

Potential for the replication of the betanodavirus redspotted grouper nervous necrosis virus in human cell lines

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Summary

The determination of the host ranges of viruses is important because of the possible emergence of infectious agents, which may result from the zoonotic transmission of animal viruses to humans. The family *Nodaviridae*, whose members are non-enveloped, positive-stranded bipartite RNA viruses, is comprised of the genera *Alphanodavirus* and *Betanodavirus*, whose members predominantly infect insects and fish, respectively. The alphanodaviruses can also infect suckling mice and suckling hamsters, resulting in paralysis and death. Pigs near the site of isolation of the Nodamura virus (NoV), an alphanodavirus, have been reported to have high levels of NoV neutralizing antibody, suggesting that they may be part of the natural host range of this virus. Betanodaviruses are the causative agents of viral nervous necrosis, which occurs in several species of fish. However, little is known regarding the mechanism of infection of these viruses. Whether betanodaviruses can infect hosts other than

fish remains unclear. In this study, we examined the possibility that a betanodavirus, redspotted grouper nervous necrosis virus (RGNNV), can infect human cell lines and showed that this virus can attach to the cells but cannot penetrate them, although human cells can support the replication of the betanodavirus when viral RNAs are transfected. The betanodavirus in its present form cannot infect human cells.

Introduction

The family *Nodaviridae* is comprised of the recently established genera *Alphanodavirus* and *Betanodavirus*, whose members predominantly infect insects and fish, respectively. Viruses belonging to the betanodavirus genus are the causative agents of viral encephalopathy and retinopathy, also known as viral nervous necrosis (VNN). VNN occurs in many species of fish and causes devastating economic effects on marine aquaculture worldwide [14]. The betanodaviruses have been isolated from more than 30 marine fish species from 14 families, striped jack and sea bass, for example. The betanodaviruses are classified into four genotypes based on the phylogenetic analysis of their genomic RNA2 (coat protein) sequences. The genotypes are: striped jack nervous necrosis virus

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(SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and redspotted grouper nervous necrosis virus (RGNNV) [25, 29, 30].

The betanodaviruses are small (25–30 nm diameter), spherical, non-enveloped viruses with a genome that is composed of two single-stranded, positive-sense RNA molecules [24]. The larger genomic segment, RNA1 (3.1 Kb), encodes the RNA-dependent RNA polymerase (RdRp) [12, 28, 37], and the smaller genomic segment, RNA2 (1.4 Kb), encodes the coat protein [29]. It has recently been determined that a subgenomic RNA transcribed from the 3' end of RNA1, termed RNA3, encodes a protein, B2. This protein is highly conserved among the betanodaviruses and functions as an RNAi antagonist [8, 9, 19].

The replication of nodaviruses has been studied in a wide range of cultured cells. Among the known alphanodaviruses, only the Nodamura virus (NoV) has the ability to lethally infect suckling mice and suckling hamsters [10, 34]. When NoV or Flock House virus (FHV) genomic RNAs are introduced by transfection, they can replicate in a variety of cell types, including mammalian, insect, yeast, and plant, though none of these support virus replication in whole virus infection [2, 13, 20, 33, 35, 36]. In BHK-21 cells, controversy exists regarding the infectivity of NoV [1, 2, 13], as the determinants of infectivity of the alphanodaviruses have not yet been established, and the specific receptors have also not been identified. The replication of betanodaviruses, on the other hand, is considered to be very temperature dependent because of the RdRp activity, the result being that fish constitute the narrow natural host range of such betanodaviruses. However, the mechanism of betanodavirus infection, which involves both viral factors and essential host factors, has not yet been elucidated. We recently suggested that betanodaviruses enter cells by endocytosis (manuscript in press). However, it is not known whether this virus can infect other animal species, particularly humans. The only report that has described the pathogenicity of the betanodaviruses in mammals, using genotypes of SJNNV and RGNNV, demonstrated that mice are not susceptible to betanodaviruses, as no clinical

signs of infection manifested, and no virus was detected in the organs targeted by the betanodaviruses [3].

