

Persistence of betanodavirus in Barramundi brain (BB) cell line involves the induction of Interferon response

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Abstract

The BB cell line derived from the brain tissue of a barramundi (*Lates calcarifer*) that survived nervous necrosis virus (NNV) infection is persistently infected with NNV. To elucidate whether interferon (IFN) plays a role in the mechanism of NNV-persistent infection in BB cell line, a virus-negative control cell line was obtained by treating BB cells with NNV-specific rabbit antiserum for 5 subcultures. After the treatment, NNV titer or RNA or capsid protein was no longer detected in the cured BB (cBB) cells. Expression of Mx gene, encoding a type I IFN-inducible antiviral protein, was found in BB cells and cBB cells following NNV infection, but not in NNV-free cBB cells. Moreover, expression of Mx gene and antiviral activity against NNV were induced in cBB cells by the treatment with MAb-neutralized BB cell supernatant. Furthermore, NNV persistent infection was induced again in cBB cell culture if multiplicity of infection (MOI) was low (≤ 1). These experimental results indicated that IFN-like cytokines existed in the culture supernatant of BB cells, and IFN-induced response played an important role in protecting the majority of cells from virus lytic infection and regulating NNV persistence in the BB cell line.

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1. Introduction

Viral nervous necrosis (VNN) disease is a worldwide disease among many economically important fish species, and causes mass mortality of fish at the larval stage [1,2]. The pathological characteristic of VNN disease is the vacuolation of the brain and retina [3]. Clinical signs of diseased fish are abnormal swimming behavior and a dark body surface. The causative agent is nervous necrosis virus (NNV), a non-enveloped icosahedral RNA virus with a diameter of 20–34 nm and two single strands of positive-sense RNA without poly A tail [4,5]. RNA1 encodes RNA-dependent RNA polymerase, and RNA2 encodes capsid protein. It belongs to the piscine nodavirus (betanodavirus) of *Nodaviridae*.

Betanodavirus has been detected in fish surviving VNN disease without clinical and pathological syndromes [6]. To date, knowledge about NNV-persistent infection is very limited. A novel cell line BB was established from the brain

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tissue of a barramundi (*Lates calcarifer*) that survived VNN disease. The BB cell line was confirmed to have NNV-persistent infection due to the detection of infectious viral particles in its culture supernatants, and the identification of capsid protein in a few BB cells of each subculture by immunochemical staining [7].

Persistent infections are usually caused by the production of defective-interfering (DI) viral particles or the existence of temperature sensitive (Ts) mutants [8]. However, DI particles and Ts mutants do not cause NNV persistent infection in BB cell line [7]. Another type of persistent infection occurs when a small fraction of cells are infected by few progeny viruses at any given time due to the presence of IFN-like substances in the culture supernatant [8,9].

Interferons are secreted proteins (cytokines) that induce an antiviral state in cells and play a major role in the defense against virus infection in vertebrates [10,11]. The antiviral action of mammalian type I IFN (IFN α/β) is mediated by a 2-step signaling pathway. During the first step, virus-infected cells recognize viral dsRNA generated during viral replication by Toll-like receptor 3 (TLR 3) which triggers activation of the transcription factors IRF-3 and NF κ B that translocate and attach to specific sites in the IFN β promoter. The first IFN β is then produced and secreted from the virus-infected cells. The second step involves the binding of the secreted type I IFN to the type I IFN receptors on the cell membrane of other uninfected cells. The antiviral effect is then established via the JAK-STAT signal transduction pathway resulting in expression of Mx and other antiviral proteins [10,11].

The cloned fish IFNs have the characteristic properties of type I IFNs, and antiviral activity due to type I IFN has been demonstrated in a number of fish species in vitro and in vivo [12]. Although little is yet known about the IFN-signaling system of fish, STAT1 has been cloned from zebrafish, and the zebrafish STAT1 was able to rescue type I IFN-signaling functions in a STAT1-deficient human cell line, indicating that cytokine-signaling mechanisms are likely to be conserved between fish and mammals [11]. Furthermore, Mx proteins have been used as putative markers for type I IFN production in fish [13–15], and antiviral activity of Mx protein from Atlantic salmon and Japanese flounder has been demonstrated [11].

