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In vitro propagation of rabies virus in mouse dorsal root ganglia cells

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Abstract

Rabies virus (RV) is highly neurotropic and migrates to the neuronal soma by retrograde axonal transport from nerve terminals, after which it is taken by anterograde axonal transport to be finally released into the central nervous system (CNS) from which it disseminates, resulting in lethal encephalitis. Dorsal root ganglia (DRG) are crucial in the initial events of the infection by RV since they can act as a gate for the viral entrance into the CNS. In the present study, we examined cell tropism of RV and the roles of neuronal cytoskeletal components in the production of viral nucleoprotein (N protein) using cultured nerve cells and non-neuronal cells from DRG of newborn mice. Our *in vitro* study demonstrated a low propagation rate of RV in nerve cells, susceptibility of non-neuronal cells to RV, and independence of cytoplasmic synthesis of viral N protein from the neuronal cytoskeleton. The present study also suggests that Schwann cells should be considered as another possible candidate supporting RV propagation.

Introduction

Rabies virus (RV) belongs to family *Rhabdoviridae,* genus *Lyssavirus*. Rhabdoviruses are enveloped with a typical bullet-or rod-shaped morphology and are characterized by an extremely broad host spectrum ranging from plants to insects and mammals[8,9]. RV is highly neurotropic and migrates to the neuronal soma by retrograde axonal transport from the periphery, and then is taken by anterograde axonal transport to be finally released into the central nervous system (CNS), resulting in lethal encephalitis[8,9,18]. Under natural conditions,

humans and animals may experience long and variable incubation periods following bite exposure. This may play a role in maintaining enzootic rabies, especially in high-density, high-contact populations where there is a tendency for the disease to burn itself out by rapidly reducing the number of susceptible animals. In humans, the incubation period is usually between 20 and 90 days, although it may rarely be as short as a few days or longer than a year[8,9].

Peripheral nerves of both motor and sensory neurons can be used for propagation of rabies virus, although sensory neurons are most probably

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involved in the transfer of the virus from the site of the primary infection to the CNS[10,14,24]. Fibers from neurons of dorsal root ganglia (DRG) are crucial in the initial events of the infection by the rabies virus since they can act as a gate for the viral entrance into the CNS[5,10,19]. In addition, they have been useful in understanding initial virus-host interactions.

In our previous report, we demonstrated that neural spread of pseudorabies virus (PRV) in cultured nerve cells obtained from DRG of newborn mice required the integrity of microtubules and intermediate filaments of the cells, whereas neural spread of influenza A virus was independent of microtubule integrity[15]. In this paper, we examined the cell tropism of RV and the roles of neuronal cytoskeletal components in the production of viral protein using cultured nerve cells and non-neuronal cells from DRG of newborn mice.

Materials and Methods

Rabies virus : The CVS strain (a gift from Dr. N. Minamoto, Gifu University) was used in this experiment. The strain was propagated in clone C 1,300 mouse neuroblastoma (NA) cells.

DRG cell culture : DRG of newborn ICR/Jcl mice (2-4 days old) were dissociated by incubation with 1mg/ml collagenase (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 37℃ for 30 min. Dissociated cells were resuspended at a concentration of 5×10^4 nerve cells (about 100 ganglia) per ml in maintenance medium (MM) comprised of Eagle's minimal essential medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD, U.S.A.), 50 U/ml penicillin (Invitrogen, Carlsbad, CA, U.S.A.) and 50 μg/ml streptomycin (Invitrogen). Collagen-coated 35 mm dishes with central 14 mm glass coverslips (Matsunami Glass, Osaka, Japan) were seeded with 100 μl of the cell suspension. Cells were then incubated in MM with 10 μM 5-fluoro-2'-deoxyuridine (Sigma-Aldrich) and 40 nM 2.5S nerve growth factor (Invi-

trogen) in a 5% CO_2 atmosphere at 37℃. The medium was changed every 2-3 days. After 7-10 days of incubation, when an axonal network of nerve cells had formed, dishes were used for the experiments. In each dish, non-neuronal cells were admixed with nerve cells. All the animal experiments were conducted in accordance with the policies of the Animal Care and Use Committee, Graduate School of Veterinary Medicine, Hokkaido University.

