ビブリオ病,連鎖球症およびBKDワクチンを投与したニジマスの白血球の化学発光反応

誌名	日本水産學會誌
ISSN	00215392
著者	酒井, 正博 小西, 正浩 厚田, 静男
巻/号	57巻2号
掲載ページ	p. 237-241
発行年月	1991年2月

Nippon Suisan Gakkaishi 57(2), 237–241 (1991)

The Chemiluminescent Response of Leucocytes from the Anterior Kidney of Rainbow Trout *Oncorhynchus mykiss* Vaccinated with *Vibrio anguillarum*, *Streptococcus* sp. or *Renibacterium salmoninarum*

Masahiro Sakai,* Masahiro Konishi, *Shizuo Atsuta,* and Masanori Kobayashi*

(Received July 2, 1990)

The activity of leucocytes isolated from the anetrior kidney of rainbow trout *Oncorhynchus mykiss* vaccinated with formalinkilled *Vibrio anguillarum*, β-haemolytic *Streptococcus* sp. or *Renibacterium salmoninarum* were investigated using the chemiluminesecnce (CL) assay. The vibrio and streptococcal bacterins were administrated by intraperitoneal injection or by the direct immersion method. The *R. salmoninarum* bacterin was inoculated intraperitoneally. The CL response of leucocytes from fish injected with vibrio or streptococcal bacterin was excellent against each opsonized bacterial cell. The leucocytes of each fish administrated by the immersion method also showed a high response level; however these emitted light levels were less than those of fish vaccinated by injection. The enhancement of CL response was not observed on the leucocytes of fish vacclnated with *R. salmoninarum* bacterin.

Vaccination is one of the essential methods for the control of infectious fish diseases, and many attempts have been reported on.[1] For fish vaccination, there are three method; injection, immersion and oral administration. Vaccination by immersion is readily applied to a large number of small fish and this method has been shown to provide adequate protection against vibriosis,[2,3] red mouth disease[4] and streptococcosis.[5] Fish vaccinated by the immersion method demonstrate excellent protection against the challenge test; however, the serum agglutinating antibody of vaccinated fish is at low levels[6] or is not detectable at all.[7,8] This fact indicates that the protective immunity of fish vaccinated by the immersion method can not be estimated by the serum agglutinating antibody levels. The results suggested that humoral immunity were not playing important roles as protective mechanisms. Sakai et al.[9,10] reported that the phagocytic activity was stimulated in fish vaccinated by the immersion method. These reports indicate that the phagocytic cells are activated in fish vaccinated by immersion.

Quantitative assessment of phagocytosis has been simplified since Allen *et al.*[11] first demonstrated that cells phagocytizing bacteria exhibit chemiluminescence (CL) of reactive oxygen intermediates such as the superoxide anion. This phenomenon was associated with respiratory burst and bactericidal activity of phagocytes. This CL assay has been applied in order to measure the phagocytosis of fish leucocytes.[12–15]

In this study, the CL response of leucocytes from the anterior kidney of vaccinated rainbow trout *Oncorhynchus mykiss* which were vaccinated with three kinds of bacterins were compared.

Materials and Methods

Fish

Rainbow trout with a mean weight of 50 g were obtained form Iwate Fisheries Experimental Station, Iwate. The fish were maintained with running water at 15°C, and fed daily with commercial pellets.

Baterin Preparation and Vaccination

In this study, *Vibrio anguillarum* PT479, β-haemolytic *Streptococcus* sp. SG8004 and *Renibacterium salmoninarum* KU8501 were used. The origin and culture media of these bacteria are shown in Table 1.

For the immersion vaccine, *V. anguillarum*

* School of Fisheries Sciences, Kitasato University Sanriku, Iwate 022–01, Japan. (酒井正博, 小西正浩, 厚田静男, 小林正典: 北里大学水産学部)

Table 1. Sources of bacteria used in this study

Bacterial Strain	Isolated in			Supplied by	Culture Media
	Place	Year	Fish		
Vibrio anguillarum PT479	Tokusima	1975	Ayu	Y. Jo	1% NaCl+TSA*1
Streptococcus sp. SG8004	Miyazaki	1980	Rainbow Trout	T. Kitao	BHI*2
Renibacterium salmoninarum KU8501	Iwate	1985	Coho Salmon	M. Sakai	KDM-C*3

*1 TSA: Trypto Soya Agar
*2 BHI: Brain Heart Infusion Agar
*3 KDM-C: Charcoal Agar

and *Streptococcus* sp. were used. These bacteria were cultured in each broth for 2 days at 30°C and were then killed by the addition of 0.3% formalin for 48 h at 4°C. This preparation was dilued with underground water to about 1×10^8 CFU/m*l* and used as the bacterin for immersion vaccination. For the injection vaccine, the formalin-killed bacteria were washed by centrifugation in sterile phosphate buffer saline (PBS, pH 7.2) which was then adjusted to a concentration of O. D. 1.0 at 520 nm.

