

ULTRASTRUCTURAL ANALYSIS OF GRUNT FIN (GF) CELLS TREATED WITH RED SEA BREAM IRIDOVIRUS (RSIV; family *Iridoviridae*, genus *Megalocyctivirus*) IN COMBINATIONS WITH INTERFERONS AND SPLENIC SUBSTANCES

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ABSTRACT

The genus *Megalocyctivirus* in the family of *Iridoviridae* encompasses isolate of red sea bream iridovirus (RSIV). In the present study, grunt fin (GF) cells were treated with red sea bream iridovirus (RSIV) in combinations with interferons (IFNs) and splenic substances. The viral titer in the combination with primary splenic substance was higher than the other combinations of 10^{-1} and 10^{-2} diluted splenic substances, and the positive control. The viral titer was not decreased by all combinations with recombinant murine interferon- α (rMuIFN- α), recombinant murine IFN- β (rMuIFN- β), and recombinant feline interferon- ω (rFeIFN- ω). Electron microscopy revealed inclusion body bearing cells (IBCs) and enlarged cells allowing virus propagation within the intracytoplasmic virus assembly site (VAS). Most were enlarged cells. These enlarged cells were divided into three cell types. Cells of Type II, which contained many mature virions within the VAS, were numerous in number in all treated cells. Cells of Type I allowing assembly of few virions and cells of Type III containing many immature viral particles were rather fewer in number. Their percentage was almost the same in all combinations with the splenic substances and IFNs. These results determined in *in vitro* treatment with IFNs did not prevent viral replication of RSIV, as well as the splenic substances which were derived from the RSIV-infected spleen of red sea bream did not contain any factors to disturb RSIV replication.

KEYWORDS: red sea bream, iridovirus, grunt fin, splenic substances, interferons, ultra structural analysis

INTRODUCTION

The genus *Megalocyctivirus* in the family *Iridoviridae* encompasses several viral isolates such as red sea bream iridovirus (RSIV), sea bass iridovirus (SBIV), African lampeye iridovirus (ALIV), dwarf gourami iridovirus (DGIV), grouper sleepy disease iridovirus (GSDIV), Taiwan grouper iridovirus (TGIV), and infectious spleen and kidney necrosis virus (ISKNV), which are genetically identical (Chinchar *et al.*, 2005).

In our previous study (Mahardika *et al.*, 2004), we performed artificial infection in juvenile humpback grouper with grouper sleepy disease iridovirus (GSDIV). As a result, the mortality was lower in the groups injected with the primary filtrate than in groups receiving the 10^{-4} diluted inoculum. Electron microscopy revealed the presence of many unusual inclusion body-bearing cells (IBCs) containing abnormal inclusion body with a small number of

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deformed virions in the former groups. This impaired virus assembly and appeared to prevent mortality in the challenged fish and was assumed to be due to an interferon-like effect of a previously unknown substance that was passed with a filtrate from the donor fish.

In order to prove this hypothesis, we performed *in vitro* tests using grunt fin (GF) cells and semi-purified RSIV (red sea bream iridovirus) derived from samples of infected red sea bream in combinations with the splenic substances derived from the red sea bream infected with RSIV, recombinant murine interferon- α (rMuIFN- α), recombinant murine IFN- β (rMuIFN- β), and recombinant feline interferon- ω (rFeIFN- ω). IFN- α and IFN- β are known to be potential for endogenous antiviral cytokines that suppress the replication of viruses such as murine leukaemia virus (MuLV; genus *Gammaretrovirus*, family *Retroviridae*) (Pitha *et al.*, 1979), human herpes simplex virus type 1 (HSV-1; genus *Simplexvirus*, family *Herpesviridae*) (Härle *et al.*, 2002; Sainz & Halford, 2002), severe acute respiratory syndrome-associated coronavirus (SARS-CoV; genus *Coronaviridae*, family *Coronaviridae*) (Sainz *et al.*, 2004). Other type of IFN such as rFeIFN- ω has clinical anti-viral activity for feline calicivirus and herpesvirus (Yamamoto *et al.*, 1990; Uchino, 1997) as well as canine parvovirus (Uchino, 1997; Ishiwata *et al.*, 1998) and akoya-virus infected Japanese pearl oysters *Pinctada fucata martensii* (Miyazaki *et al.*, 2000). This paper describes the results of the viral titer and ultrastructural features of the RSIV-infected GF cells treated with the splenic substances and various IFNs.