To determine whether the persistence of NNV in BB cell line involved the induction of IFN response, the expression of Mx gene in BB cells was examined and the antiviral activity in the culture supernatant of BB cells was assayed.

2. Materials and methods

2.1. Cell lines and viruses

The GF-1 cell line [16] and the BB cell line [7] were used in this study. GF-1 cells were used for the titration and identification of NNV in the culture supernatants from BB cells. GF-1 cells were maintained in Leibovitz's L-15 medium supplemented either with 5% fetal bovine serum (FBS) for routine subcultures or 1% FBS for NNV titration, and incubated at 28 °C. BB cells were cultured using L-15 medium supplemented with 20% FBS, and also incubated at 28 °C. An NNV strain B00GD, isolated from NNV-infected Barramundi (*Lates calcarifer*) [1], was used in this study.

2.2. Curing NNV-persistent infection in BB cells by NNV-specific rabbit antiserum

One set of BB cells was continuously subcultured for 5 generations with medium containing 0.2% NNV-specific rabbit antiserum, and restored to normal culture medium without antiserum from the 6th subculture. The BB cells after antiserum curing were designated as cBB cells.

2.3. Titration of the culture supernatants from BB and cBB cells

The culture supernatants collected from 18 subcultures of BB and cBB cells were titrated in GF-1 cells. The supernatants were serially 10-fold diluted and inoculated into 96 well-tissue culture plates pre-seeded with GF-1 cells. The 50% tissue culture infectious dose (TCID₅₀) per ml were determined on the 6th day of infection.

2.4. Detection of NNV by RT-PCR and semi-nested PCR

BB and cBB cells were separately scraped from one 25-cm² tissue culture flask and re-suspended in 200 μ l culture supernatants. The cell suspension was then mixed with 500 μ l RNA extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate dihydrate, 0.5% sodium lauroyl sarcosine), 70 μ l 3 M sodium acetate (NaOAc) (pH 4.2),

500 μ l acid phenol (pH 4.2) and 150 μ l chloroform/isoamylalcohol (24:1), and the mixture was centrifuged at $16\,440 \times g$ for 15 min at 4 °C. Then 700 μ l of the upper layer was taken, and mixed with 150 μ l chloroform/isoamylalcohol (24:1). After centrifugation, RNA precipitation was carried out by taking 600 μ l of the upper layer, and adding an equal volume of isopropanol, followed by incubation at –70 °C for more than 1 h. The RNA pellet was obtained by centrifugation at $16\,440 \times g$ for 30 min at 4 °C, washed with 70% ethanol, dried at 60 °C and re-dissolved in 40 μ l diethyl pyrocarbonate (DEPC)-treated water.

For reverse transcription (RT), 6 μ l of the extracted RNA was incubated at 42 °C for 1 h in a final volume of 30 μ l RT reaction buffer containing 0.4 mM dNTP, 50 mM Tris–HCl buffer, 75 mM KCl, 3 mM MgCl₂, 0.33 mM reverse primer R3 (5'-CGAGTCAACACGGGTGAAGA-3'), 11.7 mM DTT, 40 U ribonuclease inhibitor rRNasin (Promega), and 60 U MMLV reverse transcriptase (Promega).

Following cDNA synthesis, 5 μ l cDNA was added to the PCR mixture to a final volume of 25 μ l containing 50 mM KCl, 10 mM Tris–HCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.4 mM forward primer F1 (5'-GGATTGACGTGC GACCAA-3'), 0.4 mM reverse primer R3, 0.25 mM dNTP, and 0.5 U DyNzyme II DNA polymerase (Finnzymes). The mixture was then incubated in an automatic thermal cycler (GeneAmp PCR system 2400, Applied Biosystems, USA). Amplification was performed by an initial denaturation step for 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 45 s at 72 °C, with a final extension of 5 min at 72 °C. The target product for primer set (F1, R3) is T2 (870 bp).

Semi-nested PCR amplification was performed using the 100-fold diluted RT-PCR product as template. The PCR program is the same with that above. Forward primer F2 (5'-CGTGTCAGTGCTGTGTCGCT-3') and reverse primer R3 were used, and the target product for primer set (F2, R3) is T4 (421 bp). Primer sequences of F1, F2 and R3 were based on those described by Nishizawa et al. (1994) [17] with minor modification.

