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Localizations of NS3 and E proteins in mouse brain infected with mutant strain of Japanese encephalitis virus

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Summary. Infection with a mutant Japanese encephalitis virus (JEV) strain RP-2ms showed reduced neurovirulence than wild type or RP-9 strains after inoculation in BALB/c mice. However, higher intracellular viral titer was detected in Rp-2ms infected cultured cells. Localizations of non-structural 3 (NS3) and envelope (E) proteins were demonstrated by immunocytochemistry. NS3 protein was primarily found in the pyramidal neurons in cerebrum, in the molecular and granular layers of cerebellum. Neither E nor NS3 protein was detected in Purkinje cells of the cerebellum. Immunoelectron microscopic observations showed that E and NS3 proteins were positive in JEV-induced membranous systems, mainly hypertrophic rough endoplasmic reticulum (rER) and membrane vesicle structure (MVS) but not smooth membrane structure. Virus particles were seen in the Golgi apparatus, rER, nuclear envelope, MVS and cytoplasmic vacuoles. Different mechanisms of intracellular trapping in vivo provide a possible basis for attenuation of RP-2ms strains of JEV.

Introduction

Japanese encephalitis virus (JEV) causes major encephalopathogenesis in the host [6, 7, 9, 10, 17]. It has a single stranded positive sense genomic RNA that encodes a single polyprotein. The polyprotein is processed co-translationally and post-translationally to 10 mature viral proteins, i.e., C, prM, E, and seven non-structural proteins in the cytoplasm [20]. The complexes of viral proteins C/prM, prM/E, and E/NS1 are cleaved co-translationally in the lumen of endoplasmic reticulum by the signalase [21]. On the other hand, the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 segments are cleaved in the cytoplasm by the non-structural protein 3 (NS3) [1]. Our unpublished data indicated distributions of E

and NS3 proteins in almost all over the brain. However, the significance of the subcellular localizations of viral proteins during viral morphogenesis or pathogenesis is still poorly understood.

NS3 protein of JEV is one of the major viral proteins that play a vital role during the assembly of flaviviruses [16, 30]. Studies on molecularly expressed protein and sequence analysis, showed that NS3 protein was involved in viral RNA replication [26]. Furthermore, the presence of consensus sequences suggested that NS3 is probably bifunctional, both proteinase activity and nucleotide triphosphatase/helicase activity [23]. NS3 molecules have been found closely associated with nuclear and membranous fractions when analyzed by cellular fractionation methods [25]. Recently, biochemical and immunoelectron microscopic observations indicated that flavivirus-induced intracellular membranes were actively involved in the assembly of viruses [20, 22]. Thus the endoplasmic reticulum and NS3 protein play important roles in the morphogenesis and the processing of viral proteins in JEV infected cells. Yet, the exact locations of NS3 and other viral proteins of flaviviruses are still ambiguous.

Developing attenuated JEV strains or designing a viral protein for potential vaccine candidate is important in flaviviral studies [11, 14, 15]. However, limited information is available for the mechanism of JEV attenuation. The envelope (E) protein of flaviviruses is a glycoprotein containing 500 amino acids [8]. It has been shown that the major determinants for cell tropism and viral entry were carried in the E protein and NS3 proteins of JEV [4, 8, 12, 18]. Furthermore, E protein induces a protective immune response in the host and mediates receptor-specific virus attachment to the target cell surfaces [8, 23]. The E protein is the primary candidate for JEV vaccine development [4]. Our recent data indicated that non-structural protein NS1, could also be a vaccine candidate [15].

Two mutant strains of JEV, neurovirulent RP-9 and attenuated RP-2ms (derived from mutant RP-2 [4, 29]), have been developed in our laboratory by γ-ray irradiation of a local Taiwanese NT109 strain of JEV [3, 4]. Furthermore, a protective immune effect was induced by infection of RP-2ms strain of JEV [14]. Major pathological changes in JEV infected brain occurred in the neurons and the developing neurons [6, 7, 10, 19, 29]. However, the mechanism of encephalopathogenesis of attenuated strain of JEV is still unknown. In the present study, further evidences for higher intracellular titer of RP-2ms strains in the brain were demonstrated. We have characterized the morphogenesis of a less neurovirulent mutant strain of JEV by ultrastructural and immunocytochemical analysis in vivo. The distributions of NS3 and E proteins of attenuated virus strain and the mechanism of viral assembly in the brain could provide basic understandings for vaccine development.