MATERIALS AND METHODS

Cell Culture

GF (grunt fin) cells were used in this study. GF cells were grown in 25 cm² cell culture flasks at 25°C to confluence in Eagle's minimum essential medium (MEM: Nissui, Japan) supplemented with fetal bovine serum (FBS: Sigma, USA), L-glutamine, and buffered with NaHCO to pH 7.2-7.4. For routine passage and virus inoculation, 10% and 2% FBS was added to the medium (MEM-10 and 2), respectively.

Preparation of Viral Inoculum and Splenic Substances

Semi-purified virus was prepared from the spleens of the RSIV-infected red sea bream

from a mariculture farm in Mie Prefecture, Japan, likewise our previous preparation of semi-purified virus (Mahardika & Miyazaki, 2009) but with some modifications. The procedures were as follows: A total of 0.6 g of swollen spleens from the diseased fish was homogenized in 6 mL of MEM-2 containing antibiotics (penicillin 50 IU mL⁻¹ and streptomycin 50 μ g mL⁻¹) in a glass homogenizer. The obtained homogenate was confirmed to be RSIV-positive by PCR assay described by Kurita *et al.* (1998). The homogenate was centrifuged (CT4D Himac, Hitachi, Japan) at 1,200 x g for 15 min. at room temperature, and the supernatant was filtered (450 nm). The obtained filtrate was diluted to be a final volume of 12 mL and ultracentrifuged (L-70 Ultracentrifuge, Beckman, USA) at 100,000 x g for 10 min. at 4°C. The supernatant was collected, filtered (450 nm) and used as the primary splenic substance. This substance was diluted 10 and 100 times (10⁻¹ and 10⁻² dilution) with MEM-2.

The sediment was resuspended in 12 mL of MEM-2 containing antibiotics and ultracentrifuged at 100,000 x g for 10 min. at 4°C. Then, the recovered sediment was resuspended in 5 mL of MEM-2 containing antibiotics and filtered (450 nm). The obtained semi-purified virus was used for an inoculum although, as the result, the virus concentration was decreased in number by repeated ultracentrifugation.

Interferons (IFNs)

An amount of 10⁵ units vial⁻¹ of rMuIFN- α and rMuIFN- β (PBL Biomedical Laboratories, New Brunswick, NJ.), and 10 mega units of rFeIFN- ω (Toray, Japan) were dissolved with MEM.

Treatments

Two treatments were performed. The first was treatment with the splenic substances using five tubes: 0.1 mL of the semi-purified virus inoculum mixed into each of the four tubes containing 0.2 mL of different dilutions of the splenic substances (primary, 10⁻¹, 10⁻² dilution) and MEM-2 as positive control, respectively. In the fifth tube, the same volume of the primary splenic substances and MEM-2 were mixed as negative control. These five mixtures were kept at room temperature for 15 min., and inoculated into monolayer of GF cells for 1 hour at 25°C, followed by adding the MEM-2 to a final volume of 5 mL. This treatment was repeated two times.

The second was treatment with IFNs and set in two trials. First trial consisted of five tubes: 0.1 mL of the semi-purified virus inoculum was mixed into each of the five tubes, which contained 0.1 mL of different concentrations of rMuIFN- α (10^4 and 10^3 units mL $^{-1}$), rMuIFN- β (10^4 and 10^3 units mL $^{-1}$) and MEM as control, respectively. The second trial consisting of three tubes: 0.1 mL of the semi-purified virus inoculum was mixed into each of the three tubes which contained 0.1 mL of rMuIFN- β (10^5 units mL $^{-1}$), rFeIFN- ω (10^6 units mL $^{-1}$) and MEM as control, respectively. This second trial was repeated two times. All mixtures were kept at room temperature for 15 min., and inoculated into monolayer of GF cells for 1 hour at 25°C, followed by adding the MEM-2 to reach 5 mL. The final concentration of both rMuIFN- α and rMuIFN- β was 2×10^2 and 2×10^1 units mL $^{-1}$ in the first trial. In the second trial, the final concentration was 2×10^3 units mL $^{-1}$ of rMuIFN- β and 2×10^4 units mL $^{-1}$ of rFeIFN- ω .