2.5. Detection of NNV by immunochemical staining

Cells were first fixed with Carnoy's (CH₃COOH: CH₃OH = 1:3) for 15 min, washed 3 times with Tris buffered saline (TBS) (0.05 M Tris, 0.157 M NaCl, pH 7.5), reacted with NNV-specific monoclonal antibody (MAb) [18], and stained by Fast-Red following the procedures described by the UltraTech AP Streptavidin-Biotin Universal Detection System (Immunotech, Beckman Coulter Company, France).

2.6. Detection of Mx mRNA by RT-PCR

We have cloned and sequenced the full cDNA for a Mx protein in BB cells by RACE PCR. The primers used for the detection of Mx gene expression in the current study were all designed from this cloned Mx sequence available in Genbank (Accession number [AY821518](#)).

BB and cBB cells were cultured in 25-cm² flask (5×10^5 cells per flask). The cBB cells were either incubated with poly I:C (1 μ g ml^{–1}) or BB cell culture supernatant neutralized by NNV-specific MAb [18], or transfected with poly I:C (1 μ g ml^{–1}) using lipofectin (Invitrogen), or infected with NNV (B00GD) (MOI = 5). After 48 h incubation, total RNA of the cells was extracted and transcribed into cDNA using Mx reverse primer (5'-CTTCTGACCCCTGCACCT GACGA-3') and actin reverse primer (5'-AAAGTCCAGCGCCACGTAGCACAG-3').

Mx forward primer (5'-CACCATGAACACCCTGAACC-3') and reverse primer were then used in semiquantitative RT-PCR to amplify a 745 bp fragment of BB Mx gene. The PCR program of Mx is the same as that of NNV. Actin-specific forward primer (5'-CACTCAACCCCAAAGCCAACAGG-3') and reverse primer were used for semiquantitative RT-PCR to confirm that all tested samples contained the same amount of cDNA. Actin PCR amplification was performed by an initial denaturation step for 2 min at 94 °C, followed by 20 cycles of 20 s at 94 °C, 20 s at 68 °C, and 20 s at 72 °C, with a final extension of 5 min at 72 °C [19].

2.7. Comparison of cytopathic effect caused by NNV with high MOI and low MOI in cBB cells

Two flasks of cBB cells were separately infected with NNV (B00DG) with high MOI (100) and low MOI (1), and then observed for 14 days. The cBB cells that survived from low MOI were subcultured 14 times, and characterized by titrating culture supernatants from 14 subcultures, detecting NNV nucleic acid by RT-PCR and semi-nested PCR, and

immunochemical staining by NNV-specific MAb. The methods used here were the same as those used for characterization of BB cells described in our previous paper [7].

2.8. Antiviral activity assay

First, the culture supernatant of 4-day old BB cells was mixed with an equal volume of NNV-specific MAb [18] for 1 h in order to neutralize the activity of BBNNV. The MAb-neutralized BB cell supernatant was tested in GF-1 cells, and no CPE was observed even after 6 days of incubation.

Then, 4 different treatments were separately given to 4 wells of cBB cells pre-seeded in a 96-well tissue culture plate (1×10^4 cells per well). The treatments included (1) incubation with the MAb-neutralized BB cell culture supernatant; (2) incubation with MAb; (3) transfection with poly I:C ($1 \mu\text{g ml}^{-1}$) as a positive control; and (4) incubation with L15 medium as a negative control. After 24 h incubation, all the supernatants were removed from cBB cells, and the cells were washed 3 times by phosphate buffered saline (PBS), and NNV (B00GD) (MOI = 100) was added into each treated well. The cBB cells with neither treatment nor NNV (B00GD) infection were used as non-infected control.