Materials and methods

JEV infection to cultured cell lines

BHK-21 cells (ATCC CCL 10) and NT2 cells [29] were infected with wild type and mutant JEV strains at m.o.i. of 1.0 pfu/cell to examine the infection rates of the cells. JEV mutant

strains RP-9 and RP-2ms were obtained by γ-ray irradiation of a local Taiwanese NT109 JEV strain. Four hours after infection, the culture medium was switched to RPMI medium containing 2% fetal bovine serum. After 24 h, the infected fluid and cells were harvested to determine infection rate by one-step-growth curves of JEV [3, 4, 29]. NT2 cells (2×10^6) were infected with the virus at a m.o.i. of 5 pfu/cell. At the indicated time points after post-infection (p.i.), infected fluid and cell lysates were collected, and their virus titers were determined by plaque assay on BHK-21 cells. The burst size (pfu/cell) of viruses was determined by dividing the total pfu by the number of infected cell [4].

Infection of JEV in BALB/c mice

Suckling BALB/c mice were inoculated with JEV intracerebrally into the right hemisphere. The NT109 strain was isolated from *Cx. tritaeniorhynchus* mosquitoes. Two mutants, RP-9 with high neurovirulence and RP-2ms with low neurovirulence, were selected from NT109 strain by γ-ray irradiation. In RP-2ms strain, No. 43 amino acid in prM protein was mutated from Tyr to His, and No. 138 amino acid in E protein was changed from Glu to Lys [4]. The viral titers calculated as 50% lethal doses were [10^7 pfu for RP-2ms and 0.4 pfu for RP-9] [4]. Five out of seven inoculated animals showed neurological symptoms 5–8 days after inoculation. The non-inoculated left hemisphere or brains from mock-infected mice were used as controls. The mice showing symptoms were anesthetized and perfused with 4% paraformaldehyde in 0.1 M PBS. Brain tissues were further fixed for 3 h in 4% paraformaldehyde and 0.5% glutaraldehyde. The samples were then washed and processed for light and electron microscopy.

Light microscopy and immunohistochemistry

The paraffin sections of brain samples were prepared on slides for immunohistochemistry. The cells grown on round glass cover slips and infected with JEV strains were washed with PBS and fixed in 50% PBS-buffered methanol for 20 min. Each oligoclonal antibody containing four monoclonal antibodies specific to JEV NS3 or E protein was used as primary antibody [3, 4, 29]. After the incubations, peroxidase-or FITC-conjugated rabbit anti-mouse IgG antibody was followed. Two-heavy metal-enhanced peroxidase method (Pierce, USA) was applied for color reaction.

Electron microscopy and immunocytochemistry

The cell pellets from cultured cell samples were collected for pre- and post-embedding immuno-gold stainings [27–29]. For pre-embedding experiments, the samples were fixed, immuno-labeled, dehydrated with graded ethanol and then embedded eponate-12. The brain samples for post-embedding immunoelectron microscopy were fixed and embedded in L.R. White without post-fixation of osmium tetroxide. Thin sections were incubated with mouse anti-NS3 or anti-E oligoclonal antibodies, and then labeled by goat anti-mouse IgG and streptavidin-biotin complex gold-conjugates (ABC-gold) (5 nm in diameter) as our previous works [29].

Results

One-step-growth curves of JEV in neuronal cells

We have previously demonstrated that JEV infection was cytolytic to murine neuroblastoma N18 and human neuronal precursor NT2 cells [4, 13]. In order to further examine whether these cells could support productive replication of JEV,

