三重科技七水研報 第12号 平成17年2月
Bull. Fish. Res. Div, 12, 53-94 (2005)

Studies on viral nervous necrosis of sevenband grouper *Epinephelus septemfasciatus* at grow-out stage[*1]

Shinji TANAKA

Contents

[*1] Thesis submitted for the degree of Doctor of Philosophy at the Mie University, March 2004.

Introduction

In Japan, more than 30 marine fish species as yellowtail *Seriola quinqueradiata*, red sea bream *Pagrus major*, Japanese flounder *Paralichthys olivaceus* and tiger puffer *Takifugu rubripes* are cultured. The total production of these cultured marine fish amounted to 263 thousand metric tons and valued at 231 billion yen in 2001.

Sevenband grouper *Epinephelus septemfasciatus* is one of species of which production recently increased because of the good taste and high market price. This species has been already cultured in a small scale using the wild seedlings that were captured in Japanese coastal area since 1970's. The production of this species has increased since 1980's for using the wild seedlings imported from Korea. From the latter half of 1990's, the improvement of the seedling production technology made the mass production of hatchery-reared seedlings possible. Therefore, the production of sevenband grouper is expected to increase still more.

However, mass mortalities broke out not only in young fish but also in older fish up to harvest size causing sometimes over 50% mortality since 1984. Diseased fish showed abnormal behavior, loss of balance of the body at the water surface, and inflation of swimbladder in summer and autumn. The mass mortalities at grow-out stage have been the most important obstructive factor against the culture of sevenband grouper. In a preliminary study on natural outbreaks, the disease was characterized histopathologically by intracytoplasmic vacuolizations and necrosis in the nerve cells of the brain and the retina of diseased fish. A betanodavirus was also detected from these affected organs. Betanodaviruses have been reported as the causative agents of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) which has affected many kinds of cultured marine fishes all over the world (Munday & Nakai 1997; Munday *et al.* 2002). Although VNN has been recognized to cause mass mortalities in larvae and juveniles so far, the results of the preliminary study suggested that the mortalities of sevenband grouper at grow-out stage were caused by the betanodavirus.

In the present studies, the etiology of mass mortalities in sevenband grouper at grow-out stage and its control measure were studied. This thesis is made up of four categories: 1) pathogenicity of the betanodavirus detected from diseased sevenband grouper, 2) determination of the definite sevenband grouper nervous necrosis virus (SGNNV)-targeted cells in sevenband grouper at grow-out stage, 3) susceptibility of several marine fish species to SGNNV, 4) potential of vaccination with an *Escherichia coli*-expressed recombinant coat protein against experimental SGNNV infection.

Chapter 1: Pathogenicity of the betanodavirus detected from diseased sevenband grouper

A preliminary study on natural outbreaks in Mie Prefecture and Wakayama Prefecture from 1994 to 1995 has been performed. In histopathological examinations, affected nerve cells in the brain and the retina showed intracytoplasmic vacuolizations and necrosis in diseased fish. The indirect fluorescent antibody test (IFAT) with anti-striped jack nervous necrosis virus (SJNNV; Mori *et al.* 1992) rabbit serum revealed the viral antigens in the affected nerve cells. A betanodavirus was detected from these affected organs by the reverse transcription-polymerase chain reaction (RT-PCR) amplification with primers for detection of SJNNV coat protein gene. Based on these results, the mortalities of sevenband grouper were suggested to be caused by the betanodavirus.

In this chapter, the pathogenicity of the betanodavirus obtained from diseased sevenband grouper and the effects of rearing water temperature in the pathogenicity were demonstrated by infection experiments using two hatchery-produced marine fish species; sevenband grouper and redspotted grouper *E. akaara*. The latter species is also known to be a susceptible host of betanodavirus (Mori *et al.* 1991).

Materials and Methods

Fish

Hatchery-reared sevenband grouper and redspotted grouper were used for the infection experiments. These fish were reared in 500 L tanks with a flow-through water supply at about 15℃. Prior to experimentation, brains and retinas of each of the 5 fish randomly sampled from two fish groups were examined for betanodavirus by an IFAT procedure using an anti-SJNNV rabbit serum (Nguyen *et al.* 1996) and an RT-PCR procedure using a primer set (F2-R3) designed for detection of SJNNV (Nishizawa *et al.* 1994). All the tests were negative.

Virus inoculum

Three diseased sevenband grouper (one 1-year-old and two 3-years-old) showing upside down behavior were collected from natural outbreaks at Mie and Wakayama Prefectures. These fish were confirmed to be infected with betanodavirus by the RT-PCR test. The infected brains and eyes were pooled and then stored at −80℃. Just before infection experiments, the samples were thawed and homogenized with an equal volume of minimum essential medium (MEM; Nissui, Japan), and centrifuged at 1,500 *g* for 15 min. The supernatant was filtered through a membrane filter (0.45 μm) and further diluted with MEM when required.

Infection experiments

Fish were transferred to 15 L tanks and the tank waters were adjusted to each required water temperature by an increase of 2℃ a day. Fish were acclimated for one week before experiments were commenced. Eight sevenband grouper (33 g in average body weight) in the experimental group were given an intramuscular (i.m.) injection with 0.04 mL of serial 10-fold dilutions (10^{-1}-10^{-6}) of the virus filtrate and eight control fish were sham-challenged with MEM instead of the filtrate. After injection, fish were observed in 15 L sea water tanks at 28℃ (±0.5℃) for 20 days. Redspotted grouper (7 g) were divided into 4 groups, each consisting of 9 or 10 fish, and challenged by an i.m. injection with 0.04mL of 5-fold dilution of the filtrate (Group 1, duplicated) or by a bath method where fish were exposed to 500 mL of sea water containing 1mL of the filtrate for 1 h (Group 2). Group 3 (injection method, duplicated) and Group 4 (bath method) sham-challenged with MEM instead of the filtrate were served as controls. Fish were then transferred to 15 L tanks at 27-28℃ and

observed for 19 days (Groups 1 and 3) or 15 days (Groups 2 and 4). Each one of duplicated tanks of Groups 1 and 3 was used to sample abnormal swimming fish (n=5) and normal swimming fish (n=5), respectively, at 10 days post-inoculation (p.i.). Fish were fed commercially prepared pellet once a day. Fish just after death, fish showing signs of disease, and survivors at the termination of experiments were examined histopathologically and microbiologically.

In a separate infection experiment to examine the effect of water temperature, redspotted grouper (13 g) were divided into 6 groups, each containing 7 fish, and transferred to 15 L tanks where water temperature was raised by 1-3℃ a day to 16℃, 20℃, 24℃, and 28℃ (±0.5℃) and then maintained at each temperature for 5 days. In the fifth group (designated as the 24-28℃ group), tank water temperature was manipulated to 24℃ and 28℃ on alternate days to examine effect of fluctuation of water temperature on infection. Fish were given an i.m. injection with 0.04 mL of the virus filtrate. The sixth group was sham-challenged with MEM at 28℃. After challenge, fish were observed for 50 days at each water temperature. Fish just after death and survivors at the termination of experiments were examined as described above.

Histopathological and microbiological examinations

Brains and eyes of fish were fixed in 10% buffered formalin solution and embedded in paraffin wax. In redspotted grouper, the gills and internal organs were also examined. The sections were stained with haematoxylin and eosin (H & E). An IFAT was used for detection of the betanodavirus. Tissue sections of the brains and the eyes were immunostained with a rabbit anti-SJNNV serum and fluorescein isothiocyanate (FITC)-conjugated swine immunoglobulin to rabbit immunoglobulin (Dako A/S, Denmark) according to the procedure of Nguyen *et al.* (1996). Dead fish were also examined for the presence of ectoparasites on the gills, and bacterial isolation from the kidneys was attempted on brain heart infusion agar (Nissui, 2% NaCl) at 25℃ for 5 days.

Results

Pathogenicity of the virus in sevenband grouper

The results of experimental infection with young sevenband grouper are shown in Table 1. The abnormal behavior included loss of balance of the body at the water surface or on the tank bottom. Inflation of swimbladder was observed only in fish floating at the water surface with loss of balance. Fish inoculated with 10^{-1} and 10^{-2} dilutions of the filtrate evidenced abnormal behavior at 3 days p.i. and all died by 6 days p.i. Fish inoculated with 10^{-3} dilution also showed abnormal behavior and high mortality by 12 days p.i. but the incidence of these abnormalities in fish with 10^{-4} and 10^{-5} dilutions was low. One of the fish in 10^{-5} challenge group exhibited abnormal behavior at 8 days p.i. but recovered at 16 days p.i. No apparent abnormalities were observed in the 10^{-6} dilution and control groups.

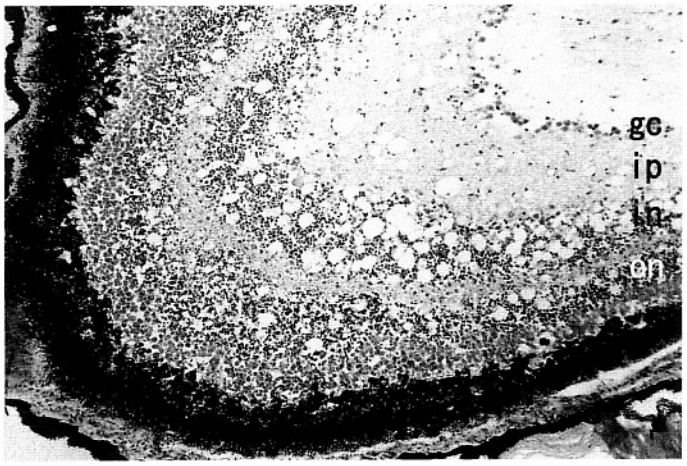

Fig. 1. Light micrograph showing extensive vacuolization in the retina of an experimentally infected sevenband grouper. gc: ganglion cell layer, ip: inner plexiform layer, in: inner nuclear layer, on: outer nuclear layer (H&E, ×33).

Histopathologically the degeneration (necrosis and vacuolization) of the brain and the retina,

Table 1. Experimental infection of the virus in young sevenband grouper

Dilution factor of the filtrate[a]	No. of fish with abnormal swimming/ examined	No. of fish dead/examined	No. of fish IFAT-positive/examined	
			Dead fish	Survivors[b]
10^{-1}	8/8	8/8	8/8	–
10^{-2}	8/8	8/8	8/8	–
10^{-3}	7/8	6/8	6/6	2/2
10^{-4}	1/8	2/8	2/2	3/6
10^{-5}	1/8	0/8	–	0/8
10^{-6}	0/8	0/8	–	0/8
Control	0/8	0/8	–	0/8

[a] Fish were injected intramuscularly with 0.04 mL of the virus-filtrate or MEM (control) and kept at 28℃.
[b] Sampled at 20 days p.i.

similar to that of the naturally diseased fish, was observed in the experimental group (Fig. 1). The brain and/or the retina of all dead fish were positive in the IFAT test using anti-SJNNV serum (Table 1). Positive IFAT reaction were also detected in 5 of the 8 survivors in the 10^{-3} and 10^{-4} challenge groups, but all the 16 survivors in the 10^{-5} and 10^{-6} challenge groups were negative in IFAT test when they were examined at 20 days p.i. No parasites were observed on the gills and no dominant bacteria were isolated from the kidney of dead fish.

Pathogenicity of the virus in redspotted grouper

The pathogenicity of the virus in juvenile redspotted grouper was also demonstrated in both the inoculation methods (Table 2). In Group 1, inoculated with the filtrate by an i.m. injection, anorexia was observed at 2 days p.i. and 8 out of 10 fish lay on the bottom or floated at the water surface at 4-9 days p.i. Only 2 of these fish died at 7 and 9 days p.i. and the other fish recovered from abnormal behavior and anorexia at 11 and 16 days p.i., respectively. In Group 2, bath-challenged with the filtrate, anorexia was observed from

Table 2. Experimental infection of the virus in juvenile redspotted grouper

Challenge[a] method	No. of fish with abnormal swimming/ examined	No. of fish dead/examined	No. of fish IFAT-positive/examined		
			Dead fish	Survivors[b]	Sacrificed fish[c]
Injection (Group 1)	8/10	2/10	2/2	8/8	5/5[d]
Control (Group 3)	0/10	0/10	–	0/5	0/5[e]
Bath (Group 2)	3/9	0/9	–	8/9	–
Control (Group 4)	0/10	0/10	–	0/5	–

[a] Fish were challenged with the virus-filtrate by intramuscular injection or bath method and kept at 27-28℃. Control fish were sham-challenged with MEM.
[b] Sampled at 19 days p.i. (Injection) or 15 days p.i.(Bath)
[c] Sampled at 10 days p.i.
[d] Fish with abnormal swimming.
[e] Fish with normal swimming.

2 days p.i. and 3 out of 9 fish lay on the bottom at 5-6 days p.i. However, fish recovered from these disease conditions at 8 days p.i. and all survived until the termination of experiment (15 days p.i.). No abnormality was observed in their control groups.

Necrosis and vacuolization, as in experimentally infected sevenband grouper, were observed in the brain and the retina of dead and abnormally-swimming fish. As shown in Table 2, specific fluorescence in the retina and/or brain was observed in both dead fish of the i.m. injected group (Group 1). The positive IFAT reaction was also observed in all the abnormally-swimming fish which were sacrificed at 10 days p.i. (Group 1), in all Group 1 survivors at 19 days p.i., and in 8 out of 9 Group 2 survivors at 15 days p.i., though the detection rate was always higher in the retina than in the brain. No specific fluorescence was observed in both brains and retinas of all fish of control groups. No parasites or dominant bacteria were detected on the gills and from the kidney of dead fish.

Effect of water temperature on infection

The results of infection experiment with redspotted grouper juveniles at different water temperatures are shown in Table 3 and Fig. 2. The appearance of abnormal behavior and mortality of fish were strongly influenced by water temperature. In the 28℃ group, all 7 fish exhibited abnormal behavior at 4-9 days p.i. and 6 fish died at 6-10 days p.i. Similarly, high incidence of abnormal behavior was also recognized in the 24-28℃ and 24℃ groups, though the mortality rates were lower than that in the 28℃ group. In the 20℃ and 16℃ groups, the onset of abnormal behavior was delayed, leading to low mortalities. Most of fish survived at 20℃ to 28℃ recovered from abnormal behavior, however fish in the 16℃ group did not (Fig. 2). Throughout the observation period of 50 days, no abnormalities was observed in the control group at 28℃. As shown in Table 3, positive IFAT reaction was observed in the retina and/or the brain of all dead fish and also 4 out of 6 survivors showing abnormal behavior when examined (50 days p.i.). Degeneration of nervous tissues was seen in these IFAT-positive sections. However, 8 survivors that recovered from abnormal behavior were all negative for IFAT. All fish in the control group were found to be negative.

Table 3. Experimental infection of the virus in redspotted grouper juveniles at different water temperatures

Water[a] temperature	No. of fish with abnormal swimming/ examined	No. of fish dead/examined	No. of fish IFAT-positive/examined	
			Dead fish	Survivors[b]
28℃	7/7	6/7	6/6	0/1
24-28℃	7/7	4/7	4/4	1/3
24℃	7/7	2/7	2/2	0/5
20℃	5/7	1/7[c]	1/1	0/4
16℃	4/7	1/7	1/1	6/6
28℃ (Control)	0/7	0/7	−	0/7

[a] Fish were injected intramuscularly with the virus-filtrate or MEM (control) and kept at each water temperature (See Materials and Methods).
[b] Sampled at 50 days p.i.
[c] Except two fish which died by cannibalism.

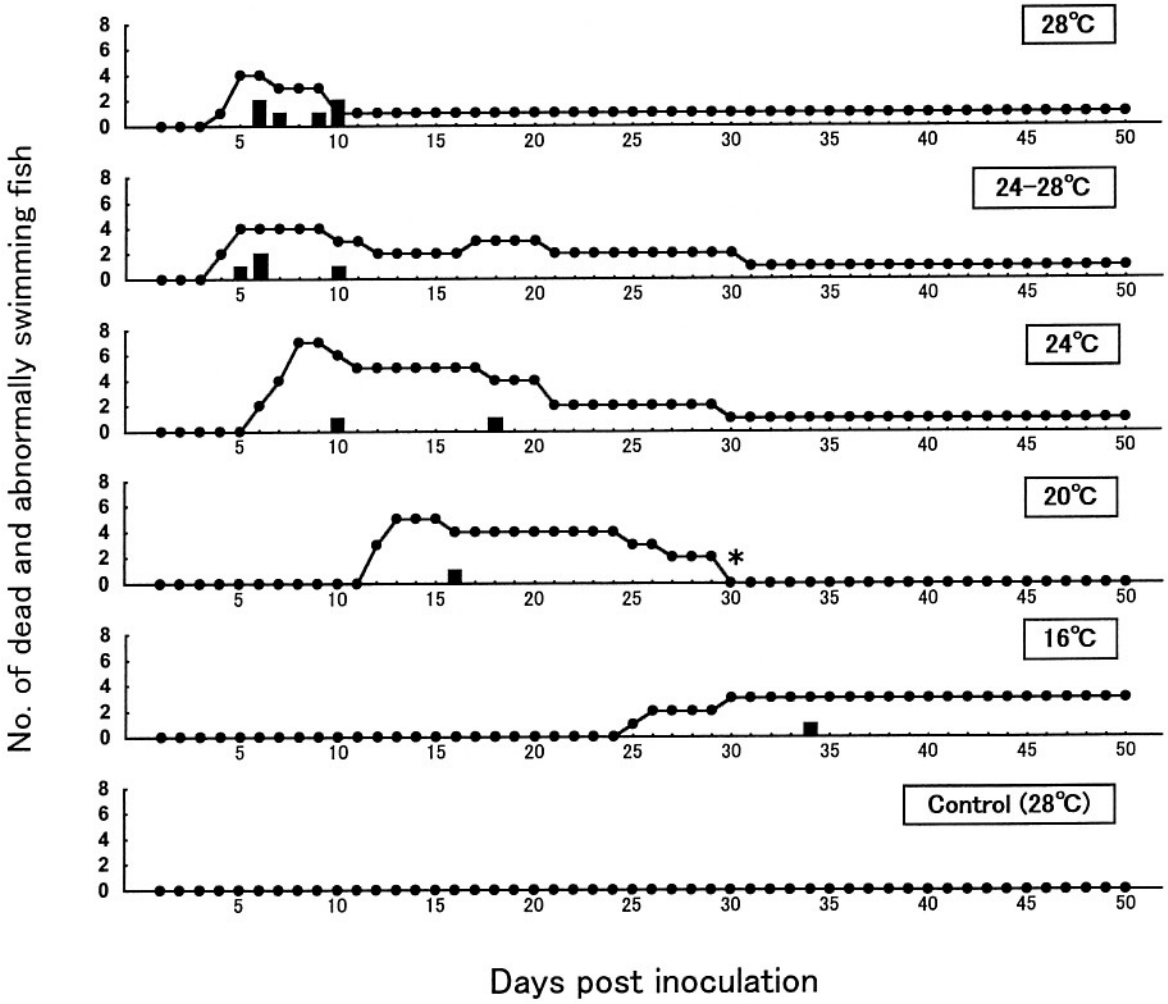

Fig. 2. Appearance of dead and abnormal swimming fish in redspotted grouper challenged with the virus at different water temperatures. ■: Number of dead fish, —●—: Cumulative number of fish showing abnormal swimming (dead and recovered fish were counted out). *Death by cannibalism.