The determination of the host ranges of viruses is an important research subject because of the possible emergence of infectious agents, which can result from zoonotic transmission of animal viruses to humans [23, 27, 31]. The host range switch of the SARS coronavirus from animal species to humans was recently reported [21, 32]. The acquisition of the ability of this switch in viruses may constitute a permanent threat to human health. In light of these events, there exists an urgent need to better understand the factors involved in the infectivity of poorly characterized viruses, including the betanodaviruses.

In this study, we examined the possibility of infection of betanodavirus (RGNNV genotype) in human HeLa, 293T, and A549 cell lines *in vitro*. The results demonstrated that RGNNV possesses the ability to bind to human cells. However, penetration of the betanodavirus into the human cells was not detected, although human cells support the replication of the betanodavirus when transfected with betanodavirus RNAs. Our data therefore suggest that human cells lack specific host receptors for the infection of betanodaviruses.

Materials and methods

Cells, viruses and antibody

E-11 cells, cloned from the SSN-1 cell line, which was isolated from a striped snakehead [16], were maintained in Leibovitz's L-15 medium supplemented with 5% fetal bovine serum (FBS) at 25 °C. HeLa, 293T, and A549 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37 °C in 5% CO₂. Fish nodavirus (betanodavirus) was originally isolated in 2001 from a seven-band grouper in Nagasaki, Japan. This virus belongs to the RGNNV genotype, as determined by our own RNA2 nucleotide sequence analysis. E-11 cells were infected with the betanodavirus for propagation, and viruses were harvested when nearly all of the cells in the monolayer showed a cytopathic effect (CPE). The viral titer was determined by tissue culture infectious dose of 50% (TCID₅₀) assay. Polyclonal anti-RGNNV coat protein antiserum was produced by immunizing a guinea pig with a total of 8 µg of purified coat protein with Freund's complete adjuvant (DIFCO).

Virus infection

Inoculation was performed in the presence or absence of 5% CO₂ for E-11 cells and HeLa, 293T, and A549 cells, respectively. The cells were inoculated with virus for 1 h and then were washed to remove any unbound virus and incubated in each medium containing FBS.

Detection of viral RNA by RT-PCR

Total RNA was prepared from RGNNV-infected cells (3 to 4 × 10⁵) using Trizol reagent (Invitrogen). To detect viral genomic (+) RNA1, (+) RNA2, template (-) RNA1, and 18S rRNA, the RNA samples were reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) using RGRNA1-2490R (5' GTCAGTGTAGTCTGCATACTG 3'), RGRNA2-1310R (5' CACTGGCACCCAATTAAGCA 3'), RGRNA1-1868F (5' TGCGTGAGTTCGTCGAG TTT 3'), and 18S rRNA-R (5' GCTGGAATTACCGCG GCT 3'), respectively. PCR amplification was performed with a primer pair (RGRNA1-1868F and RGRNA1-2490R for (+) and (-) RNA1, SJRNA2-615F [5' CGTGTCAGT CATGTGTCGCT 3'] and RGRNA2-1310R for RNA2, and 18S rRNA-F [5' CGGCTACCACATCCAAGGAA 3'] and 18S rRNA-R for 18S rRNA). The PCR products were analyzed on agarose gel. The band intensities were semi-quantitated by Image J software (NIH).

Neuraminidase treatment and virus infection of cells

The cells were grown in 12-well plates and were washed twice with the culture medium without FBS and incubated with *Clostridium perfringens* neuraminidase (SIGMA), using medium as a diluent. After incubation at 37°C for 4 h, the cells were washed twice with culture medium and inoculated with virus at 4°C for 1 h. After washing four times, total RNA was prepared and subjected to RT-PCR as described above.

Immunofluorescence analysis

Indirect immunofluorescence assays were performed with infected or transfected cells grown on glass coverslips. The reactions were performed at room temperature. Cells were fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then permeabilized by treatment with 0.1% NP-40 in PBS for 20 min. The cells were immersed in 1% dehydrated skimmed milk powder in PBS for 1 h and treated with a 1:500 dilution of polyclonal anti-RGNNV coat protein antiserum for 1 h. After washing with 0.1% NP-40 in PBS, the cells were treated with a 1:1000 dilution of Alexa Fluor 546 goat anti-guinea pig IgG (Invitrogen) for 1 h. The cells were washed again and then observed using an Axiophoto fluorescence microscope (Carl Zeiss).

