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Phagocytic Response of Peritoneal Exudate Cells of the Japanese Eel against *Vibrio vulnificus*

Teruo MIYAZAKI and Keikichi KURATA

Faculty of Fisheries, Mie University

We studied histopathological features of the Japanese eel *Anguilla japonica* that were experimentally infected by the intraperitoneal injection with attenuated and virulent *Vibrio vulnificus* (3.2×10^7 CFU / 100 g body weight), and also studied the vitro phagocytic activity of peritoneal exudate cells (PEC) of eels, unvaccinated and vaccinated with the sonicated bacterin, against the viable bacteria. Eels that were infected with virulent bacteria were moribund within 24 hrs, and showed the systemic infection followed by necrosis and hemorrhage in visceral organs. Eels that were infected with attenuated bacteria survived for 7 days and showed bacterial phagocytosis of the peritoneal exudate cells, and slight necrosis and hemorrhage with bacterial invasions in the intestine on the third day.

PEC that were induced by intraperitoneal injection with protease peptone solution, contained macrophages and neutrophils. PEC of unvaccinated fish showed slight phagocytosis against attenuated bacteria but heavy phagocytosis and intracytoplasmic multiplication of virulent bacteria. PEC of vaccinated fish displayed significantly increased phagocytic activity against attenuated bacteria on the 7th and 14th days. They showed significantly increased phagocytic activity against virulent bacteria on the 14th day, and the preventive activity of bacterial intracytoplasmic multiplication on 7th day and it was significantly increased on 14th day.

Key words : PEC. Eel. *V. vulnificus*

Vibrio vulnificus causes vibriosis of the Japanese eel *Anguilla japonica* (MUROGA *et al.* 1976, MIYAZAKI *et al.* 1977). Histopathological features of both natural and experimental cases of this vibriosis were characterized by extensive bacterial invasions and multiplication, edema, hemorrhage, tissue necrosis and very poor response of inflammatory cells in the lesions (MIYAZAKI *et al.* 1977, MIYAZAKI 1980). On the other hand, eels that had been

vaccinated by the sonicate of *V. vulnificus* could resist the viable bacterial injection and exhibited bacteria-phagocytosis of inflammatory cells (MIYAZAKI and KUBOTA 1981). This result indicated that phagocytic response of inflammatory cells was an important defence mechanism of eels. This study examined histopathological features of eels that were injected with attenuated and virulent strain of V. *vulnificus*, and phagocytic response of peritoneal exudate cells of fish, unvaccinated and vaccinated, against the attenuated and virulent bacteria.

Materials and Methods

Bacterium

Examined bacterium was *Vibrio vulnificus* ET-7617 which was obtained through the courtesy of Professor K. MUROGA (Hiroshima Univ.). The primary strain (attenuated strain) was so less virulency that intraperitoneal injection with the viable bacteria at the rate of 35 mg per 100 g body weight of eel could kill the fish within 24 hrs. The bacteria were passed six times in eels and acquired the virulence as strong as the intraperitoneal injection at the rate of 0.5 mg per 100 g body weight of eel could kill the fish within 24 hrs (virulent strain). The bacteria were grown for 24 hrs at 20 ℃ in BHI agar plates and broth (Nissui) containing 1.5 % NaCl.

Fish

Japanese eels weighing 100-200 g were used and held in 60 l aquaria at a water temperature of 23-25 ℃ during the experiment.

Bacterial injection and vaccination

Virulence of attenuated and virulent bacteria against eel was evaluated as follows. Cultures of both attenuated and virulent bacteria were suspended in sterilized saline and intraperitoneally injected at the rate of 0.5 mg (3.2×10^7 CFU/ml) per 100 g body weight of eel. These experimental fish were held in aquaria and observed for 7 days at the longest. To make the sonicated bacterin, the culture of virulent bacteria was washed three times with saline by centrifugation at 3600 rpm, and the packed cells weighing 300 mg were resuspended in 10 ml saline and then sonicated (Ultra sonic disruptor, Tomy-Seiko) with 30 W/hr for 20 min. The sonicated bacterin was diluted with sterilized saline and intramuscularly injected at the rate of 15 mg/0.5 ml an eel.