Discussion

Fukuda *et al.* (1996) preliminarily studied the mass mortalities in the cultured populations of grow-out stages of sevenband grouper in Oita Prefecture and suggested that the mortalities were caused by the betanodavirus from their histopathological signs and electron microscopic features. However, pathogenicity of the virus was not examined. The present infection experiments confirmed that the filtered homogenate of organs infected with the betanodavirus produced the disease similar to that of naturally infected sevenband grouper, accompanying abnormal swimming behavior and mortality. Necrosis and vacuolization of the brain and retinal tissues characteristic to VNN (Munday & Nakai 1997) were found in the dead and abnormally-swimming fish and the viral antigens were detected in the degenerated nervous tissues by IFAT with the anti-SJNNV serum, demonstrating that the betanodavirus caused VNN in young sevenband grouper. The high IFAT-positive results of bath challenge in redspotted grouper (Table 2), though mortalities were not produced, indicate a possible water-born transmission of the virus, seemingly causing spread of the disease among cultured fish populations. In addition to VNN in this sevenband grouper, Le Breton *et al.* (1997) recently reported VNN in cage-cultured European sea bass *Dicentrarchus labrax*, ranging 10 to 580 g in body weight,

though infection experiment to fulfill Koch's postulates was not carried out in their report.

Natural infections of betanodavirus in marine fish species occur in a wide range of rearing water temperature, for example 28-30℃ in juvenile brownspotted grouper *E. malabaricus* (Danayadol *et al.* 1995), 20-26℃ in striped jack *Pseudocaranx dentex* larvae (Arimoto *et al.* 1994) and 4-15℃ in barfin flounder *Verasper moseri* juveniles (Japan Sea-Farming Association, 1995)*[2]. Arimoto *et al.* (1994) reported in an experiment using striped jack larvae naturally infected with SJNNV that the earliest mortality was observed at 24℃ of water temperature in a test range of 18-27℃. The VNN outbreaks in sevenband grouper occur from July to October, water temperature ranging 21-30℃, and Fukuda *et al.* (1996) suggested higher water temperature as a possible predisposing factor of the disease. In the present study, the pathogenicity of the virus was found to be associated with rearing water temperature and the highest pathogenicity was displayed at 28℃. The virulence of the virus was reduced with the decrease of water temperature, though fish was susceptible to the virus even at 16℃. This supports the fact that a similar abnormality was observed in January (about 16℃ water temperature) among a cultured population of sevenband grouper which had experienced heavy mortalities in the previous August to September in Mie Prefecture. As the disease often becomes more severe when there is daily fluctuation of water temperature in addition to high water temperature (Dr. Y. Fukuda personal communication), this fluctuation may affect the defense mechanisms of fish against the virus. However, the effect of fluctuation of water temperature on the susceptibility was not clear in this study (Table 3).

Interestingly, some fish survived with abnormal swimming for a long time or recovered, and the virus antigens disappeared from brain and retina of all of these recovered fish (Table 3). Fukuda *et al.* (1996) reported that the virus was not detected from some naturally affected fish and suggested that these fish were in the convalescent stage. As there exists age or adult resistance of fish in VNN (Arimoto *et al.* 1994; Munday & Nakai 1997), the infection using sevenband and redspotted groupers will be useful to elucidate the resistance mechanisms of fish against the betanodaviruses. Another interesting finding was that the virus antigen was detected frequently in survivors (50 days p.i.) at 16℃, indicating long lasting survival or slow multiplication in the host at low water temperature. These findings may help to identify the source and etiology of the virus in cultured groupers and to control the disease.

*[2] Japan Sea-Farming Association (1995): Diseases of hatchery reared larvae and juveniles, Kyokai Kenkyu Shiryo No.66 (in Japanese)

Chapter 2: Histopathological studies on viral nervous necrosis of sevenband grouper at grow-out stage

In Chapter 1, mass mortalities in sevenband grouper at grow-out stage were determined to be caused by a betanodavirus (SGNNV). The previous studies reported that the distinctive histopathological sign of naturally betanodavirus-infected larvae and juveniles was necrosis with vacuolization of the nerve cells in the central nervous system (CNS) and the retina. These necrotic nerve cells displayed intracytoplasmic inclusion bodies consisted of betanodavirus particles with diameters of 25-30 nm (Office International des Epizooties (OIE) 2000; Munday *et al.* 2002). Immunohistopathological studies were performed in naturally diseased larval striped jack and bath-challenged larval Atlantic halibut *Hippoglossus hippoglossus* (Nguyen *et al.* 1996; Grotmol *et al.* 1999). These affected fish displayed specific immunolabeling in the epidermal cells or the intestinal epithelial cells simultaneously with the nerve cells of the CNS in an early stage of betanodavirus infection, suggesting that the epithelium was a possible portal of entry. On the other hand, VNN outbreaks have been also reported in grow-out and adult stages of several fish species such as brownspotted grouper (Danayadol *et al.* 1995), sevenband grouper (Fukuda *et al.* 1996), greasy grouper *E. tauvina* (Chew *et al.* 1998), European sea bass (Le Breton *et al.* 1997), and Atlantic halibut (Aspehaug *et al.* 1999). In larvae and juveniles, differentiation of nerve cells is incomplete (Miyazaki *et al.* 1991). Therefore, betanodavirus-targeted cells in the CNS and the retina have not been definite. As well as larvae and juveniles, definite betanodavirus-targeted cells have not been determined although grow-out and adult stages of fishes with VNN have the well developed CNS and retina.

In this chapter, the diseased sevenband grouper at grow-out stage in natural outbreaks were collected and used for histopathological studies. The histopathological features suggested a putative invasion way of the pernasal infection of SGNNV where virus penetrated the nasal epithelium of nostril, passed through the olfactory nerve and the olfactory bulb, and invaded the olfactory lobe. In order to verify this hypothesis, infection experiments with SGNNV were performed and the pernasal infection was established. In this study, I describe histopathological and electron microscopic features of SGNNV-targeted cells in the CNS and the retina in diseased fish from the natural outbreaks and infection experiments.

Materials and Methods

Diseased fish in natural outbreaks

A total of 96 diseased sevenband grouper weighing 45-1,400 g (0-3 years old) were collected at various fish farms in Mie Prefecture from 1994 to 2002. In order to observe the mortality character of diseased grow-up fish, a total of 60 naturally diseased fish (150 g in average body weight, 1 year old) showing abnormal behavior were captured from pens, and 30 fish were held in a small net cage (2×2×2 m, covered with sunproof net) and the other 30 fish were held in a land-based 1 m^3 tank with a flow-through system at 22-24℃ for 15 days. Moribund fish, fish just after death, and all survivors at the termination of the experiment were used for histopathological examinations.

Histopathological and electron microscopic examinations

The tissues of the brain, spinal cord, eyes, optic nerve, olfactory nerve, nostril regions, liver, spleen, kidney, heart, gills, stomach, intestine, pyloric caecum, pancreas and swimbladder were dissected and fixed in 10% buffered formalin solution for histopathological examinations. The fixed tissues were prepared according to standard techniques and tissue sections were stained with H & E. For IFAT, tissue sections including the

brain, spinal cord, eyes, optic nerve, olfactory nerve, nasal epithelium and swimbladder were also immunostained as described previously in Chapter 1. Piece of the remaining brain, spinal cord and retina were fixed in 70% Karnovsky solution, postfixed in 1% OsO_4 and processed for electron microscopy (EM) according to standard techniques.

Virus inoculum and infection experiments

A diseased sevenband grouper (260 g) had been taken during a mass mortality in Mie Prefecture in 1995. The infected brain and eyes were homogenized with an equal volume of MEM and centrifuged at 1,500 *g* for 15 min. The supernatant was passed through a 0.45 μm membrane filter and stored at −80℃. The virus stock designated as SGMie95. Twenty sevenband grouper (33 g) were given an i.m. injection with an SGMie95 isolate at a dose of $10^{5.4}$ $TCID_{50}$ (median tissue culture infective dose) per fish, and held at 28℃. The brains and eyes removed from moribund fish were homogenized with four volumes of phosphate buffered saline (PBS), and the filtrate was prepared as described above and stored at −80℃. The stock solution of virus contained $10^{7.8}$ $TCID_{50}$ mL^{-1} as determined by the cell culture assay with E-11 cell line (Iwamoto *et al.* 2000), which was cloned from SSN-1 cells (Frerichs *et al.* 1991).

Hatchery-reared sevenband grouper (200-405 g, 1 year old) which had not experienced an VNN epizootic were used for the infection experiments. Prior to experimentation, brains of 10 fish were randomly sampled from the experimental fish stock and were examined for betanodavirus by the RT-PCR procedure. All the tests were negative. Fish were acclimated at 28℃ for 1 week before the infection experiments.

Experiment 1 (expt 1) was designed to establish pernasal infection of SGNNV. Fifteen fish were anesthetized in sea water containing ethylene glycol monophenyl ether (200 ppm) for 1 min. Anesthetized fish were removed and enclosed with wet papers, and 0.06 mL of the virus suspension ($10^{6.6}$ $TCID_{50}$ per fish) was inserted into the nostrils. After keeping fish in the air for 10 min, the snout region was carefully washed away with flowing sea water for 1 min. Treated fish were held in a tank containing 500 L of sea water for 5 min to remove the remaining virus from the nostrils and moved into another 500 L tank with a flow-through water supply at 28℃. Negative control fish (15 fish) were sham-challenged with PBS instead of the virus suspension. For positive control, another 15 fish were given an i.m. injection at a dose of $10^{7.1}$ $TCID_{50}$ per fish. These fish were reared in 500 L tanks with a flow-through water supply at 28℃. Fish showing abnormal behavior were removed from the tank, and the CNS, eyes and nostril regions were used for histopathological examination, IFAT and EM.

Experiment 2 (expt 2) was designed to confirm a way of SGNNV invasion into the CNS and the retina. Twenty fish were subjected to the same way as in expt 1 and held in a 500 L tank. Five fish were randomly sampled at 1, 2, 3, and 5 days p.i. The CNS, eyes and nostril regions were used for histopathological examination, IFAT and EM.

Results

Natural outbreaks

Outbreak, external and internal signs

The outbreaks of VNN in sevenband grouper at grow-out stage are summarized in Table 4. The mortalities were observed in fish populations of 0-3 years old (from young fish just after introduction into the culturing sites to older fish up to harvest size) at 18-30℃. The cumulative mortalities varied from 1% to 70% that high mortality depended on the high water temperature but not on the age. Diseased fish showed abnormal behavior, loss of balance of the body at the water surface or on the bottom of the net pen

Table 4. Outbreaks of VNN in sevenband grouper at grow-out stage in Mie prefecture from 1994 to 2002

Year	Site	Age	Mean body weight(g)	Disease occurrence		Mortalities (%)		
				Months	Water temperature(℃)	Daily Max.	Daily Av.	Total
1994	A	2	570	Jul.−Oct.	30−24	1.0	0.4	40
	B	2	500	Aug.−Oct.	29−23	0.6	0.4	25
1995	C	1	260	Aug.−Oct.	27−21	0.2	0.1	3
1997	D	3	1,400	Oct.	24−23	0.1	0.1	1
1998	D	2	930	Sep.−Oct.	27−23	0.2	0.1	7
1999	E	1	300	Aug.−Oct.	27−23	1.0	−	−
	F	1	560	Oct.−Nov.	25−22	0.3	0.03	1
	D	0	45	Nov.−Dec.	24−20	4.0	1.4	70
	D	3	1,325	Nov.	24−22	0.8	0.2	4
	G	0	80	Nov.−Dec.	22−20	0.2	0.1	2
2000	H	1	350	Jul.−Aug.	27−25	10.0	−	−
	D	1	350	Jul.−Sep.	28−25	1.0	0.4	13
	I	0	55	Nov.	20	5.0	−	−
	J	0	45	Nov.	21	0.1	0.05	1
	J	1	560	Nov.−Dec.	21−19	0.1	0.03	1
2001	G	3	1,280	Aug.−Oct.	27−23	0.7	0.2	10
	K	0	45	Oct.	25−23	2.0	−	−
	G	0	65	Oct.−Nov.	25−21	2.0	1.2	60
2002	D	1	150	May−Jul.	20−23	4.0	0.6	30
	B	1	240	Sep.−Oct.	27−23	2.5	1.1	65
	B	3	1,150	Aug.−Nov.	27−18	2.5	0.6	50
	D	1	310	Oct.−Nov.	25−18	0.5	0.25	30

(Fig. 3A). Some fish sometimes displayed listless spiral swimming. These diseased fish often showed such abnormal behavior for several days or more. Only slight abrasion and hemorrhage due to net damage were observed on the skin and the fin of some diseased fish. Dead fish opened their mouth and opercula. All of the fish floating at the water surface internally displayed expansion of the swimbladder (Fig. 3B). Parasitological examination revealed the presence of a few monogeneans such as *Benedenia epinepheli* and *Neobenedenia girelle* on the skin and the fin in a few fish. Bacteriological examination resulted in negative in all of examined fish.

Histopathology and IFAT

Definite histopathological signs were necrosis of nerve cells accompanied by the formation of intracytoplasmic inclusion bodies and vacuoles. The IFAT using the specific antibody revealed specific fluorescence in these affected cells, indicating SGNNV propagation. Based on histopathological signs and results of IFAT, we determined SGNNV-infected nerve cells in the CNS. The area of the CNS was determined referring Takashima & Yokote (1982) and Yoshimoto & Ito (2002).

Most of diseased fish displayed a large number of SGNNV-infected cells in the olfactory lobe. Many small nerve cells with a round shape and a paucity of dendrites were necrotized with vacuolization in the granule cell layer of the pars posterior and pars lateralis in the dorsal area of the olfactory lobe. In the ventral area of the olfactory lobe, many small nerve cells form a granule cell layer. These small nerve cells were also extensively affected and vacuolized. Vacuolated small nerve cells had an appearance of seal ring cell with a marginally compressed nucleus or a pyknotic nucleus. Necrotized and fragmented nerve cells were replaced by a vacuolar space including cellular debris (Fig. 4A). The regions that had many vacuolized nerve cells

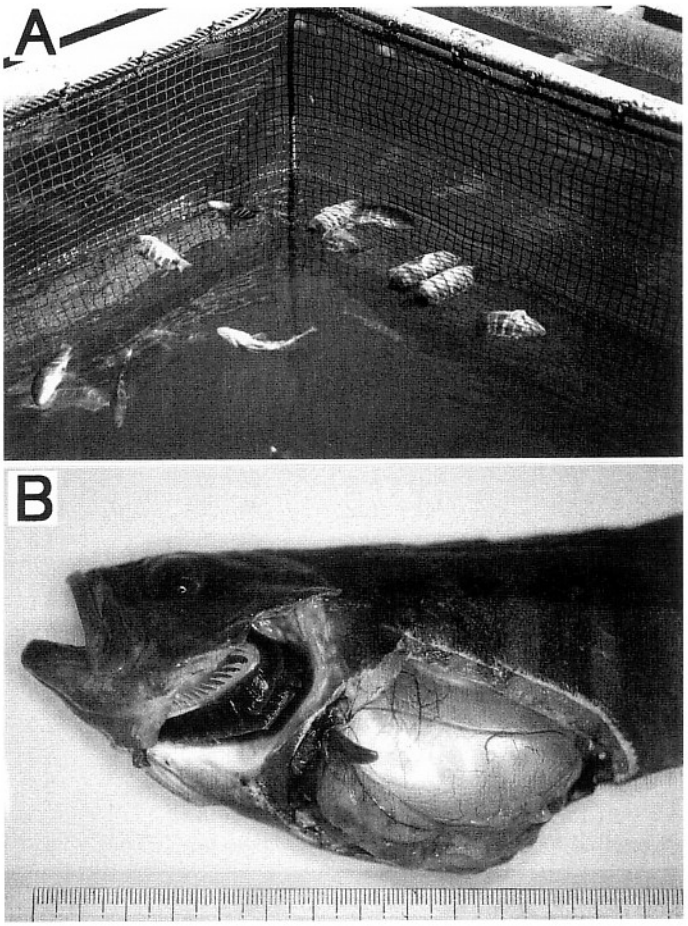

Fig. 3. Naturally diseased sevenband grouper. (A) Fish show abnormal behavior, loss of balance of the body at the water surface. (B) Dissected view of fish floating at the water surface. Mouth and opercula are opened. Swimbladder is markedly expanded.

developed to spongy lesions in which microglia having a small basophilic cytoplasm were proliferated. In some spongy lesions, perivasculitis occurred or many macrophages infiltrated (Fig. 4B). The IFAT revealed fluoresced spots in a cytoplasm or an entirely fluoresced cytoplasm of affected nerve cells. Fluoresced spots were observed in vacuoles while there was no fluorescence in axon (Fig. 4C). No meningeal cells adjacent to extensively affected granule cell layer showed necrosis or fluorescence. In the pars centralis of the dorsal area, nerve cells with a stellar shape and large nerve cells extending dendrites form the paleostriatum. These nerve cells were affected and showed a darkly stained cytoplasm or a vacuolated cytoplasm (Fig. 4D). The IFAT revealed specific fluorescence not only in the cytoplasm but also the dendrites (Fig. 4E).

The olfactory bulb is composed of the primary olfactory nerve layer, glomerular layer, mitral cell layer and granule cell layer from outer to central. A small number of nerve cells in the granule cell layer were affected and vacuolized which were fluoresced with IFAT (Fig. 4F). The olfactory nerve and the olfactory epithelium in the nostrils did not show any change and specific fluorescence with IFAT, indicating no propagation of SGNNV.

The area from the preoptic area to the thalamus contains some nuclei for vision, audition and sense of taste. These are magnocellular nuclei which are composed of magnocells and microglia, and also parvocellular nuclei consisting of small nerve cells. In the fasciculus longitudinalis medialis and the commissura ansulata, a small number of magnocells displayed many small vacuoles (Fig. 5A). In IFAT of affected magnocells, the

cytoplasm and dendrites were entirely fluoresced and often showed spots with strong fluorescence (Fig. 5B). Small nerve cells forming parvocellular nuclei were extensively vacuolated and fluoresced with IFAT. The surrounding fibrous layer consisted of axons was loosen. The regions that had many vacuolized cells displayed spongy lesions in which macrophages sometimes infiltrated. Necrotized and IFAT-positive small nerve cells were also observed at lateral recessus and beneath the meninges of the inferior lobe but not in the meninges. The saccus vasculosus displayed some necrotized and IFAT-positive small cells. In some fish, proliferation of a large number of microglia was observed in various regions of the thalamus.

In the optic tectum of most fish, only a small number of nerve cells were affected and vacuolated, and fluoresced with IFAT in the stratum griseum periventriculare. Some fish also displayed a few of fluoresced nerve cells and dendrites in the areas between stratum album centrale and stratum opticum. Because of a small number of SGNNV-infected cells, EM could not be performed in those cells.

The cerebellum is composed of the corpus cerebelli and the valvula cerebelli which is extended into the IVth ventricle. The corpus cerebelli consists of the outer molecular layer, Purkinje cell layer and the inner granule cell layer. Purkinje cells have well developed dendrites extending into the outer molecular layer. Although the Purkinje cell layer also contains eurydendroid cells, both cell types were indistinguishable and here, simply represented by Purkinje cells. The granule cell layer consists of granule cells with a darkly stained nucleus and Golgi cells with a bigger size and a lightly stained nucleus. Most diseased fish showed infected lesions in the Purkinje cell layer and the just underlying granule cell layer. SGNNV-infected Purkinje cells showed many of fine vacuoles or enlarged vacuoles in a cytoplasm and a compressed nucleus. The IFAT revealed specific fluorescence in dendrites as well as in the cytoplasm (Figs. 5C-5E). Golgi cells are mainly located in the outer area of the granule cell layer and partially in the Purkinje cell layer. Many Golgi cells showed vacuolization and specific fluorescence. A small number of granule cells were also affected with vacuolization while most of granular cells were spared of SGNNV infection (Figs. 5C, 5D). Proliferation of a large number of microglia was observed in every regions of corpus cerebelli in some fish. In a few of these fish, macrophages also infiltrated in the Purkinje cell layer. In the valvulla cerebelli, only a few granule cells fluoresced with IFAT.