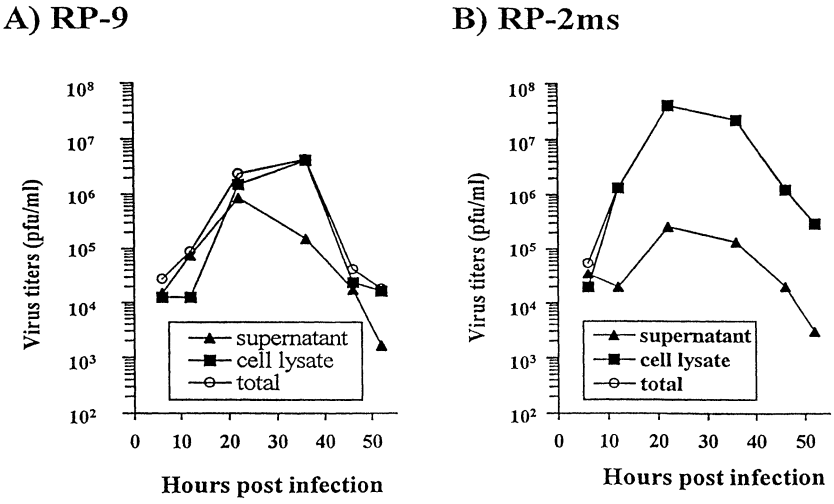
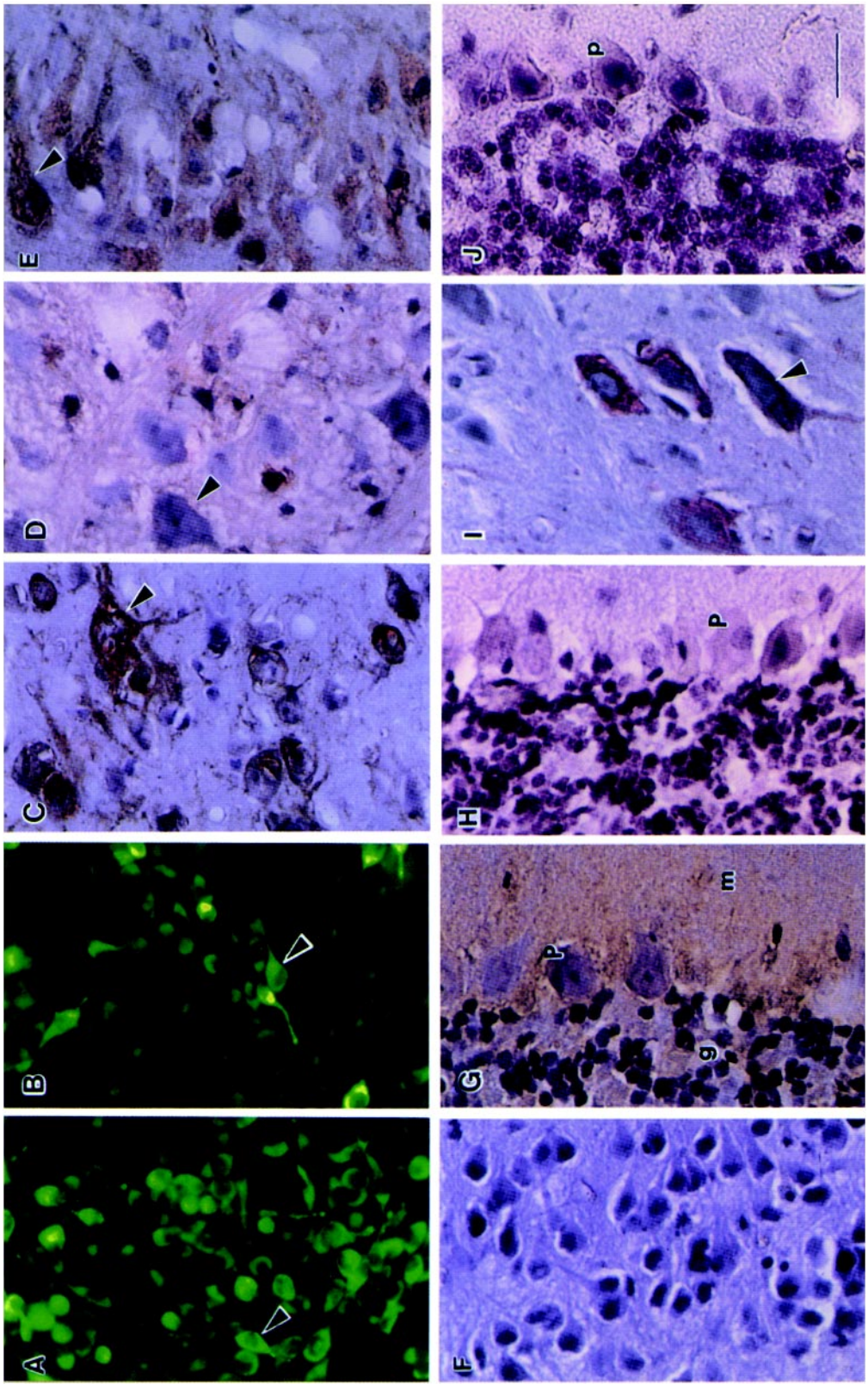


