*l* of physiological saline and pooled. Those samples of the serum and body surface mucus were used to determine agglutinating antibody titer using microtiter method[12] against formalin-killed *V. anguillarum* cells (FKC) and passive hemagglutinating antibody titer using sheep red blood cells (SRBC) sensitized with the crude LPS, purified LPS, polysaccharide or lipid A by Neter's method.[13]

Challenge

Three weeks after immunization, the immunized and control fish were challenged by bathing in a bacterial suspension of strain PT-84060 at a concentration of 5.2×10^3 CFU/m*l* for 15 min. To confirm death from vibriosis, reisolation of *V. anguillarum* from dead fish and slide agglutination using anti-*V. anguillarum* rabbit serum were performed.

Electrosyneresis

For comparison of precipitating antibody production in the fish immunized under different conditions, pooled serum was analyzed by electrosyneresis[14] against crude LPS applying an electric current at 2 mA/cm for 40 min.

Results

Agglutinating antibody titers against FKC in the serum of ayu immunized and cultured at different temperatures in exp. 1 are shown in Fig. 1. Increase of the titer was observed 1 week after immunizaion only in the fish cultured at 25°C and 3 weeks after immunization in the fish reared at 10, 15 and 20°C. Five weeks after the immunization, almost same titer was shown in the fish reared at 15, 20 and 25°C, while the fish cultured at 10°C showed lower titer. Agglutinating antibody titers in the body surface mucus are shown in Table 1. There was a slight increase of the titer in the fish cultured at 15, 20 and 25°C 3 and 5 weeks after immunization, but not in the fish cultured at 10°C.

Passive hemagglutination titers against 4 antigen preparations in the serum of the fish cultured at

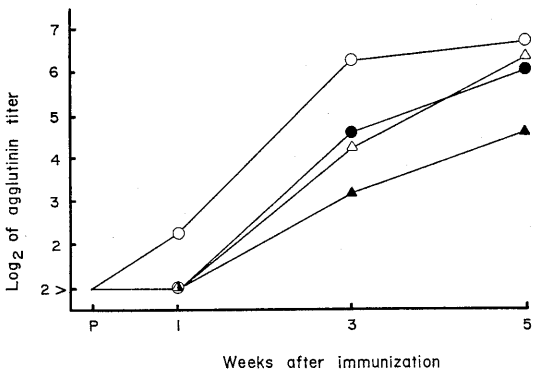

Fig. 1. Change in the agglutinating antibody titer against formalin-killed *Vibrio anguillarum* cell antigen in the serum of ayu cultured at various temperatures. P: pre-immunization, ▲: 10°C, ●: 15°C, △: 20°C, ○: 25°C.

Table 1. Agglutinating antibody titer against formalin-killed *Vibrio anguillarum* cell antigen in the body surface mucus of ayu immunized and cultured at various temperatures

Temperature (°C)	Weeks after immunization			
	0	1	3	5
10	<4	<4	<4	<4
15	<4	<4	4	16
20	<4	<4	4	8
25	<4	<4	4	8

Mucus was pooled from five fish of each group.

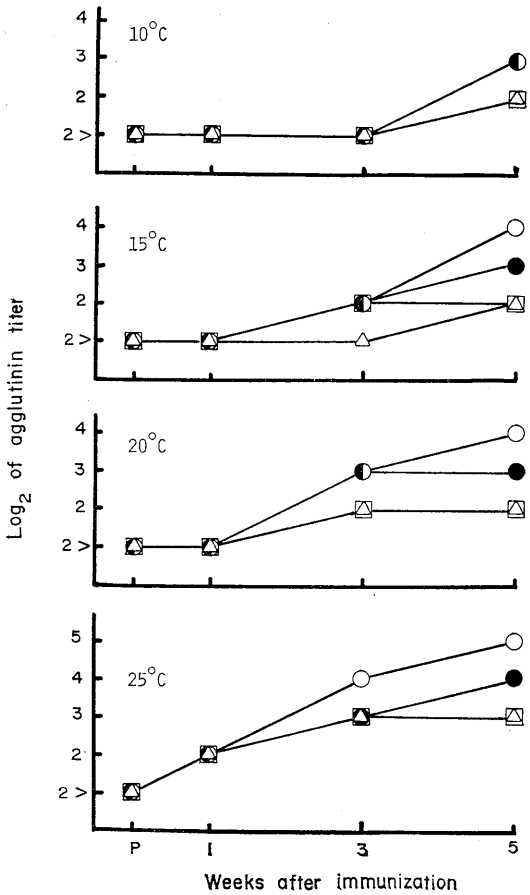

Fig. 2. Change in the passive hemagglutination titers against four antigen preparatins in the serum of ayu immunized with crude lipopolysaccharide (LPS) and cultured at various temperatures, ○: crude LPS, ●: purified LPS, □: polysaccharide, △: lipid A.