Monolayer of GF cells were infected with semi-purified virus inoculum at a multiplicity of infection (MOI) of approximately 0.5-1. Infected cells were incubated at 25°C. The rounded and enlarged cells were harvested by shaking the culture medium in order to separate these cells from the monolayer at 6 days post infection (p.i.). The recovered cells were used for virus titration and electron microscopy (EM).

Virus Titration

Viral titration in RSIV-infected GF cells was conducted using the TCID $_{50}$ method with GF cells grown in 96-well tissue culture plates. This assay was analyzed in duplicate.

Electron Microscopy

For transmission electron microscopy, a 3.5 mL of harvested medium containing RSIV-infected GF cells were centrifuged at $1,200 \times g$ for 10 min. The obtained pellets were fixed with 70% Karnovsky's solution (1.6 g paraformaldehyde in 20 mL distilled water, 2 mL of 70% glutaraldehyde, 35 mL of 0.2 M phosphate-buffered saline, and 13 mL distilled water), post-fixed in 1% OsO $_4$ and processed for electron microscopy, as previously described by Mahardika *et al.* (2008). Rounded and enlarged cells contained within 800 mm 2 of the semi-thin section per sample were examined. These cells were counted at an instrument magnification of 5,000X to 10,000X. The data were expressed

as percentage per cells. All of obtained data were evaluated using fisher's exact test.

RESULTS AND DISCUSSION

Viral Propagation in Combination With The Splenic Substances

The viral titers in combination with the primary splenic substance were higher ($10^{4.93}$ and $10^{5.2}$ TCID $_{50}$ mL $^{-1}$) than the other combinations with splenic substances at 10^{-1} dilution: $10^{4.6}$ and $10^{4.9}$ TCID $_{50}$ mL $^{-1}$, and 10^{-2} dilution: $10^{4.6}$ and $10^{4.8}$ TCID $_{50}$ mL $^{-1}$, positive control ($10^{4.6}$ and $10^{4.8}$ TCID $_{50}$ mL $^{-1}$), and negative control ($10^{1.3}$ and $10^{1.7}$ TCID $_{50}$ mL $^{-1}$) in the two examinations at 6 d p.i. (Figure 1).

Viral Propagation in Combination with IFNs

The viral titers were $10^{4.8}$ TCID $_{50}$ mL $^{-1}$ in both combinations with 2×10^2 and 2×10^1 units mL $^{-1}$ of rMuIFN- α at 6 d p.i. In the combination with 2×10^2 and 2×10^1 units mL $^{-1}$ of rMuIFN- β , the viral titers were $10^{4.8}$ and $10^{4.9}$ TCID $_{50}$ mL $^{-1}$. These viral titers were similar to the control ($10^{4.9}$ TCID $_{50}$ mL $^{-1}$) (Figure 2a). In combination with 2×10^3 units mL $^{-1}$ of rMuIFN- β , the viral titers were $10^{4.9}$ and $10^{5.1}$ TCID $_{50}$ mL $^{-1}$ in the two examinations. In combination with 2×10^4 units mL $^{-1}$ of rFeIFN- ω , the viral titers were $10^{4.8}$ and $10^{5.1}$ TCID $_{50}$ mL $^{-1}$ in the two examinations. These viral titers were similar to the control ($10^{4.9}$ and $10^{5.1}$ TCID $_{50}$ mL $^{-1}$) (Figure 2b).

Electron Microscopic Features of GF Cells Treated with RSIV and Splenic Substances

Electron microscopic examination of GF cells, which were treated with mixture of semi-purified virus and different dilution of the splenic substances revealed IBCs and necrotic cells allowing virus propagation within the intracytoplasmic virus assembly site (VAS) at 6 d p.i. IBCs were observed a few in the number (2.1%-5.7%) (Table 1). Some small cells were also observed forming the intracytoplasmic VAS containing a small number of virus particles (10.8%-12.7%). Most were enlarged cells (83.0%-86.6%) and had fragmented nuclei whose components were assimilated with the cytoplasm, resulting in the disappearance of the nuclei. These cells developed intracytoplasmic VAS within which many virions were propagated. These enlarged cells were divided into three cell types. Cell type-I allowed development of

