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Insufficient amounts of nervous necrosis virus used for immunization might be one of the factors that causes preferential suppression of generation of neutralizing antibodies

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Surface protrusions on nervous necrosis virus (NNV) particles play crucial roles in NNV antigenicity and infectivity. Pocket structures at the apex of surface protrusions are most likely responsible for initiating NNV infection by functioning as cell receptor binding sites. To create a scientifically robust model, we developed mouse monoclonal antibodies (MAbs) that recognize common epitopes on pocket structures shared among serologically distinct NNVs. We obtained 108 hybridoma clones that produced NNV-specific MAbs by immunizing eight mice using highly purified red-spotted grouper NNV (RGNNV) with and without physicochemical treatments. Finally, 13 hybridoma clones that produced MAbs which recognized different epitopes on RGNNV and/or striped jack NNV (SJNNV) were obtained. NNV-neutralizing activity was not observed in any of the MAbs. We believe that insufficient amounts of NNV used for immunization might be one of the factors responsible for preferential suppression of the generation of NNV-neutralizing antibodies.

Key words: Mouse monoclonal antibody (MAb), Nervous necrosis virus (NNV), Neutralizing antibodies, Antigenicity, Pocket structures of surface protrusion

Introduction

Nervous necrosis virus (NNV) of the *Betanodavirus* genus in the *Nodaviridae* family is one of the simplest spherical RNA viruses and is well known to be pathogenic to more than 170 fish species (Munday et al., 2002; Costa and Thompson, 2016; Bandín and Souto, 2020). NNV particles consist of a single coat protein (CP, M_r 42,000) and encapsulate two molecules of positive-sense single-stranded RNA (Schneemann et al., 2005). Surface protrusions on NNV particles play crucial roles in NNV antigenicity and infectivity (Tang et al., 2002; Chen et al., 2015; Gye et al., 2018b; Gye and Nishizawa, 2019a; 2021). NNV-neutralizing antibodies are directed against conformational epitopes on surface protrusions (Gye et al., 2018b; Gye and Nishizawa, 2019a, 2021), and upon binding, suppress NNV infectivity. Several sites of surface protrusions with amino-acid residue variations among serologically distinct NNVs have been reported to bind NNV-neutralizing antibodies (Chen et al., 2015; Panzarin et al., 2016; Lin et al., 2018; Costa et al., 2007). However, the receptor binding sites remain functional even after NNV-neutralizing antibody binding sites are denatured by specific chemical treatments (Gye and Nishizawa, 2021). Recently, N-glycan antennae with terminal Neu5Ac-Gal-Glc NAc moieties (*N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine) have been reported to function as one of the cellular receptors for NNVs (Gye and Nishizawa, 2022). Furthermore, serologically distinct

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NNVs can bind to the terminal Neu5Ac-Gal-GlcNAc moieties *in vitro*, suggesting that there may be common sites for binding to cellular receptors (Gye and Nishizawa, 2022). Subsequently, serologically distinct NNVs were shown to share common pocket structures with highly conserved amino-acid residues at the apex of surface protrusions. NNV-neutralizing antibodies that recognize common epitopes on the pocket structures were detected in anti-NNV rabbit sera. Moreover, molecular docking analysis suggested that the terminal moiety of Neu5Ac-Gal-GlcNAc antennae are fully competent to bind pocket structures (Nishizawa et al., 2023). Thus, the pocket structures at the apex of surface protrusions may most likely be responsible for initiating viral infection by functioning as a cell receptor binding site (Nishizawa et al., 2023).

To create a scientifically robust model for binding NNV to cellular receptors, developing NNV-neutralizing monoclonal antibodies (MAbs) that recognize common epitopes on the pocket structures shared among serologically distinct NNVs may be useful. Thus, we developed MAbs by immunizing mice with highly purified NNV particles.