Fourteen days after the NNV (B00GD) infection, when complete CPE appeared in the wells of negative control (treatment 4), the entire 96-well plate was stained by crystal violet (0.5% in methanol). After 30 min of staining, the plate was washed by tap-water and dried in the air. The optical density (OD) of each well was measured at 595 nm by an ELISA reader (MRXplus, DYNEX Technologies). The highest OD₅₉₅ values appeared in non-infected control. For statistical analysis, quadruplicate wells were performed for each treatment. The relative survival rate of cBB cells after each treatment and subsequent NNV (B00GD) infection was calculated by the formula: (the average OD₅₉₅ value of 4 wells of each treatment/the average OD₅₉₅ value of 4 wells of the non-infected control) $\times$ 100%.

3. Results

3.1. Curing the NNV-persistent infection in BB cells by NNV-specific antiserum

During the 95th to the 99th subcultures, one set of BB cells was cultured in medium containing NNV-specific rabbit antiserum. From the 100th subculture onward, this set of BB cells was no longer treated with antiserum. Fig. 1 revealed the effect of antiserum curing. Viral titers of the culture supernatants from cBB cells declined drastically to an undetectable level, whereas the titers of the supernatants from non-treated BB cells remained at

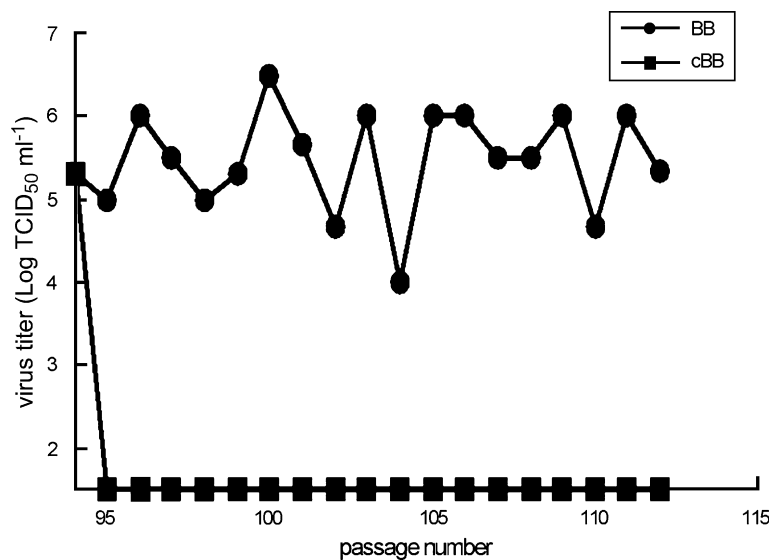


Fig. 1. Viral titers of culture supernatants from BB and cBB cells.

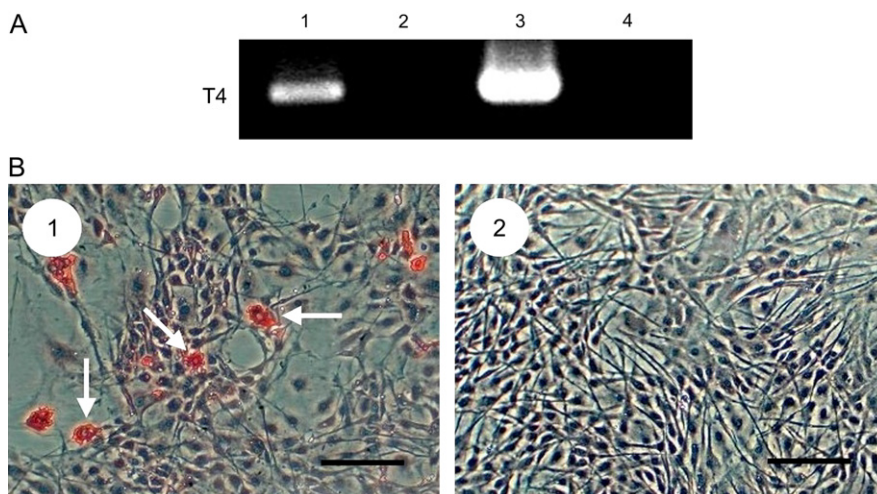


Fig. 2. Detection of NNV in BB and cBB cells. (A) Detection of NNV RNA by RT-PCR and semi-nested PCR. The target fragment of semi-nested PCR is T4. Lane1, BB cells; 2, cBB cells; 3, NNV-infected grouper larvae as positive control; 4, template-free negative control. (B) Detection of NNV capsid protein by immunochemical staining using NNV-specific MAb. The arrowheads indicated the positive signals revealed by fast-red. 1, BB cells; 2, cBB cells. Bar = 100 μ m.