Fig. 1. One step growth curve of JEV, RP-9 (**A**) and RP-2ms (**B**) strains in NT2 cells. NT2 cells were infected with RP-9 and RP-2ms strains at m.o.i. of 5 pfu/cell and cultured for various time periods. The infected culture fluid and cell lysates were collected at indicated time points and the virus titers were determined by plaque assay on BHK-21 cells

both RP-9 and RP-2ms strains were located to NT2 cells at m.o.i. 5, and one-step growth curves were determined. For NT2 cells infected with RP-9, progeny virus was first detected in the supernatants approximately 8 h p.i., and the intracellular virus titer reached its peak at 36-h p.i. nevertheless, declined drastically thereafter (Fig. 1A). At 36 h p.i., the burst size of RP-9-infected NT2 cells was 2.6 pfu/cells for cells infected with RP-2ms, the intracellular virus titer peaked at 20 h p.i. and the burst size was 17.3 pfu/cell (Fig. 1B). In addition, intracellular virions appeared to constitute the majority of both JEV strains during the 50 h infection period. However, only about 1% of total infectious virions was released into the extracellular fraction from RP-2ms-infected cells (Fig. 1B). This observation was consistent with the results from our previous study where attenuated RP-2ms

Fig. 2. Localization of NS3 and E proteins in BHK-21 cells and the neurons of mouse brain infected with JEV. After 24 h infection with JEV RP-9 strain, NS3-immunofluorescence positive BHK-21 cells (arrowheads) (**A**) indicated higher infection rates than those infected with RP-2ms strain (**B**). Neurovirulent RP-9 and attenuated JEV RP-2ms strains of JEV were inoculated intracerebrally in suckling mice. Five to 8 days later, the pyramidal neurons in brain cortex showed higher neurotropism of RP-9 (**C**) than RP-2ms strains (**D**) when detected by anti-NS3-antibodies (arrowhead). NS3 protein-peroxidase deposits were found in the neurons in the hippocampus (**E**). The control samples were negative (**F**). NS3 proteins were distributed in the molecular (*m*) and granular layer (*g*) of the cerebellum, but were not found in Purkinje cells (*P*) after infection with RP-9 strain of JEV (**G**). The control samples (**H**) were negative of NS3 deposits. E protein-peroxidase deposits were detected in the cytoplasm of pyramidal neuronal cells (arrowhead) (**I**). There were no E proteins found in the cerebellum by immunohistochemistry (**J**). Arrowheads: NS3- or E-positive cells; *g* granular layer; *m* molecular layer; *P* Purkinje cells; **A–J** 500 ×, bar: 20 μm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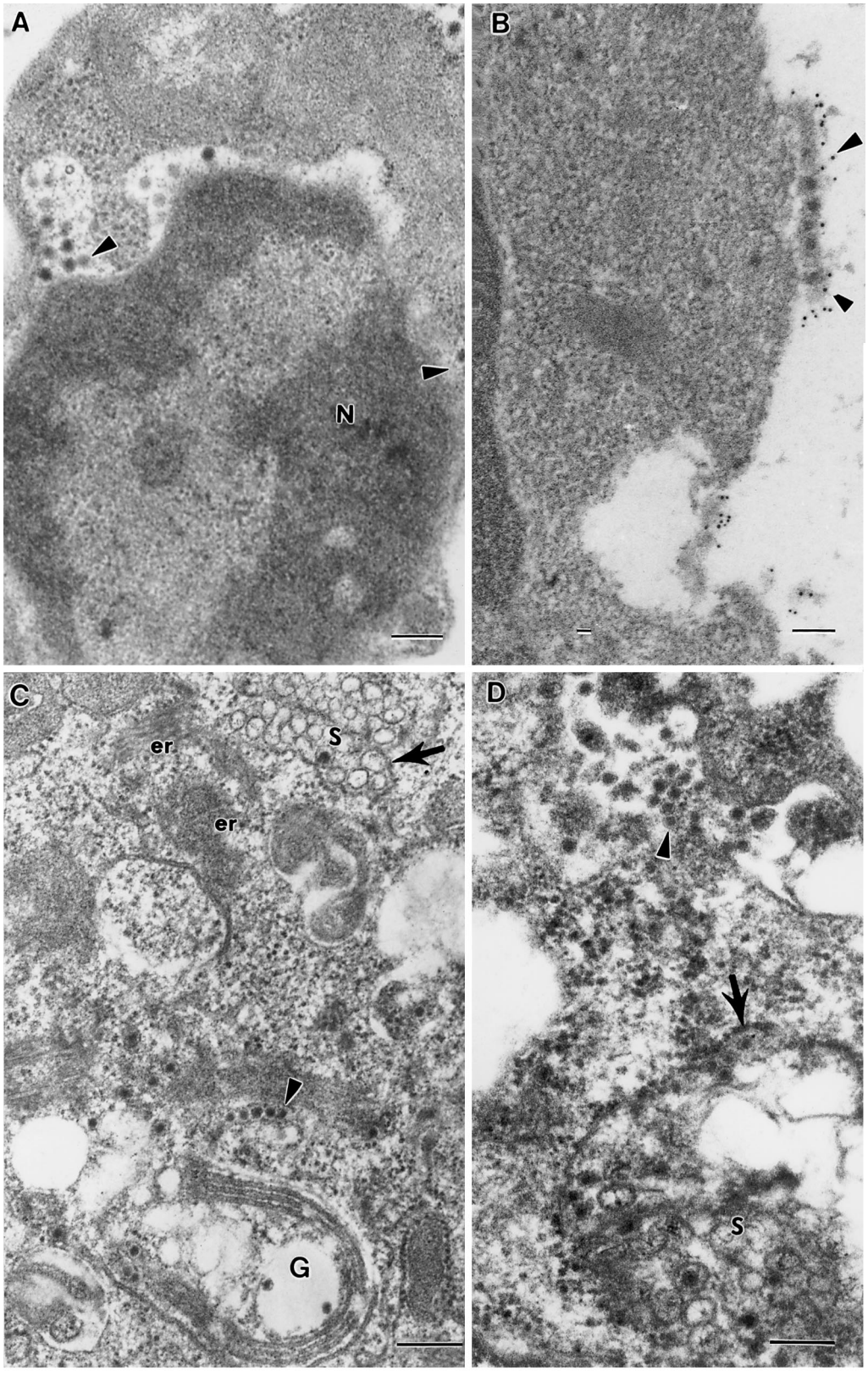
displayed cell-associated properties but could grow to a higher titer than virulent RP-9 in BHK-21 cell [4]. The above data indicate that neuronal cells can support productive replication of JEV in vitro leading to severe cytopathic effects (CPE) of the infected cells.