R. salmoninarum was only used for the injection vaccine. Bacterium was cultured in KDM–C[16] for 15 days at 15°C and a vaccine was prepared by the same method for other species as well.

In the vaccine injected group, rainbow trout were anesthetized by MS–222 and injected intraperitoneally with 0.1 m*l* of each formalin-killed bacterin. Another group of fish was immersed in *V. anguillarum* or *Streptococcus* sp. formalin-killed bacterin for 3 min. Control fish were injected intraperitoneally with the same volume of PBS. These fish were maintained in tanks at 16°C and fed daily. The CL activity of leucocytes was examined 25 days after the vaccination.

Collection of Fish Sera

Fish serum was prepared by the method of Sakai *et al.*[9] The serum of 9 fish, from each fish group was pooled and used for the opsonin of each bacteria for the stimulants of the CL assay.

CL assay

The CL assay was examined by the previously described method.[17] Briefly, the leucocytes were isolated from the anterior hidney of fish and suspended in Hank's balanced salt solution without phenol red (HBSS). The cells were washed three times by centrifugation at 1200 rpm and adjusted to 5×10^6 cells/m*l*. Formalin-killed *V. anguillarum*, *Streptococcus* sp. or *R. salmoninarum* and vaccinated or nonvaccinated fish sera were mixed and incubated at 20°C for 30 min. These opsonized bacteria were then centrifuged three times and adjusted to a concentration of 10^7 cells/m*l* in HBSS.

The CL of leucocytes was determined by using Luminometer 1250 (Walic) at 20°C. A mixture of 0.1 m*l* luminol solution (1.4 mg/m*l*) and 0.3 m*l* of kidney leucocyte suspension in a glass tube was placed in the reaction chamber and 0.1 m*l* of the opsonized bacteria was added after the background count became constant. The emitted light was measured for 30 min. As in the preliminary experiment, individual variations were observed in the same group of fish, the kidneys from three fish of the same group were pooled before obtaining the leucocytes, and the CL response was tested. This experiment was repeated three times and the statistical difference (Student's t-test) was determined by comparing the peak emitted light of leucocytes sampled from each fish group.

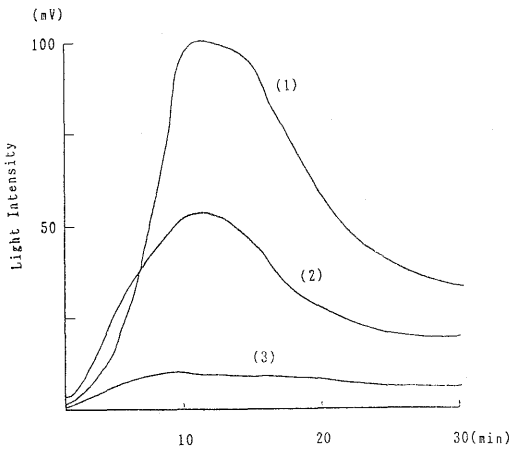

Fig. 1. The chemiluminescent responses in leucocytes of rainbow trout vaccinated with formalin-killed *Vibrio anguillarum*. Injection (1), Immersion (2), Control (3).

Table 2. The peak chemiluminesecnt responses in leucocytes of rainbow trout vaccinated with formalin-killed *Vibrio anguillarum*, β-haemolytic *Streptococcus* sp. or *Renibacterium salmoninarum*.

Vaccine	Method	Peak Emission (mV)	Significance
Vibrio anguillarum	Injection	97.5± 9.3*	$P<0.005$
	Immersion	51.9±10.2	$P<0.005$
	Control	12.1± 3.9	
Streptococcus sp.	Injection	179.6±55.1	$P<0.005$
	Immersion	98.1±15.1	$P<0.005$
	Control	39.0±10.3	
Renibacterium salmoninarum	Injection	11.2± 2.6	No Significance
	Control	9.8± 3.6	

* Mean±standard deviation.

Results

CL response

For vibrio vaccine, the high CL response was observed in the leucocytes of fish vaccinated by the injection method (Fig. 1), and the mean maximum levels reached 97.5 mV (Table 2). In the cells of fish vaccinated by the immersion method, the mean maximum CL level was 51.9 mV. However, in nonvaccinated fish leucocytes, which phagocytized with formalin-killed *V. anguillarum* opsonized by nonvaccinated fish sera, showed a low level of CL response. The statistical differences indicated between vaccinated and nonvaccinated fish leucocytes are shown in Table 2.