10^3 – 10^7 TCID₅₀ ml⁻¹. Furthermore, NNV RNA was not detected in cBB cells by RT-PCR and semi-nested PCR (Fig. 2A), but was detected in BB cells. The NNV capsid protein was not found in cBB cells by immunochemical staining (Fig. 2B) while 1–5% of BB cells showed positive signals. The results revealed that cBB cells were completely cured by the NNV-specific antiserum, and became NNV-free.

3.2. Detection and analysis of Mx mRNA in BB cells

The expression of the Mx gene was examined by semi-quantitative RT-PCR. The Mx mRNA was detected in the BB cells, and cBB cells transfected with poly I:C, infected with NNV (B00GD), or treated with MAb-neutralized BB cell supernatant (Fig. 3); however, Mx mRNA was not found in the cBB cells, nor in the poly I:C-incubated cBB cells. Expression of Mx gene in the BB cells implied that type I IFN response existed in the BB cells.

3.3. Comparison of cytopathic effect caused by NNV with high MOI and low MOI in cBB cells

Expression of Mx mRNA was detected in NNV-infected cBB cells either with high MOI (100) or low MOI (1) two days post infection. After 14 days, total cell lysis occurred in cBB cells with high MOI (100); however, most (>95%)

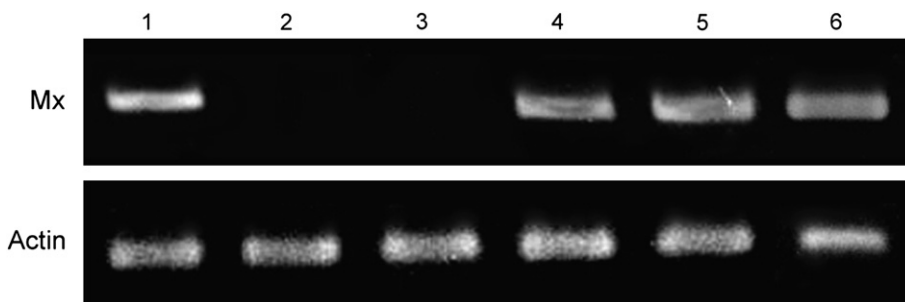


Fig. 3. Detection of Mx mRNA by semiquantitative RT-PCR. Lane 1, BB cells; 2, cBB cells; 3, poly I:C-incubated cBB cells; 4, poly I:C-transfected cBB cells; 5, NNV (B00GD)-infected cBB cells; 6, MAb-neutralized BB cell supernatant-treated cBB cells. The amount of cDNA in each sample was verified by actin-specific RT-PCR.

cells following low MOI (1) had normal morphology. The cBB cells after low MOI (1) became persistently infected after subculture for 14 times. The cell supernatants of the 14 subcultures were titrated, and the titer of the first subculture was 10^8 TCID₅₀ ml⁻¹, and gradually declined to 10^5 TCID₅₀ ml⁻¹ in the 5th subculture. Finally, the titers fluctuated at 10^4 – 10^7 TCID₅₀ ml⁻¹ in the 6th–14th subcultures. The NNV RNA was detected in the nucleic acids extracted from these subcultures. Moreover, positive immunochemical staining was observed in 1–5% of cells using NNV-specific MAb.

3.4. Antiviral activity assay

An antiviral activity assay was carried out to reconfirm the presence of IFN-like substances in the supernatant of BB cells, and to examine whether IFN-induced protection existed in the cBB cells after incubation with the BB cell supernatant. In order to eliminate the disturbance of NNV in the BB cell supernatant, NNV-specific MAb was used to neutralize the viral activity. The MAb-neutralized BB cell supernatant had no viral activity because it did not induce CPE in GF-1 cells.