Materials and Methods

Viruses

Two different NNVs, namely red-spotted grouper NNV (RGNNV) (SgNag05 isolate, serotype C) (Kokawa et al., 2008) and striped jack NNV (SJNNV) (SJ93Nag isolate, serotype A) (Nishizawa et al., 1997), were cultured with SSN-1 cells maintained at 25ºC using Leibovitz's L-15 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 100 U/ml penicillin-streptomycin solution (Gibco) ($L\text{-}15_{10}$ medium). NNV was purified by following previously described methods (Gye and Nishizawa, 2016, 2019a, 2021, Nishizawa et al., 2023). The cultured NNV supernatant produce by centrifugation at 12,000×g for 20 min at 4°C was dialyzed using a Biotech cellulose ester (CE) membrane tube with a molecular weight cut off (MWCO) of 10^6 (Spectrum Laboratories). After anion-exchange chromatography using a Hi-trap Q column (GE Healthcare), NNV particles were eluted with 700 mM sodium chloride (NaCl). They were then desalted and concentrated by centrifugal ultrafiltration using a membrane at MWCO of 10^5 (Vivaspin, Sartorius). Finally, approximately 10^{10} 50% tissue culture infectious doses ($TCID_{50}$)/ml (7.6 μg/ml) of highly purified NNV particle suspension was obtained.

Physical and chemical treatments of purified NNV

Two procedures were performed to alter the structural elements of purified NNV particles. In the first method, the particles were heated at 45ºC for 16 h, as described previously (Gye and Nishizawa, 2019a). Under these conditions, surface protrusions of NNV particles disappear completely, although the viral particle structure is maintained (Gye and Nishizawa, 2019a). In the second method, the particles were treated with 2.0 M urea at 25ºC for 16 h, as described previously (Gye and Nishizawa, 2021). Under these conditions, NNV antigenicity declines owing to partial denaturation of surface protrusions, but infectivity is not reduced (Gye and Nishizawa, 2021).

Mouse monoclonal antibodies

One-month-old female mice (BALB/c) were immunized by intraperitoneal injection of 100 μl naïve or urea-treated NNV particle suspension (7.6 μg/ml) emulsified with equal parts of Freund's complete adjuvant. The mice were intraperitoneally re-injected three times with booster doses of 100 μl of the purified naïve or urea treated NNV suspension at 10-day intervals. The immunized spleen cells were harvested 3 days after the final immunization and fused with mouse myeloma cells (SP2/0-Ag14) at a 10:1 ratio using polyethylene glycol (average at M_r 1,500). Fused cells were maintained in eRDF medium (Kyokuto Co., LTD.) supplemented with 10% FBS and

incubated under 5% carbon dioxide (CO_2) at 37ºC. After hypoxanthine-aminopterin-thymidine (HAT)-selection, hybridoma colonies that reacted with purified NNV were selected and cloned by limited dilution.

Enzyme-linked immunosorbent assay (ELISA)

Reactivity of each MAb before and after physicochemical treatment with NNV particles were compared using ELISA. Naïve and heat-treated NNV particles (approximately 7.6 ng/ml) suspended in deionized water (DIW) were aliquoted into wells of ELISA plates (Greiner Bio-one) at 100 µl/well and fixed by drying at 37°C overnight. Subsequently, urea treatment was performed. NNV antigens were detected according to previously published methods (Gye and Nishizawa, 2016, 2019a, 2019b, 2020, 2021). The ELISA plate wells were blocked with 5% skim milk in PBS (SM-PBS) at 25°C for 30 min. After washing three times with PBS, MAb suspension was added into each well and incubated at 25°C for 30 min. Antibodies bound to NNV antigens fixed on ELISA plates were detected using horseradish peroxidase (HRP) conjugated antisera against mouse immunoglobulins (Ig, Dako) (diluted with SM-PBS) and OPD substrate solution (1 mg/ml *o*-phenylenediamine, 0.03% hydrogen peroxide [H_2O_2], 100 mM disodium phosphate [Na_2HPO_4], and 50 mM citric acid). The reaction was halted using 100 µl of 2 N sulfuric acid (H_2SO_4). Absorbances at 490 nm (A_{490}) were measured using a microplate reader (SpectraMax ™ 340PC^{384}, Molecular Devices), and data were presented as averages of duplicate well values.

NNV-neutralization test of MAb suspension

MAb suspensions obtained by culturing each hybridoma at 37°C for 14 days were subjected to a quantitative neutralization test as follows: two-fold serial dilutions of MAb suspensions were prepared and mixed with equal volumes of purified NNV suspension at 10^2 $TCID_{50}$/50 µl. After incubation at 25°C for 1 h, aliquots of each mixture (100 µl/well) were transferred in duplicate to wells of a microtiter plate seeded with SSN-1 cells. After incubation at 25°C for 10 days, the maximum dilution of antiserum neutralizing 10^2 $TCID_{50}$ NNV was defined as the NNV-neutralization titer.