The medulla oblongata contains nuclei for motor neurons in the ventral zone. There are nuclei consisting of megalocells with dendrites and microglia, and nuclei of many small nerve cells and a few of megalocells. Small nerve cells are also embedded among myelinated axons. Beside the fasciculus longitudinalis medialis, a small number of megalocells were sometimes affected, vacuolated and infiltrated by microglia (Fig. 5F). IFAT revealed specific fluorescence in the entire cytoplasm and dendrites (Fig. 6A). Small nerve cells were often affected and vacuolated, and fluoresced with IFAT.

The spinal cord contains nuclei of megalocells with dendrites. Fasciculus cells and internal cells are embedded among myelinated axons. In the columna anterior, some megalocells were infected and necrotized with infiltration of microglia. Infected megalocells fluoresced in the entire cytoplasm and dendrites or in their fragments. EM of infected megalocells could not be performed because of a small number. In the myelinated axon zone, a small number of small nerve cells were vacuolated and fluoresced with IFAT (Figs. 6B, 6C). The surrounding axon zone was loosen.

The retina of some diseased fish displayed marked lesions in the outer nuclear layer, inner nuclear layer and ganglion cell layer. The cells in the inner nuclear layer and ganglion cell layer were most severely necrotized and vacuolized. The spaces formed by fragmentation of necrotized cells were fused to each other and formed large spaces containing cellular debris (Fig. 6D). SGNNV-infected cells displayed specific fluorescence in these layers, especially in the inner nuclear layer. Some corn cells of the rod and cone layer also displayed specific fluorescence (Fig. 6E). No specific fluorescence was observed in the optic nerve.

No distinct histopathological changes associated with SGNNV infection were observed in other organs.

In observation of the mortality character of naturally diseased fish, fish in a net pen group had a 33% mortality (10 of 30) at 2-14 days p.i. Fish in a land-based tank group showed a 13% mortality (4 of 30) at 2-11 days p.i. In each group, some fish recovered from abnormal behavior. In histopathological examinations, all moribund and dead fish showed distinct histopathological signs of VNN mentioned above. Most of survivors which contained fish showing abnormal behavior and fish being recovered from abnormal behavior, had only a few necrotized and IFAT-positive nerve cells with many microglia proliferating in the CNS while macrophages infiltrated in the olfactory lobe and thalamus in some survivors. The retina of survivors showed many large spaces and fluorescence mainly in the inner nuclear layer and ganglion cell layer.

Infection experiments

Expt 1

Sevenband grouper subjected a pernasal challenge with SGNNV displayed anorexia at 4 days p.i. and all of 15 fish exhibited the abnormal behavior as loss of balance at the water surface or on the tank bottom at 4-7 days p.i. These fish often displayed pale or dark body coloration. Fish showing abnormal behavior often moved their eyes in response to the movement of the thing outside the aquarium which did not suggest total blindness. One of diseased fish died at 6 days p.i., and alive 14 fish showing abnormal behavior were sampled by 8 days p.i.

All of diseased fish suffering from loss of balance of the body at the water surface had an expanded swimbladder. However, the heart beat, peristalsis of the stomach and intestine, and the shape of red gland of the swimbladder looked like normal. Histopathological examinations of all of sampled fish with a pernasal infection revealed large numbers of necrotized and IFAT-positive nerve cells in the olfactory lobe and the thalamus (Figs. 6F, 7A-7C). The fish that were sampled at an earlier period (4-6 days p.i.) displayed a small number of necrotized nerve cells in the Purkinje cell layer of the cerebellum and in the spinal cord while the fish that were sampled at a later period (7 and 8 days p.i.) showed a large number of necrotized cells with proliferation of microglia in these regions as in the naturally diseased fish (Fig. 7D). The olfactory bulb contained only a few necrotized nerve cells. No distinct fluorescence was observed in the nasal epithelium, olfactory nerve and optic nerve. The retina showed necrotized and IFAT-positive nerve cells in the inner nuclear layer and ganglion cell layer.

On the other hand, all of 15 fish that were challenged by i.m. injection displayed anorexia and abnormal behavior at 3 days p.i. Six of these fish died at 4 days p.i., and alive 9 fish showing abnormal behavior were sampled at 3-4 days p.i. All of diseased fish showing abnormal behavior exhibited many necrotized and IFAT-positive nerve cells in the medulla oblongata and the spinal cord while the olfactory lobe, thalamus, optic tectum and cerebellum slightly showed affected nerve cells. The retina also displayed only a small number of necrotized and IFAT-positive nerve cells.

Negative controls exhibited no abnormal behavior and mortality during the 20 days observation period, and showed no histopathological changes.

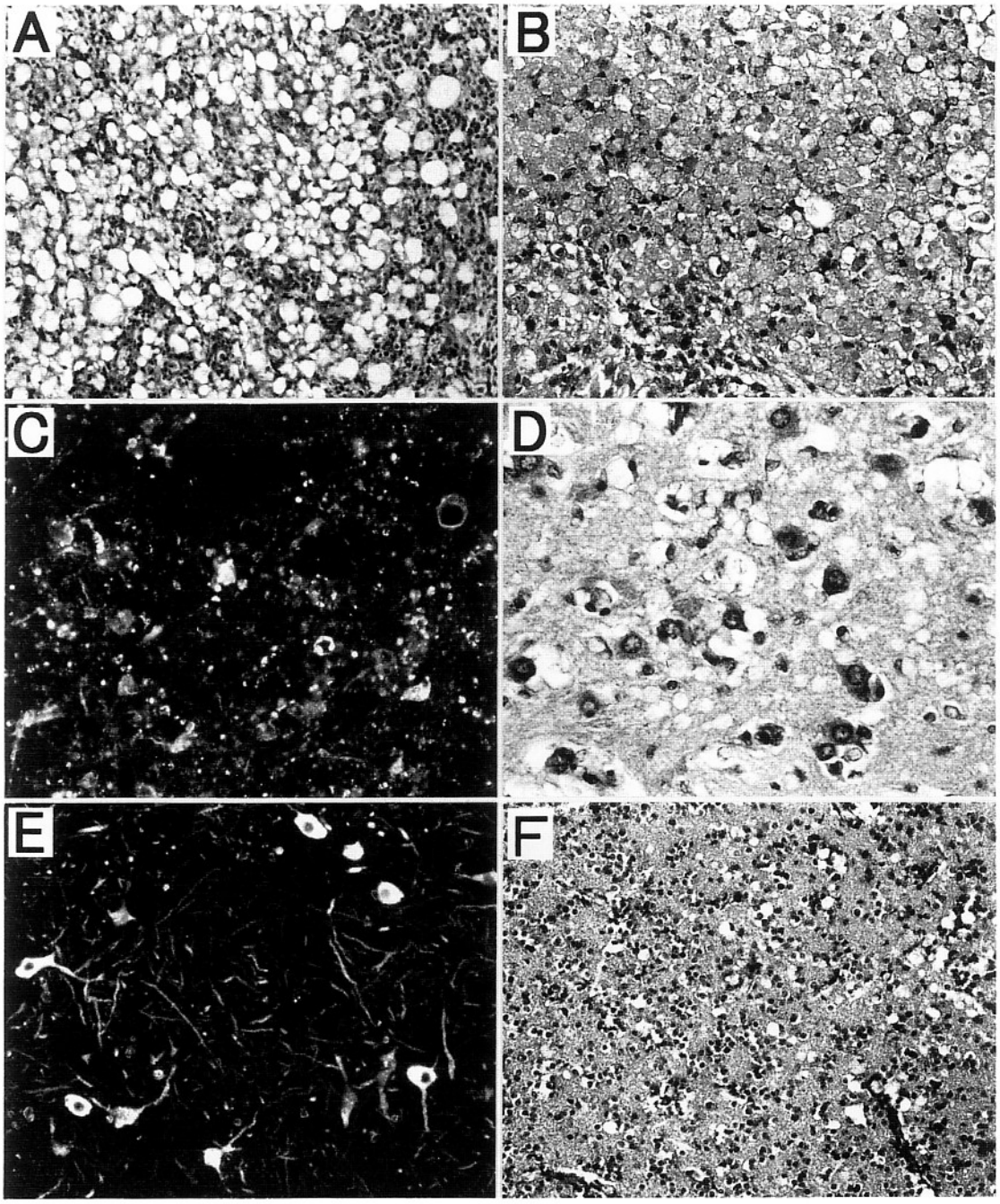

Fig. 4. Histological signs of naturally diseased fish. (A-C) Granule cell layer of the olfactory lobe. (A) SGNNV-infected small nerve cells are vacuolized and fragmented cells are replaced by a vacuolar space, sometimes containing cellular debris (H&E, ×160). (B) Macrophages infiltrate in the affected lesion (H&E, ×100). (C) SGNNV-infected nerve cells show specific fluorescence with IFAT (×100). (D, E) Pars centralis of the dorsal area of the olfactory lobe. (D) SGNNV-infected nerve cells have a vacuolized cytoplasm. (H&E, ×320). (E) SGNNV-infected nerve cells and dendrites show specific fluorescence with IFAT (×100). (F) Granule cell layer of the olfactory bulb has small nerve cells showing necrosis and vacuolation (H&E, ×160).

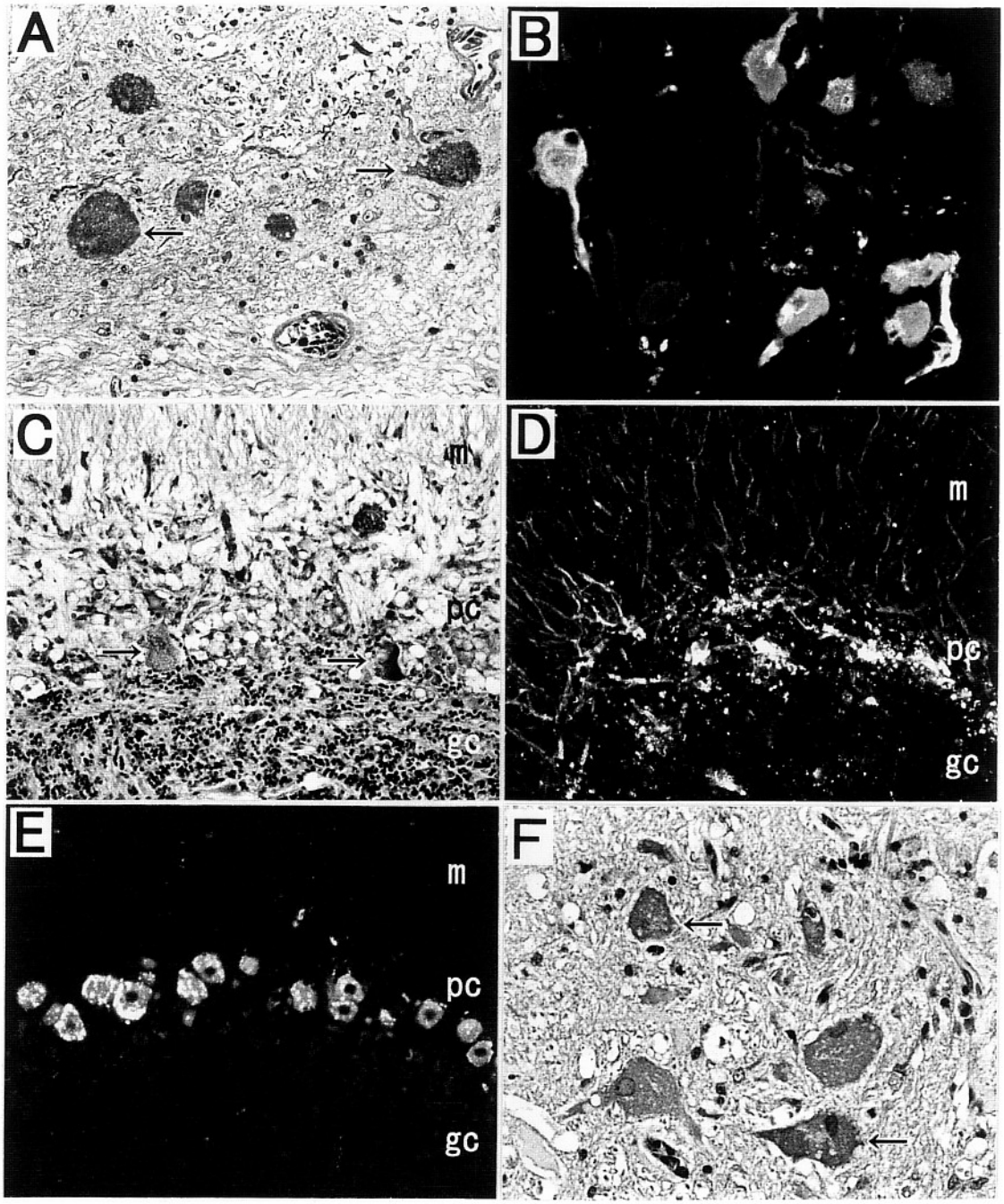

Fig. 5. Histological signs of naturally diseased fish. (A, B) Thalamus. (A) Commissura ansulata displays magnocells showing many small vacuoles (arrows show representative ones) (H&E, ×100). (B) SGNNV-infected magnocells and dendrites show specific fluorescence with IFAT (×100). (C-E) Cerebellum. m: molecular layer, pc: Purkinje cell layer, gc: granule cell layer. (C) SGNNV-infected Purkinje cells and Golgi cells show necrosis and vacuolation. Infected Purkinje cells also display many small vacuoles (arrows) (H&E, ×160). (D) SGNNV-infected nerve cells in the Purkinje cell layer and dendrites in the molecular layer show specific fluorescence with IFAT (×50). (E) SGNNV-infected Purkinje cells exhibit specific fluorescence with IFAT (×100). (F) SGNNV-infected megalocells display many small vacuoles (arrows show representative ones) beside the fasciculus longitudinalis medialis of the medulla oblongata (H&E, ×100).

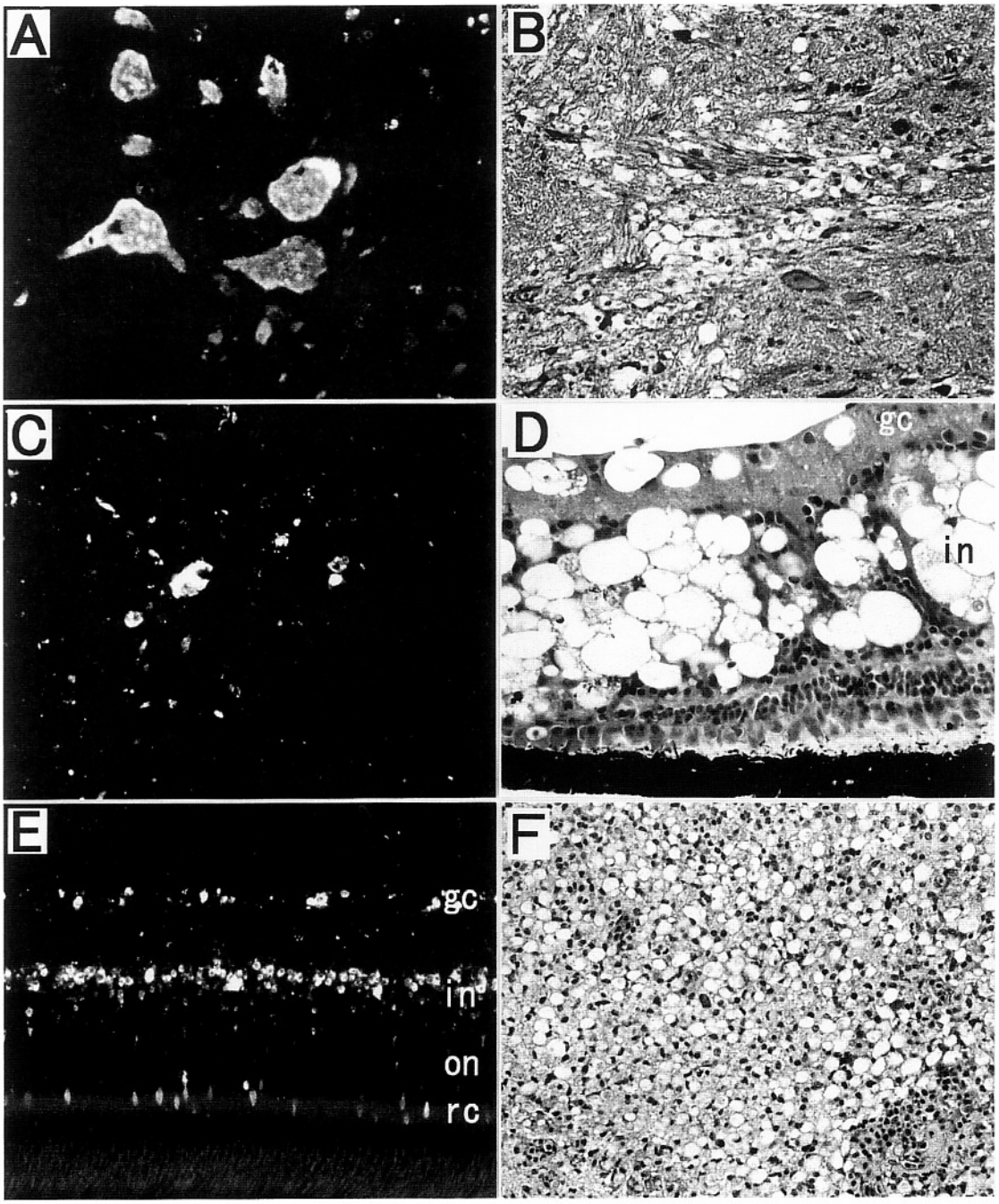

Fig. 6. Histological signs of naturally diseased fish (A-E) and diseased fish with a pernasal challenge with SGNNV (F). (A) Tissue same to Fig. 5F displays megalocells and dendrites showing specific fluorescence with IFAT (×100). (B, C) Spinal cord (B) SGNNV-infected small nerve cells show necrosis and vacuolation (H&E, ×160). (C) SGNNV-infected nerve cells show specific fluorescence with IFAT (×100). (D, E) Retina. (D) Inner nuclear layer (in) and ganglion cell layer (gc) have large spaces containing cellular debris. (H&E, ×320). (E) SGNNV-infected nerve cells in the inner nuclear layer (in), ganglion cell layer (gc), outer nuclear layer (on) and rod and cone layer (rc) show specific fluorescence with IFAT (×50). (F) Granule cell layer of the olfactory lobe has fragmented small nerve cells replaced by a vacuolar space (H&E, ×100).