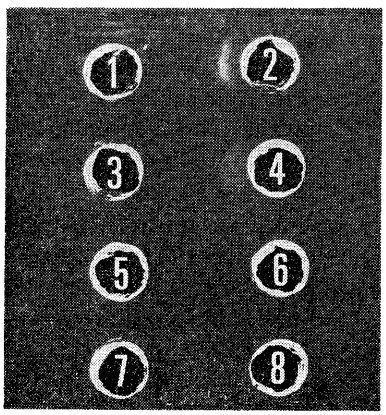

Fig. 3. Electrosyneresis of the serum of ayu immunized with *Vibrio anguillarum* crude lipopolysaccharide (LPS) and cultured at 25°C. 1,3 and 5: serum of ayu obtained 5, 3 and 1 week after immunization. 7: serum of ayu obtained before immunization. 2, 4, 6 and 8: crude LPS.

Table 2. Agglutinating antibody titer against formalin-killed *Vibrio anguillarum* cell antigen in the serum and body surface mucus of ayu immunized with various amount of crude lipopolysaccharide (LPS)

LPS injected (mg/fish)	Serum	Body surface*1 mucus
0.50	73.5*2 (128–32)*3	16
0.25	42.2 (128–16)	16
0.05	21.1 (32–16)	8
Control	<4	<4

*1 Muscus was pooled from five fish of each group.
*2 Figures indicate geometric mean of the titer.
*3 Figures in parentheses indicate range of the titer.

Table 3. Passive hemagglutination titer in the serum* of ayu immunized with various amount of crude lipopolysaccharide (LPS)

LPS injected (mg/fish)	Antigens			
	Crude LPS	Pure LPS	Polysaccharide	Lipid A
0.50	8	8	4	4
0.25	8	8	4	4
0.05	4	4	<4	<4
Control	<4	<4	<4	<4

* Serum was pooled from five fish of each group.

various temperatures are shown in Fig. 2. The highest titer was shown against the crude LPS then purified LPS in all groups of the fish cultured at different temperatures.

Precipitation patterns in electrosyneresis of the pooled serum of the fish cultured for 1, 3 and 5 weeks at 25°C after immunization was shown in Fig. 3. Precipitin line was produced from 1 week after immunization then the density of the precipitin line increased as the time elapsed. Same result was observed in the fish cultured at 10, 15 and 20°C though the density of the prescipitin lines were thinner than in the fish cultured at 25°C (data are not shown).

Agglutinating antibody titers agains FKC in the serum and body surface mucus of the fish immunized with different amount of the crude LPS in exp. 2 are shown in Table 2. Higher titer was shown according as the amount of immunizing antigen was increased. Passive hemagglutination titers of the serum are shown in Table 3. Increase of the titer was observed against four reactive antigens in the fish immunized with 0.50 and 0.25 mg crude LPS/fish and the minimum increase occurred against the crude LPS and pure LPS in the fish immunized with 0.05 mg crude LPS/fish.

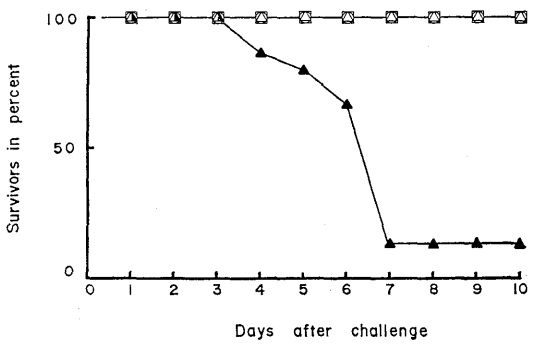

Fig. 4. Change in the survival rate of ayu immunized with various amount of crude lipopolysaccharide. □: 0.50 mg/fish, ○: 0.25 mg/fish, △: 0.05 mg/fish, ▲: control.

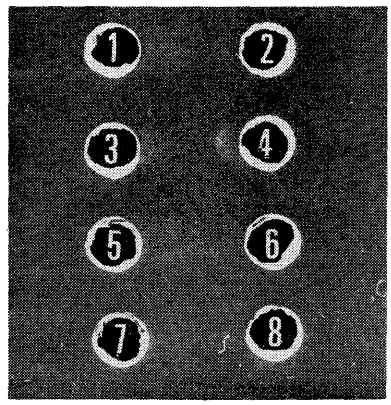

Fig. 5. Electrosyneresis of the serum of ayu immunized with various amount of crude lipopolysaccharide (LPS). 1, 3 and 5: serum of ayu immunized with 0.50, 0.25 and 0.05 mg crude LPS, respectively. 7: serum of control ayu. 2, 4, 6 and 8: crude LPS.

Changes in the survival rate in the fish immunized with various amount of the crude LPS after challenge were shown in Fig. 4. All the immunized groups showed 100% survival rate, while the control group showed 13.3% in 10 days after challenge.

Precipitation patterns of electrosyneresis of the pooled serum of the fish immunized with various amount of the crude LPS was shown in Fig. 5. Precipitin line was observed in the serum of the fish immunized with 0.50 and 0.25 mg crude LPS/fish.