Ethics statement

Experiments using mice were carried out in strict accordance with the recommendations of the Institutional Animal Care and Use Committee of Chonnam National University (permit number: CNU IACUC-YS-2019-3).

Results and Discussion

A total of 548 hybridomas were obtained by immunization of six mice with purified naïve RGNNV particles. Among them, 58 clones of hybridomas produced MAbs that showed positive reaction with purified RGNNV particles, and 39 clones were selected because of the productivity and reactivity of each MAb. Finally, 5 clones of hybridomas (RG/6E11, RG/30E10, RG/15A9, RG/9A1 and RG/29D11) were selected based on reaction patterns of MAbs with naïve RGNNV, urea-treated RGNNV, heat-treated RGNNV and naïve SJNNV particles (Fig. 1A). The MAb RG/6E11 showed reactivity with naïve RGNNV but not with urea-treated RGNNV, heat-treated RGNNV or naïve SJNNV, suggesting that this MAb may recognize a heat- and urea-sensitive epitope specific to RGNNV. MAbs RG/30E10 and RG/15A9 showed reactivity with heat-treated RGNNV and SJNNV; therefore, these MAbs may recognize a heat-resistant epitope commonly specific to RGNNV and SJNNV. The MAbs RG/9A1 and RG/29D11 reacted relatively well with urea-treated RGNNV, although the reactivity was low with heat-treated RGNNV or naïve SJNNV, suggesting that these MAbs may recognize a urea-resistant epitope on RGNNV.

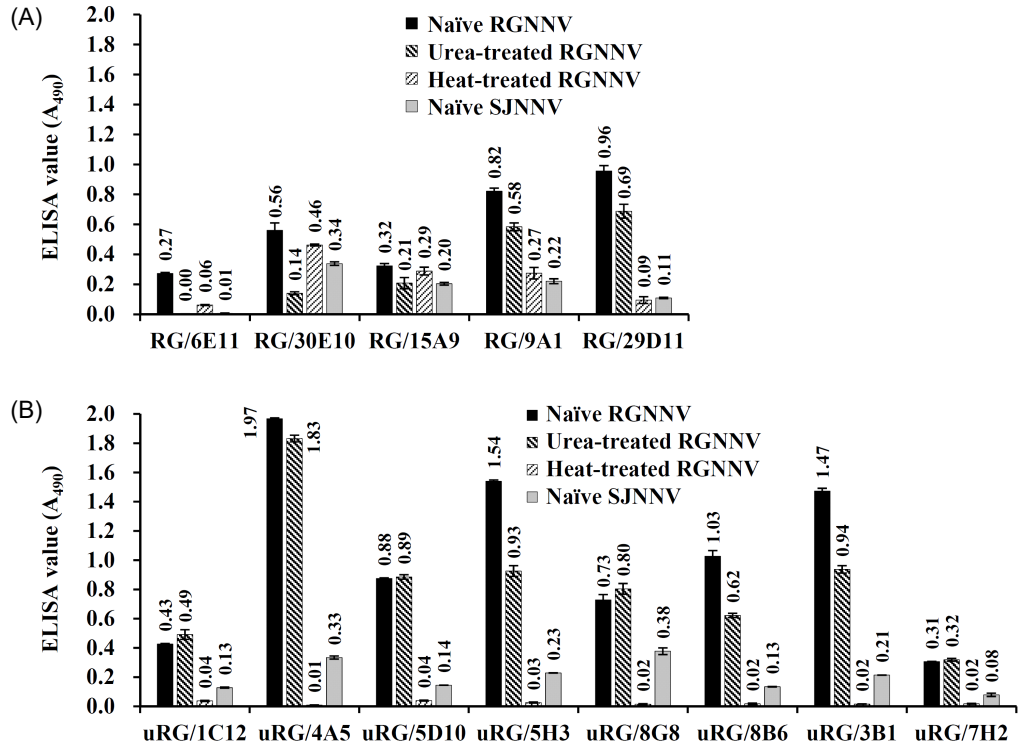

Fig. 1. Reactivity of each MAb with purified NNV before and after physicochemical treatments. Reactivity of each MAb was investigated using ELISA plate wells immobilized with naïve, urea-treated and heat-treated RGNNV particles as well as naïve SJNNV particles. (A) MAbs produced by hybridomas fused with mouse splenocytes immunized using naïve RGNNV and (B) urea-treated RGNNV. Error bars indicate standard deviation (SD).