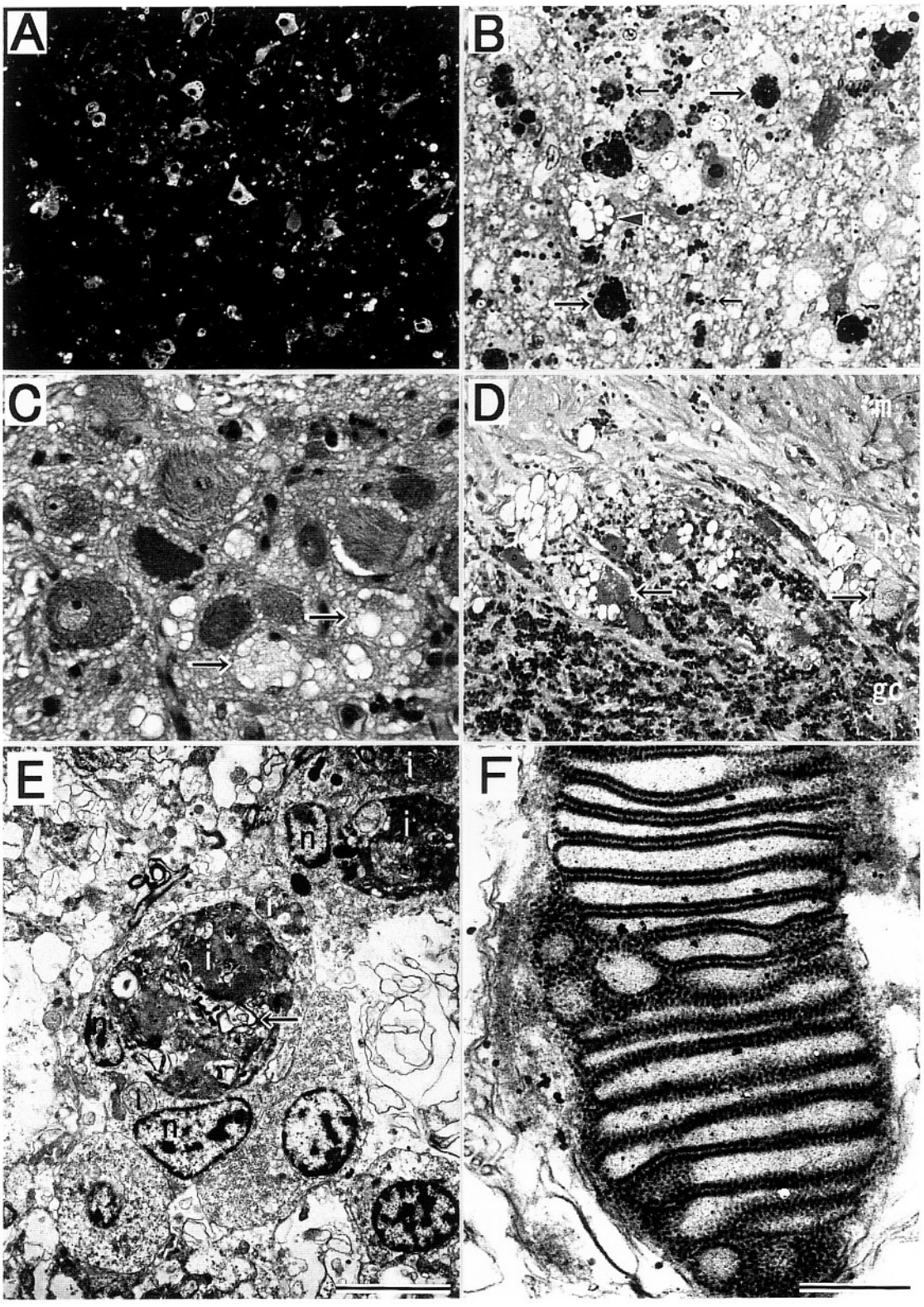

Fig. 7. Histological signs of diseased fish with a pernasal challenge with SGNNV (A-D) and Electron micrographs of SGNNV-infected nerve cells of diseased fish (E, F). (A) Granule cell layer of the olfactory lobe has small nerve cells and dendrites showing specific fluorescence with IFAT (×100). (B) Pars centralis of the dorsal area of the olfactory lobe has necrotized nerve cells showing darkly stained cytoplasm (big arrows show representative ones) or vacuolized cytoplasm (arrowhead shows representative one) and lipid droplets (small arrows show representative ones) (Semi-thin section. Toluidine blue stain, ×480). (C) Commissura ansulata of the thalamus has magnocells showing intracytoplasmic vacuoles (arrows show representative ones) (H&E, ×320). (D) Cerebellum has Purkinje cells and Golgi cells showing necrosis and vacuolation. SGNNV-infected Purkinje cells also display many small vacuoles (arrows show representative ones). m: molecular layer, pc: Purkinje cell layer, gc: granule cell layer (H&E, ×160). (E) SGNNV-infected small nerve cell in the granule cell layer of the olfactory lobe displays viroplasmic inclusion bodies (i) containing SGNNV virions and myelin-like structures (arrow shows representative one) in the cytoplasm. n: nucleus. Bar=5 μm. (F) Viroplasm has rosary arrangements of virions. Bar=500nm.

Expt 2

Each 5 fish that were sampled at 1, 2 and 3 days p.i. exhibited no abnormal behavior. Remained 5 fish showed abnormal behavior at 5 days p.i. The results of detection of virus antigen by IFAT in the brains are shown in Fig. 8. No IFAT-positive fluorescence was observed in any regions of the brain in all of fish sampled at 1 and 2 days p.i. In 3 out of 5 fish that were sampled at 3 days p.i., the olfactory lobe displayed some IFAT-positive nerve cells without intracytoplasmic vacuoles. In one of these 3 fish, a few of IFAT-positive nerve cells were also observed in the olfactory bulb and the thalamus. In all of 5 fish that were sampled at 5 days p.i., necrotized and IFAT-positive nerve cells extensively occurred in all regions of the brain, spinal cord and retina. No fluorescence was observed in the nasal epithelium of all fish that were sampled between 1-5 days p.i.

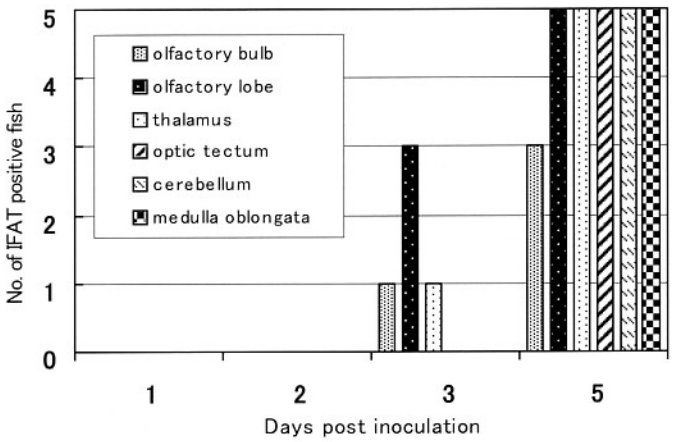

Fig. 8. IFAT detection of SGNNV from infected sevenband grouper. Fish were challenged by inserting the virus suspension into the nostrils.

Electron microscopy

Based on histopathological and IFAT findings, electron microscopic features of SGNNV-infected cells were observed. Both fish in natural outbreaks and an artificial pernasal infection displayed the almost same electron microscopic features.

In the olfactory lobe, small nerve cells were extensively affected with SGNNV at the granule cell layer of the pars posterior and pars lateralis in the dorsal area, and the granule cell layer in the ventral area. Affected nerve cells displayed either the formation of viroplasmic inclusion bodies in a cytoplasm or the cytoplasm occupied by the viroplasm. The viroplasm contained the electron dense matrix, subviral particles and virions with no envelope and diameters of 25-30 nm, and myelin-like structures (Fig. 7E). Organelles of these affected cells were degenerated and fragmented. Highly vacuolated cells displayed the fragmented cytoplasm and viroplasm in the space. In the fragmented viroplasm, virions were decreased in the number and showed crystalline array, rosary arrangement, and concentric circle which were delimited by a thin membrane and contained massive virions or a myelin-like structure in the central core (Figs. 7F, 9A). The surrounding axon zone was extensively damaged. The markedly damaged area was proliferated by many microglia with a small cytoplasm having a few of mitochondria and a paucity of organelles which were spared of SGNNV infection (Fig. 9B). In the pars centralis of the dorsal area, nerve cells with a stellar shape and large nerve cells were also infected with SGNNV and showed viroplasmic inclusion bodies in a cytoplasm or the cytoplasm occupied by the viroplasm (Figs. 9C, 9D). Their dendrites also contained viroplasmic inclusion bodies with a small size.

In the area from the preoptic area to the thalamus, an early stage of infected magnocells displayed the formation of the viroplasm entirely in the cytoplasm with fine vacuoles and in dendrites (Fig. 10A). In the cytoplasm, virions were propagated and invaded the degenerated mitochondria and endoplasmic reticula. Degenerating cells exhibited enlarged vacuoles and the formation of myelin-like structures in the viroplasm which was occupied by huge numbers of virions (Fig. 10A). However, microglia beside vacuolated magnocells were spared of SGNNV infection. In the area abundant in myelinated nerve fibers oligodendrocytes holding a myelinated axon located and some of them were infected. The infected oligodendrocytes displayed

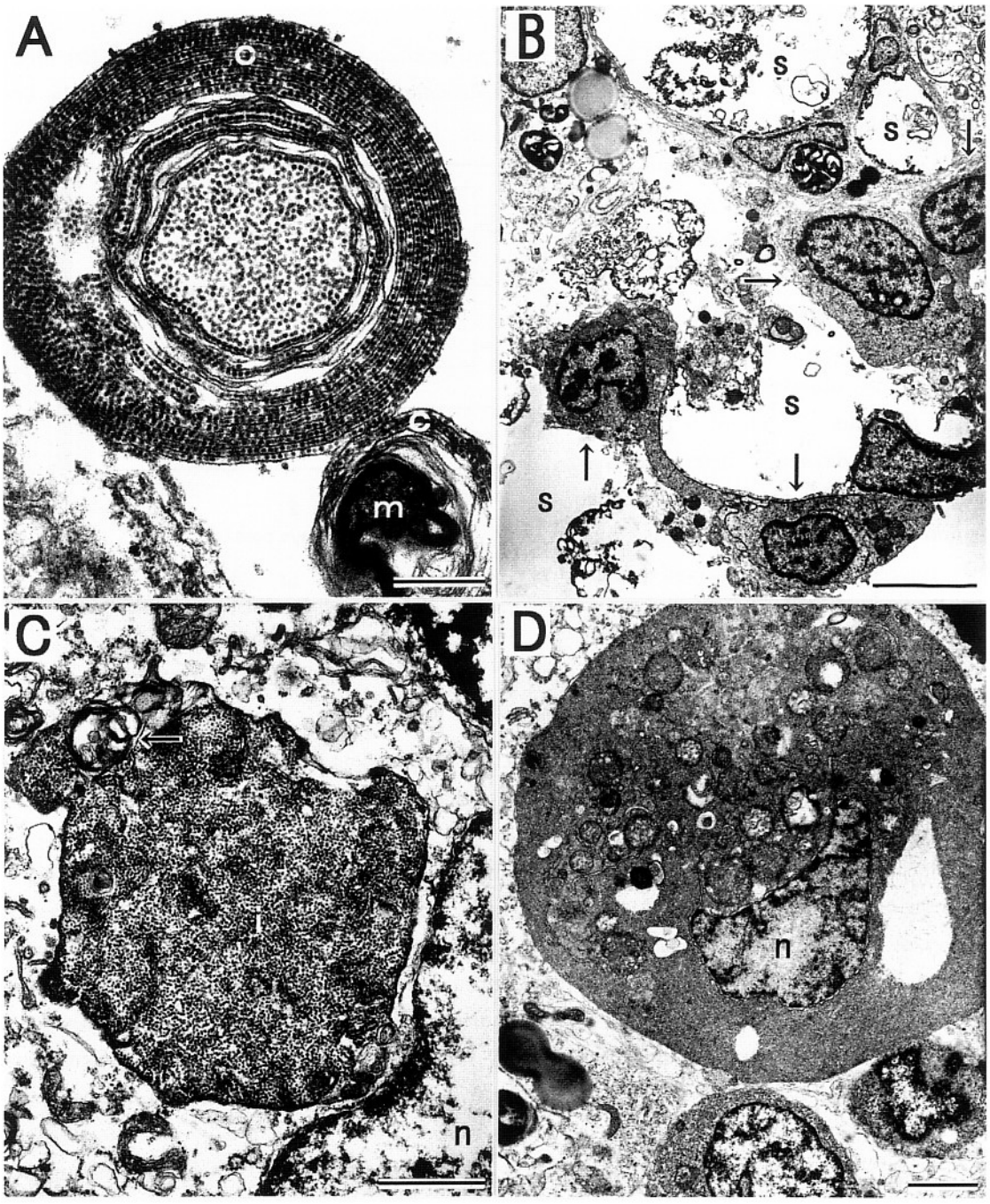

Fig. 9. Electron micrographs of SGNNV-infected nerve cells in the granule cell layer (A, B) and the pars centralis of the dosal area (C, D) of the olfactory lobe. n: nucleus. (A) Viroplasm displays concentric circle (c) delimiting by a thin membrane and containing massive virions or myelin-like structure (m). Bar=500nm. (B) SGNNV-infected and destroyed cells are replaced by a space (s) containing cellular debris and proliferated by microglia (arrows). Bar=5μm. (C) SGNNV-infected nerve cell with a stellar shape displays viroplasmic inclusion body (i) containing SGNNV virions and myelin-like structures (arrow shows representative one) in the cytoplasm. Bar=1μm. (D) SGNNV-infected large nerve cell shows a cytoplasm occupied by the viroplasm. Bar=2μm.

viroplasmic inclusion bodies (Fig. 10B). The myelinated axon contains several mitochondria which helps us to tell the myelinated axon from a myelin-like structure due to SGNNV infection.

In the cerebellum, infected Purkinje cells showed a cytoplasm and dendrites which were replaced by the viroplasm containing a huge numbers of virions, fine vacuoles and degeneration of mitochondria and endoplasmic reticula (Figs. 10C, 11A). After virus release, Purkinje cells formed many enlarged vacuoles and

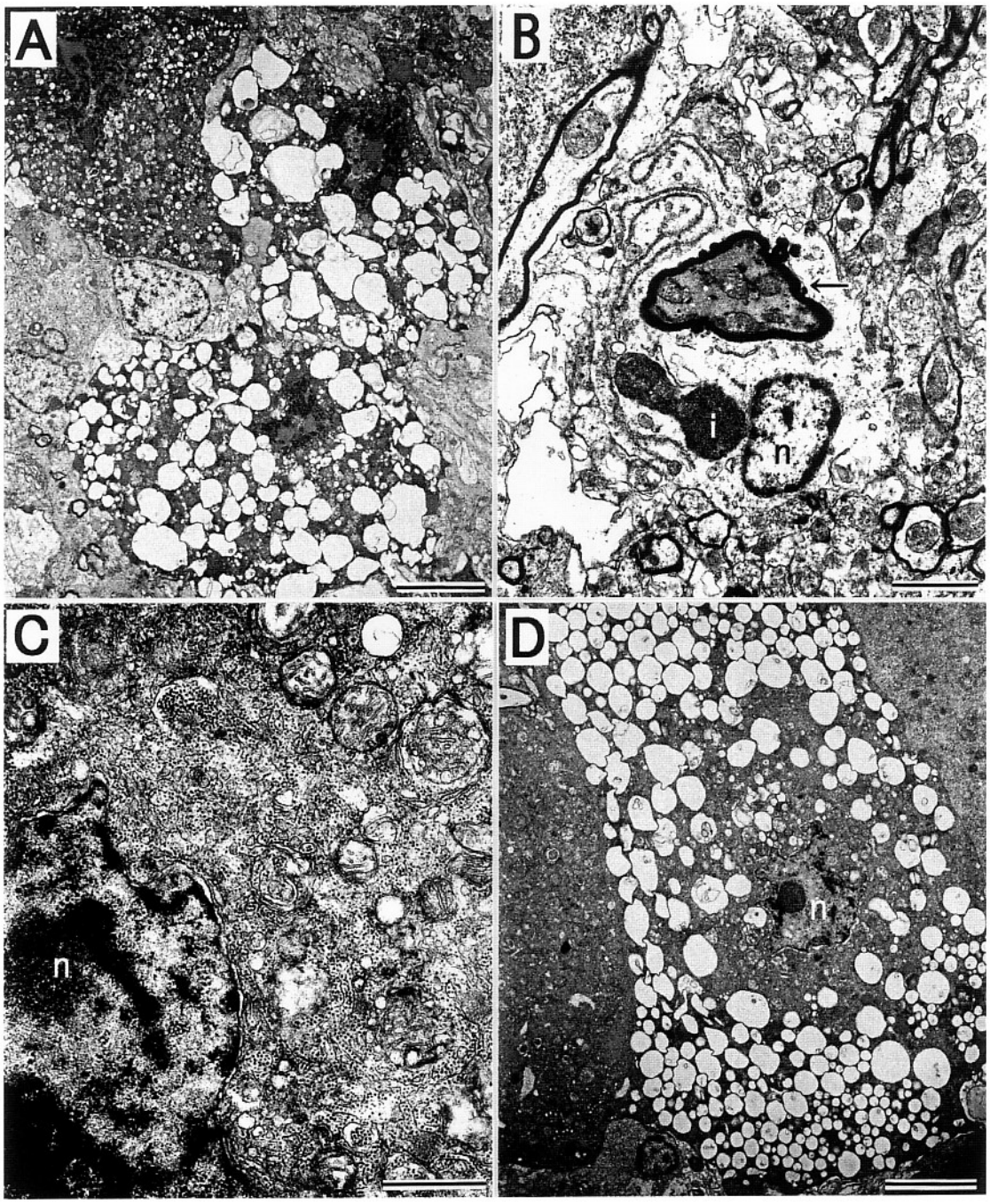

Fig. 10. Electron micrographs of SGNNV-infected nerve cells in the thalamus (A, B) and the cerebellum (C, D). n: nucleus. (A) SGNNV-infected magnocells show viroplasm entirely in the cytoplasm with vacuoles. Bar=5 μm. (B) SGNNV-infected oligodendrocyte displays viroplasmic inclusion (i). arrow: myelinated axon. Bar=2 μm. (C) SGNNV-infected Purkinje cell shows a cytoplasm replaced by the viroplasm containing virions. Bar=1 μm. (D) SGNNV-infected Purkinje cell forms enlarged vacuoles and a degenerated nucleus. Bar=5 μm.

a degenerated nucleus (Fig. 10D). Infected Golgi cells formed viroplasmic inclusion bodies (Fig. 11A). After virus release from the viroplasm, the cytoplasm and viroplasm were destroyed.

In the medulla oblongata, infected megalocells showed a cytoplasm and dendrites which were replaced by the viroplasm containing a huge numbers of virions, and degenerated mitochondria and endoplasmic reticula (Fig. 11B). Infected small nerve cells formed viroplasmic inclusion bodies. After virus release, the cytoplasm was highly vacuolized and contained fragments of viroplasm and organelles.