Infection of cultured DRG cells with RV : Cultured DRG cells on the 14 mm coverslip were inoculated with RV at 6.3×10^6 FFU/ml or 6.3×10^5 FFU/ml in 100 μl of MM (126 MOI or 12.6 MOI, respectively) and incubated for 1 hr at 37℃. The viral suspension was removed and cells were washed three times with MM, then incubated in MM for 24, 48 and 72 hr. At least three samples were examined for each time point. Mock-infected DRG cells were used as a negative control.

Cytoskeletal interference and viral propagation in cultured DRG cells : Nocodazole (NOC, Sigma-Aldrich) was used for the disruption of microtubules, acrylamide (ACR, Sigma-Aldrich) for the perturbation of intermediate filaments and cytochalasin D (CYD, Sigma-Aldrich) for the disruption of microfilaments. Fully grown nerve cells with axonal networks were prepared on 14 mm coverslips in 35 mm dishes. The cultured DRG cells were treated with NOC at 1, 5 and 10 μM or ACR at 1, 2 and 3 mM for 23 hr before the infection. In the case of CYD, DRG cells were treated at 1, 2.5 and 5 μM for 2 hr before the infection. After that, the cells were inoculated with RV at 6.3×10^6 FFU/ml or 6.3×10^5 FFU/ml in 100 μl of MM and incubated for1hr at 37℃. The inoculum was then removed by washing with MM three times and MM was applied with each drug. At 2 hr post-inoculation (p.i.), the inoculum of CYD was removed by washing with MM three times and MM was applied without drugs and incubated until 48 hr p.i. At the withdrawal of each drug, cells were washed with MM three times. At 48 hr p.i., in-

fected DRG cells in all dishes were fixed for morphological examination. Stock solutions of NOC (10 mM) and CYD (1 mM) in DMSO and ACR (1 M) in double-distilled water were prepared and diluted to the final concentration in MM. The maximum concentrations of inhibitors were chosen to maximize the drug effects without any apparent cytotoxic effect on cell morphology. CYD concentrations of more than 5 μM caused detachment of cultured DRG cells. The intermediate and minimum concentrations were used to confirm the effects of inhibitors. Controls were treated with an equal volume of DMSO or double-distilled water. At least three dishes were used for each treatment. Student's t-test was used to assess the statistical significance of differences between the groups ($P < 0.01$). Each experiment was performed at least three times.

Immunocytochemical analysis: DRG cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 10 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 5 min. Nonspecific binding of antibodies was blocked by incubation with 2% bovine serum albumin (Sigma-Aldrich) for 30 min. A mouse monoclonal antibody against rabies virus N protein clone 6-4 (a gift from Dr. N. Minamoto) was used for the detection of viral antigens. A rabbit polyclonal anti-tubulin βIII isoform antibody (Abcam, Cambridge, U.K.), rabbit polyclonal anti-pan neurofilament antibody (BIOMOL International, Plymouth Meeting, PA, U.S.A.) and Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR, U.S.A.) were used to visualize microtubules, neurofilaments and microfilaments, respectively. Alexa Fluor 488-conjugated anti-rabbit IgG donkey serum (Molecular Probes) and Alexa Fluor 555-conjugated anti-mouse IgG donkey serum (Molecular Probes) were used as secondary antibodies. Hoechst 33258 (Wako Pure Chemical Industries, Osaka, Japan) was used for nuclear staining. Analyses were performed with an Olympus Fluoview FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan) and Fluoview software version 5.0 (Olympus).

For calculating the infectivity of RV, images of 5 fields were viewed at random, and the number of antigen-positive DRG cells was counted. At least three dishes were used for each treatment. Student's t-test was used to assess the statistical significance of differences between the groups. Each experiment was repeated at least three times.

Results

Infectivity of RV in nerve cells in cultured DRG

To evaluate the propagation of RV in newborn mouse DRG nerve cells, the percentage of antigen-positive nerve cells in the 14 mm coverslip was counted at 0, 24, 48, 72 hr p.i. Nerve cells were always stained with the anti-tubulin βIII antibody in our cultured DRG cells. Viral antigen-positive nerve cells were not detected at 0 hr p.i. in DRG cells infected with 6.3×10^6 FFU/ml of RV (Fig. 1A). At 24 hr p.i., the viral antigens were localized in perikarya of nerve cells (Fig. 1B). At 48 hr p.i., viral antigen was observed in small round accumulations in the cytoplasm (Fig. 1C). From 24 to 72 hr p.i., the number of viral antigen-positive nerve cells slightly increased time dependently (Fig. 2). However, the maximum percentage of antigen-positive nerve cells was about 45% at 72 hr p.i. (Fig. 1D). In DRG cells infected with 6.3×10^5 FFU/ml of RV, the viral antigen-positive nerve cells gradually increased time dependently and about 40% of nerve cells were antigen-positive at 72 hr p.i. (Fig. 2). No severe damage due to virus infection was observed in nerve cells except for mild swelling of cell bodies. Mock-infected nerve cells showed no staining for viral antigens.