Virion RNAs preparation and transient transfection

Viral purification was performed as described previously [18, 24] with minor modifications. Briefly, the culture supernatants of the RGNNV-infected E-11 cells were harvested and centrifuged at 100,000 × g for 2.5 h at 4°C. The resultant pellets were suspended in TE buffer, and then the virus solution was layered onto 10–40% (wt/vol) sucrose gradients and centrifuged at 80,000 × g for 2 h at 4°C. Each fraction was collected, and its virus content was analyzed by Coomassie brilliant blue (CBB) staining and Western blot analysis with anti-RGNNV coat protein antiserum. RNA was extracted from purified virus preparations using Trizol reagent. RNA transfection was performed as described previously [17] with minor modifications. The cells were grown on glass coverslips to 70–80% confluence and washed twice in fresh L-15 medium without FBS for the E-11 cells, and in DMEM for the human cells. The cells were transfected at a ratio of virus RNAs to lipofectin (Invitrogen) of 1 μg to 3 μl at 28°C. After 2 h of incubation, the medium was removed, and the cells were washed once with medium and then incubated with medium containing FBS at 28°C. After transfection, culture supernatant was harvested, and the viral titer was assayed by TCID₅₀ using E-11 cells. For the detection of coat protein expression, the transfected cells were subjected to an immunofluorescence assay with the anti-RGNNV coat protein antiserum described above.

Results

Binding activity of RGNNV to human cell lines

To investigate the ability of RGNNV to attach to human cells, we initially examined the ability of RGNNV to bind at 4°C to the human cell lines HeLa, 293T, and A549, compared to the fish E-11 cell line. As shown in Fig. 1A, the genomic (+) RNA1 was detected in the E-11 cells from multiplicity of infection (M.O.I.) of 100–0.1 with an M.O.I.-dependent band intensity, and that of each of the human cell lines was detected in the same manner. The intensity of (+) RNA1 varied among the cell lines used in the experiment; a significant intensity of (+) RNA1 was detected in each human cell line. The normalized (+) RNA1 intensity of the infected human cells was closely comparable to that of infected E-11 cells (Fig. 1B). These results suggest that RGNNV can attach not only to the fish cell line but also to human cells at a comparable level.

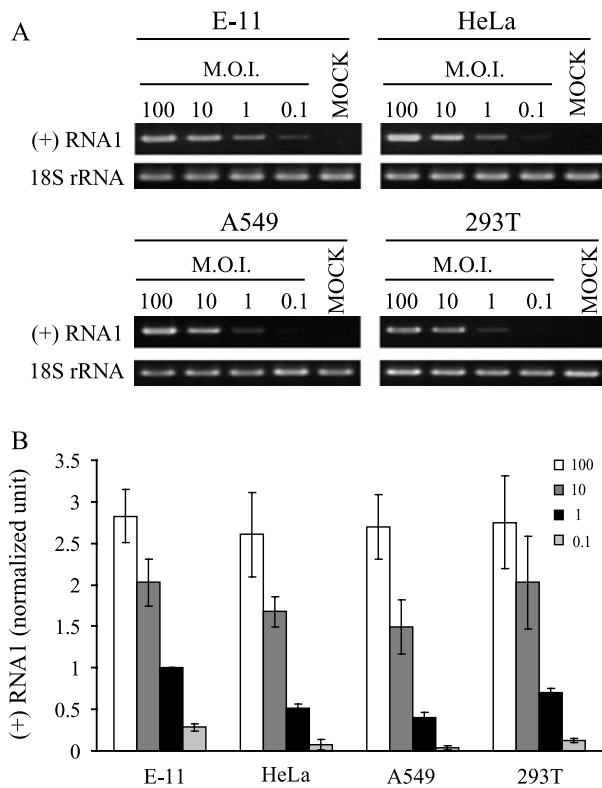


Fig. 1. Betanodavirus attachment on various human cells. (A) Cells (3 to 4×10^5) were incubated with RGNNV at the indicated M.O.I. for 1 h at 4°C . The cells were washed four times to remove unbound virus, and then the total RNA was prepared and (+) RNA1 and 18S rRNA were detected by RT-PCR as described in Materials and methods. (B) The band intensity of (+) RNA1, normalized to cellular 18S rRNA, is indicated as the ratio of (+) RNA1 at an M.O.I. of 100 (white bar), 10 (dark gray bar), 1 (black bar), and 0.1 (light gray bar) compared to that of the E-11 at an M.O.I. of 1. Data represent the means and standard deviations from three independent experiments