In the spinal cord, internal cells were infected, and displayed a vacuolated cytoplasm containing

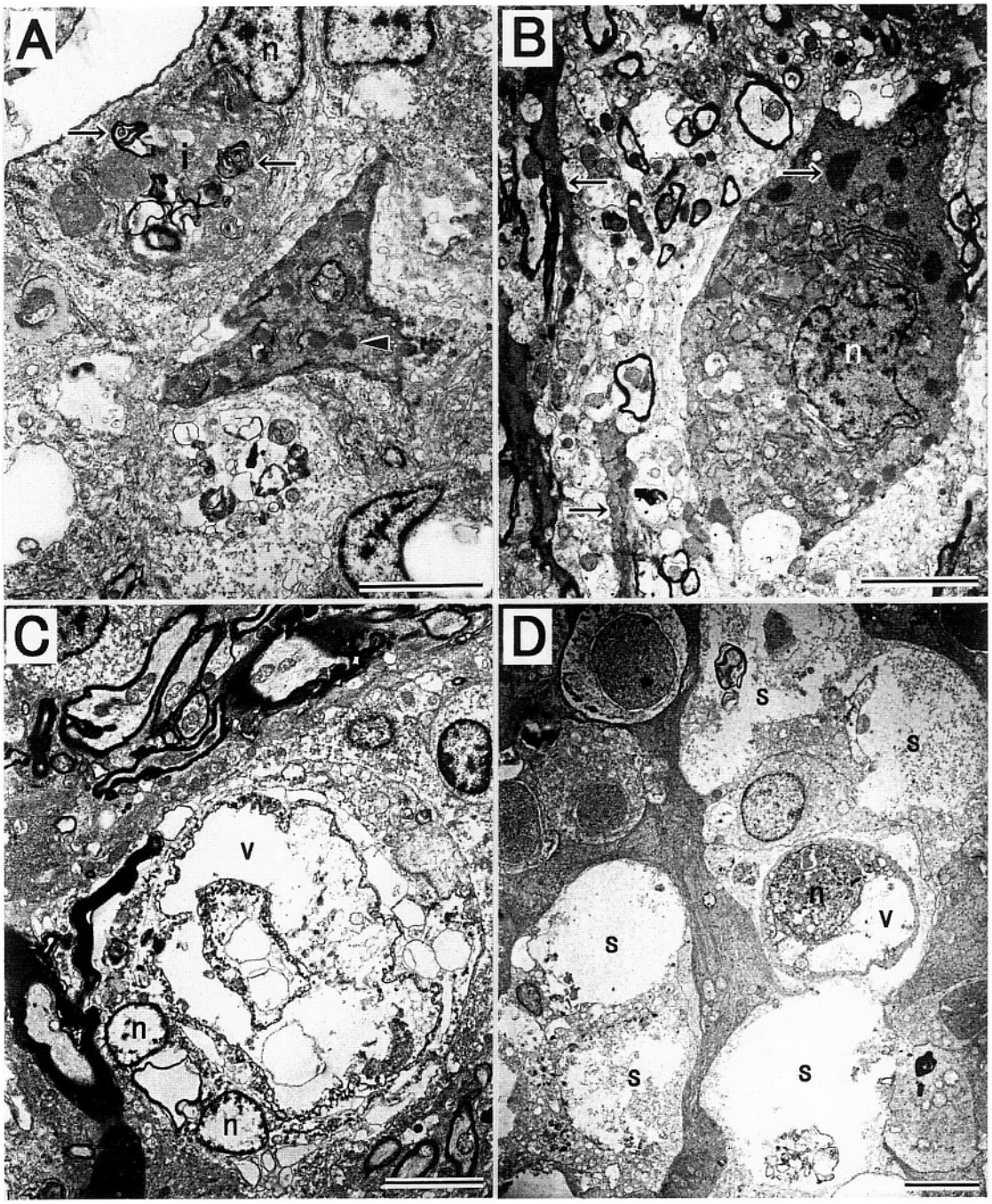

Fig. 11. Electron micrographs of SGNNV-infected nerve cells. n=nucleus, v=vacuole. Bars=5 μm. (A) SGNNV-infected Golgi cell of the cerebellum displays viroplasmic inclusion (i) containing myelin-like structures (arrows show representative ones) in the cytoplasm. SGNNV-infected dendrites of Purkinje cell show viroplasm (arrowhead shows representative one). (B) SGNNV-infected megalocell shows viroplasm (arrows show representative ones) in the cytoplasm and dendrites in the medulla oblongata. (C) SGNNV-infected internal cell has a vacuolated cytoplasm (v) containing fragmented organelles and viroplasm in the spinal cord. (D) SGNNV-infected and destroyed bipolar cells are replaced by a space (s) containing cellular debris.

fragmented organelles and the viroplasm (Fig. 11C).

In the inner nuclear layer of the retina, bipolar cells were mainly affected and displayed a cytoplasm containing vacuoles, degenerated and fragmented organelles and the viroplasmic inclusions. Markedly damaged cells showed fragmented organelles and the viroplasm, and fused neighboring cells together (Fig. 11D). Muller cells appeared to be spared of SGNNV infection.

Discussion

Grow-out stages of sevenband grouper have the well differentiated nerve cells in the CNS and the retina. In the present study, grow-out stages of sevenband grouper with SGNNV infection were studied. Both fish in natural outbreaks and pernasal infection experiments showed the almost same pathological changes. Most of diseased fish extensively displayed SGNNV-infected nerve cells in the olfactory lobe, small nerve cells forming nuclei in the preoptic area, magnocells and parvocells in the thalamus, Purkinje cells, Golgi cells and granule cells in the cerebellum, megalocells forming nuclei in the medulla oblongata, and megalocells and small nerve cells forming nuclei in the spinal cord. The olfactory bulb and optic tectum contained a small number of SGNNV-infected nerve cells. The retina displayed many SGNNV-infected cells in bipolar cells of the inner nuclear layer and ganglion cells in some of diseased fish. On the other hand, meningeal cells in the olfactory lobe and the thalamus, and Muller cells in the retina beside extensively affected nerve cells were spared of SGNNV infection. In the neuroglia, oligodendrocytes allowed SGNNV infection. However, microglia were usually spared of SGNNV infection whereas they proliferated and invaded the necrotic lesions in the CNS. In naturally diseased larvae and juveniles of Japanese parrotfish *Oplegnathus fasciatus* and Atlantic halibut, viral multiplications have been observed in oligodendrocytes, astrocytes and microglia as well as nerve cells (Yoshikoshi & Inoue 1990; Grotmol *et al.* 1997). Because of incomplete differentiation of these cells in larval and juvenile stages, microglia would be infected with betanodavirus unlike fish at grow-out stage. Thus, in sevenband grouper at grow-out stage, SGNNV was determined to target mainly nerve cells, and also oligodendrocytes at the situation that SGNNV markedly propagated in nerve cells.

In some diseased fish, many macrophages infiltrated in necrotic lesions in the olfactory lobe, thalamus and cerebellum, indicating the inflammatory response. Infiltration of inflammatory cells in the CNS and the retina was also observed in naturally diseased larvae and juveniles of several fish species such as barramundi *Lates calcarifer* (Munday *et al.* 1992), Japanese sea bass *Lateolabrax japonicus* (Jung *et al.* 1996), brownspotted grouper (Boonyaratpalin *et al.* 1996) and Atlantic halibut (Grotmol *et al.* 1995; Grotmol *et al.* 1997). Based on histopathological changes in this and above mentioned studies, the disease name should be renamed viral necrotic encephalitis and retinitis.

Determination of the portal of entry and initial multiplication site of betanodavirus is an important subject. There have been reports suggesting the putative portal of entry and initial multiplication site of betanodavirus in larvae and juveniles. The spinal cord and spinal ganglia were proposed to be the primary sites of betanodavirus multiplication in naturally diseased larvae and juvenile of Japanese parrotfish (Yoshikoshi & Inoue 1990). Jung *et al.* (1996) also reported that many affected nerve cells were observed in the thalamus, medulla oblongata and spinal cord but not in the olfactory lobe in naturally diseased juvenile of Japanese sea bass. Nguyen *et al.* (1996) reported that necrosis and specific immunolabeling of the nerve cells were first observed in the spinal cord, later in the brain, and then in the retina of naturally diseased larval striped jack. Larval Atlantic halibut received a bath-challenge also displayed initial immunolabeling in the medulla oblongata and the spinal cord (Grotmol *et al.* 1999). These affected fish of larval striped jack and Atlantic halibut displayed immunolabeling in the epidermal cells or the intestinal epithelial cells simultaneously with the nerve cells of the CNS in an early stage of betanodavirus infection, which suggested that the epithelium was a possible portal of entry for betanodavirus. They also implied that betanodavirus invaded the medulla oblongata and the spinal cord via sensory and/or motor nerve linking to the under epithelium. Many cases of successful bath challenge and cohabitation experiments have been also reported in larvae and juveniles of several fish species (Glazebrook *et al.* 1990; Mori *et al.* 1991; Tanaka *et al.* 1998; Arimoto *et al.* 1993; Nguyen *et al.* 1994; Peducasse *et al.* 1999). Thus, in larvae and juveniles, the epithelial cells of the skin or the

intestine were implied to be the possible portal of entry. However, the nasal epithelium has not been regarded being a portal of entry for betanodavirus.

In the present study, because only some fish showed the affected retina and slight affection in nerve cells of the optic tectum, it was speculated that SGNNV did not invaded the retina via the conjunctiva or cornea in the natural outbreaks. However, in infection experiments using grow-out stages of sevenband grouper, a pernasal infection with SGNNV was established and diseased fish displayed the almost same pathological changes as those found in the natural outbreaks. The nasal epithelium and olfactory nerve did not show any sign of evident SGNNV propagation. This result indicated these tissues were unsusceptible to SGNNV. Nerve cells in the olfactory bulb were slightly infected with SGNNV and the onset of necrotized cells was later than in the olfactory lobe, which indicated that the olfactory bulb did not have so many cells susceptible to SGNNV. These findings determined that SGNNV penetrated the nasal epithelium, passed through the olfactory nerve and olfactory bulb, and invaded the olfactory lobe to markedly propagate. On the other hand, diseased fish by an i.m. injection with SGNNV displayed distinct SGNNV-infected nerve cells in the medulla oblongata and spinal cord while the olfactory lobe, thalamus, optic tectum and cerebellum slightly showed affected nerve cells. The retina also showed only a small number of affected nerve cells. These findings indicated axonal transport of injected SGNNV from the peripheral nervous system in the lateral musculature to the medulla oblongata and spinal cord. If SGNNV invaded the CNS via the blood circulation from the SGNNV-injected site, nerve cells at various regions of the CNS and the retina are expected to be equally affected. These results of infection experiments indicated that grow-out stages of sevenband grouper had a pernasal invasion route of SGNNV.

Diseased fish displayed abnormal behavior and the expanded swimbladder. Because of many affected nerve cells in the cerebellum, the damage of cerebellum appears to cause the abnormal behavior. Although the nucleus for gas regulatory function of the swimbladder could not be determine in the medulla oblongata and the spinal cord, one of the nuclei consisted of affected nerve cells would disturb gas regulation, resulting in expansion of a swimbladder. The expanded swimbladder also induced abnormality of behavior.

In expt 1, pernasal infection group showed later appearance of abnormal behavior and lower mortality than i.m. injection group. In an infection experiment designed to observe the mortality character of fish with pernasal SGNNV infection, mortality remained only 10% (1 of 10) during the 20-day experimental period while most of fish showed abnormal behavior (data not shown). Naturally diseased fish of the groups for the observation of the mortalities also exhibited low mortalities (13% and 33%). In natural outbreaks, mortalities (1-70%) usually included not only dead fish but also sacrificed fish that had been still alive and showing abnormal behavior. Thus, low mortality of the fish with experimental pernasal SGNNV infection was consistent with natural outbreaks.

Chapter 3: Susceptibility of cultured juveniles of several marine fish to the sevenband grouper nervous necrosis virus

Betanodaviruses have a wide range of host marine fish species (Munday & Nakai 1997; OIE 2000). Genomic classification of betanodaviruses, based on partial nucleotide sequences (about 430 bases) of the coat protein gene RNA2, has revealed four major types: SJNNV-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type and redspotted grouper nervous necrosis virus (RGNNV)-type (Nishizawa *et al.* 1997). The SJNNV or TPNNV genotype is composed of virus isolates from a single host fish species: SJNNV from striped jack (Mori *et al.* 1992), and TPNNV from tiger puffer (Nakai *et al.* 1994), while BFNNV and RGNNV genotypes have a variety of host species. The BFNNV genotype was isolated from cold water fish such as barfin flounder (Watanabe *et al.* 1999), Atlantic halibut (Grotmol *et al.* 1997), turbot *Scophthalmus maximus* (Bloch *et al.* 1991), and Japanese flounder reared in a cold water area (Hokkaido, Japan, Dr. T. Nakai personal communication). Almost all other virus isolates, including those from warmwater fish belong to the RGNNV genotype (Nishizawa *et al.* 1997; Iwamoto *et al.* 1999). These genotypic variants have their own optimal growth temperature in SSN-1 cells or E-11 cells (Iwamoto *et al.* 1999; Iwamoto *et al.* 2000). Furthermore, a clear difference in pathogenicity between a striped jack isolate (SJNNV genotype) and an Atlantic halibut isolate (BFNNV genotype) was recognized when larvae of both striped jack and Atlantic halibut were bath-challenged with each virus isolate (Totland *et al.* 1999). These results suggest that the genotypic variation of betanodaviruses is closely linked to the phenotypic variations, finally leading to the observed host specificity. However, it is premature to discuss a relationship between genotypes and host specificity because of limited information about the pathogenicity of betanodaviruses.

In this chapter, pathogenicity of an SGNNV isolate that identified to the RGNNV genotype (Tanaka *et al.* 1998; Iwamoto *et al.* 1999) was evaluated in six cultured marine fish species: sevenband grouper, kelp grouper *E. moara*, Japanese flounder, tiger puffer, red sea bream and rock fish *Sebastiscus marmoratus*. The first four species are known as natural hosts of betanodaviruses (Munday & Nakai 1997; OIE 2000), but the latter two are not. In addition to these fish species, hybrids of kelp grouper (female) and sevenband grouper (male) were also examined in the infection experiment.

Materials and Methods

Fish

Hatchery-reared juveniles (0.3-0.9 g average body weight) of seven marine fish species were used for the infection experiments. Fish were reared in 100 L tanks with a flow-through water supply at 15-25℃. Prior to experimentation, brains and retinas of each of the 10 fish randomly sampled from the experimental fish stocks were examined for betanodavirus by RT-PCR. All the tests were negative. Fish were acclimatized at 28℃ for 1 week before infection experiments.

Virus inoculum and infection experiments

Virus inoculum described in Chapter 2 (SGMie95 isolate) was used for infection experiments. Juvenile fish of all species were challenged by a bath method with the SGMie95 isolate. A group of 15-50 juveniles of each species was exposed to 500 mL of sea water containing virus ($10^{5.1}$ $TCID_{50}$ mL^{-1}) for 2 h and then kept in 15 L tanks with a flow-through system. Controls were sham-challenged with PBS instead of the virus. For comparison, 20 sevenband grouper (13.6 g) were given an i.m. injection at a dose of $10^{6.1}$ $TCID_{50}$ per fish and held

in 100 L tanks with a flow-through system. Control fish received an i.m. injection with PBS. Water temperature in all experiments was 28℃. Challenged fish and the controls were fed commercially prepared pellets one to three times a day and mortalities were observed for 15 days.

Histopathology and IFAT

Moribund fish, fish just after death, and all survivors at the termination of experiments were examined by histopathological and immunological procedures. Fish were fixed in 10% buffered formalin solution, processed according to standard histological methods and tissue sections were stained with H & E. Tissue sections including the brain and eyes were also immunostained as described previously.

Virus titration

The E-11 cell line was used for the virus titration. The cells were cultured in 96-well tissue culture plates (Corning, USA) in Leibovitz L-15 medium (Gibco BRL, USA) with 3% fetal bovine serum prior to the assay. Brains and eyes of dead fish were homogenized with 99 volumes of Hanks' balanced salt solution (HBSS; Gibco) and centrifuged at 1,500 *g* for 15 min. The supernatants were filtered (0.45 μm). Virus samples were further serially 10-fold diluted with HBSS and 50 μL from each dilution was inoculated into the wells. Four wells were used for each diluted sample. Cytopathic effect (CPE) was observed daily at 25℃ for 10 days and the $TCID_{50}\,g^{-1}$ was calculated by the method of Reed & Muench (1938).

Results

Mortality, histopathology, and IFAT

Mortalities of fish during the 15-day observation period after challenge with SGNNV and results of the IFAT are shown in Table 5 and Fig. 12. Mortalities were observed in juveniles of sevenband grouper, Japanese flounder, and tiger puffer that were bath-challenged with SGNNV. Bath-challenged sevenband grouper displayed anorexia and abnormal behavior, loss of balance of the body on the tank bottom at 3 days p.i., but the cumulative mortality was only 4% (one of 25). Japanese flounder showed anorexia at 6 days p.i., loss of balance on the tank bottom and spiral swimming at 9 days p.i., and 42% of fish (21 of 50) died at 6-13 days p.i. Tiger puffer exhibited anorexia and lethargic behavior on the tank bottom at 7 days p.i. Some fish had an expanded swimbladder and floated upside down at 9 days p.i., and 32% of fish (16 of 50) died at 8-13 days p.i. All the sevenband grouper that were given an i.m. injection showed anorexia and abnormal behavior, loss of balance on the tank bottom at 2 days p.i. At 3 days p.i., some of these fish had expanded swimbladders and floated at the water surface with loss of balance. All 20

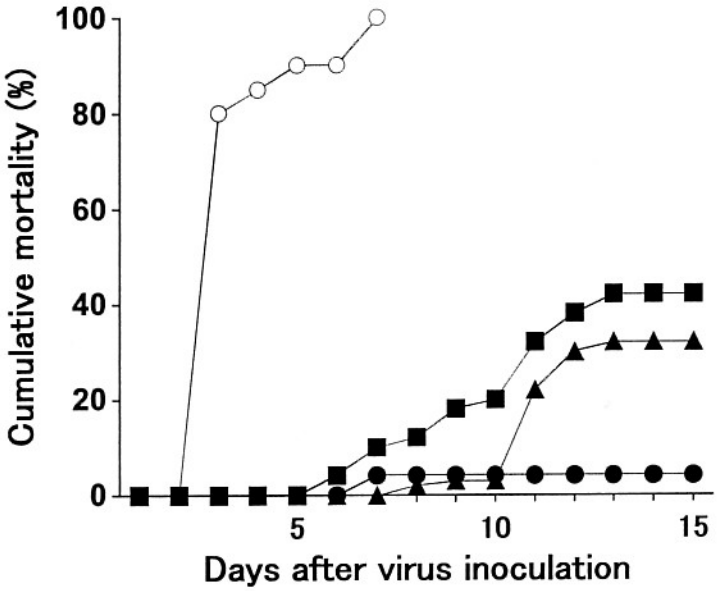

Fig. 12. Cumulative mortalities in juvenile marine fish species challenged with SGNNV. Bath-challenge: sevenband grouper (●), Japanese flounder (■), tiger puffer (▲), i.m.-injection: sevenband grouper (○).

Table 5. Mortalities in juvenile fish after challenge with SGNNV and the detection of virus antigens by IFAT

Challenge method[a]	Fish species	Body weight(g)	No. of fish	Mortality (%)	No. of fish IFAT-positive/examined			
					Dead fish		Survivor[b]	
					CNS[c]	Retina	CNS	Retina
Bath	Sevenband grouper	0.8	25	4	1/1	1/1	19/24	24/24
	Japanese flounder	0.3	50	42	21/21	21/21	20/29	27/29
	Tiger puffer	0.7	50	32	16/16	16/16	9/34	9/34
	Rock fish	0.5	30	0	–	–	13/30	21/30
	Grouper hybrid[d]	0.4	15	0	–	–	4/15	15/15
	Kelp grouper	0.9	30	0	–	–	0/30	0/30
	Red sea bream	0.6	30	0	–	–	0/30	0/30
Injection	Sevenband grouper	13.6	20	100	20/20	20/20	–	–

[a] Fish were challenged with SGNNV by bath method at $10^{5.1}$ $TCID_{50}$ mL^{-1} or intramuscular injection at $10^{6.1}$ $TCID_{50}$ per fish and kept at 28℃. Fish of every control group were all negative in IFAT.
[b] Sampled at 15 days p.i.
[c] Central nervous system (brain and spinal cord).
[d] Hybrid of kelp grouper (female) and sevenband grouper (male).

fish died at 3-7 days p.i.

