Vaccination Against Red Sea Bream Iridoviral Disease in Red Sea Bream

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The effectiveness of vaccination against red sea bream iridoviral disease (RSIVD) was evaluated using two kinds of vaccines in red sea bream (*Pagrus major*). For one vaccine, RSIV-infected GF cells were inactivated with formalin (1.0% v/v) for 10 days at 4°C and for the other the cell culture supernatant of RSIV-infected GF cells was inactivated with formalin (0.3% v/v) for 10 days at 4°C. Juvenile red sea bream were intraperitoneally injected with vaccines and after 10 days the fish were RSIV-challenged by intraperitoneal injection. Statistical analysis showed significantly high survival rates in the vaccinated groups than that of the respective control groups, indicating the protection is induced by the vaccination.

In addition, the expression of the virus specific antigens in the spleen was examined for vaccinated and control fish after RSIV challenge by immunofluorescence test. The result that the expression of antigens was weaker in the vaccinated fish compared with the control fish supported the efficacy of the vaccination.

Outbreak of red sea bream iridoviral disease (RSIVD) first occurred in cultured red sea bream (*Pagrus major*) in Shikoku Island, Japan in 1990. Since then, RSIVD has been reported for 20 species of cultured marine fishes (Matsuoka *et al.*, 1996) and become one of the most threatening viral disease in cultured marine fishes such as red sea bream, yellowtail (*Seriola quinqueradiata*), sea bass (*Lateolabrax* sp.) and Japanese parrot fish (*Oplegnathus fasciatus*). For disease diagnosis, Giemsa staining and immunofluorescence (IF) test using a monoclonal antibody (MAb) have been used (Inouye *et al.*, 1992, Nakajima and Sorimachi, 1995, Nakajima *et al.*, 1995). However, no control measures for the disease have been reported. In this report, we describe the efficacy of vaccination against RSIVD in juvenile red sea bream. We evaluated it both from the protection and the appearance of viral antigens in the spleen after experimental challenge with RSIV.

Materials and Methods

Virus and cells

The grunt fin (GF) cell line (Clem *et al.*, 1961) was grown at 25°C in Basal Medium Eagle (BME) supplemented with 10% fetal calf serum, penicillin 100 I.U./ml and streptomycin 100 µg/ml. RSIV Ehime-1 strain was propagated in GF cells at 25°C.

Vaccine preparation

Two kinds of vaccines, formalin inactivated RSIV-infected GF cells and formalin inactivated cell culture supernatant of RSIV-infected GF cells were prepared. For the preparation of the vaccine from RSIV-infected GF cells, RSIV was inoculated onto GF cells and incubated for 7 days until a distinct CPE developed. Then the RSIV-infected cells were collected by scraping and centrifuged at 2,500×g for 10 min at 4°C. The cells were suspended in PBS (–) and formalin 1% (v/v) was added. The suspension was kept at 4°C for 10 days for inactivation. After centrifugation, cells were collected, suspended in PBS (–), adjusted to a final concentration of about 10⁸ cells/ml and used for vaccination. For the control, formalin inactivated non-infected GF cells were prepared and used for injected control in the same manner.

For the preparation of a vaccine from the supernatant of RSIV-infected GF cells, RSIV-infected GF cells and non-infected GF cells were separately cultured from those for the preparation of the vaccine from RSIV-infected GF cells. The culture medium of RSIV infected GF cells was centrifuged at 2,500 × g for 10 min at 4°C. Formalin (0.3% v/v) was then added to the supernatant which contained 10^{3.5} TCID₅₀/0.1 ml of the virus, and the virus was inactivated for 10 days at 4°C. The cell culture supernatant of non-infected GF cells was treated in the same manner and used for the injected control.

Experimental fish

Hatchery reared red sea bream of 2–10 g body weight were used for the experiment.