Collection of peritoneal exudate cells

2 ml of 10 % protease peptone was intraperitoneally injected into fish and after 20 hrs, the peritoneal cavity was washed carefully with 2 ml of cold sterilized saline ten times and peritoneal exudate cells (PEC) were collected by centrifugation at 1800 rpm at 4 ℃, for 5 min.

Table 1. Methods to collect peritoneal exudate cells (PEC)

Vaccination : Intramuscular injection with sonicated Vibrio at the rate of 15 mg/ 0.5 ml a fish (100-200 g)

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Holding for 1, 3, 7 and 14 days at 23-25 ℃ in aquaria

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Induction of PEC : Intraperitoneal injection with 10 % protease peptone solution at the rate of 2 ml a fish

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Holding for 20 hrs at 23-25 ℃ in aquaria

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Collection of PEC : Intraperitoneal injection with 2 ml of sterilized cold saline and withdrawal of the cell-suspending saline 10 times

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Washing PEC with cold sterilized salin and centrifugation (1800 rpm, 5 min.) 3 times

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Phagocytosis : PEC suspension in 1.5 ml of Hank's solution and addition of 0.005 mg of viable Vibrio

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Incubation for 3 and 24 hrs at 21 ℃

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Collection and washing of PEC with 1.5 ml of sterilized cold saline and centrifugation (1800 rpm, 5 min.) 3 times, and cell suspension in sterilized cold saline

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Smear. Fixation with methanol. Staining with Giemsa. Observation.

Collection of peritoneal exudate cells

2 ml of 10 % protease peptone was intraperitoneally injected into fish and after 20 hrs, the peritoneal cavity was washed carefully with 2 ml of cold sterilized saline ten times and peritoneal exudate cells (PEC) were collected by centrifugation at 1800 rpm at 4 ℃, for 5 min. When PEC contained erythrocytes, PEC were separated with Histopaque (1.077 density, Sigma) and centrifugation. PEC were resuspended in cold sterilized saline and washed with centrifugation three times. PEC of one fish were suspended in 1.5 ml of cold Hank's solution (pH 7.2, Nissui) in two glass tubes.

Phagocytic activity of PEC

Phagocytic activity of PEC was evaluated as follows. Viable bacteria (0.005 mg, 3.2×10^5 CFU/ ml) were inoculated into 1.5 ml of PEC suspension. After incubation at 20 ℃ for 3 and 24 hrs, PEC were washed three times with sterilized cold saline and centrifugation. PEC were resuspended into cold saline, smeared on slide glasses and after fixation with methanol, stained with Giemsa and PAS. Unfixed specimen were treated with the peroxidase

reaction (Sato-Sekiya method). Bacteria-laden cells were counted per 500 cells of total cell count under microscope.

Histopathological examination

Eels injected with attenuated bacteria were killed on the third day and visceral organs were fixed with 10 % formalin solution. Eels injected with virulent bacteria became moribund between 18 and 24 hrs and the moribund fish were taken up and visceral organs were fixed with 10 % formalin solution. These specimens were thin-sectioned and stained with Hematoxylin-eosin (H-E) and Giemsa.

Results

Experimental infection

Eels injected with attenuated bacteria showed slight hemorrhage in the injected sites on the second day and survived for at least 7 days. Some of them were taken on the third day and showed partial hemorrhage in the stomach, intestine and peritoneum. The stomach and intestine histopathologically showed bacterial invasions, hemorrhage and necrosis in the muscle layers and submucosa. The peritoneum displayed infiltration of bacteria-laden inflammatory cells (**Plate I-1**). The liver, spleen, kidney, heart and gills histopathologically showed no evidence of bacterial invasions and pathological change.