The CL responses of leucocytes in fish vaccinated by streptococcal bacterin were indicated in Fig. 2. The fish vaccinated by the injection method showed the highest stimulation in leucocytes phagocytized with *Streptococcus* sp. and the middle stimulation was observed in fish vaccinated by the immersion method. This pattern of CL response in leucocytes of fish administered with streptococcal vaccine was the same as the vibrio vaccine. However, the peak emitted levels in streptococcal vaccine were higher than that in the vibrio vaccine.

Vaccination with *R. salmoninarum* did not stimulate the CL of leucocytes (Fig. 3).

Statistical differences of the peak emitted light were indicated in the leucocytes of fish vaccinated with vibrio or streptococcal bacterin (Table 2).

Discussion

The CL assay is applied as a bioindicator of fish health and the effects of exposure to enviro-

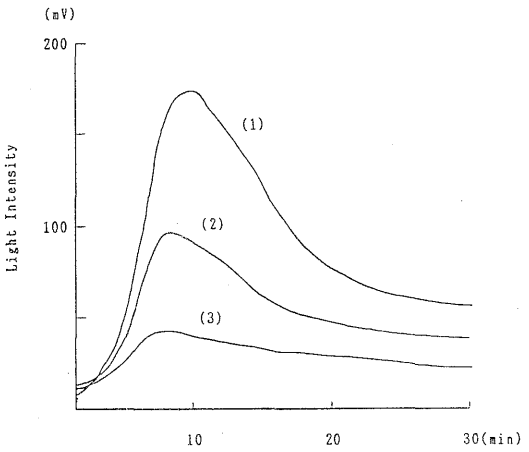

Fig. 2. The chemiluminecent responses in leucocytes of rainbow trout vaccinated with formalin-killed β-haemolytic *Streptococcus* sp. Injection (1), Immersion (2), Control (3).

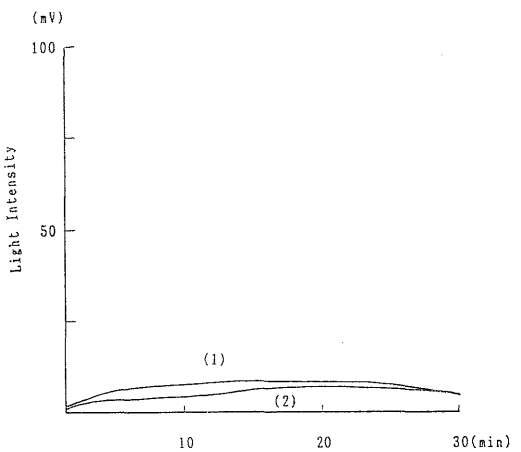

Fig. 3. The chemiluminescent responses in leucocytes of rainbow trout vaccinated with formalin-killed *Renibacterium salmoninarum*. Injection (1), Control (2).

mental stress. Stave and Roverson[18] indicated that phagcytic cells of striped bass *Morone saxatilis* exposed by hydrocortisone acetate reduced the CL responses. Wishkovsky *et al*[14]. reported that tetracycline and oxytetracycline caused the suppresion of CL responses emitted by phagocytes from the kidney of rainbow trout. Although these reports did not examine the relationship between the suppresion of the CL response and the susceptibility to infection, the increase of susceptibility to infection might occur in these stressed fish. The CL response also relates to the virulence of certain pathogens. Stave *et al*.[18,19] reported that the virulence strains of *Vibrio* spp. or *Yerisinia ruckeri* circumvented the stimulation of CL response generating metabolism of fish phagocytes. Thus, the CL assay is a useful method to determine the immune function of fish phagocytes.

The role of phagocytic cells in vaccinated fish is still unclear. It is well known that salmonid fish immersed in vibrio bacterin attained the protective immunity against vibrio infection. However, the mechanism of this protective immunity is still unknown. Cory and Amend[7] and Aoki *et al*[8]., reported that in the vibrio immersion vaccine, the vaccinated fish the serum agglutinating antibody titer was not detected. Thus, the role of agglutinating antibodies is still unclear in the fish that received vibrio immersion vaccine. On the other hand, the cell-mediated immunity (CMI) in vaccinated fish has not been yet discussed. In this study, we examined the activation of phagocytic cells in vaccinated fish using CL assay. The CL resposes significantly increased in leucocytes of vaccinated fish. These results suggest that CMI is stimulated by the vaccination with vibrio vaccine.

The β-haemolytic streptococcal disease is widely distributed in freshwater fish. Succcssful vaccination was already reported by Sakai *et al*.[5] In this study, vaccination was examined by the method of Sakai *et al*.[10] and rainbow trout immunized with streptococcal bacterin also increased in the CL responses of leucocytes. Sakai *et al*.[10] reported that the phagocytic activities increased in fish vaccinated by injection or immersion with streptococcal bacterin and antibodies worked as opsonin. In the case of streptococcal vaccine, the importance of CMI is indicated in these experiments.