Vaccination experiments

Three kinds of experiments were carried out. Experiment 1 (Exp. 1) was designed to determine the efficacy of different types of vaccines. The experiment consisted of 2 sets of 5 groups. Each group had 30 fish and were kept in a 60l aquarium supplied with running sea water of controlled temperature at 25°C. In each set, four groups were intraperitoneally injected with 0.1 ml of one of the following preparations: RSIV-infected GF cells, cell culture supernatant of RSIV-infected GF cells, non-infected GF cells and cell culture supernatant of non-infected GF cells. The other group received no injection. Ten days after the vaccination all the fish were challenged by intraperitoneal injection with 0.1 ml of RSIV (Ehime-1 strain) solution. Challenge test was conducted at 2 levels of infection. In Exp. 1A, one set of 5 group fish were challenged with RSIV solution of $10^{3.5}$ TCID₅₀/0.1 ml and in Exp. 1B another set of 5 group fish were challenged with $10^{4.5}$ TCID₅₀/0.1 ml. Other experimental conditions were the same for both experiments. After the challenge, mortality was examined over 14 days.

Experiment 2 (Exp. 2) was designed to determine the optimum concentration of vaccine of cell culture supernatant of RSIV-infected GF cells. The supernatant was diluted with BME to make virus titers of $10^{4.5}$, $10^{3.5}$ and $10^{2.5}$ TCID₅₀/0.1 ml and inactivated by formalin as described above. The experiment consisted of 5 groups. Each group had 25 fish and were kept in a 60l aquarium as in Exp. 1. Fish of 3 experimental groups were injected with 0.1 ml of vaccine of the three respective concentrations and one of the remaining groups received cell culture supernatant of non-infected GF cells as injected control. Another control group received no injection. Ten days after the vaccination, all the fish were injected with 0.1 ml of RSIV solution of $10^{4.0}$ TCID₅₀/0.1 ml and thereafter mortality was recorded daily for 14 days.

Experiment 3 (Exp. 3) was conducted to determine the effect of vaccination on the appearance of viral antigens in RSIV-infected fish. The experiment consisted of 2 groups. Each group had 25 fish kept in a 60l aquarium as in Exps. 1 and 2. The fish of experimental group was injected with vaccine of cell culture supernatant of RSIV-infected GF cells and the fish of control group with that of the cell culture supernatant of

non-infected GF cells. Ten days after vaccination all the fish were injected with 0.1 ml of RSIV solution of $10^{3.0}$ TCID₅₀/0.1 ml and 1, 3, 5 and 9 days after the challenge, three fish were randomly sampled from both groups. From each fish the spleen was removed, bisected and imprinted onto a clean slide glass. The virus antigen was detected by indirect IF test using a MAB against RSIV as described previously (Nakajima *et al.*, 1995).

Statistical analysis was carried out using χ^2 test. The relative percentage survival (RPS: Amend, 1981) was also determined by the formula $RPS = (1 - (\% \text{ loss of immunization fish} / \% \text{ loss of control})) \times 100$.

Results

Exp. 1

Cumulative mortalities during Exp. 1 are shown in Fig. 1A and 1B and statistical analyses of cumulative mortalities at the end of the experiment are summarized in Table 1. Mortality occurred between 4 and 13 days after the viral challenge and the results of experiments of 2 different challenge doses showed good agreement. As shown in Table 1, the group vaccinated with RSIV-infected GF cells showed significantly ($P < 0.01$) lower mortality compared to the two control groups: injected with non-infected GF cells and non-injected groups. Similarly, the group vaccinated with cell culture supernatant of RSIV-infected GF cells showed significantly ($P < 0.01$) lower mortality compared to the two control groups: injected with cell culture supernatant of non-infected GF cells and non-injected groups. RPS values for both types of vaccines were also high ranging from 68 to 100. Thus, both types of vaccines showed significantly high levels of protection against experimental RSIV infection.