Neurotropism examined by immunohistochemistry

The RP-9 infected BHK-21 cells (Fig. 2A) showed higher infection rate (3.1 folds) than RP-2ms-infected cells (Fig. 2B) when detected by anti-NS3 antibodies. When the BALB/c mice were inoculated with RP-2ms and RP-9 JEV strains [4], different degrees of symptoms were shown after 5–8 days. Higher infection rates of neuronal cells were demonstrated in RP-9-infected brain cortex (Fig. 2C). NS3 proteins, shown as brown deposits by immunoperoxidase stains, distributed at the perinuclear cytoplasm infected of neurons (Fig. 2C). In contrast, an overall reduced infection of neuronal cell was observed in RP-2ms-infected brains (Fig. 2D). The nuclei were slightly blue after counter-stained by haematoxylin. Positive NS3-staining indicates that JEV had replicated in the neurons of infected brains. Similar results were found in the hippocampus. Strong NS3 protein-positive deposits were seen after infection with RP-9 strains (Fig. 2E). Whereas, weak reactions were found with RP-2ms infections (data not shown). Negative controls did not show staining of viral protein in the non-inoculated left hemisphere; mock infected samples, or samples omitting the primary antibody incubation (Fig. 2F).

NS3 proteins were found in the molecular and granular layers of the cerebellum after infection with RP-9 strain, however, the Purkinje cells (Fig. 2G) as well as the controls (Fig. 2H) were negative for NS3 expressions. When detected with anti-E protein oligoclonal antibodies, RP-2ms strain showed weak reactions while the wild type or RP-9 strains indicated a strong deposits for E-protein reaction in the cerebrum (Fig. 2I). Similarly, E proteins were not present in the Purkinje cells of cerebellum (Fig. 2J). The negative results of NS3 or E proteins in Purkinje cells indicated that cerebrum, but not cerebellum, is the main target for JEV infections.