Eels injected with virulent bacteria became moribund between 18 and 24 hrs displaying the swollen abdomen with hemorrhage in the surface, the prolapsed anus, extensive hemorrhage in the digestive tracts and peritoneum, bloody ascitic fluid, a congested liver, a swollen spleen and kidney. The intestine extensively displayed bacterial invasions and multiplication in the muscle layers and submucosa, in which hemorrhage and necrosis were observed to involve the muscle layer, submucosa and villi (**Plate I-2**). The stomach extensively showed bacterial invasions, hemorrhage and necrosis in the muscle layers and submucosa, and separation of the mucous epithelium. The spleen was completely necrotized and hemorrhagic with bacterial invasions. The liver showed congestion and atrophied hepatic cells. The kidney showed necrosis involving glomeruli, renal tubules and hematopoietic tissue with bacterial invasions. Infiltraton of inflammatory cells could not be observed in the peritoneum.

Phagocytosis of PEC

PEC contained neurtophils and macrophages. Both cells exhibited bacterial phagocytosis and the subsequent degeneration and destruction due to intracytoplasmic multiplication of the bacteria when they were inoculated with viable bacteria. The evaluation of phagocytic activity of PEC included the data of these two types of cells because these cells of the phagocytic condition were indistinguishable.

When PEC of two unvaccinated control fish were inoculated with attenuated bacteria, 5.8-7.2 % of PEC phagocytized bacteria, in which 1 to 4 bacterial cells were contained

after 3 hrs incubation (**Plate I-3**). The other 1.0-1.6 % of PEC showed the destruction and intracytoplasmic multiplication of bacteria. After 24 hrs incubation, 8.4-9.6 % of PEC displayed bacterial phagocytosis and the other 2.2-2.4 % of PEC showed the destruction with intracytoplasmic bacterial multiplication (**Table 2**). Phagocytic activity of PEC of vaccinated fish against attenuated bacteria were shown in **Table 2**. Most phagocytic PEC included 1 to 4 bacterial cells and even after 24 hrs incubation, destroyed PEC were in small numbers. Their phagocytic activity markedly increased to 16.4 % on the 7th day and to 24.2-25.4 % on the 14th day, and the rates of degenerated PEC tended to slightly decrease on the 14th day post vaccination.

Table 2. Phagocytic response of peritoneal exudate cells against viable *Vibrio vulnificus*

Strains	attenuated strain				virulent strain			
Incubation (hrs)	3		24		3		24	
Unvaccinated fish	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2
Phagocytosis	7.2*	5.8	8.4	9.6	18.2			
Destruction**	1.6*	1.0	2.2	2.4	37.6		100	
Vaccinated, lst day	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2
Phagocytosis	8.8		11.6		19.6			
Destruction	2.8		3.6		35.0		100	
Vaccinated, 3rd day	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2
Phagocytosis	8.6		9.8		17.4			
Destruction	2.4		2.0		22.2		100	
Vaccinated, 7th day	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2
Phagocytosis	16.4		17.0		24.4	20.8		
Destructin	2.4		3.0		22.6	18.2	100	100
Vaccinated, 14th day	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2
Phagocytosis	25.4	24.2	21.2	22.7	24.8	40.2		
Destruction	2.2	0.4	2.2	1.4	15.2	16.4	100	100

* : percentage of cells. Cell count : 500 cells

* * : destruction of bacteria-laden cells due to bacterial multiplication in cells

When PEC of an unvaccinated control fish were inoculated with virulent bacteria, they phagocytized many bacterial cells and were subsequently destroyed by intracytoplasmic multiplication of bacteria (**Plate I-4**). 18.2 % of PEC exhibited bacterial phagocytosis and the other 7.6 % of PEC were destroyed after 3 hrs incubation and all PEC were destroyed after 24 hrs incubation (**Table 2**). Hank's solution as medium of PEC changed color from pink to yellow indicating bacterial multiplication in the solution and actually many bacteria were observed in the smeared specimens after 3 and 24 hrs incubation. Phagocytic activity of PEC of vaccinated fish against virulent bacteria was shown in **Table 2**. In this combination, the phagocytic activity mostly tended to be constant from the lst to 14th day post vaccination. However, the rates of destroyed cells decreased to 18.2 % on the 7th day and 15.2 - 16.4 % on the 14th day post vaccination (**Plate I-5**).