In histopathological examinations of all i.m. injected sevenband grouper that were sampled when moribund or just after death showing abnormal behavior, the brain, the spinal cord and the retina displayed the distinct signs of VNN. Many nerve cells in the olfactory lobe, thalamus, optic tectum, medulla oblongata and the spinal cord showed intracytoplasmic vacuolization, followed by nuclear degeneration and cellular fragmentation. Necrotized cells were often replaced by spaces containing debris after fragmentation of the cells (Fig. 13A). The IFAT revealed positive fluorescence in the cytoplasm of vacuolized nerve cells and in the debris from necrotized nerve cells. The IFAT-positive spots were also observed in nerve cells showing no clear intracytoplasmic vacuolization (Fig. 13B). Many Purkinje cells showed specific fluorescence in the cerebellum (Fig. 13C). Sevenband grouper, Japanese flounder and tiger puffer that were bath-challenged, displayed histopathological signs and IFAT-positive spots similar to those of i.m. injected sevenband grouper (Fig. 14), but had only a few Purkinje cells showing fluorescence in the cerebellum. In every group, the retina of moribund or dead fish also displayed many necrotized cells that were subsequently replaced by spaces containing debris derived from fragmented cells in the inner nuclear layer (Fig. 15A). These affected cells markedly fluoresced with IFAT. On the other hand, surviving bath-challenged sevenband grouper, Japanese flounder and tiger puffer, although displaying abnormal behavior during the experimental period, showed only a small number of nerve cells with vacuolar degeneration in the olfactory lobe, thalamus and optic tectum which were fluorescent with IFAT. However, their retinal lesions were severe, as seen in dead fish. In moribund, dead or surviving fish, no distinctive pathological signs were found in other tissues such as the heart, spleen, kidney, liver, skin, lateral musculature, gills, and digestive tract.

Bath-challenged rock fish and hybrid of kelp grouper (female) and sevenband grouper (male) (grouper hybrid) did not display any behavioral abnormality and mortality during the experimental period. Some fish from these groups showed a small number of nerve cells with karyopyknosis and fluorescence with IFAT in the thalamus and the optic tectum. The retina of all grouper hybrids showed many spaces containing debris after fragmentation of the necrotized cells and fluorescence with IFAT in the inner nuclear layer (Fig. 15B), while rock fish showed some necrotic cells with karyopyknosis and fluorescence with IFAT.

Kelp grouper and red sea bream showed no behavioral abnormality after the bath challenge, and no histopathological changes or no fluorescence with IFAT. All control groups displayed no abnormalities.

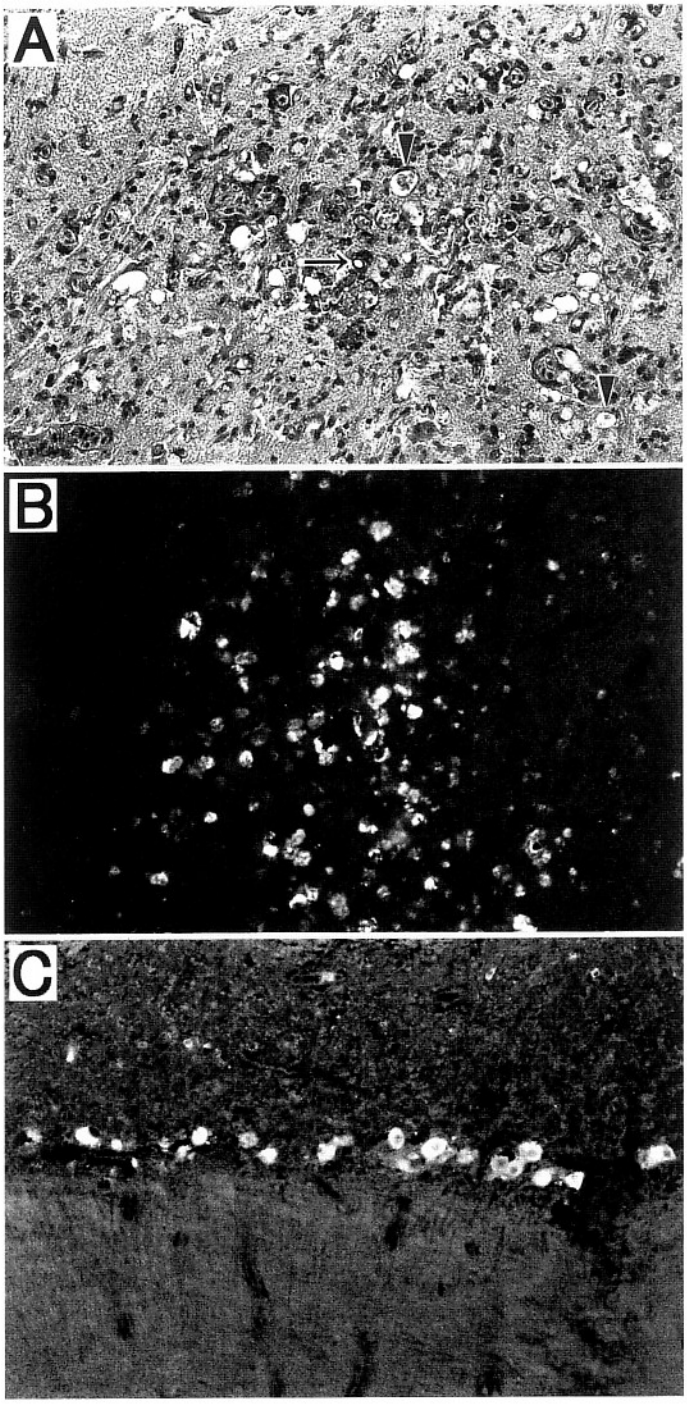

Fig. 13. Histological signs of sevenband grouper. (A) SGNNV-infected nerve cells are vacuolized (arrow shows representative one) and fragmented cells are replaced by a vacuolar space, sometimes containing cellular debris (arrowheads show representative ones) in the olfactory lobe (H&E, ×100). (B) SGNNV-infected nerve cells in the olfactory lobe show specific fluorescence with IFAT (×100). (C) SGNNV-infected Purkinje cells in the cerebellum show specific fluorescence with IFAT (×100).

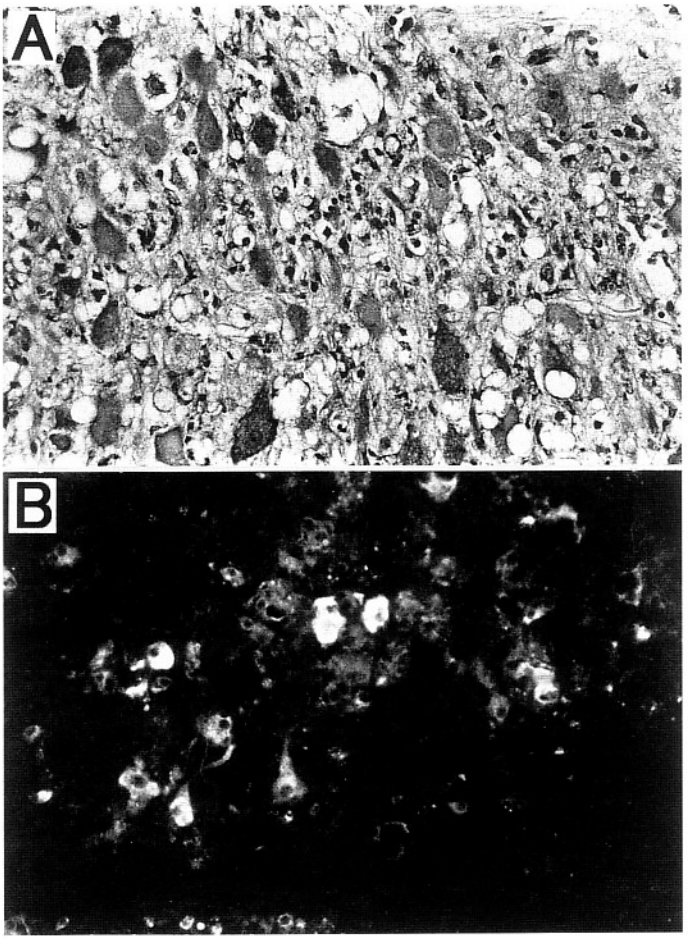

Fig. 14. Histological signs of the spinal cord of moribund Japanese flounder. (A) SGNNV-infected nerve cells show necrosis and vacuolation and fragmented cells are replaced by a vacuolar space, sometimes containing cellular debris (H&E, ×100). (B) SGNNV-infected nerve cells show specific fluorescence with IFAT (×100).

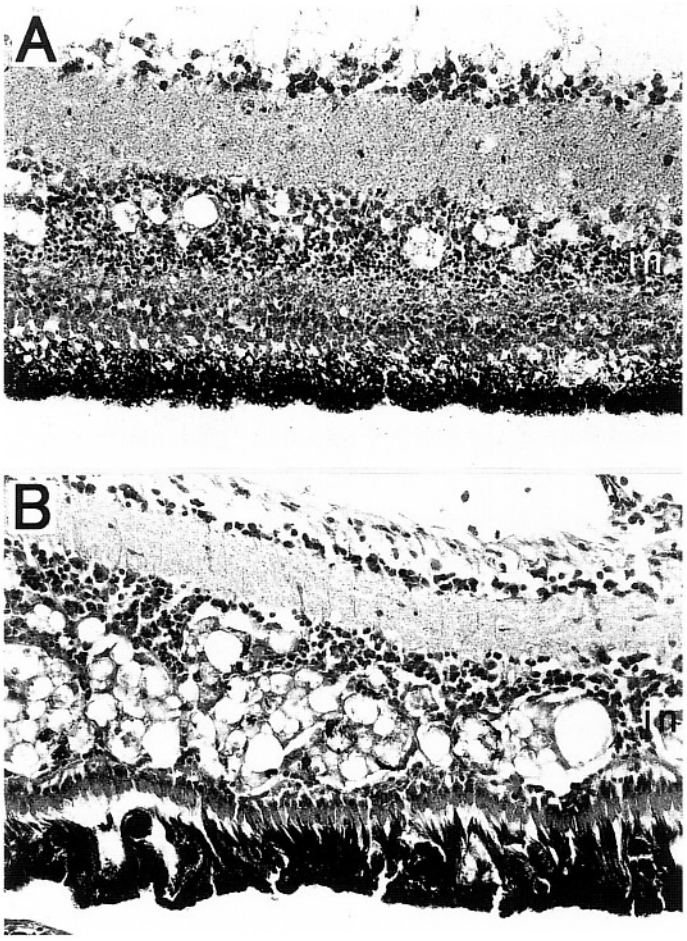

Fig. 15. Large spaces containing cellular debris in the inner nuclear layer (in) (H&E, ×100). (A) Retina of moribund tiger puffer. (B) Retina of survivor of grouper hybrid.

Virus titration

The results of virus titrations in dead i.m. injected sevenband grouper, bath-challenged Japanese flounder and tiger puffer are shown in Table 6. CPE characterized by intracytoplasmic vacuole formation followed by intensive disintegration, similar to that mentioned by Iwamoto *et al.* (2000), was observed within 3-5 days p.i. Virus titers ranged from $10^{7.80}$ to $10^{9.97}$ $TCID_{50}$ g^{-1}. No remarkable difference was observed in the titer among these three species.

Table 6. Titration of virus in dead fish challenged with SGNNV

Fish species (challenge method)	Virus titer (average) in $\log_{10}$ $TCID_{50}$ g^{-1}	
	Brain	Eye
Sevenband grouper (N=5) (injection)	8.63−9.30 (8.80)	7.80−8.97 (8.57)
Japanese flounder (N=5) (bath)	8.80−9.97 (9.21)	7.97−8.97 (8.70)
Tiger puffer (N=3) (bath)	8.97−9.30 (9.08)	7.80−8.97 (8.47)

Discussion

In the present study, an SGNNV isolate belonging to the RGNNV genotype was used for bath-challenge infection experiments on hatchery-reared juveniles of several marine fish species. Its pathogenicity was determined by histopathological and immunological examination in addition to the appearance of clinical signs and mortalities. The SGNNV was pathogenic not only to sevenband grouper and Japanese flounder that are known to be natural hosts for the RGNNV genotype, but also to species previously unaffected by this genotype (tiger puffer, rock fish and grouper hybrid). There were no significant differences in immunohistological changes and virus titers in the CNS and the retina of affected fish between these fish species or by challenge method. High mortality (32%) in bath-challenged tiger puffer suggests the possibility of natural outbreaks of VNN in this species caused by the RGNNV genotype. Although neither mortality nor behavioral abnormalities were recorded in rock fish and grouper hybrid, virus antigens were detected at high levels in the brain or the retina of challenge survivors of these fish species, as in sevenband grouper, Japanese flounder and tiger puffer, indicating the neuro-invasiveness of the virus. In contrast, no behavioral or immunohistological abnormalities in bath-challenged kelp grouper and red sea bream were observed, indicating no or little susceptibility of these species to SGNNV. Grouper hybrid showed intermediate susceptibility between that of the parent species, i.e. sevenband grouper and kelp grouper, suggesting that grouper hybrids

inherited elements of the susceptibility of both parent species to SGNNV.

There have been some reports on the susceptibility of non-natural host species to betanodaviruses. An SJNNV genotype isolate caused mortality only in larval striped jack, but not in larvae of yellowtail, goldstriped amberjack *Seriola lalandi*, and red sea bream when fish were bath-challenged (Arimoto *et al.* 1993). As mentioned previously, two betanodavirus strains, striped jack isolate SJNag93 (SJNNV genotype) and Atlantic halibut isolate AH95NorA (BFNNV genotype), differed in their pathogenicity to larval striped jack and larval Atlantic halibut using bath-challenges (Totland *et al.* 1999). In the present study, the experimental results are partly consistent with the occurrence of natural outbreaks of VNN in sevenband grouper and Japanese flounder and the luck of natural outbreaks in red sea bream caused by the RGNNV genotype. On the other hand, SGNNV showed high pathogenicity to tiger puffer that is known as a natural host of TPNNV, but not to kelp grouper that is known as a natural host of the RGNNV genotype. These results are inconsistent with the reported natural host range of RGNNV. Husgard *et al.* (2001) also reported that i.m. injection of SJNNV could result in the development of VER with significant mortality in juvenile turbot, a species that is known as a natural host of the BFNNV genotype. These results suggest that the biological properties underlying the host specificity of betanodaviruses are complicated and may not follow the four genotypes designated by Nishizawa *et al.* (1997). In order to elucidate a relationship between genotype and host specificity, the genotypes should be classified in more detail, and cross-infection experiments should be carried out. As demonstrated in this and other studies (Husgard *et al.* 2001), betanodavirus infections in fish are evidenced by multiplication of the virus in the CNS and the retina which can be detected by immunohistopathological methods, even in fish showing no clinical signs. Immunohistopathological examinations are necessary in addition to the observation of clinical signs and mortalities, in order to determine the pathogenicity of betanodaviruses in infection experiments.

Chapter 4: Protective immunity of sevenband grouper against experimental viral nervous necrosis

In the former chapters, mass mortalities in sevenband grouper at grow-out stage were confirmed to be caused by SGNNV. Target cells of SGNNV were also determined and pernasal invasion route of SGNNV into the CNS was indicated. Moreover, the relation between genotype and host specificity of SGNNV was discussed. Thus, valuable information on the etiology of the disease was presented. However, at present, no control measures have been established for VNN of sevenband grouper, or for other fish except for striped jack in which the effective measures to cut off vertical transmission of betanodavirus were established (Munday & Nakai 1997). Because of the global spread of VNN in groupers, a way to control the disease is urgently needed. It is known that survived sevenband grouper from natural infection are resistant to recurrence of the disease, suggesting a protective immune response of fish to the infection. In this chapter, the presence of protective virus-neutralizing antibodies in survivors from experimental infections and the potential of vaccination with an *E. coli*-expressed recombinant coat protein against experimental infection were described.

Materials and methods

Fish

Wild sevenband grouper (90 g in average weight) and hatchery-reared redspotted grouper (9 g) were used for the preparation of immune sera and for an *in vivo* neutralizing activity test, respectively. Hatchery-reared sevenband grouper (28 g) were used for active immunization with recombinant coat protein. Fish were reared in 1 m^3 tanks with a flow-through system at about 25℃. Prior to experimentation, brains and retinas of each of 10 fish randomly sampled from fish stocks were examined for betanodavirus by RT-PCR and all were negative.

Virus inoculum

The virus filtrate designed as SGMie95 isolate described in Chapter 2 was used for infection experiments and virus neutralization assays.

Preparation of serum from surviving fish

A total of 20 wild sevenband grouper received two i.m. injections of the virus filtrate at a dose of $10^{4.4}$ $TCID_{50}$ per fish at 10-day intervals. Fifteen days after the second injection, the fish were i.m.-injected with the virus filtrate at $10^{4.4}$ $TCID_{50}$ per fish and observed at 28℃ for 12 days. During the observation period, cumulative mortality of fish caused by specific SGNNV infection was 50%. Blood samples were collected from five survivors. After being kept at 4℃ overnight, serum was obtained by centrifugation at 1,500 *g* for 15 min, and stored at −80℃ until use. Control sera were obtained from five untreated sevenband grouper. Virus-neutralizing activity of these sera was examined by *in vitro* cell culture assay and *in vivo* assay using redspotted grouper, as described below.

Virus neutralization assays

The E-11 cell line was used for the virus-neutralization assay. The cells were cultured in 96-well tissue culture plates (Corning) in Leibovitz L-15 medium (Gibco) with 5% fetal bovine serum prior to the assay. All fish sera were diluted with 15 volumes of HBSS (Gibco) and sterilized through a 0.45 μm membrane filter.

Serum samples were further serially 2-fold diluted with HBSS and mixed with an equal volume of the virus ($10^{4.7}$ $TCID_{50}$ 0.025 mL^{-1}). After incubation at 25℃ for 1 h, the mixture was inoculated into the wells. Four wells were used for each diluted sample. CPE was observed daily at 25℃ for 10 days and the virus-neutralizing titer of serum was calculated according to Reed and Muench (1938).

In the *in vivo* assay, the pooled serum was mixed with an equal volume of the virus ($10^{7.8}$ $TCID_{50}$ mL^{-1}) and incubated at 25℃ for 2 h and then at 4℃ overnight. A total of 20 redspotted grouper were i.m.-injected with 0.04 mL of the virus treated with either immune or control serum. Fish were observed in 100 L tanks at 27-28℃ for 10 days. Mortalities during this period and survivors at the termination of the experiment were examined histopathologically and by IFAT.

Histopathology and IFAT

Brains and eyes of fish were fixed in 10% buffered formalin and embedded in paraffin wax. The sections were stained with H & E. They were also immuno-stained (IFAT) as described previously.