Exp. 2

Cumulative mortality in Exp. 2 is shown in Fig. 2. Mortality occurred between 6 and 13 days after the viral challenge. Statistical analysis of the final cumulative mortality is summarized in Table 2. As shown in Table 2, those groups vaccinated with cell culture supernatant of RSIV-infected GF cells at doses of $10^{2.5}$ and $10^{3.5}$ TCID₅₀/0.1 ml showed a significantly lower mortality ($P < 0.01$) than the control group. However, the mortality in the group vaccinated at the dose of $10^{1.5}$ TCID₅₀/0.1 ml was the same to the control group. Thus, the minimum effective concentration of the vaccine is considered to be between $10^{2.5}$ and $10^{1.5}$ TCID₅₀/0.1 ml.

Fig. 1. Mortality of vaccinated fish challenged with different doses RSIV. Juvenile red sea bream were vaccinated by intraperitoneal injection either with formalin inactivated RSIV-infected GF cells (○), formalin inactivated cell culture supernatant of RSIV-infected GF cells (●), formalin inactivated non-infected GF cells (□) or formalin inactivated cell culture supernatant of non-infected GF cells (■). One group of fish (△) received no injection. Ten days after vaccination, all the fish were challenged with RSIV at doses of $10^{3.5}$ TCID₅₀/0.1 ml (A) or $10^{4.5}$ TCID₅₀/0.1 ml (B).

Table 1. Protection against experimental RSIV infection by two types of vaccines in juvenile red sea bream

	Experimental group (Vaccines)	No of challenged fish	% Mortality	χ^2 test	RPS
Exp. 1A	RSIV-infected GF cells	30	13	$P < 0.01$	81
	Cell culture supernatant of RSIV-infected GF cells	30	0	$P < 0.01$	100
	Non-infected GF cells	30	67		
	Cell culture supernatant of non-infected GF cells	30	50		
	Non-injected	30	43		
Exp. 1B	RSIV-infected GF cells	30	7	$P < 0.01$	86
	Cell culture supernatant of RSIV-infected GF cells	30	20	$P < 0.01$	68
	Non-infected GF cells	30	50		
	Cell culture supernatant of non-infected GF cells	30	63		
	Non-injected	30	70		

Fish were challenged by intraperitoneal injection with RSIV 10 days after the vaccination.

Challenge doses were $10^{3.5}$ TCID₅₀/0.1 ml/fish for Exp. 1A and $10^{4.5}$ TCID₅₀/0.1 ml/fish for Exp. 1B.

Mean total length and body weight of fish at the beginning of vaccination: 5.5 cm, 3.2 g (Exp. 1A), 7.5 cm, 7.8 g (Exp. 1B).

Exp. 3

Appearance of viral antigen in the spleen in Exp. 3 is summarized in Table 3. In the vaccinated group, viral antigen was detected in only 1 fish 5 days after the challenge. In contrast, in the control group the viral antigen was detected in all the fish examined both 5 and 9 days after the challenge. In addition, enlarged IF positive cells were observed only in the control group.

Discussion

The present study clearly shows the effectiveness of

vaccines of both formalin-inactivated RSIV-infected GF cells and cell culture supernatant of RSIV-infected GF cells against RSIVD. Most vaccine experiments against viral diseases have also been conducted using inactivated viruses such as IPNV (Dorson 1988, Tatner 1993), IHN (Leong *et al.* 1988, Tatner 1993), VHS (de Kinkelin 1988, Tatner 1993), CCV (Plumb 1988, Tatner 1993) and SVC (Fijan 1988), though some trials have recently been made using component vaccines (Gilmore *et al.*, 1988, Lecocq-Xhonneux *et al.*, 1994). Difficulty of mass production of viral antigens sometimes become an obstacle for practical application of vaccines. The re-

Fig. 2. Effect of vaccine concentrations on the protection against RSIV infection. Juvenile red sea bream were vaccinated with different doses of formalin inactivated cell culture supernatant of RSIV-infected GF cells. The virus titers of the vaccine before inactivation were $10^{3.5}$ TCID₅₀/0.1 ml (○), $10^{2.5}$ TCID₅₀/0.1 ml (●) or $10^{1.5}$ TCID₅₀/0.1 ml (□). The fish of a control groups were injected with formalin inactivated cell culture supernatant of non-infected GF cells (■) and the fish of another groups were not injected (△). Ten days after vaccination, all the fish were challenged with RSIV.