Discussion

In results on experimental infection, eels injected with virulent bacteria became moribund between 18 and 24 hrs and showed systemic infection followed by necrosis and hemorrhage in visceral organs. On the other hand, eels that were injected with attenuated bacteria survived for at least 7 days and showed bacteria-phagocytosis of peritoneal exudate cells in the peritoneum on the third day. This finding indicated that peritoneal exudate cells (PEC) phagocytized attenuated bacteria and prevented the systemic bacterial dissemination. Results on phagocytic activity of PEC against attenuated bacteria also showed small numbers of bacteria-laden PEC being destroyed by the bacterial multiplication. These results indicated that attenuated bacteria could not multiply in PEC and failed in establishing the infection. On the other hand, although virulent bacteria might be phagocytized by PEC, the bacteria could multiply inside them, destroy them and establish the infection.

PEC of vaccinated fish showed increased phagocytic activity against attenuated bacteria on the 7th and 14th days post vaccination. The rates of PEC phagocytizing virulent bacteria were mostly constant after vaccination but the ratio of destroyed cells significantly decreased on the 7th and 14th days post vaccination. These results indicated that PEC of vaccinated fish increased the phagocytic activity and preventive activity of intracytoplasmic bacterial multiplication on the 7th to 14th days. Phagocytic activity of blood leukocytes and PEC of vaccinated fishes were examined between *Edwardsiella tarda* and Japanese eel (SONG and KOU 1981), between *Aeromonas salmonicida* and rainbow trout (SAKAI 1984), and between *Vibrio anguillarum* and rainbow trout (HONDA *et al.* 1985). SAKAI (1984) reported that PEC of vaccinated rainbow trout did not show any evidence of increased phagocytic activity at least after 2 weeks. HONDA *et al.* (1985) reported that macrophages (glass adherent cells) which were provided from PEC and peripheral blood of vaccinated rainbow trout showed the significantly increased activity of phagocytosis as long as 5 weeks after. They also described that in vaccinated fish, some protective reaction excepting antibody and macrophage phagocytosis, could work to inhibit Vibrio infection as fast as in one week. In

their study, neutrophils were not examined. On the other hand, in our study, PEC included both macrophages and neutrophils and they showed significantly increased preventive activity of intracytoplasmic bacterial multiplication on the 7th day post vaccination. MIYAZAKI *et al.* (1981) observed neutrophils of vaccinated eels acquiring Vibrio-phagocytic activity in the tissues. Although it might be difficult to compare results on eel and rainbow trout, there is the possibility that neutrophils will act to inhibit vibriosis by their phagocytosis.

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Explanation of Plate I

Fig. 1. Peritoneal exudate cells phagocytizing bacterial cells in the peritonium that attenuated bacteria were injected. Giemsa, X800.

Fig. 2. Intestine infected by the intraperitoneal injection with virulent bacteria. The intestine exhibited hemorrhage in the muscle layer, and necrosis in the muscle layer, submucosa and villi. H-E,X80.

Fig. 3. Peritoneal exudate cells, isolated from unvaccinated fish, phagocytizing attenuated bacterial cells. Macrophage (big cell) and neutriphils (smaller cells) included one to four bacterial cells. Giemsa, X800.

Fig. 4. Peritoneal exudate cells, isolated from unvaccinated fish, phagocytizing virulent bacterial cells. They showed destruction with intracytoplasmic bacterial multiplication. The type of each cell was indistinguishable. Giema, X800.

Fig. 5. Peritoneal exudate cells, isolated from vaccinated fish on the 14 th day post vaccination, phagocytizing virulent bacterial cells. Although bacteria multiplied in neutrophils, the cells were stable. Giemsa, X800.

Plate I