Preparation of recombinant coat protein

Two oligonucleotide primers were designed from the RNA2 gene sequence of SJNNV (Nishizawa *et al.* 1995). The sense NNV-F5 (5'-gactccATGGTACGCAAAGGTGA-3': nucleotide position 17-33) and antisense NNV-R3 (5'-cagctcgaGGCCATTTAACCACATG-3': nucleotide position 1371-1387) with recognition sites in the linker sequences (lowercase letters) were used for amplification of a target region containing the open reading frame of the SGMie95 isolate. The RT-PCR amplification was carried out by the method of Nishizawa *et al.* (1994). The PCR amplicons were digested with NcoI and XhoI restriction enzymes and ligated in a pET-16b plasmid (Novagen, Germany) which was cleaved with the same enzymes and treated with alkaline phosphatase (Takara Shuzo, Japan) at 16℃ overnight. After cloning in *E. coli* DH5α, *E. coli* BL21 (DE3) (Novagen) as an expression host was transformed with a cloned recombinant plasmid.

The cells of recombinant *E. coli* were cultured in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg mL^{-1}) at 37℃ with agitation until the mid-log phase (OD=0.6). Isopropylthio-β-D-galactoside (IPTG) was added to the culture at 1 mM final concentration and the mixture cultured for a further 3 h. After centrifugation at 3,000 *g* for 20 min, the bacterial cells were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel and Western blot using the anti-SJNNV rabbit serum, carried out according to the methods described by Laemmli (1970) and Towbin *et al.* (1979), respectively. The bacterial cells were suspended in a buffer (0.2 mM EDTA-2Na, 0.1% TritonX-100, 50 mM Tris-HCl, pH8.0), sonicated, and centrifuged at 3,000 g for 20 min. After three washings with centrifugation, the insoluble fraction obtained was suspended in 10 mM phosphate-buffered saline and used as an immunogen for fish.

Vaccination experiments

Two separate vaccination trials were carried out using hatchery-reared sevenband grouper weighing 28 g. Sixty fish (expt 1) or 40 fish (expt 2) were twice i.m.-injected with 60 μg of the recombinant coat protein at 10-days intervals. Another 60 or 40 fish received the insoluble fraction (6 μg protein per fish) extracted from *E. coli* before IPTG induction as a control. The fish were transferred to 500 L tanks with a flow-through water supply at 25℃ and fed with commercially prepared pellets once a day. Ten days after the second injection, groups of 20 fish were i.m.-injected with the virus at doses of $10^{3.4}$, $10^{4.4}$, or $10^{5.4}$ $TCID_{50}$ per fish (expt 1), or $10^{3.4}$ or $10^{4.4}$ $TCID_{50}$ per fish (expt 2), and observed in 100 L tanks with a flow-through water supply at 28℃ for 2 weeks. Mortalities and all survivors at the termination of experiments were examined histopathologically and by IFAT. Statistical analysis was performed by the χ^2-test. Relative percent survival (RPS) was

calculated from the cumulative mortality by the following: RPS= |1−(% mortality of vaccinated group/% mortality of control group)| ×100.

Apart from these experiments, two groups of 30 fish were used to assay virus-neutralizing titers in serum after vaccination. Fish received two i.m.-injections with the same recombinant coat protein or *E. coli* extract (control) as described above and were reared in 200 L tanks with a flow-through water supply at 20 -25℃. At 0, 20, 30, 50, 70, and 110 days after the first injection, serum was collected from five fish randomly sampled from each group and the virus-neutralizing titers were examined using the E-11 cell line as described above.

Results

Virus-neutralizing activity of serum from surviving fish

A total of five individual serum samples obtained from survivors after virus infection were examined for neutralizing activity against betanodavirus SGMie95 isolate. All serum samples from survivors showed virus-neutralizing activity at titers of 1:158-1:1257, while those of uninfected control fish were lower than 1:32 (Table 7). In the *in vivo* assay, treatment of SGMie95 isolate with pooled serum from survivors resulted in significantly ($P<0.01$) lower mortality of fish, compared with that in fish which received virus treated with normal serum (Table 8). Mortality occurred at 3-8 days p.i. with abnormal swimming and loss of balance at the water surface or on the tank bottom. All dead fish exhibited positive-IFAT reactions in the degenerated (necrosis and vacuolization) brain and retinal tissues. The IFAT-positive rate in survivors at 10 days p.i. was 19.2% (5/26).

Table 7. Detection of neutralizing antibodies against betanodavirus in sevenband grouper surviving virus infection

Fish serum no.	Neutralizing titer (1:)[a]
Survivor	
1	1000
2	852
3	1257
4	158
5	1170
Normal	
1	<32
2	<32
3	<32
4	<32
5	<32

[a] Assayed by using E-11 cell line.

Table 8. *In vivo* betanodavirus-neutralizing test of svenband grouper serum

Virus treatment with[a]	No. of fish dead/examined	Mortality (%)	χ^2-test	RPS	No. of fish IFAT-positive/examined	
					Dead fish	Survivor[b]
Immune serum	3/20	15	P<0.01	73	3/3	2/17
Normal serum	11/20	55			11/11	3/9

[a] Fish were intramuscularly challenged by pretreated betanodavirus (SGMie95 isolate) with serum of surviving or control fish.
[b] Sampled at 10 days p.i.

Recombinant coat protein

The SDS-PAGE and Western blot profiles of the recombinant *E. coli* cells are shown in Fig. 16. A 42 kDa protein of the same size as the SJNNV coat protein was detected from recombinant *E. coli* cells which reacted with the anti-SJNNV rabbit serum. The expressed 42 kDa protein was observed as inclusion bodies in the induced bacterial cells and obtained as insoluble fraction at a concentration of ca. 0.2 mg mL^{-1} from bacterial cultures.

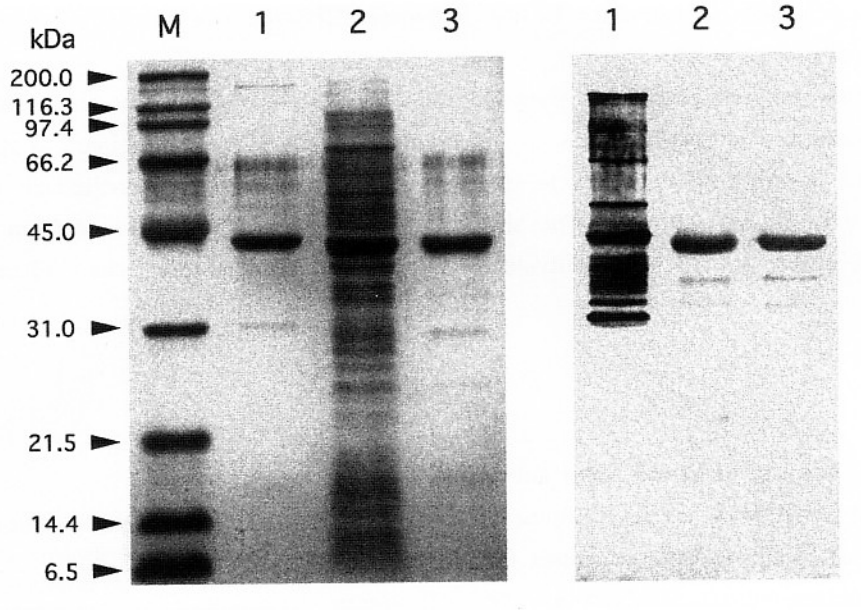

Fig. 16. SDS-PAGE and Western blot analyses of *E. coli*-expressed recombinant coat protein. Left: SDS-PAGE. Right: Western blot. M: molecular weight marker, Lane 1: purified SJNNV, 2: *E. coli*-expressed coat protein of SGMie95, 3: insoluble fraction of the expressed protein.

Vaccination experiments

The two different vaccination trials (expts 1 and 2) gave almost identical results (Fig. 17, Table 9). Vaccinated fish groups showed significantly ($P<0.01$ or 0.001) lower mortalities at every challenge dose of virus than the control groups. The RPS values of the vaccinated group challenged with a minimum dose of virus ($10^{3.4}$ $TCID_{50}$ per fish) were particularly high, 88 in expt 1 and 69 in expt 2. The virus etiology in all dead fish was demonstrated by histopathological and IFAT examinations. The IFAT-detection of nodavirus in survivors at 14 days p.i. was 22.9% (8/35) in expt 1 and 18.8% (6/32) in expt 2.

Table 9. Betanodavirus-challenge of sevenband grouper vaccinated with the recombinant coat protein

Expt	Fish group[a]	Challenge dose ($TCID_{50}$ /fish)[b]	No. of fish dead/examined	Mortality (%)	χ^2-test	RPS	No. of fish IFAT-positive/examined: Dead fish	No. of fish IFAT-positive/examined: Survivor[c]
1	Vaccinated	$10^{5.4}$	13/20	65	$P<0.01$	35	13/13	4/7
		$10^{4.4}$	13/20	65	$P<0.01$	35	13/13	2/7
		$10^{3.4}$	2/20	10	$P<0.001$	88	2/2	1/18
	Control	$10^{5.4}$	20/20	100			20/20	–
		$10^{4.4}$	20/20	100			20/20	–
		$10^{3.4}$	17/20	85			17/17	1/3
2	Vaccinated	$10^{4.4}$	12/20	60	$P<0.01$	37	12/12	2/8
		$10^{3.4}$	4/20	20	$P<0.01$	69	4/4	1/16
	Control	$10^{4.4}$	19/20	95			19/19	1/1
		$10^{3.4}$	13/20	65			13/13	2/7

[a] Fish were twice intramuscularly injected with *E. coli*-expressed recombinant coat protein of betanodavirus (vaccinated) or *E. coli*-cell extract (control).
[b] Fish were intramuscularly challenged with betanodavirus (SGMie95 isolate) 20 days after 1st immunization.
[c] Sampled at 14 days p.i.

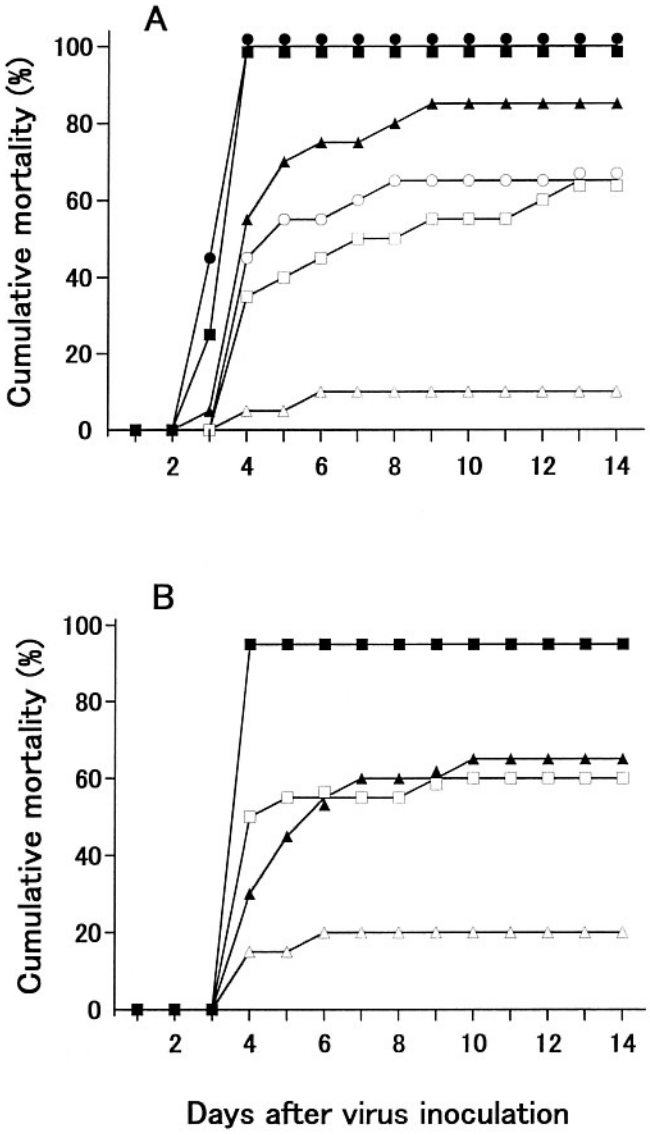

Fig. 17. Cumulative mortalities of vaccinated and control sevenband grouper after SGNNV-challenge in expt 1 (A) and expt 2 (B).
○: vaccinated, challenged with $10^{5.4}$ $TCID_{50}$ per fish
□: vaccinated, challenged with $10^{4.4}$ $TCID_{50}$ per fish
△: vaccinated, challenged with $10^{3.4}$ $TCID_{50}$ per fish
●: control, challenged with $10^{5.4}$ $TCID_{50}$ per fish
■: control, challenged with $10^{4.4}$ $TCID_{50}$ per fish
▲: control, challenged with $10^{3.4}$ $TCID_{50}$ per fish

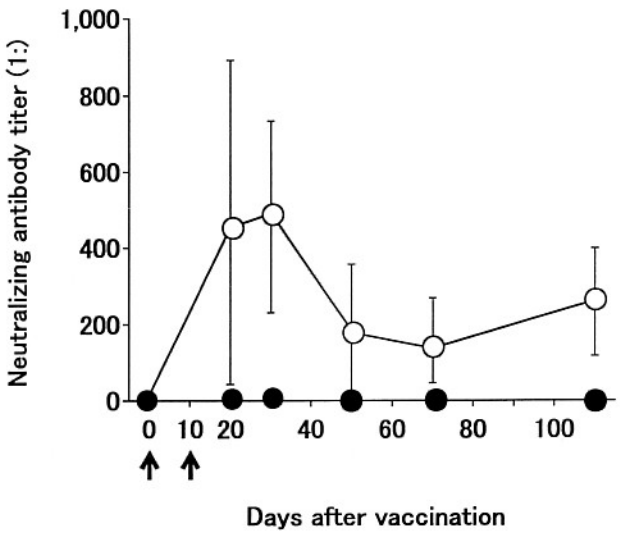

Fig. 18. Neutralizing antibody titers against SGNNV in sevenband grouper. Fish were twice (arrows) injected intramuscularly with *E. coli*-expressed recombinant coat protein (vaccinated ○) or *E. coli* cell extract (control ●). Titer 0 indicates titer lower than 1:32. Data are shown as mean with standard deviation (n=5).

The kinetics of neutralizing antibody titers are shown in Fig. 18. Neutralizing antibody titers of control fish at day 0, 20, 30, 50, 70, or 110 were all lower than 1:32. At day 20 and 30 of the first vaccination, neutralizing antibodies were detected at mean titers of 1:470 and 1:480 and remained at mean values of 1:160-1:260 throughout the experimental period (110 days p.i.).

Discussion

Indirect enzyme-linked immunosorbent assay (ELISA) has been developed to detect antibodies against betanodaviruses from striped jack (Mushiake *et al.* 1992) and sea bass (Breuil & Romestand 1999). Earlier studies on striped jack broodstock, however, did not show correlation between the presence of antibody in the serum and the presence of virus antigen in the ovary (Mushiake *et al.* 1992), and the use of an antibody test to select low-risk broodstocks was replaced by direct detection of virus by RT-PCR (Mushiake *et al.* 1994). The first successful culture of betanodavirus was reported on a betanodavirus from diseased sea bass using the SSN-1 cell line (Frerichs *et al.* 1996). Iwamoto *et al.* (1999) demonstrated that the SSN-1 cell line is suitable for isolation and proliferation of all genetic variants of betanodavirus, and Iwamoto *et al.* (2000) reported that a clonal cell line E-11 from SSN-1 cells is more useful for qualitative and

quantitative analyses based on the cytopathic effects expression. The present study is the first report of detection of betanodavirus-neutralizing antibody from fish. The assay with the E-11 cell line demonstrated that virus-neutralizing antibody was produced at high titers in sevenband grouper survived experimental infection. Such virus-neutralizing activity of the survivor's serum was also confirmed by an *in vivo* test. These results indicate the establishment of acquired immunity in the survivors, which explains their resistance against a natural recurrence of the infection.

Undoubtedly, vaccination is an ideal method to prevent infectious diseases in fish as well as in other animals and humans. Recombinant DNA technology was introduced for the development of vaccines against fish diseases during the last decade (Leong *et al.* 1997; Lorenzen 1999). Although there have been many experimental studies for recombinant vaccine development, only one recombinant vaccine, i.e. *E. coli*-expressed recombinant VP2 protein against infectious pancreatic necrosis virus (IPNV) is commercially available (Christie 1997). A variety of vector/host expression systems have been used in the production of fish vaccine antigens, as reviewed by Leong *et al.* (1997). In order to facilitate the preparation of the coat protein, plasmid containing the coat protein gene was constructed directly into the ATG start codon under control of the T7 promoter. The resulting coat protein was expressed as an intact 42 kDa native protein, of the same size as the virion coat protein, which reacted with anti-SJNNV serum. Expressed coat protein was observed as inclusion bodies in *E. coli*. The inclusion bodies were highly productive, ca. 0.2 mg mL^{-1} of fermentation, and easily isolated from disrupted bacterial cells by centrifugation to yield almost pure protein. In the present study, the production of virus-neutralizing antibody in sevenband grouper was quickly (20 days p.i.) induced by injections of the recombinant coat protein and lasted at least for 110 days. The injection of recombinant coat protein also gave fish high protection against experimental infection. No adjuvant was required for induction of such protective immunity.

Betanodavirus infection is a threat generally prevalent in hatchery-reared larvae and juveniles of marine fish because of heavy mortalities, often causing complete destruction of fish populations (Munday & Nakai 1997; OIE 1997). In striped jack, the disease which is caused by the SJNNV genotype betanodavirus occurs in larvae up to approximately 10 days after hatching and experimental infection demonstrated that striped jack is susceptible to the virus only at the larval stage (Arimoto *et al.* 1993). Broodstock was proved to be an important virus-reservoir (Arimoto *et al.* 1992). Therefore, elimination or segregation of virus-carrying spawners by RT-PCR and disinfection of fertilized eggs with residual oxidants were efficacious in preventing the disease in striped jack (Mushiake *et al.* 1994; Mori *et al.* 1998). It has been already confirmed that *E. coli*-expressed recombinant coat protein of SJNNV induces virus-neutralizing antibodies in broodstocks of striped jack (Dr. K. Mori, personal communication), but efficacy of the recombinant protein as vaccine was not evaluated in active immunization because of the lack of a good vaccination and challenge model. On the other hand, betanodavirus infection at grow-out stages has been often reported in some species of groupers, including sevenband grouper (Nakai *et al.* 1994; Danayadol *et al.* 1995; Fukuda *et al.* 1996; Tanaka *et al.* 1998) and sea bass (Le Breton *et al.* 1997). At present, there is no way to control the disease at these grow-out stages. Injection vaccination will be an effective method to prevent the disease. However, some problems may be encountered in vaccine development against VNN. Some reports suggested different host specificity among genotypic variants (Iwamoto *et al.* 1999, 2000; Totland *et al.* 1999). Furthermore, infection experiments in Chapter 3 suggest that such host specificity may exist even among strains belonging to the same genotype (RGNNV). This will require cross protection experiments with recombinant coat protein from a variety of betanodavirus strains.