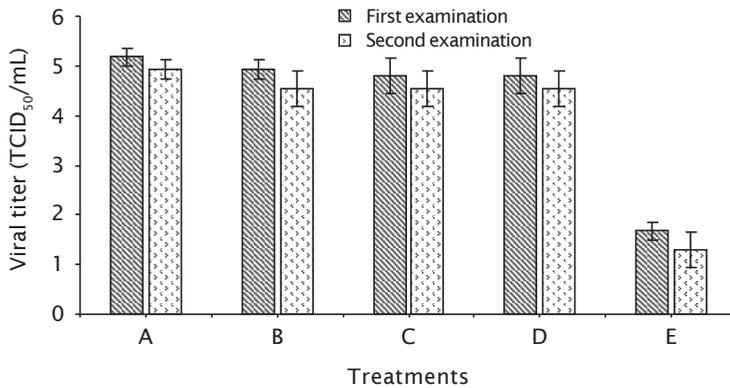


Figure 1. Viral titers of RSIV in combination with splenic substances in two examinations at 6 d p.i. (A) In combination with primary splenic substance, (B) In combination with 10^{-1} dilution of splenic substance, (C) In combination with 10^{-2} dilution of splenic substance, (D) Positive control, (E) Negative control. Data was analyzed in duplicate and error bars show standard deviation (SD)

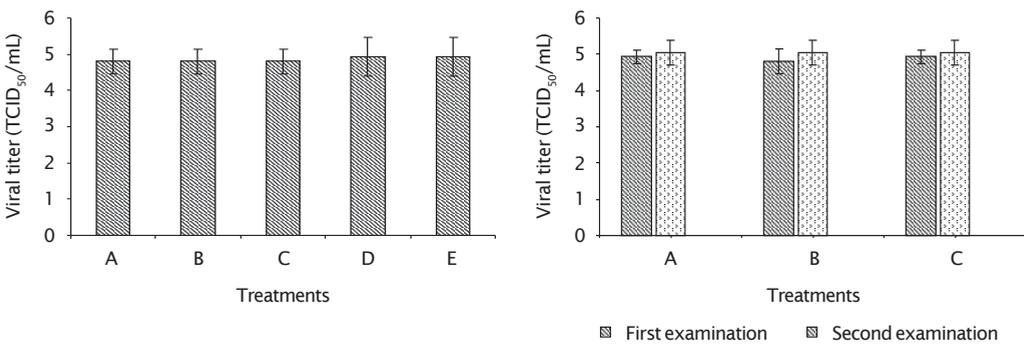


Figure 2. Viral titers of RSIV in combination with IFNs in two set trials. A: Viral titers of RSIV in combination with two different concentrations of rMuIFN- α and rMuIFN- β at 6 d p.i. (A) In combination with 2×10^2 unit mL^{-1} of rMuIFN- α , (B) In combination with 2×10^1 unit mL^{-1} of rMuIFN- α , (C) In combination with 2×10^2 unit mL^{-1} of rMuIFN- β , (D) In combination with 2×10^1 unit mL^{-1} of rMuIFN- β , and (E) Control. B: Viral titers of RSIV in combination with rMuIFN- β and rMuIFN- ω in the two examinations at 6 d p.i. (A) In combination with 2×10^3 unit mL^{-1} of rMuIFN- β , (B) In combination with 2×10^4 unit mL^{-1} of rMuIFN- ω , and (C) Control. Data was analyzed in duplicate and error bars show standard deviation (SD)

the VAS, which showed the assembly of a few number of virions (less than 20). Cell type-II had assembled many mature virions (more than 20) within the VAS. Cell type-III contained many immature viral particles (more than 20) within the VAS. In the GF cells treated with the mixture of semi-purified virus inoculum and different dilution of the splenic substances (primary, 10^{-1} and 10^{-2}), the percentages of cell type-I and cell type-III were 11.6%-18.8% and 2.9%-10.6%, respectively, which were lesser than

cell type-II (75.8%-85.5%). The percentages of these cell types were similar ($P > 0.05$) to the positive control (Table 2, Figure 3 & 4).