Effect of neuraminidase treatment on RGNNV attachment to human cells

In a previous report, cell-surface sialic acid was suggested to be involved in betanodavirus binding to fish SSN-1 cells because of its sensitivity to neuraminidase and tunicamycin treatment [22]. We therefore analyzed whether sialic acid is required for the attachment of RGNNV to human cells. The E-11 cells and the human cells were treated with *Clostridium perfringens* neuraminidase prior to infection with RGNNV. Pre-treat-

ment of each of the cell types with 4 or 40 mU of neuraminidase was sufficient to reduce the intensity of the band of (+) RNA1 (Fig. 2, left panel). The band intensity of the normalized (+) RNA1 clearly indicated that the amount of the bound virus decreased in comparison to the untreated cells (Fig. 2, right panel). These results suggest that cell-surface sialic acid is involved in RGNNV attachment, irrespective of the species from which the cell line is derived.

Lack of viral replication in RGNNV-infected human cells

To further analyze the infectivity of RGNNV, we detected viral genomic (+) RNA1, template (–) RNA1, and (+) RNA2 accumulation in infected cells (Fig. 3). (–) RNA1 was synthesized from (+) RNA1 as the replication template. The presence of (–) RNA1 indicated that the replication of viral genomes had occurred. RGNNV-infected E-11 cells and human cells were incubated at 28°C for the number of hours indicated. Total RNA was subsequently prepared and RT-PCR was conducted. As shown in Fig. 3A, (+) RNA1 bands in infected E-11 cells gradually increased in intensity and reached a maximum level at 18 h post-infection. No significant (–) RNA1 bands were detected at 1 h post-infection; however, their intensity increased until 9 h post-infection, and significant accumulation was observed from 9 to 12 h post-infection. The (+) RNA2 bands were detected from 9 h post-infection onward. These data imply that RGNNV had entered the cells and that the synthesis of the (–) RNA1 had begun immediately, reaching its maximum level between 9 and 12 h after infection. On the other hand, no significant (–) RNA1 and (+) RNA2 bands were detected in the infected HeLa, A549, and 293T cells, even at 36 h post-infection (Fig. 3B–D, left panel). In addition, the (+) RNA1 level did not change until 36 h post-infection (Fig. 3B–D, right panel). No coat protein expression was detected in the human cells at 5 days post-inoculation (Fig. 3E). These results indicate that the replication of the viral genomes was strongly suppressed and/or inhibited in the RGNNV-infected human cells.