Bacterial kidney disease, caused by *R. salmoninarum*, is a systemic and clonic infection of salmonid fishes characterized by the presence of granulomatous lesions. The efficacy of the vaccine against BKD is still unclear. Sakai *et al*.[20] reported that the serum agglutinating antibody titers were detected in rainbow trout injected by *R. salmonarum* bacterin. Leucocyte phagocytosis also increased in these fish. However, protection was not evident in these vaccinated fish.[20] In this study, rainbow trout was vaccinated by the method of Sakai *et al*.[20] and the CL response did not increase in leucocytes of these vaccinated fish. Young and Chapman[21] reported that the phagocytized *R. salmoninarum* cells could survive into fish macrophage. To evaluate the protective immunity against *R. salmoninarum*, the stimulation of bactericidal activity into macrophage might be required.

In this study, we demonstrated that rainbow trout immunized with vibrio and streptococcal vaccine by the injection or immersion method increased their CL response of leucocytes. In the casc of *R. salmoninarum*, the CL was not stimulated by the injection vaccination. A further study is neccessary to explain the correlation between CL response and protection.

Acknowledgements

We thank Mrs. M. Kitajima and K. Tsuchidate for their technical assistance.

References

1) A. E. Ellis: in "Fish vaccination" (ed. by A. E. Ellis), Academic Press, London, 1988, pp. 1–247.
2) R. Antipa and D. F. Amend: *J. Fish. Res. Bd. Can.*, **34**, 203–208 (1977).
3) R. W. Gould, R. Antipa and D. F. Amend: *J. Fish. Res. Bd. Can.*, **36**, 222–225 (1979).
4) K. A. Johnson, J. K. Fiynn, and D. F. Amend: *J. Fish. Dis.*, **5**, 207–223 (1982).
5) M. Sakai, R. Kubota, S. Atsuta, and M. Kobayashi: *Nippon Suisan Gakkaishi*, **53**, 1376–1376 (1987).
6) M. Sakai, T. Aoki, T. Kitao, J. S. Rohovec, and J. L. Fryer: *Nippon Suisan Gakkaishi.*, **50**, 1187–1192 (1984).
7) T. R. Cory and D. F. Amend: *Aquaculture*, **12**, 317–325 (1977).
8) T. Aoki, T. Kitao, M. Fukudome, S. Takahashi, and S. Egusa: *J. Fish. Dis.*, **7**, 149–156 (1984).
9) M. Sakai, S. Atsuta, and M. Kobayashi: *Fish Path.*, **3**, 169–173 (1989a).
10) M. Sakai, S. Atsuta, and M. Kobayashi: in "The Second Asian Fisheries Forum" (ed. by R.

Hirono and I. Hanyu), The Asian Fisheries Society, Manila, 1990, pp. 689–691.
11) N. Allen, R. C. Dtjernholm, and R. E. Steele: *Biochem. Biophys. Res. Commum.*, **47**, 679–684 (1972).
12) A. L. Scott and P. H. Klesius: *Dev. Biol. Standard.*, **49**, 243–254 (1981).
13) J. W. Stave and B. S. Roberson: *Dev. Com. Immun.*, **9**, 77–84 (1985).
14) A. Wishkovsky, B. S. Roberson, and F. M. Hetrick: *J. Fish Biol.* **31**, Supplement A., 61–65 (1987).
15) M. Cossarini-Dunier: *J. Fish Biol.* **31**, Supplement A, 67–73 (1987).
16) J. G. Daly and R. M. W. Stevenson: *Appl. Environ. Microbiol.*, **50**, 6868–871 (1985).
17) M. Sakai, S. Atsuta, and M. Kobayashi: in "Current Topics in Marine Biotechnology" (ed. by S. Miyachi, I. Karaube, and Y. Ishida) The Japanese Society for Marine Biotechnology, Tokyo, 1989, pp. 345–348.
18) J. W. Stave, B. S. Roberson and F. M. Hetrick: *J. Fish Dis.*, **8**, 479–483 (1985).
19) J. W. Stave, T. M. Cook and B. S. Roberson: *J. Fish Dis.*, **10**, 1–10 (1987).
20) M. Sakai, S. Atsuta, and M. Kobayashi: *Nippon Suisan Gakkaishi*, **55**, 2105–2109 (1989b).
21) C. L. Young and G. B. Chapman: *J. Fish. Res. Bd. Can.*, **35**, 1234–1248 (1978).

Nippon Suisan Gakkaishi : Formerly *Bull. Japan. Soc. Sci. Fish.*