After 14-day of NNV (B00GD) infection, the survival rates of cBB cells pre-treated with MAb-neutralized BB cell supernatant or transfected with poly I:C were 81.8% and 87.5%, respectively. Meanwhile, the survival rates of cBB cells pre-treated with NNV-specific MAb or L15 medium were 1.9% and 2.3%, respectively (Fig. 4). Therefore, the BB cell supernatant contained IFN-like substances that conferred protection to cBB cells against the NNV (B00GD) infection.

4. Discussion

The BB cell line is the first cell line reported to be persistently infected with NNV, and is an important system for studying the mechanism of NNV persistence. In the previous study, NNV persistent infection was shown not to be related to DI particles or Ts mutants [7]. Herein, the relationship between IFN and the persistent infection of NNV in BB cell line was investigated.

In cultured cells with persistent viral infection, the virus can be released at a low level from all cells, or from only a small fraction of the total cells. In the latter situation, the few infected cells release lytic viruses and are killed, but the

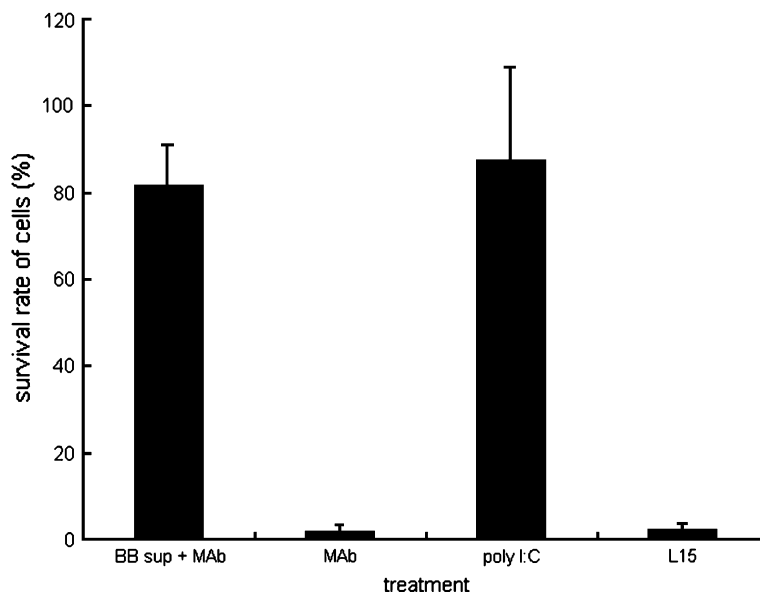


Fig. 4. Antiviral activity in cBB cells stimulated for 24 h with BB supernatant Mab-neutralized, Mab and L15 medium, or transfected with poly I:C against NNV (B00GD) infection. The cells were fixed and stained with crystal violet. Results are presented as the percentage of surviving cells, calculated as the OD₅₉₅ of infected cells ($n = 4$) divided by the OD₅₉₅ of cells with-neither treatment nor viral infection. Bars represent standard deviations.

progeny viruses go on to infect only a small number of the total cells, while the majority of the cells remains uninfected [9]. A similar phenomenon was observed in BB cell line. Only 1–5% of BB cells in each subculture showed positive signal after immunochemical staining with NNV-specific MAb, and most BB cells showed negative result. Moreover, the medium containing NNV-specific antiserum was applied to neutralize the viruses released from NNV-infected BB cells and minimize the chance of NNV infection of other BB cells. This treatment proved effective for curing NNV persistent infection, and no viral titer, RNA or capsid protein was further detected in the cured BB (cBB) cells (Figs. 1 and 2). These results demonstrated that only a few cells in each BB culture were lytically infected with NNV and released viruses into the culture supernatant.

Lytic viruses can persist *in vitro* under conditions in which only a small fraction of cells is infected at any one time, and this restriction can be due to the presence of soluble inhibitors such as interferon in the culture medium [9]. In mammalian cells, IFN is reportedly involved in the maintenance of viral persistence in L cells persistently infected with vesicular stomatitis virus (VSV) [20]. Pretreatment of L cells with IFN suppressed CPE caused by VSV infection, and led to the establishment of L cells persistently infected with the virus. Only 5–30% of the persistently infected L cells were stained with VSV-specific fluorescent antibodies. Therefore, we hypothesized that IFN was involved in the restriction of NNV replication in BB cell line.