Infectivity of RV in non-neuronal cells in cultured DRG

In our cultured DRG cells, non-neuronal cells coexisted with nerve cells. The non-neuronal cells were not stained with the anti-tubulin βIII antibody. The non-neuronal cells were spindle-shaped with fusiform nuclei or irregularly shaped with large flattened nuclei. The non-neuronal cells that
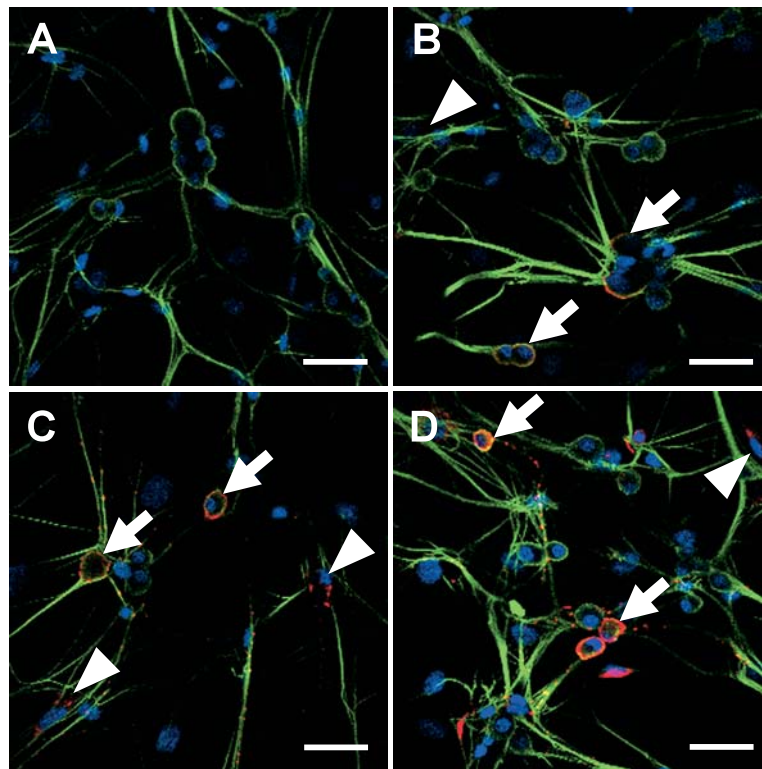
Fig. 1. Confocal images of cultured DRG cells after RV infection at 6.3×10⁶ FFU/ml in 100 μl of MM.
The DRG cells are immunostained for viral antigens (red), tubulin βIII (green) and nuclei (blue). Confocal images of cultured DRG cells at 0 hr p.i. (A), at 24 hr p.i. (B), at 48 hr p.i. (C) and at 72 hr p.i. (D). Nerve cells are immunostained by tubulin βIII and non-neuronal cells are not stained by tubulin βIII. Arrows indicate nerve cells and arrowheads indicate non-neuronal cells. Bars=100 μm.
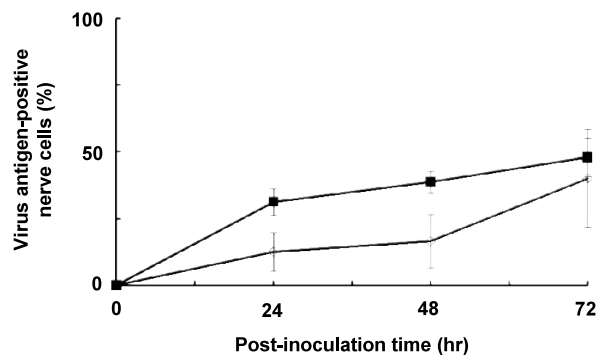