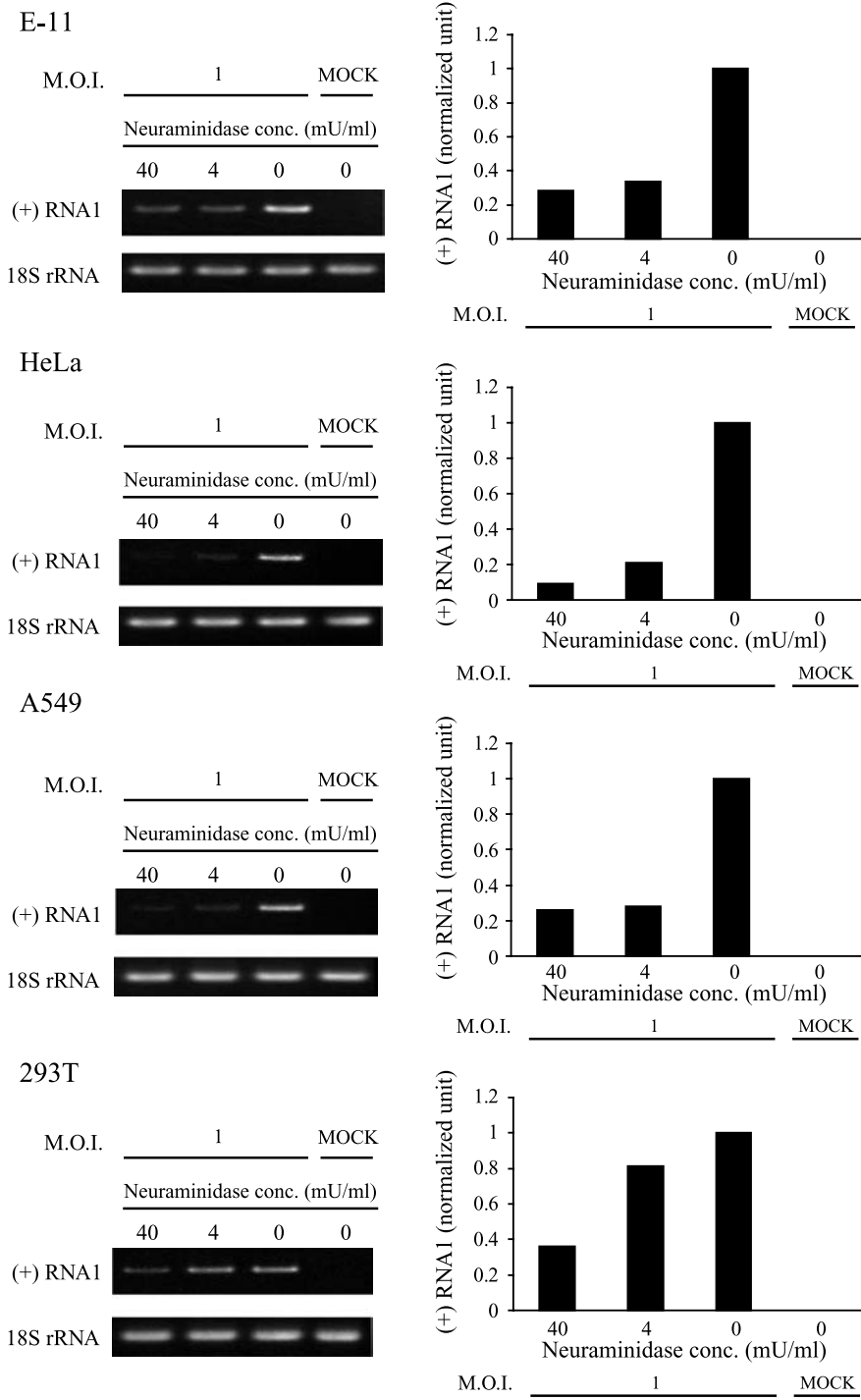


Fig. 2. Effect of neuraminidase treatment of cells on betanodavirus attachment. E-11, HeLa, A549, and 293T cells were treated with the indicated amounts of neuraminidase for 4 h at 37°C prior to inoculation. Then, they were inoculated with RGNNV at an M.O.I. of 1 at 4°C. At 1 h post-inoculation, total RNA was prepared and (+) RNA1 and 18S rRNA were detected by RT-PCR. PCR products were analyzed by agarose gel electrophoresis (*left panel*). The normalized (+) RNA1 band intensity is indicated as the ratio of (+) RNA1 to that of no treatment (*right panel*). Data represent the means from two independent experiments

Expression of the coat protein and virus production in virion-RNA-transfected human cells

To analyze whether human cells support RGNNV RNA replication, we examined virus production in

virus-RNAs-transfected cells. E-11, HeLa, A549, and 293T cells were transfected with RNAs prepared from sucrose gradient-purified virus, and coat protein expression and virus production were ana-