Discussion

It has been demonstrated that the antibody production by poikilothermic vertebrates depends on the animal's normal temperature environment and a higher temperature brings a better immune response within a suitable range of the animal.[15] In the present study, ayu showed good antibody production at a range from 15 to 25°C 3 weeks after immunization. The value and the rate of increase of the titer are the highest at 25°C, and this coincides well with the optimum temperature for growth of this fish species.[16]

Paterson and Fryer[17] reported that 7 g coho salmon produced antibody when injected intraperitoneally with 1 μg of *Aeromonas salmonicida* endotoxine. Saeed and Plumb[8] reported that 0.2 mg of *E. ictaluri* LPS is the optimum immunizing dose for 60 g channel catfish by intraperitoneal injection. The results of exp. 2 showed that 100% survival rate was induced by injecting with at least 0.05 mg of the crude LPS. Considering that the challenge was strong enough to result in low survival rate in the control fish, 0.05 mg of antigen is enough to induce protection in the fish. The minimum dose of LPS used in this study is the same or less than those reported by Paterson and Fryer[17] or Saeed and Plumb[8] in consideration of the body weight of the fish.

It has been reported that the effective amount of *V. anguillarum* LPS to induce protection in ayu were 0.021 mg×10 times/kg body weight by oral administration against waterborne infection[5] and at a concentration of 10 μg/*l* for 3 min by immersion administration against intraperitoneal injection.[6] Effective administered dose of LPS in the present study can not be compared with those of oral or immersion administration because the mechanisms of immune stimulation and the following immune response may be different according to the administration method. Nevertheless, it is confirmed that the intraperitoneal injection with 0.05 mg crude LPS provided the fish acquires with the protective immunity against infection of *V. anguillarum*.

The passive hemagglutination showed a similar degree of antibody titer between using the crude LPS-coated SRBC and the purified LPS-coated SRBC. This could be an indirect confirm that LPS is the main immunogenisc substance in the crude LPS preparation. Electrosyneresis showed almost the same result as the passive hemagglutination against crude LPS with a little better sensitivity suggesting that it is a good method to detect precipitating antibody. As it is already known that LPS induces not only specific immune response but also nonspecific immune response that

contributes protective immunity, in fish,[18] further investigations especially on the latter response are still needed.

References

1) R. Kusuda, K. Kawai, Y. Jo, T. Akizuki, M. Fukunaga, and N. Kotake: *Nippon Suisan Gakkaishi*, **44**, 21–25 (1978).
2) T. Aoki and T. Kitao: *Fish Pathol.*, **13**, 19–24 (1978).
3) T. Itami and R. Kusuda: *Nippon Suisan Gakkaishi*, **44**, 1413 (1978).
4) K. Kawano, T. Aoki, and T. Kitao: *Fish Pathol.*, **18**, 185–190 (1984).
5) K. Kawai and R. Kusuda: *Nippon Suisan Gakkaishi*, **49**, 511–514 (1983).
6) T. Aoki, M. Sakai, and S. Takahashi: *Fish Pathol.*, **19**, 181–185 (1984).
7) F. Salati, K. Kawai, and R. Kusuda: *Fish Pathol.*, **19**, 187–192 (1984).
8) M. O. Saeed and J. A. Plumb: *Dis, aquat. Org.*, **2**, 21–25 (1987).
9) O. Westphal: *Int. Archs. Allergy Appl. Immun.*, **49**, 1–43 (1975).
10) Y. Ezura, K. Tajima, M. Yoshimizu, and T. Kimura: *Fish Pathol.*, **14**, 167–179 (1980).
11) O. Westphal and K. Jann: in "Methods in Carbohydrate Chemistry" (ed. by R. L. Whistler, J. N. BeMiller and M. L. Wolfrom), Vol. V, Academic Press, New York, 1969, pp. 139–143.
12) G. Talatsy: in "Basic Exercises in Immunochemistry" (ed. by A. Nowotny), Springer-Verlage, New York, 1969, pp. 139–143.
13) E. Neter, L. F. Bertram, D. A. Zak, M. R. Murdok, and C. E. Arbesman: *J. Exp. Med.*, **96**, 1–15 (1952).
14) A. Bussard and J. Huet: *Biochem. Biophys. Acta*, **34**, 258 (1959).
15) R. A. Good and B. W. Papermaster: in "Advance in Immunology" (ed. by F. J. Dixon, Jr. and J. H. Humphrey), Academic Press, New York, 1964, pp. 72–74.
16) R. Ishida: in "Yogyogaku Kakuron" (ed. by N. Kawamoto), Koseisha Koseikaku, Tokyo, 1967, pp. 229–251.
17) W. D. Paterson and J. L. Fryer: *J. Fish Res. Board Can.*, **31**, 1743–1749 (1974).
18) A. E. Ellis: *Dis. aquat. Org.*, **4**, 159–164 (1988).