sult of the present study that vaccine of the cell culture supernatant of RSIV-infected cells is as equally potent as that of RSIV-infected cells in inducing protection indicates efficacy of the whole cell culture of RSIV-infected GF cells. A continuous cell culture system may efficiently provide enough amount of RSIV culture. In

addition, it is found that red sea bream smaller than 50 g in body weight usually show high sensitivity to RSIVD under experimental conditions. This means that injection to small fish, requiring only a small amount of vaccine, may be practical for the vaccination in the hatchery.

In a preliminary experiment to select vaccination period, fish were vaccinated for 10, 20, 30, 40 or 60 days and then challenged with RSIV (data not shown). The effectiveness of the vaccine was shown in the fish vaccinated for 10, 20 or 30 days, but the effectiveness was not evaluated in the fish vaccinated for 40 or 60 days by low mortality in the control fish. This is because that red sea bream usually become increasingly resistant to RSIVD after the vaccination for 40 or 60 days under experimental conditions. Therefore, fish were vaccinated for 10 days in this experiment.

We conveniently expressed the quantity of the antigen in the vaccine preparation as TCID₅₀ of RSIV, however immune protection is not always induced with only the complete viral virion. It is possible to consider that some peptides such as virus-induced polypeptides in the RSIV-infected cell culture also had potent substances to induce immunity. In fact we have shown that the MAb which was used for the detection of viral antigens in the present experiment recognized 230–180 kDa polypeptide which may possibly be a virus-induced polypeptide rather than the structural protein of the virus (Nakajima & Sorimachi, 1995). What kind of epitope in the vaccine contributes to induce the immunity is now being studied in our laboratory.

Exp. 3 showed the decrease in the appearance of RSIV antigen in the spleen of vaccinated fish, probably indicating the depression of viral replication in the organ. This may show that the protection by the vaccination is

Table 2. Effect of vaccine concentrations on the protection against experimental RSIV infection in juvenile red sea bream

Vaccine concentration administered ^{a)}	No. of challenged fish	Specific fish losses	% Mortality	χ^2	RPS
$10^{3.5}$	25	11	44	$P < 0.01$	50
$10^{2.5}$	25	12	48	$P < 0.01$	45
$10^{1.5}$	25	22	88		
Injected control ^{b)}	25	22	88		
Non-injected	25	22	88		

^{a)} TCID₅₀/0.1 ml/fish of cell culture supernatant of RSIV-infected GF cells.

^{b)} Cell culture supernatant of non-infected GF cells.

Fish were challenged by intraperitoneal injection with RSIV ($10^{4.0}$ TCID₅₀/0.1 ml/fish).

Mean total length and body weight at beginning of vaccination: 7.2 cm, 7.0 g.

Table 3. Detection of viral antigen in the spleen of vaccinated juvenile red sea bream after challenge of RSIV by indirect immunofluorescence test

Days after infection	Vaccinated ^{a)}			Control ^{b)}		
1	–	–	–	–	–	–
3	–	–	–	–	–	–
5	+	–	–	+	++	+
9	–	–	–	++	+	+

^{a)} Cell culture supernatant of RSIV-infected GF cells.

^{b)} Cell culture supernatant of non-infected GF cells.

–: Immunofluorescence-positive cells were not observed.

+: Immunofluorescence-positive cells were observed.

++: Immunofluorescence-positive cells including enlarged cells were observed.

virus specific rather than nonspecific protection because specific antibodies against virus structural and virus induced polypeptides were detected in the serum of vaccinated fish by radio-immunoprecipitation (unpublished data).

How the vaccination induces immunity or what is the potential antigen of the virus are not known. Determination of the virus neutralizing antibody titer in the serum of vaccinated fish is necessary to identify protective antigen or to specify specific immune response. These studies may be carried out using purified antigens.

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