In this study, expression of Mx gene which is one of the IFN-inducible genes, was detected in both BB cells and NNV-infected cBB cells, but not in non-infected cBB cells (Fig. 3), indicating that IFN response was active in NNV-persistently infected BB cell culture, and NNV infection was able to induce an IFN response in cBB cells. Expression of Mx gene was also found in cBB cells treated with the MAb-neutralized BB cell supernatant (Fig. 3), indicating the existence of IFN-like cytokines in BB cell supernatant. Furthermore, the survival rate of cBB cells following infection with NNV (MOI = 100) was much higher if they were pre-treated with the MAb-neutralized BB cell supernatant than when pre-treated with L15 medium or MAb (Fig. 4), indicating that IFN-like cytokines in BB culture supernatant could induce an antiviral activity against NNV in the pre-treated cBB cells. Additionally, the cBB cells being infected with NNV of low MOI (≤ 1) finally became an NNV-persistently infected cell line, and regained all the characteristics of BB cell line. Therefore, we suggested that NNV persistent infection in BB cell line involved 2-step induction of IFN response. The first IFN response occurred in some initially NNV-infected BB cells, and led to the release of IFN into the cell culture supernatant. The released IFN then reacted with other non-infected BB cells, and induced the second IFN-induced response that gave rise to antiviral activity and restricted NNV lytic infection in the BB cell line. However, the molecular level of the mechanism of NNV persistence in BB cells needs further study.

Expression of type I IFN-related genes can be induced by virus infection, dsRNA (e.g. poly I:C) stimulation, and type I IFN ligand-receptor signal transduction. Double-stranded RNA can directly activate IRF-3, which then activates the IFN-inducible genes [21]. However, expression of Mx gene in cBB cells was only induced by transfection with poly I:C, but not by incubation with poly I:C. A similar situation exists for CHSE-214 cells that have Mx expression and resistance to IPNV infection only by transfection with poly I:C [22]. Conversely, RTG-2 cells can express Mx gene by incubation with poly I:C [23]. It is suggested that BB and CHSE-214 cells may have defects in the mechanisms mediating poly I:C uptake and/or the cells lacking TLR-3 surface receptor.

Multiplicity of infection (MOI) is used to measure the average amount of virus added per cell in an infection. Infection with virus at high MOI (≥ 10) will ensure that all cells in the culture are infected and the infection is as synchronous as possible, but low MOI (≤ 1) will not [9]. When cBB cells infected with NNV of low MOI, asynchronous infection occurred in the cell culture and only some cells would be infected at the beginning. In this case, the first IFN released from the virus-infected cells may in time induce an antiviral activity in other uninfected cells before they contact with the progeny viruses released by the virus-infected cells. Conversely, high MOI caused synchronous infection of NNV in cBB cells, and all the cells were initially infected with ≥ 1 viruses. Under these conditions, even though IFN would be released from the virus-infected cells, there are no uninfected cells left for induction of antiviral activity.

Although Mx mRNA was detected in NNV-infected cBB cells both with low MOI (1) and high MOI (100), complete CPE of cell culture was only observed under high MOI, and persistent infection of the culture was derived by low MOI. It remains unclear why NNV lysed the cells even when Mx mRNA was detected during viral infection of high MOI. It is likely that the cells would be lysed if the entry of NNV was ahead of the IFN induction of antiviral activity, and the cells would remain alive if the induction of IFN response was ahead of the entry of NNV. A possible explanation is that IFN and the subsequent antiviral proteins were induced in insufficient amounts and/or too late to prevent virus replication. In addition to inducing antiviral activity, IFN can also mediate apoptosis and/or necrosis in

virus-infected cells [10]. In this study, no cell was identified with characteristics of apoptosis (data not shown), and the cells with NNV-specific CPE showed a rounded morphology before lysis. It is also possible that viral protein might block the translation of Mx mRNA into Mx protein and, thus, viral replication might continue and eventually lyse the cells. It remains unclear whether cell lysis was caused simply by the rapid replication of NNV, or by IFN-induced cell necrosis at the same time. All these speculations will be clarified in future, and the role of BB Mx protein in the antiviral activity against NNV will be examined in following works.

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