Fig. 3. Ultrastructure and immunoelectron microscopy of E protein expressed in cultured BHK-21 cells after infection of RP-9 JEV strain. JEV particles (arrowheads) were found in the cisternae of the nuclear envelope, and were found on the cell surfaces after 24 h infection with RP-9 strain (**A**). Viral particles were labeled by anti-E protein antibodies conjugated with 5 nm gold granules (arrowheads) (**B**). JEV particles were found in the Golgi cisternae (*G*), nearby endoplasmic reticulum (arrowhead), and in smooth membrane structure (SMS) (*S*) formed in rER-derived vacuoles (arrow) (**C, D**). Proliferation of membrane systems shown as dense bodies (*er*) in the cytoplasm (**C**). Proliferation of SMS membrane systems in a vacuole derived from rER (arrow) was found in the cytoplasm near the virus particles (arrowhead) (**D**). Arrowheads: virus particles; *N* nucleus; *G* Golgi apparatus; *v* vacuoles; *S* smooth membrane structure; **A**. × 38 000, bar: 0.3 μm; **B** × 77 500, bar: 0.1 μm; **C** × 28 000, bar: 0.3 μm; **D** × 48 800, bar: 0.2 μm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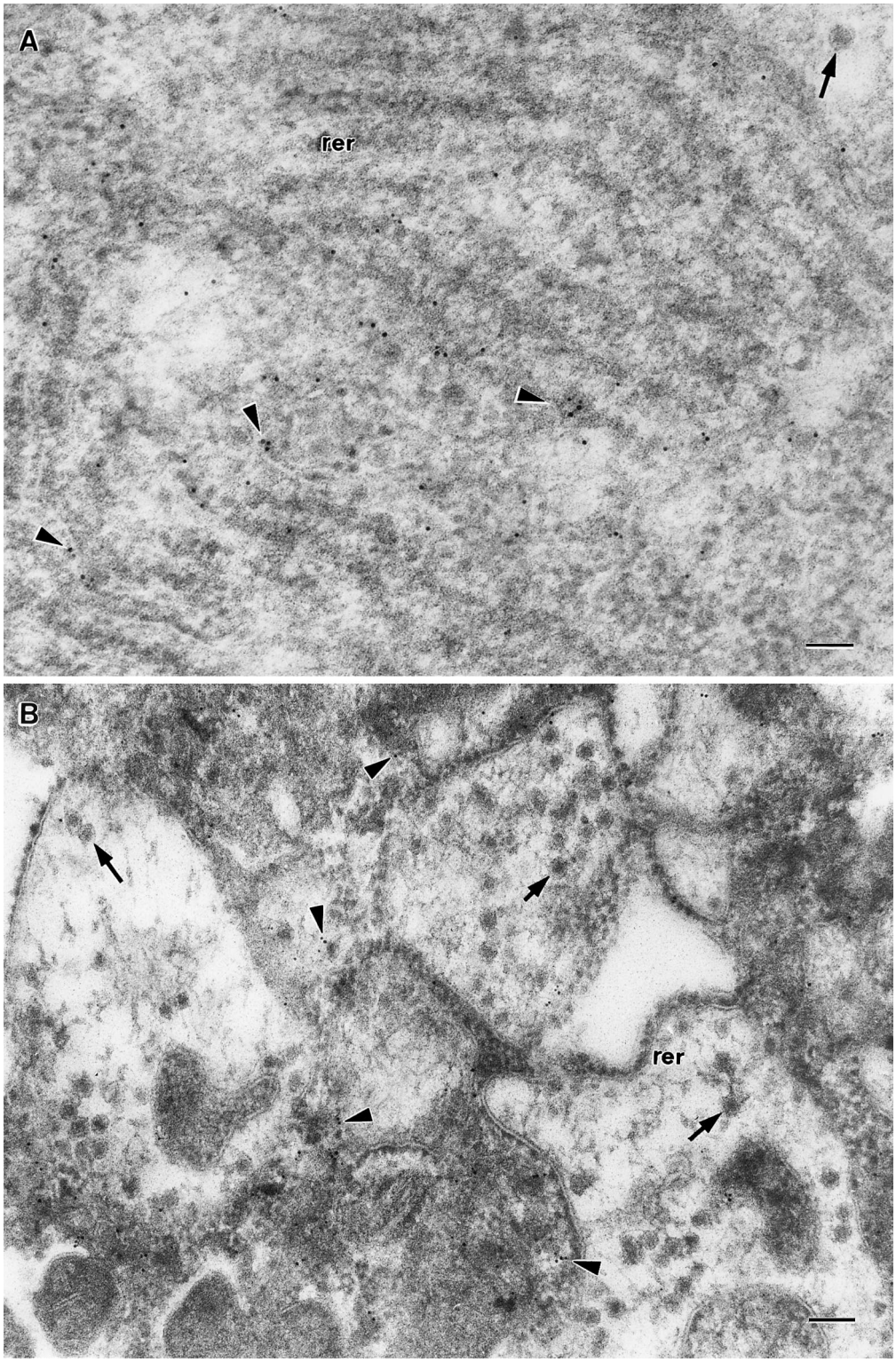
Ultrastructure and immunocytochemistry