The pocket structures at the apex of NNV surface protrusion are known to be maintained after chemical treatment with 2.0 M urea (Gye and Nishizawa, 2021; Nishizawa et al., 2023). Thus, in the next experiment, two mice were immunized with urea-treated RGNNV. A total of 145 hybridoma clones were obtained, of which 50 produced MAbs that positively reacted with purified RGNNV. Based on the productivity and reactivity of each MAb, 15 clones were selected initially (Table 1). Finally, 8 clones remained (hybridomas uRG/1C12, uRG/4A5, uRG/5D10, uRG/5H3, uRG/8G8, uRG/8B6, uRG/3B1 and uRG/7H2) (Fig. 1B). The MAbs produced by these hybridomas commonly showed negligible reaction with heat-treated

Table 1. Reaction of MAbs produced by hybridomas with purified NNV particles and their NNV-neutralizing activity

Antigen	Mouse (n)	Hybridoma	Positive reaction	1st selection	2nd selection	NNV-neutralizing activity
Naïve RGNNV	6	548 clones	58 clones	39 clones	5 clones	0% (0/58)
Urea-RGNNV	2	145 clones	50 clones	15 clones	8 clones	0% (0/50)
Total	8	693 clones	108 clones	54 clones	13 clones	0% (0/108)

MAbs, monoclonal antibodies; NNV, nervous necrosis virus; RGNNV, red-spotted grouper nervous necrosis virus; SJNNV, striped jack nervous necrosis virus.

RGNNV. Thus, heat-sensitive epitopes on RGNNV particles may respond to treatment with 2.0 M urea. The MAbs uRG/1C12, uRG/4A5, uRG/5D10, uRG/8G8 and uRG/7H2 demonstrated only a minor difference in reactivity to naïve- and urea-treated RGNNV. In contrast, reactivity of the MAbs uRG/5H3, uRG/8B6 and uRG/3B1 with urea-treated RGNNV was substantially lower than that with naïve RGNNV (Fig. 1B). All these MAbs showed reactivity with SJNNV; however, their reaction intensity varied. Based on these results, at least 13 hybridomas that produced MAbs which recognized epitopes on the surface of NNV particles were obtained.

MAbs produced by the 108 hybridoma clones were subjected to neutralization tests using purified NNV particle suspension; however, no NNV-neutralizing activity was observed in any of them (Table 1). Therefore, we were unable to create NNV-neutralizing MAbs that recognize common epitopes on pocket structures shared among serologically distinct NNVs. We believe that absence of NNV-neutralizing MAbs was not accidental, and they might have not been generated for a specific reason. In our previous study, sufficient NNV-neutralizing antibodies were generated in rabbits and mice immunized with 30~40 μg NNV particles, which were purified to the same extent as in this study (Gye et al., 2018b). In contrast, the amount of purified NNV particles used for immunization of mice in this study was approximately 3 μg, which was ≤ 1/10 of that used in the previous study. The results of specific antibody generation through immune responses are variable because they are highly dependent on antigen size, structure, dose and composition. Therefore, one of the reasons why no NNV-neutralizing antibodies were generated in our study may be the insufficient amount of NNV particles used for immunization.