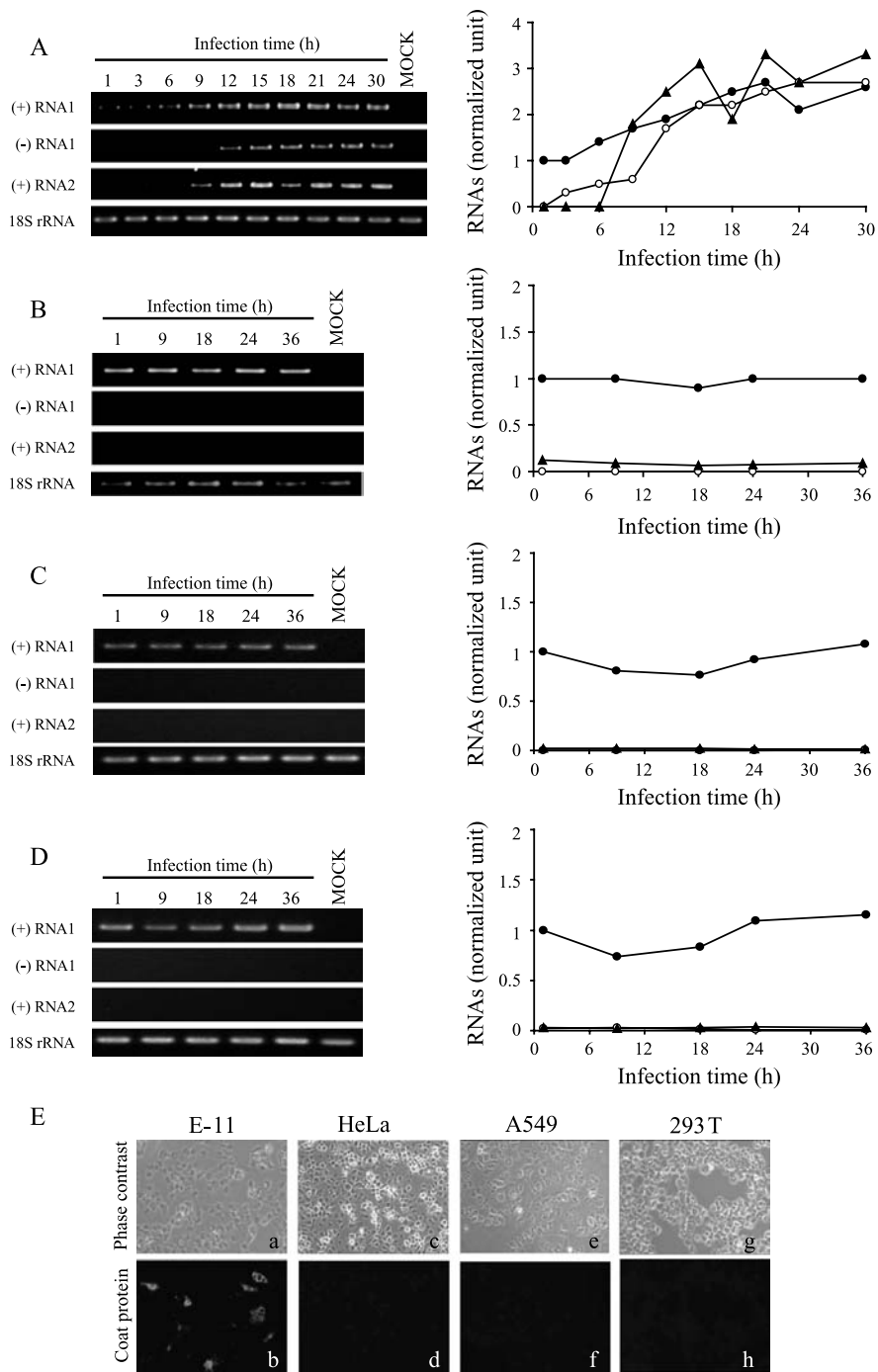


Fig. 3. RGNNV replication is restricted in human cells. E-11 cells (**A**) were infected at an M.O.I. of 1, and HeLa (**B**), A549 (**C**), and 293T (**D**) cells were infected at an M.O.I. of 100 for 1 h at 28 °C. Next, the cells were washed to remove any unbound viruses and incubated at 28 °C. At the indicated times after infection, total RNA was extracted, and the genomic (+) RNA1, template (-) RNA1, (+) RNA2 and 18S rRNA were detected by RT-PCR (*left panel*). Quantitative data for (+) RNA1 (*closed circle*), (-) RNA1 (*open circle*), and (+) RNA2 (*triangle*) accumulation in the infected cells are indicated as the relative value of the normalized band intensity to that of the (+) RNA1 detected 1 h post-infection (*right panel*). The data represent the means from two independent experiments. (**E**) E-11 cells infected with RGNNV at an M.O.I. of 1 were fixed at 3 days postinfection, and HeLa, A549, and 293T cells were infected at an M.O.I. of 100 and fixed at 5 days postinfection, then subjected to indirect immunofluorescence assay with anti-RGNNV coat protein. The images represent the phase contrast of the infected cells (a, c, e, g), and the fluorescent cells (b, d, f, h)