The ultrastructural changes induced by JEV infections were consistent with the observation by immunocytochemistry. JEV particles measuring 40–50 nm in diameter were found in the cisternae of nuclear envelope and vacuoles, and on the cell surfaces of a majority of BHK-21 cells (Fig. 3A) infected by RP-9 strain. Anti-E protein antibodies (Fig. 3B) positively labeled the virus particles associated with the cell surfaces. On the contrary, the cell membranes near the virus particles were negative for the E protein staining, similar to the intracellular virus particles also showing weak or negative E protein staining. These results suggest that E protein appeared to have assembled into virions, probably at the late stages of morphogenesis, mainly near the cell membrane, but did not accumulate on the cell surfaces.

Virus particles were observed at the trans-face of Golgi complex and at the ends of Golgi cisternae in most of the cells (Fig. 3C). Vesicles near Golgi vacuoles and smooth membrane structures (SMS) were found in rough endoplasmic reticulum (rER) and in vacuoles possibly derived from rER. Similar large vacuoles containing SMS and dense viral cores surrounded by rER membranes could be found adjacent to the virus particles releasing to the cell surfaces (Fig. 3D). Rows of rough endoplasmic reticulum (rER) were positively labeled with NS3-gold particles at the cytoplasmic side when infected with RP-2ms strain in BHK-21 cells (Fig. 4A). Numerous viral particles were seen in the cisternae of swollen rER. However, these viral particles were negative of NS3 protein (Fig. 4B).

In those positively infected cells, similar ultrastructure and immunocytochemical labeling were found in neurons of both RP-2ms and RP-9-inoculated BALB/c mice (Figs. 5–7). The JEV-induced whirl-like proliferating membranes derived from endoplasmic reticulum (er) were NS3-positive in cells infected with RP-2ms (Fig. 5A) and with RP-9 strains (Fig. 5B). Similarly, swollen rER containing SMS and virus-like particles were observed in both samples (Figs. 5C, 5D). The dense body composed of multivesicles, called membrane vesicle structure (MVS), were positive for NS3 protein (Figs. 6, 7). The increasing SMS vesicles and viral particles were observed among MVS infected with RP-2ms strain (Fig. 6A), and the viral particles and SMS were later found within the rER cisternae (Fig. 6B). The swollen rER of various sizes containing virus-like particles were found quite often in both the RP-2ms and RP-9-infected pyramidal neurons (Figs. 6C, 6D, 7). Some electron-dense filamentous materials, in which virus particles were embedded, were found in the SMS (Figs. 6A, 6C). This structure may represent accumulation of abundant membranous constituents in response