Concluding remarks

VNN has been causing severe losses in hatchery-reared larvae and juveniles of a variety of marine fish species all over the world. In striped jack, broodstock was proved to be an important virus-reservoir and the control measures to cut off vertical transmission, elimination or segregation of virus-carrying spawners by RT-PCR and disinfection of fertilized eggs with residual oxidants, were established (Arimoto *et al.* 1992; Mushiake *et al.* 1994; Mori *et al.* 1998). Watanabe *et al.* (1998) also suggested a possible vertical transmission of betanodavirus and effectiveness of above mentioned control measures against VNN in seedling production of barfin flounder. In sevenband grouper, Tsuchihashi *et al.* (2002) reported that betanodavirus was detected in the gonadal materials of broodstock, and implied the possibility of the vertical transmission and efficacy of selection of spawners by RT-PCR. Thus, in hatcheries, vertical transmission is recognized as an important infection route of betanodavirus and its control measures are successful in some fish species including sevenband grouper. However, as demonstrated in this study, VNN has been frequently occurring in sevenband grouper not only at early developmental stages but also at all other life stages. VNN outbreaks at grow-out stage have been reported in some fish species while the infection route of betanodavirus has not been investigated in these fishes. This study indicated a possible water-borne transmission of the virus via the nasal epithelium in grow-out stages of sevenband grouper. In order to prevent the outbreak of VNN in sevenband grouper, not only exclusion of the virus from hatchery but also improvement of defense mechanism against horizontal transmission at grow-out stage are needed. In this study, the potential for vaccination using *E. coli*-expressed recombinant coat protein against VNN was suggested. Active immunization using vaccine will be an important factor of control strategies against VNN. Further studies are necessary in order to make practicable a vaccine. Moreover, in sevenband grouper, the physiological property is still partially investigated for short history as culture industry. Development of health management techniques based on the physiological property will be useful to improve the efficacy of vaccination.

Acknowledgements

I would like to express my deepest gratitude to Professor Dr. Teruo Miyazaki, Laboratory of Fish Pathology, Faculty of Bioresources, Mie University for his wise guidance, kindness and encouragement.

I gratefully acknowledge Professor Dr. Toshihiro Nakai, Laboratory of Aquatic Pathobiology, Graduate School of Biosphere Science, Hiroshima University for his valuable guidance and kindness. I also wish to thank Dr. Minoru Sorimachi, National Research Institute of Aquaculture, Fisheries Research Agency (Present address: Japan Sea National Fisheries Research Institute, Fisheries Research Agency) for his valuable suggestions.

I am particularly grateful to Dr. Misao Arimoto and Dr. Koh-ichiro Mori, National Center for Stock Enhancement, Fisheries Research Agency, Dr. Tokinori Iwamoto, Laboratory of Aquatic Pathobiology, Faculty of Applied Biological Science, Hiroshima University (Present address: Department of Microbiology, University of Alabama at Birmingham) and Ms. Mizuho Takagi, Laboratory of Fish Pathology, Faculty of Bioresources, Mie University for their help in conducting the experiments.

Many thanks to the staff of the Okayama Prefectural Farming Fisheries Center, Ehime Prefectural Fisheries Experimental Station, National Center for Stock Enhancement, Fisheries Research Agency and Mie Prefectural Fish Farming Center for kindly providing fish used in this study.

I greatly appreciate the staff of Owase Fisheries Laboratory, Fisheries Research Division, Mie Prefectural Science and Technology Promotion Center for their useful support and kindness.

Finally, I wish to express my sincere thanks to everyone in my family for their love and support.

Summary

Mass mortalities have been frequently occurring in grow-out stages (0-3 years old) of sevenband grouper in summer and autumn since 1984. Diseased fish showed abnormal behavior, loss of balance of the body at the water surface, and inflation of swimbladder. In the present studies, the etiology of mass mortalities in sevenband grouper at grow-out stage and its control measure were studied.

In Chapter 1, infection experiments using the filtered homogenate of infected organs (brain and eyes) of diseased sevenband grouper were performed to determine the pathogenicity of a betanodavirus found in diseased fish. Young sevenband grouper and juvenile redspotted grouper that were given an intramuscular injection showed abnormal behavior and mortalities while some affected fish recovered after exhibiting the abnormal behavior. In histopathological examinations, affected nerve cells in the brain and the retina showed intracytoplasmic vacuolizations and necrosis of definite signs of VNN in the dead and abnormally-swimming fish. IFAT with an anti-SJNNV serum revealed the viral antigens in the affected nerve cells. The infection experiment with redspotted grouper at different water temperature (16-28℃) indicated that the higher water temperatures enhanced the early onset of the disease signs and higher mortality.

In Chapter 2, pernasal infection experiments using grow-up fish were performed based on histopathological features of the CNS in naturally diseased fish. The pernasal infection was established as a putative invasion way of SGNNV. The definite SGNNV-targeted cells were determined by histopathological studies including IFAT and electron microscopy. Nerve cells in the olfactory lobe were most extensively necrotized with vacuolization followed by proliferation of microglia and infiltration of macrophages. Purkinje cells and Golgi cells were extensively infected in the cerebellum. Megalocells and small nerve cells forming nuclei were also infected in the preoptic area, thalamus, medulla oblongata and spinal cord. Only a few small nerve cells were infected in the olfactory bulb and optic tectum. The retina of some diseased fish extensively displayed vacuolated cells in bipolar cells of the inner nuclear layer and in the ganglion cell layer. These SGNNV-infected nerve cells displayed viroplasmic inclusion containing virions, vacuoles and myelin-like structures. Based on histopathological changes, the lesion of the CNS was characterized by encephalitis but not encephalopathy.

In Chapter 3, SGNNV belonging to the RGNNV genotype, to which most betanodaviruses from warm water fish are identified, was evaluated for its pathogenicity to hatchery-reared juveniles of several marine fish species. Betanodaviruses have been tentatively divided into four genotypes (SJNNV, RGNNV, TPNNV, and BFNNV) and it is suggested that host specificity is different among these genotypes. In infection experiments by a bath method with SGNNV ($10^{5.1}$ $TCID_{50}$ mL^{-1}), sevenband grouper, Japanese flounder and tiger puffer displayed behavioral abnormalities and mortalities with distinct histopathological signs of VNN and heavily immunostained cells were observed in the CNS and the retina. Bath-challenged rock fish and a hybrid of sevenband grouper and kelp grouper did not display any behavioral abnormality or mortality during the experimental period and many fish showed slight signs of virus infection in nerve cells. Kelp grouper and red sea bream showed no behavioral abnormality, mortality or immunohistopathological changes after the virus challenge. These results are, in part, consistent with the natural host range of RGNNV, indicating the complexity in the host specificity of betanodaviruses.

In Chapter 4, the protective immune responses of sevenband grouper immunized with live SGNNV or the *E. coli*-expressed recombinant coat protein was described. Betanodavirus-neutralizing antibodies were detected at titers ranging from 1:158 to 1:1257 in serum of sevenband grouper, which survived i.m. injection with the virus, by a cell culture assay system. The virus-neutralizing ability of immune serum was also confirmed by injecting virus previously treated with serum into fish. This indicates establishment of acquired immunity in

survivors and thus explains why survivors from natural infection are resistant to recurrence of the disease. Young sevenband grouper were immunized twice by intramuscular injections with the recombinant coat protein. Immunized fish produced neutralizing antibodies at high titers for at least 110 days and showed significantly lower mortalities in virus challenge tests. These results suggest the potential for vaccination against VNN in sevenband grouper, which is susceptible to betanodavirus at all life-stages.

References

Arimoto, M., Mushiake, K., Mizuta, Y., Nakai, T., Muroga, K. and Furusawa, I. (1992): Detection of striped jack nervous necrosis virus (SJNNV) by enzyme-linked immunosorbent assay (ELISA). *Fish Pathology* **27**, 191-195.

Arimoto, M., Mori, K., Nakai, T., Muroga, K. and Furusawa, I. (1993): Pathogenicity of the causative agent of viral nervous necrosis disease in striped jack, *Pseudocaranx dentex* (Bloch & Schneider). *Journal of Fish Diseases* **16**, 461-469.

Arimoto, M., Maruyama, K. and Furusawa, I. (1994): Epizootiology of viral nervous necrosis (VNN) in striped jack. *Fish Pathology* **29**, 19-24.

Aspehaug, V., Devold, M. and Nylund, A. (1999): The phylogenetic relationship of nervous necrosis virus from halibut (*Hippoglossus hippoglossus*). *Bulletin of the European Association of Fish Pathologists* **19**, 196-202.

Bloch, B., Gravningen, K. and Larsen, J.L. (1991): Encephalomyelitis among turbot associated with a picornavirus-like agent. *Diseases of Aquatic Organisms* **10**, 65-70.

Boonyaratpalin, S., Supamattaya, K., Kasornchandra, J. and Hoffmann, R.W. (1996): Picorna-like virus associated with mortality and a spongious encephalopathy in grouper *Epinephelus malabaricus*. *Diseases of Aquatic Organisms* **26**, 75-80.

Breuil, G. and Romestand, B. (1999): A rapid ELISA method for detecting specific antibody level against nodavirus in the serum of the sea bass, *Dicentrarchus labrax* (L.): application to the screening of spawners in a sea bass hatchery. *Journal of Fish Diseases* **22**, 45-52.

Chew-Lim, M., Chong, S.Y. and Yoshimizu, M. (1998): A nodavirus isolated from grouper (*Epinephelus tauvina*) and seabass (*Lates calcarifer*). *Fish Pathology* **33**, 447-448.

Christie, K.E. (1997): Immunization with viral antigens: infectious pancreatic necrosis. In: Fish Vaccinology (ed. by R. Gudding, A. Lillehaug, P.J. Midtlyng and F. Brown), pp. 191-199. Developments in Biological Standardization 90, International Association of Biological Standardization, Karger, London.

Danayadol, Y., Direkbusarakom, S. and Supamattaya, K. (1995): Viral nervous necrosis in brownspotted grouper, *Epinephelus malabaricus*, cultured in Thailand. In: Diseases in Asian Aquaculture II (ed. by M. Schariff, J.R.Arthur and R.P. Subasinghe) pp. 227-233. Fish Health Section, Asian Fisheries Society, Manila.

Frerichs, G.N., Morgan, D., Hart, D., Skerrow, C., Roberts, R.J. and Onions, D.E. (1991): Spontaneously productive C-type retrovirus infection of fish cell lines. *Journal of General Virology* **72**, 2537-2539.

Frerichs, G.N., Rodger, H.D. and Peric, Z. (1996): Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*. *Journal of General Virology* **77**, 2067-2071.

Fukuda, Y., Nguyen, H.D., Furuhashi, M. and Nakai, T. (1996): Mass mortality of cultured sevenband grouper, *Epinephelus septemfasciatus*, associated with viral nervous necrosis. *Fish Pathology* **31**, 165-170.

Glazebrook, J.S., Heasman, M.P. and de Beer, S.W. (1990): Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* (Bloch). *Journal of Fish Diseases* **13**, 245-249.

Grotmol, S., Totland, G.K., Kvellestad, A., Fjell, K. and Olsen, A.B. (1995): Mass mortality of larval and

juvenile hatchery-reared halibut (*Hippoglossus hippoglossus* L.) associated with the presence of virus-like particles in vacuolated lesions in the central nervous system and retina. *Bulletin of the European Association of Fish Pathologists* **15**, 176-180.

Grotmol, S., Totland, G.K., Thorud, K. and Hjeltnes, B.K. (1997): Vacuolating encephalopathy and retinopathy associated with a nodavirus-like agent: a probable cause of mass mortality of cultured larval and juvenile Atlantic halibut *Hippoglossus hippoglossus*. *Diseases of Aquatic Organisms* **29**, 85-97.

Grotmol, S., Bergh, O. and Totland, G.K. (1999): Transmission of viral encephalopathy and retinopathy (VER) to yolk-sac larvae of the Atlantic halibut *Hippoglossus hippoglossus*: occurrence of nodavirus in various organs and a possible route of infection. *Diseases of Aquatic Organisms* **36**, 95-106.

Husgard, S., Grotmol, S., Hjeltnes, B.K., Rodseth, O.M. and Bierling, E. (2001): Immune response to a recombinant capsid protein of striped jack nervous necrosis virus (SJNNV) in turbot *Scophthalmus maximus* and Atlantic halibut *Hippoglossus hippoglossus*, and evaluation of a vaccine against SJNNV. *Diseases of Aquatic Organisms* **45**, 33-44.

Iwamoto, T., Mori, K., Arimoto, M. and Nakai, T. (1999): High permissivity of the fish cell line SSN-1 for piscine nodaviruses. *Diseases of Aquatic Organisms* **39**, 37-47.

Iwamoto, T., Nakai, T., Mori, K., Arimoto, M and Furusawa, I. (2000): Cloning of the fish cell line SSN-1 for piscine nodaviruses. *Diseases of Aquatic Organisms* **43**, 81-89.

Jung, S.J., Miyazaki, T., Miyata, M. and Oishi, T. (1996): Histopathological studies on viral nervous necrosis in a new host, Japanese sea bass *Lateolabrax japonicus*. *Bulletin of the Faculty of Bioresources, Mie University* **16**, 9-16.

Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685.

Le Breton, A., Grisez, L., Sweetman, J. and Ollevier, F. (1997): Viral nervous necrosis (VNN) associated with mass mortalities in cage-reared sea bass, *Dicentrarchus labrax* (L.). *Journal of Fish Diseases* **20**, 145-151.

Leong, J.C., Anderson, E., Bootland, L.M., Chiou, P.-W., Johnson, M., Kim, C., Mourich, D. and Trobridge, G. (1997): Fish vaccine antigens produced or delivered by recombinant DNA technologies. In: Fish Vaccinology (ed. by R. Gudding, A. Lillehaug, P.J. Midtlyng and F. Brown), pp.267-277. Developments in Biological Standardization 90, International Association of Biological Standardization, Karger, London.

Lorenzen, N. (1999): Recombinant vaccines: experimental and applied aspects. *Fish & Shellfish Immunology* **9**, 361-365.

Miyazaki, T., Fujiwara, K., Oka, H.P. and Yoshikawa, M. (1991): Histological studies on development of tissues and organs of larval and juvenile red sea bream. *The Bulletin of the Faculty of Bioresources, Mie University* **5**, 97-116.

Mori, K., Nakai, T., Nagahara, M., Muroga, K., Mekuchi, T. and Kanno, T. (1991): A viral disease in hatchery-reared larvae and juveniles of redspotted grouper. *Fish Pathology* **26**, 209-210.

Mori, K., Nakai, T., Muroga, K., Arimoto, M., Mushiake, K and Furusawa, I. (1992): Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology* **187**, 368-371.

Mori, K., Mushiake, K. and Arimoto, M. (1998): Control measures for viral nervous necrosis in striped jack. *Fish Pathology* **33**, 443-444.

Munday, B.L., Langdon, J.S., Hyatt, A. and Humphrey, J.D. (1992): Mass mortality associated with a viral-induced vacuolating encephalopathy and retinopathy of larval and juvenile barramundi, *Lates calcarifer* Bloch. *Aquaculture* **103**, 197-211.

Munday, B.L. and Nakai, T. (1997): Special topic review: nodaviruses as pathogens in larval and juvenile

marine finfish. *World Journal of Microbiology and Biotechnology* **13**, 375-381.

Munday, B.L., Kwang, J. and Moody, N. (2002): Betanodavirus infections of teleost fish: a review. *Journal of Fish Diseases* **25**, 127-142.

Mushiake, K., Arimoto, M., Furusawa, T., Furusawa, I., Nakai, T. and Muroga, K. (1992): Detection of antibodies against striped jack nervous necrosis virus (SJNNV) from brood stocks of striped jack. *Nippon Suisan Gakkaishi* **58**, 2351-2356.

Mushiake, K., Nishizawa, T., Nakai, T., Furusawa, I. and Muroga, K. (1994): Control of VNN in striped jack: Selection of spawners based on the detection of SJNNV gene by polymerase chain reaction (PCR). *Fish Pathology* **29**, 177-182.

Nakai, T., Nguyen, H.D., Nishizawa, T., Muroga, K., Arimoto, M. and Ootsuki, K. (1994): Occurrence of viral nervous necrosis in kelp grouper and tiger puffer. *Fish Pathology* **29**, 211-212.

Nguyen, H.D., Mekuchi, T., Imura, K., Nakai, T., Nishizawa, T. and Muroga, K. (1994): Occurrence of viral nervous necrosis (VNN) in hatchery-reared juvenile Japanese flounder *Paralichthys olivaceus*. *Fisheries Science* **60**, 551-554.

Nguyen, H.D., Nakai, T. and Muroga, K. (1996): Progression of striped jack nervous necrosis virus (SJNNV) infection in naturally and experimentally infected striped jack *Pseudocaranx dentex* larvae. *Diseases of Aquatic Organisms* **24**, 99-105.

Nishizawa, T., Mori, K., Nakai, T., Furusawa, I. and Muroga, K. (1994): Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Diseases of Aquatic Organisms* **18**, 103-107.

Nishizawa, T., Mori, K., Furuhashi, M., Nakai, T., Furusawa, I. and Muroga, K. (1995): Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fish. *Journal of General Virology* **76**, 1563-1569.

Nishizawa, T., Furuhashi, M., Nagai, T., Nakai, T. and Muroga, K. (1997): Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Applied and Environmental Microbiology* **63**, 1633-1636.

Office International des Epizooties (OIE) (1997): Viral encephalopathy and retinopathy or viral nervous necrosis. In: *Diagnostic Manual for Aquatic Animal Diseases*, pp. 99-107. OIE, Paris.

Office International des Epizooties (OIE) (2000): Viral encephalopathy and retinopathy. In: *Diagnostic Manual for Aquatic Animal Diseases*, pp. 69-73. OIE, Paris.

Peducasse, S., Castric, R., Thiery, R., Jeffroy, J., Le Ven, A. and Baudin Laurencin, F. (1999): Comparative studies of viral encephalopathy and retinopathy in juvenile sea bass *Dicentrarchus labrax* infected in different ways. *Diseases of Aquatic Organisms* **36**, 11-20.

Reed, L.J. and Muench, H. (1938): A simple method of estimating fifty percent end points. *American Journal of Hygiene* **27**, 493-497.

Takashima, F. and Yokote, M. (1982): Nervous system. In: *Monograph of Fish Histology* (ed. by T. Hibiya), pp. 32-41. Kodansha, Tokyo.

Tanaka, S., Aoki, H. and Nakai, T. (1998): Pathogenicity of the nodavirus detected from diseased sevenband grouper *Epinephelus septemfasciatus*. *Fish Pathology* **33**, 31-36.

Totland, G.K., Grotmol, S., Morita, Y., Nishioka, T. and Nakai, T. (1999): Pathogenicity of nodavirus strains from striped jack *Pseudocaranx dentex* and Atlantic halibut *Hippoglossus hippoglossus*, studied by waterborne challenge of yolk-sac larvae of both teleost species. *Diseases of Aquatic Organisms* **38**, 169-175.

Towbin, H., Staehelin, T. and Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Science,*

USA **76**, 4350-4354.

Tsuchihashi, Y., Kuriyama, I., Kuromiya, Y., Kashiwagi, M. and Yoshioka, M. (2002): Control of viral nervous necrosis (VNN) in seedling production of sevenband grouper, *Epinephelus septemfasciatus*. *Suisanzoshoku* **50**, 355-361.

Watanabe, K., Suzuki, S., Nishizawa, T., Suzuki, K., Yoshimizu, M. and Ezura, Y. (1998): Control strategy for viral nervous necrosis of barfin flounder. *Fish Pathology* **33**, 445-446.

Watanabe, K., Yoshimizu, M., Ishima, M., Kawamata, K. and Ezura, Y. (1999): Occurrence of viral nervous necrosis in hatchery-reared barfin flounder. *Bulletin of the Faculty of Fisheries, Hokkaido University* **50**, 101-113.

Yoshikoshi, K. and Inoue, K. (1990): Viral nervous necrosis in hatchery-reared larvae and juveniles of Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel). *Journal of Fish Diseases* **13**, 69-77.

Yoshimoto, M. and Ito, H. (2002): Organization and function of the telencephalon. In: *Neuroscience of Fishes* (ed. by K. Uematsu, Y. Oka and H. Ito), pp. 178-195. Kouseisha-kouseikaku, Tokyo.