Fig. 2. Percentages of virus antigen-positive nerve cells after RV infection. Closed squares represent antigen-positive nerve cells infected with 6.3×10⁶ FFU/ml of RV and open diamonds those cells infected with 6.3×10⁵ FFU/ml. Each value is expressed as the mean±S.D. (n=3).

coexisted with the nerve cells were also infected with RV (Fig. 1). The percentages of viral antigen-positive cells increased with time. At 72 hr p.i., those of antigen-positive non-neuronal cells reached about 30% of all non-neuronal cells with 6.3×10⁶ FFU/ml inoculation and about 20% with 6.3×10⁵ FFU/ml inoculation (Fig. 3).

Effects of cytoskeletal perturbation on viral N protein synthesis

In the untreated nerve cells confocal images showed granular to fibrillary tubulin, neurofilaments and phalloidin staining. Nerve cells treated with NOC lost normal tubulin characteristics depending on the concentration of the inhibitor, and only a few fine linear microtubules were observed at the maximum concentration (10 μM). The number of viral antigen-positive cells increased signifi-
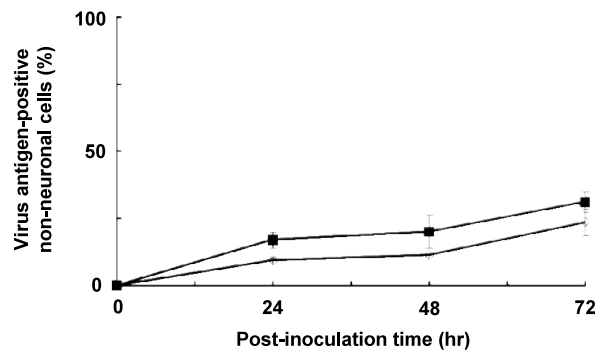
Fig. 3. Percentages of virus antigen-positive non-neuronal cells after RV infection. Closed squares represent antigen-positive non-neuronal cells infected with 6.3×10^6 FFU/ml of RV and open diamonds those cells infected with 6.3×10^5 FFU/ml. Each value is expressed as the mean$\pm$S.D. (n=3).

cantly at the maximum concentration but the increase did not show dose dependency at the low concentrations (Fig. 4A). With ACR treatment, neurofilament protein characteristically accumulated at the axon terminals and this change became evident with increased ACR concentrations. The percentage of antigen-positive nerve cells was not significantly different from the control (Fig. 4 B). The morphological changes of the cells treated with CYD were few, but the percentage of antigen-positive nerve cells was decreased, although there was no significant difference from the control (Fig.4C).

Discussion

The cultured newborn mouse DRG cells used in our study were composed of nerve cells and non-neuronal cells. Our findings were consistent with previous studies on cultured mouse DRG cells[1,7,15,22]. Nerve cells were identified by their characteristic spherical cell bodies, fine axonal projections, small spherical nuclei, and immunohistochemical staining for the neuron-specific tubulin βIII antibody. Non-neuronal cells were composed of spindle-shaped cells with fusiform nuclei and irregularly shaped cells with flattened nuclei. Based on the morphology of these non-neuronal cells, the former

were considered to be Schwann cells and the latter fibroblasts[1,5].

In the present study, the percentages of RV antigen-positive nerve cells were about 15 and 35% at 48 hr p.i., depending on the viral titer in the inoculum, and the maximum percentages were about 40 and 45% at 72 hr p.i. The propagation rate of RV in DRG nerve cells was low compared to those of pseudorabies virus (PRV) and swine hemagglutinating encephalomyelitis virus (HEV), for which the percentages of antigen-positive nerve cells were 100% and about 85% at 48 hr p.i., re-
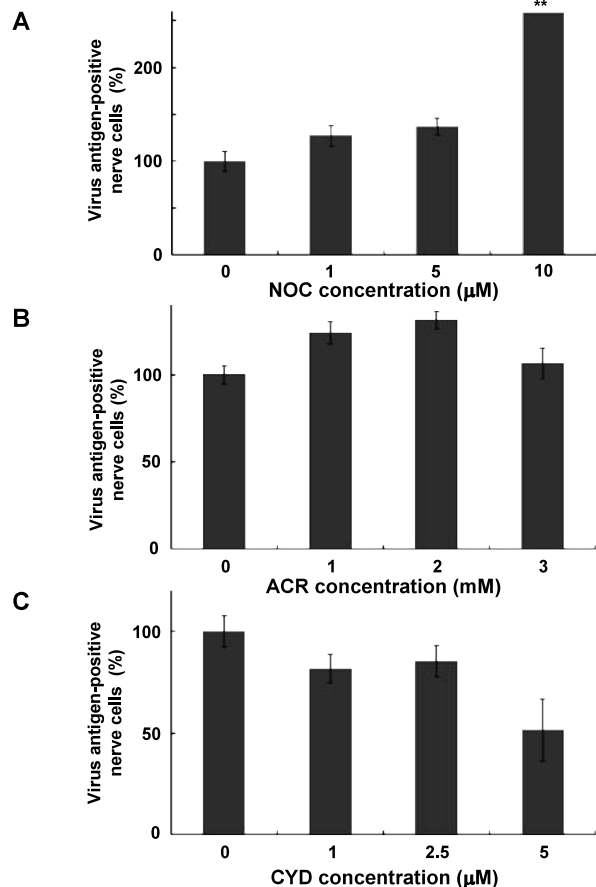