Electron Microscopic Features of GF Cells Treated with RSIV and IFNs

Electron microscopic examination of the GF cells treated with mixture of semi-purified virus and various IFNs focused to the enlarged cells. In combination with rMuIFN- α and rMuIFN-

Table 1. Electron microscopic cell count of different types of cells with virus particles derived from RSIV-infected GF cells in combination with splenic substances *in vitro*

Samples	Different types of cells with virus particles (%)			
	Enlarged cells	IBCs	Small round cells	
In combination with primary splenic substance	I	83.8	5.4	10.8
	II	86.6	2.1	11.3
In combination with 10 ⁻¹ dilution of splenic substance	I	86.1	3.2	10.8
	II	83.0	5.7	11.3
In combination with 10 ⁻² dilution of splenic substance	I	85.2	3.1	11.7
	II	85.3	2.9	11.8
In combination with MEM-2 (positive control)	I	84.1	3.5	12.4
	II	83.6	3.6	12.7

I: First examination, II: Second examination

Table 2. Electron microscopic cell count of different types of enlarged cells with virus particles derived from RSIV-infected GF cells in combination with splenic substances *in vitro*

Samples	Different types of enlarged			Total ECs (%)	Total small round cells with virus particles (%)	
	ECs with many mature virions	ECs with many immature virus particles	ECs with a few number of virions			
In combination with primary splenic substance	I	77.6	9.5	12.9	87.9	12.1
	II	75.8	10.6	13.6	85.7	14.3
In combination with 10 ⁻¹ dilution of splenic substance	I	77.9	5.4	16.8	89.8	10.2
	II	84.7	3.7	11.7	88.4	11.6
In combination with 10 ⁻² dilution of splenic substance	I	76.7	4.5	18.8	89.3	10.7
	II	85.5	2.9	11.6	89.6	10.4
In combination with MEM-2 as positive control	I	80.7	3.9	15.5	87.1	12.9
	II	84.9	3.2	11.8	86.9	13.1

IBCs were included in the enlarged cells with virus particles. I: First examination, II: Second examination

β (2×10^2 and 2×10^1 units mL⁻¹), the percentages of cell type-I and cell type-III were lesser (13.5%–14.9% and 3.4%–4.4%) than cell type-II (81.0%–82.7%), which was almost same ($P > 0.05$) as the control (Table 3, Figure 5). Some of cell type-I in combination with 2×10^2 units mL⁻¹ of rMuIFN- α contained tubule structures among immature viral particles within the VAS.

In the combinations with rMuIFN- β and rFeIFN- ω (2×10^3 and 2×10^5 units mL⁻¹), the percentages of cell type-I and cell type-III were 17.7%–18.1% and 5.9%–6.9%, respectively, which were lesser than cell type-II (75.3%–76.0%). The percentages of these cell types were similar ($P > 0.05$) to the positive control (Table 4).

Table 3. Electron microscopic cell count of different types of enlarged cells with virus particles derived from RSIV-infected GF cells in combination with interferons (rMuIFN- α and rMuIFN- β) *in vitro*

Samples	Different types of enlarged cells (ECs) (%)			Total ECs (%)	Total small round cells with virus particles (%)
	ECs with many mature virions	ECs with many immature virus particles	ECs with a few number of virions		
In combination with 2×10^2 U mL ⁻¹ of rMuIFN- β	81.0	4.4	14.6	87.8	12.2
In combination with 2×10^2 U mL ⁻¹ of rMuIFN- α	81.6	3.4	15.0	88.0	12.0
In combination with 2×10^1 U mL ⁻¹ of rMuIFN- β	82.7	3.9	13.5	84.6	15.4
In the combination with 2×10^1 U mL ⁻¹ of rMuIFN- α	82.3	3.9	13.9	85.0	15.0
In the combination with MEM (control)	82.7	3.6	13.6	85.9	14.1