Several studies have reported the development of NNV-neutralizing MAbs (Lai et al., 2001; Shieh and Chi, 2005; Gye et al., 2018b; Kim et al., 2018; Zhang et al., 2022), and in most of them, ≥ 100 μg NNV antigens were used for mice immunization (Table 2). NNV-neutralizing antibodies are generated in fish that received ≥ 100 μg recombinant CPs (Tanaka et al., 2001; Húsgarð et al., 2001; Yuasa et al., 2002; Vimal et al., 2014) (Table 2), although there were a few exceptions (Thiéry et al., 2006; Lai et al., 2014). In cases of inactivated NNV vaccine, NNV-neutralizing antibodies are generated by immunization with antigens corresponding to ≥ $10^{6.5}$ $TCID_{50}$/fish (Yamashita et al., 2005, 2009; Kai et al., 2010; Pakingking et al., 2010) (Table 2). However, while NNV-specific antibodies are generated in fish that have naturally convalesced from NNV infection, NNV-neutralizing antibodies are not. Moreover, these convalescent fish are strongly protected from re-infection by NNV (Nishizawa et al., 2012; Oh et al., 2013; Gye et al., 2018a, 2021; Kim et al., 2021). The dose of NNV required to induce convalescent fish is approximately $10^{4.3}$ $TCID_{50}$/fish (Gye et al., 2018a, 2021; Kim et al., 2021), which is substantially lower than that of inactivated NNV vaccines. Thus, we believe that insufficient amounts of NNV used for immunization might be one of the factors related to preferential suppression of the generation of NNV-neutralizing antibodies. Although scientific evidence for our hypothesis is lacking, this new perspective can aid in understanding the infection mechanism of NNV and improving efficacy of NNV vaccines.

In conclusion, at least 13 hybridoma clones that produced MAbs which recognized different epitopes on the NNV particle surface were obtained. However, none of them induced NNV neutralization. Nevertheless, these MAbs may be utilized for future research involving the analysis of epitopes on NNV particle surfaces.

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Table 2. Required doses of live, inactivated and recombinant NNV antigens for mouse and fish immunization

Immunized animal	Antigen	Immunization dose	Reference
Mouse immunized with NNV or VLP			
Mouse	Yellow grouper NNV	100 μg/mouse	Lai et al., 2001
Mouse	Grouper NNV	75 μg/mouse	Shieh and Chi, 2005
Mouse	RGNNV	30~40 μg /mouse	Gye et al., 2018b* Kim et al., 2018
Mouse	RGNNV VLP	100 μg/mouse	Zhang et al., 2022
Fish immunized with rCP of NNV			
Red-spotted grouper (*Epinephelus akaara*)	rCP of RGNNV	120 μg/fish	Tanaka et al., 2001
Turbot (*Scophthalmus maximus*) Atlantic halibut (*Hippoglossus hippoglossus*)	rCP of SJNNV	100 μg/fish	Húsgarð et al., 2001
Humpback grouper (*Cromileptes altivelis*)	rCP of RGNNV	420 μg/fish	Yuasa et al., 2002
Giant grouper (*E. lanceolatus*)	tandem-repeated NNV CP epitopes	30~300 μg/fish	Vimal et al., 2014
Fish immunized with inactivated NNV			
Sevenband grouper (*E. septemfasciatus*)	RGNNV	$10^{8.1}$ $TCID_{50}$/fish	Yamashita et al., 2005
Sevenband grouper	RGNNV	$\geq 10^{7.5}$ $TCID_{50}$/fish	Yamashita et al., 2009
Orange-spotted grouper (*E. coioides*)	HGNNV (RGNNV type)	$10^{10.7}$ $TCID_{50}$/fish	Kai et al., 2010
Brown-marbled grouper (*E. fuscogutattus*)	RGNNV	$10^{6.5}$ $TCID_{50}$/fish	Pakingking et al., 2010
Convalescent fish by NNV infection			
Sevenband grouper (*Hyporthodus septemfasciatus*)	RGNNV	$10^{4.3}$ $TCID_{50}$/fish	Gye et al., 2018a
Sevenband grouper (*E. septemfasciatus*)	RGNNV	$10^{4.3}$ $TCID_{50}$/fish	Kim et al., 2021
Sevenband grouper (*H. septemfasciatus*)	RGNNV	$10^{4.3}$ $TCID_{50}$/fish	Gye et al., 2021

* Gye et al. (2018b) described that NNV suspension of approximately 100 μg/ml was inoculated three times for mouse immunization. Considering the usual inoculum volume might be 100 μl/mouse or a little more, the immunization dose was estimated as approximately 30 μg/mouse.

NNV, nervous necrosis virus; VLP, viral like particles; rCP, recombinant coat protein; RGNNV, red-spotted grouper NNV; SJNNV, striped jack NNV.

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