Fig. 4. Effects of cytoskeletal perturbation on viral propagation in cultured nerve cells treated with the cytoskeletal inhibitors indicated at the bottom of each panel. Results are expressed as the percentage of antigen-positive nerve cells for each concentration of the cytoskeletal inhibitors. Values of the control group are adjusted to 100%. Columns and vertical bars show mean$\pm$S.D. (n=3).$^{**}p<0.01$ vs. controls.

spectively (these data will be published elsewhere). The low viral propagation rate of RV was consistent with previous *in vitro* studies[14,16]. In the adult rat DRG, not all the nerve cells were infected, in spite of the fact that the culture conditions employed allowed close contact between the virus and cells[14]. An *in vitro* experiment using embryonic neurons also revealed a low propagation rate for RV[16]. A similar experiment using a compartmentalized cell culture system demonstrated that the viral transport of RV in DRG nerve cells was slow (25 mm/day) compared to herpes simplex virus (50 mm/day)[14,24]. Therefore, the low propagation rate of RV in cultured DRG nerve cells in our experiment might have been due not only to low infectivity but also to slow transport in the cultured nerve cells. These phenomena may be related in part to the long incubation period of the virus in animals and humans.

Non-neuronal cells that coexisted with the nerve cells were also susceptible to RV in this study. The susceptibility of non-neuronal cells has been reported in previous *in vivo* and *in vitro* studies[2,18,20]. Intra-axonal transport by fast axonal flow is a well-documented route of RV propagation from the periphery to the CNS and vice versa[8,23]. The significance of the infection of non-neuronal cells by RV is not fully elucidated, but the present results and a previous *in vitro* study[14,16] may suggest that neural spread via Schwann cells of peripheral nerves should be considered as another possible route for RV propagation as was evidenced for herpes simplex virus[21]. Non-neuronal cells infected with RV may also support entry and replication of the virus[14,18]. Thus, non-neuronal cells may play a substantial role in the development of rabies virus infection.

To understand the relationship between RV propagation and neuronal cytoskeletal components (microtubules, intermediate filaments and microfilaments), cultured nerve cells disrupted selectively for these components with specific inhibitors were exposed to RV. NOC binds the fast-growing ends of microtubules and prevents monomer addition[3,11]. ACR is an intermediate filament

disruption agent[6] and inhibits neurotransmission and membrane turnover in nerve terminals[12,13]. CYD destabilizes microfilaments by binding to the fast-growing ends of the filaments[4]. Previous studies have indicated that RV requires microtubules and actin for intracellular transport and propagation among the nerve cells[3,12,14]. Those studies used polyclonal antibodies for the detection of the viral antigen and a compartmentalized culture system for RV infection via the axonal ends. The inoculated virus titer in the present study was higher than those in a previous study[14] and we used a mouse monoclonal antibody against rabies virus N protein to detect the viral antigens[17]. The signals of N protein produced in the nerve cells were not affected by the disruption of the cytoskeleton. These results may suggest that an intact nerve cell cytoskeleton is required for axonal transport and packaging of proteins and the genome of RV into viral particles, but the whole process of N protein synthesis in the cytoplasm is independent from the cytoskeleton of the host cell.

Acknowledgements

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