IBC were included in the enlarged cells with virus particles

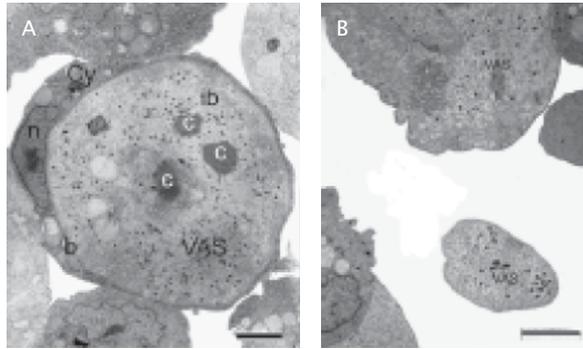


Figure 3. (A-B) Electron micrographs of IBC and cells derived from RSIV-infected GF cells in combination with splenic substances at 6 d p.i. (A) Mature IBC in the combination with primary splenic substance has a large inclusion body with a VAS containing a mass of coarse granules (c) and multiple virus particles (scale bar = 2,900 nm). (B) Enlarged cells in combination with 10^{-2} dilution of splenic substance. One enlarged cell (upper) has a disappearance of the nucleus and development of an intracytoplasmic VAS in which many virus particles are present. Another is small cell. This cell forms an intracytoplasmic VAS containing a small number of virus particles (scale bar = 2,400 nm). f: mass of fine granules; n: nucleus of host cell; VAS: virus assembly site

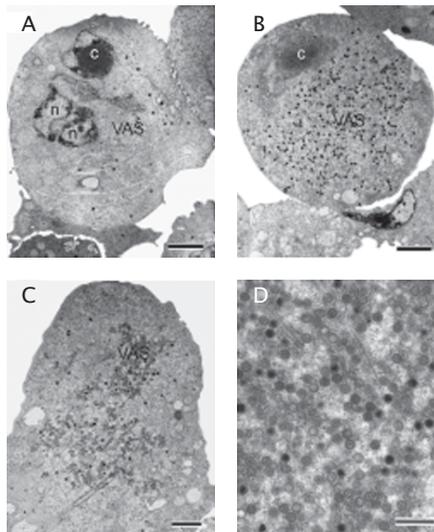


Figure 4. Electron micrographs of enlarged cells derived from RSIV-infected GF cells in the combination with splenic substances at 6 d p.i. (A) Cell type-I in control allows development of the VAS, which shows the assembly of a few number of virions (less than 20) (scale bar = 2,400 nm). (B) Cell type-II in the combination with primary splenic substance has a disappearance of the nucleus and development of an intracytoplasmic VAS that propagation of many mature virions (more than 20) takes place (scale bar = 2,400 nm). (C) Cell type-III in the combination with 10^{-1} dilution splenic substance has an intracytoplasmic VAS, within which many immature viral particles (more than 20) takes place (scale bar = 1,500 nm). (D) Detail of an intracytoplasmic VAS in a cell type-III. Assembly of immature RSIV particles is evident within the VAS comprising granules and microfilaments. Tubular structures are also formed (scale bar = 600 nm)

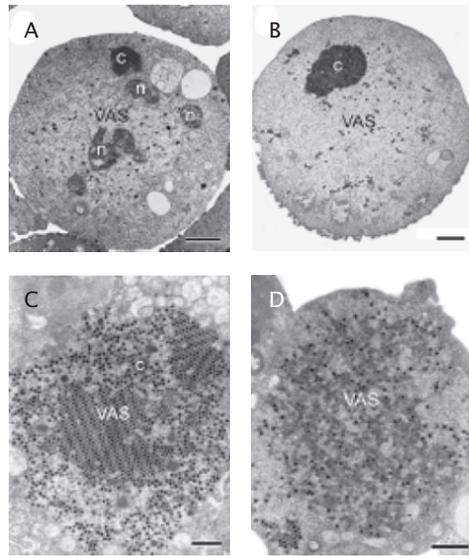


Figure 5. Electron micrographs of enlarged cells derived from RSIV-infected GF cells in combination with IFNs at 6 d p.i. (A) Cell type-I in combination with 2×10^2 unit mL^{-1} of rMuIFN- α has a developed intracytoplasmic VAS with a small number of virus particles as well as a disappearance of the nucleus (scale bar = 1,500 nm). (B) Cell type-I in combination with 2×10^2 unit mL^{-1} of rMuIFN- α has a developed intracytoplasmic VAS in which masses of coarse (c), a small number of virus particles and tubule structures takes place (scale bar = 2,000 nm). (C) Cell type-II in combination with 2×10^2 unit mL^{-1} of rMuIFN- α has a disappearance of the nucleus and development of an intracytoplasmic VAS, within which masses of coarse (c) and fine granules, and many mature virions take place (scale bar = 1,200 nm). (D) Cell type-III in combination with 2×10^1 unit mL^{-1} of rMuIFN- α has an intracytoplasmic VAS, within which many immature viral particles take place (scale bar = 1,300 nm)