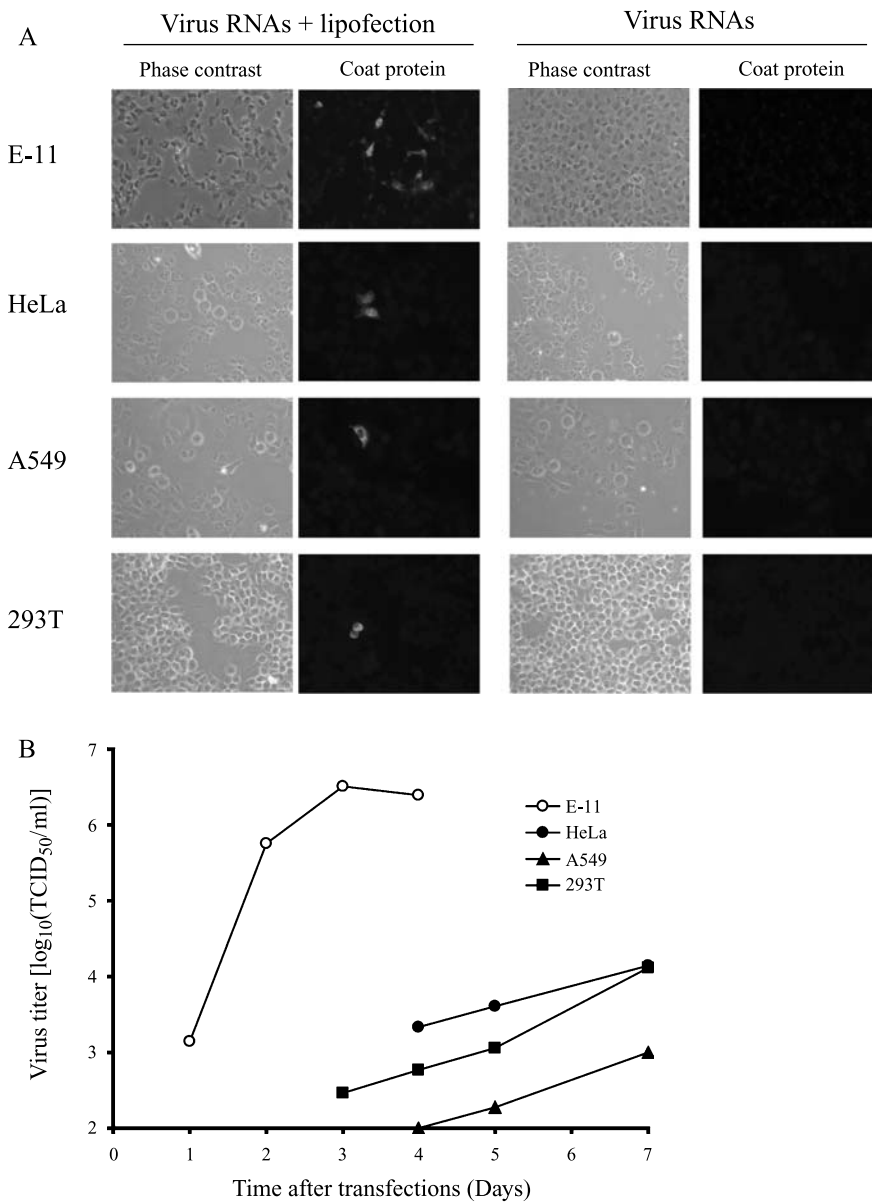


Fig. 4. Coat protein expression and virus release from virion-RNA-transfected human cells. **(A)** Cells were transfected with 2 μ g of purified virus RNAs and incubated at 28 °C for 1 day, then fixed after the indicated day and stained for virus coat protein antigen by immunofluorescence. **(B)** The culture supernatants of transfected E-11 (open circle), HeLa (closed circle), A549 (closed triangle), 293T (closed square) cells were harvested and the infective titer was determined by TCID₅₀ assay. The calculation of the mean values was based on data derived from two independent experiments performed in duplicate

lyzed. Coat protein expression was detected in the RNA-transfected E-11 cells at 1 day post-transfection. No coat protein expression was seen in E-11 cells that were incubated with virus RNAs only (Fig. 4A). In transfected HeLa, A549, and 293T cells, coat protein expression was also detected at 1 day post-transfection (Fig. 4A). Virus production in the RNA-transfected E-11 cells reached a maximum titer of 10^6 TCID₅₀/ml at 4 days after transfection. In the HeLa, A549, and 293T cells,

10^3 – 10^4 TCID₅₀/ml of released virus was detected by 7 days. These results indicate that human cells support the replication of RGNNV RNAs when they are introduced into the cells.