Table 4. Electron microscopic cell count of different types of enlarged cells with virus particles derived from RSIV-infected GF cells in combination with interferons (rMuIFN- β and rFeIFN- ω) *in vitro*

Samples	Different types of enlarged			Total ECs (%)	Total small round cells with virus particles (%)	
	ECs with many mature virions	ECs with many immature virus particles	ECs with a few number of virions			
In combination with 2×10^3 U mL^{-1} of rMuIFN- β	I	75.4	6.9	17.7	87.3	12.7
	II	76.0	5.9	18.1	85.5	14.5
In combination with 2×10^4 U mL^{-1} of rMuIFN- ω	I	75.3	6.6	18.1	86.0	14.0
	II	75.5	6.5	17.9	88.0	12.0
In combination with MEM (control)	I	77.9	4.6	17.6	88.5	11.5
	II	77.8	4.0	18.2	87.1	12.9

IBC's were included in the enlarged cells with virus particles. I: First examination, II: Second examination

In the present study, inoculated RSIV infected GF cells and produced cellular enlargement as a definite cytopathic effect in all combinations with the splenic substances and IFNs. The viral titer in combination with primary splenic substances was higher than the other combinations of 10^{-1} and 10^{-2} diluted splenic substances, and positive control. The viral titer was not decreased by all combinations with rMuIFN- α , rMuIFN- β , and rFeIFN- ω . These results determined *in vitro* treatment with IFNs did not prevent viral replication of RSIV, as well as the splenic substances which were derived from the RSIV-infected spleen of red sea bream, did not contain any factors that could disturb RSIV replication. In EM examination, IBCs seen in the GF cells that were infected with RSIV in combination with different dilutions of the splenic substances were same to those seen in the previous study (Mahardika & Miyazaki, 2009) as well as megalocytivirus-infected cells, *in vivo* (Jung *et al.*, 1997; Sudthongkong *et al.*, 2002a; 2002b; Mahardika *et al.*, 2004; 2008; Miyazaki, 2007). Enlarged cells were also derived from RSIV-infected GF cells and allowed assembly of viral particles in the intracytoplasmic VAS. Cells of type II, which contained many mature virions within the VAS, were numerous in number in all treated cells. Cells of type I allowing assembly of a few virions and cells of type III containing many immature viral particles were rather small in the number. Their percentages were almost the same in all combinations with the splenic substances and IFNs. IFN- α and IFN- β are known to be potential as endogenous antiviral cytokines that suppress the replication of viruses (Pitha *et al.*, 1979; Härle *et al.*, 2002; Sainz & Harford, 2002; Sainz *et al.*, 2004). rFeIFN- ω has clinical anti-viral activities (Yamamoto *et al.*, 1990; Uchino, 1997; Ishiwata *et al.*, 1998; Miyazaki *et al.*, 2000). In the present study, there were cells with the formation of many immature viral particles within the VAS as cell type III, and cells with slight virion assembly as cell type I. However, because these cells were small in number, they did not appear to be resulted in prevention of viral replication. These results indicate that the *in vitro* treatment with IFNs (rMuIFN- α , rMuIFN- β , and rFeIFN- ω) did not affect on the RSIV propagation in GF cells. Our *in vivo* study (Mahardika *et al.*, 2004) revealed that the mortality rate in the fish injected with the primary filtrate of the spleen of GSDIV-infected grouper were lower than the fish received the

10^{-4} diluted inoculum. Results of our study *in vivo* and *in vitro* resembled results of studies by Sainz & Halford (2002) who revealed that T-cell derived gamma interferon (IFN- γ) inhibited the replication of herpes simplex virus type 1 (HSV-1; genus *Simplexvirus*, family *Herpesviridae*) *in vivo*, while *in vitro*, HSV-1 was highly resistant to the antiviral activity of IFN- α/β or IFN- γ . Ellis (2001) reported that the cells after *in vivo* infection produced interferon to induce anti-viral defenses in neighboring cells, which able to lyse virally infected cells and reduce the rate of multiplication of virus within them.

It was concluded that the examined IFNs did not have antiviral efficacy to megalocytivirus *in vitro*. Further *in vivo* studies using IFNs will be needed to reveal real antiviral effects of IFNs on RSIV.

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