Discussion

We herein examined the possibility that RGNNV can infect human cells. We initially tested whether RGNNV could bind to several human cell

lines at RGNNV replication-permissive temperature. RGNNV attached not only to E-11 cells but to each of the human cell lines tested in this experiment. The extent of binding of RGNNV to the cells was essentially the same with the E-11 and human cell lines. Treatment of the cells with neuraminidase before viral infection clearly reduced viral binding to the cells. Therefore, these results clearly suggest that human cell lines can support the binding of the betanodavirus, possibly by sialic acid, as is the case with fish E-11 cells.

Desler et al. reported that viral coat protein expression was detected in HeLa cells when these cells were infected with *Dicentrarchus labrax* encephalitis virus (DIEV) [7], a member of the betanodavirus genus belonging to the RGNNV genotype [15, 30]. However, the data presented by Desler et al. did not confirm that the human cell line HeLa is susceptible to betanodavirus infection, as their results showed only a limited number of cells which were diffusely stained with antibody against the betanodavirus. They reported that HeLa cells infected with the betanodavirus displayed no CPE. They did not analyze the replication form of viral RNAs. It is therefore possible that they detected virus which was bound to the cell membrane but which had not replicated in the cells. It has been reported that (+) RNA1 from the greasy grouper nervous necrosis virus (GGNNV), which belongs to the RGNNV genotype, could replicate and accumulate in HeLa and BSRT7/5 cells when transfected with *in vitro*-transcribed-(+) RNA1 [9]. Our data also confirm the previous observation that human cell lines can support the replication of the betanodavirus when cells are transfected with viral RNAs. In addition, we clearly established for the first time that human cells can produce progeny viruses when transfected with viral RNAs. The cell receptors for the betanodaviruses have not been identified yet, although it has been suggested that sialic acid is involved in RGNNV binding to SSN-1 cells [22]. Our data suggest that human cells may lack specific receptors for the betanodavirus and that the penetration of the virus into the cells did not occur, although human cells can support the replication of the betanodavirus when viral RNAs are used for transfection.

Not all fish cells are susceptible to betanodaviruses infection [4, 11, 17, 26], and the determinants of the host range of betanodavirus infectivity have not yet been clearly identified. It has been demonstrated that the RNA2-encoded coat protein controls host specificity. Indeed, a variable region of RNA2, encoding the C-terminal region of the coat protein, is used to divide betanodaviruses into four genotypes [25, 29, 30]. In addition, the C-terminal sequences of the coat protein are displayed on the surface of the virion [38], suggesting that this region interacts with the cell surface. This is the only viral factor so far identified as a possible determinant of host specificity in the family *Nodaviridae*. Further detailed studies are needed to determine whether human cells lack the specific receptors for the betanodavirus.

It has also been suggested that the replication of the betanodavirus is temperature dependent, because neither viral coat protein nor viral genome replication could be detected in infected cells that were incubated at a non-permissive temperature [5, 6]. The optimal temperature supporting betanodavirus infection differs between genotypes [15]. The RNA-dependent RNA polymerase activity of the alphanodaviruses FHV and NoV [2] was affected less when the assay was conducted at a temperature above 30 °C. However, almost no replication was detected with FHV and NoV when they were incubated at 33.5 °C or 39 °C. In our experiment, no (–) RNA1 synthesis was detected at 37 °C in RGNNV-infected E-11 cells even though this temperature has no effect on the attachment of the virus to the cells (data not shown). Therefore, the betanodavirus in its present form cannot infect human cells maintained at 37 °C, probably due to the absence of receptors for the betanodavirus and the non-permissive temperature for RNA-dependent RNA polymerase.

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