Fig. 4. Immunoelectron microscopy of NS3 protein expressed in cultured BHK-21 cells after infection of RP-2ms JEV strain. Viral particles (arrow) were seen among parallel raw of rough endoplasmic reticulum (*rer*). NS3 molecules were found on the membranes of rER (arrowheads) (**A**). Swollen rER containing viral particles were labeled by NS3-gold on the membranes but not on the viral particles (**B**). Note the ribosomal granules on the rER. **A** $\times$ 78 000, bar: 0.1 μm; **B** $\times$ 77 000; bar: 0.1 μm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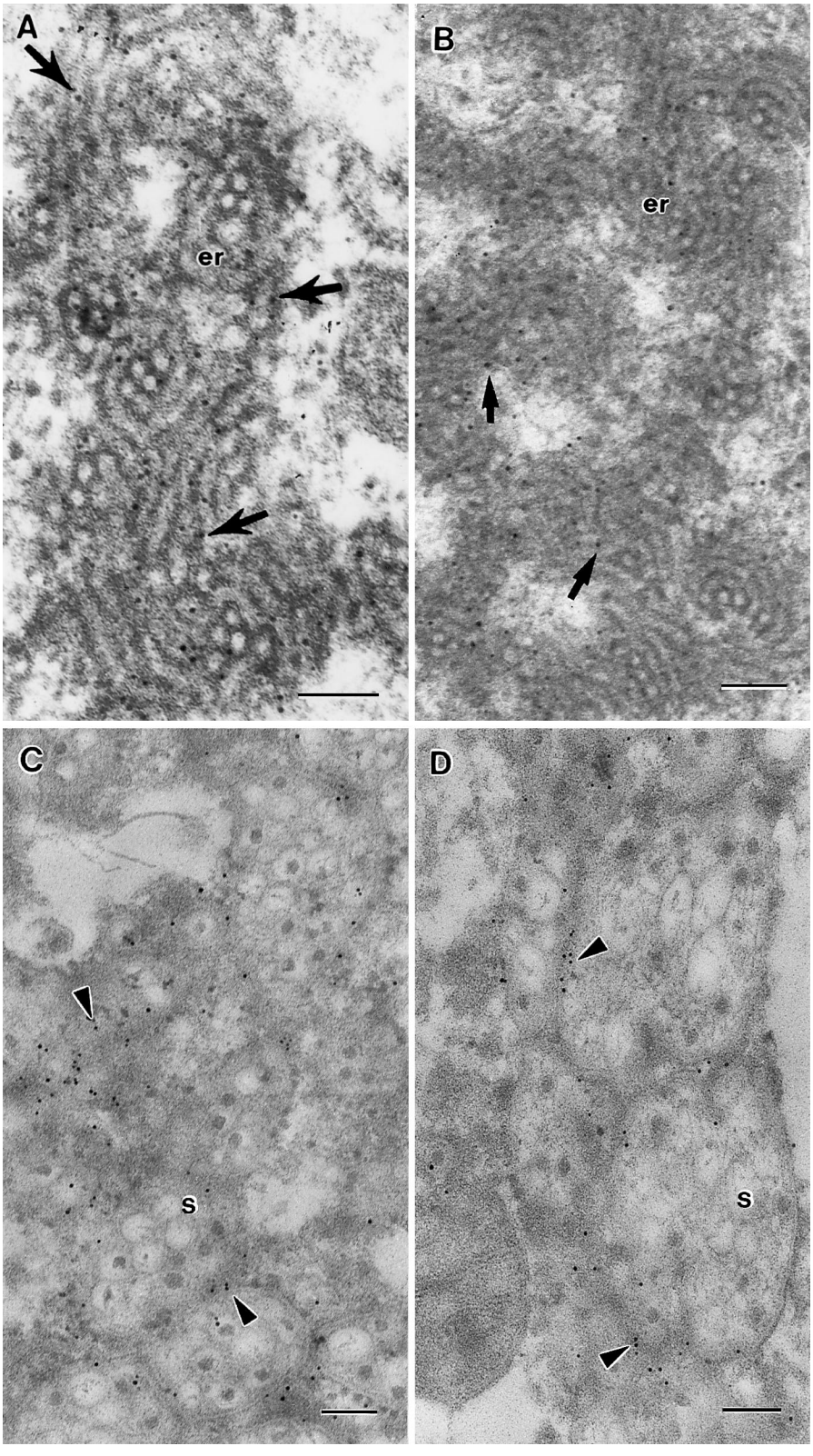
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to JEV infection. The membranes of rER connected with MVS were rich in NS3 proteins (Figs. 6A, 6B, Fig. 7). Dilated rER membranes in associate with the MVS were NS3-positive, and again, SMS as well as virus-like particles, were negative of NS3 labeling (Figs. 6, 7). Taken together, these results suggest that the MVS might be the major site for storage of viral precursors and replication of viral particles.

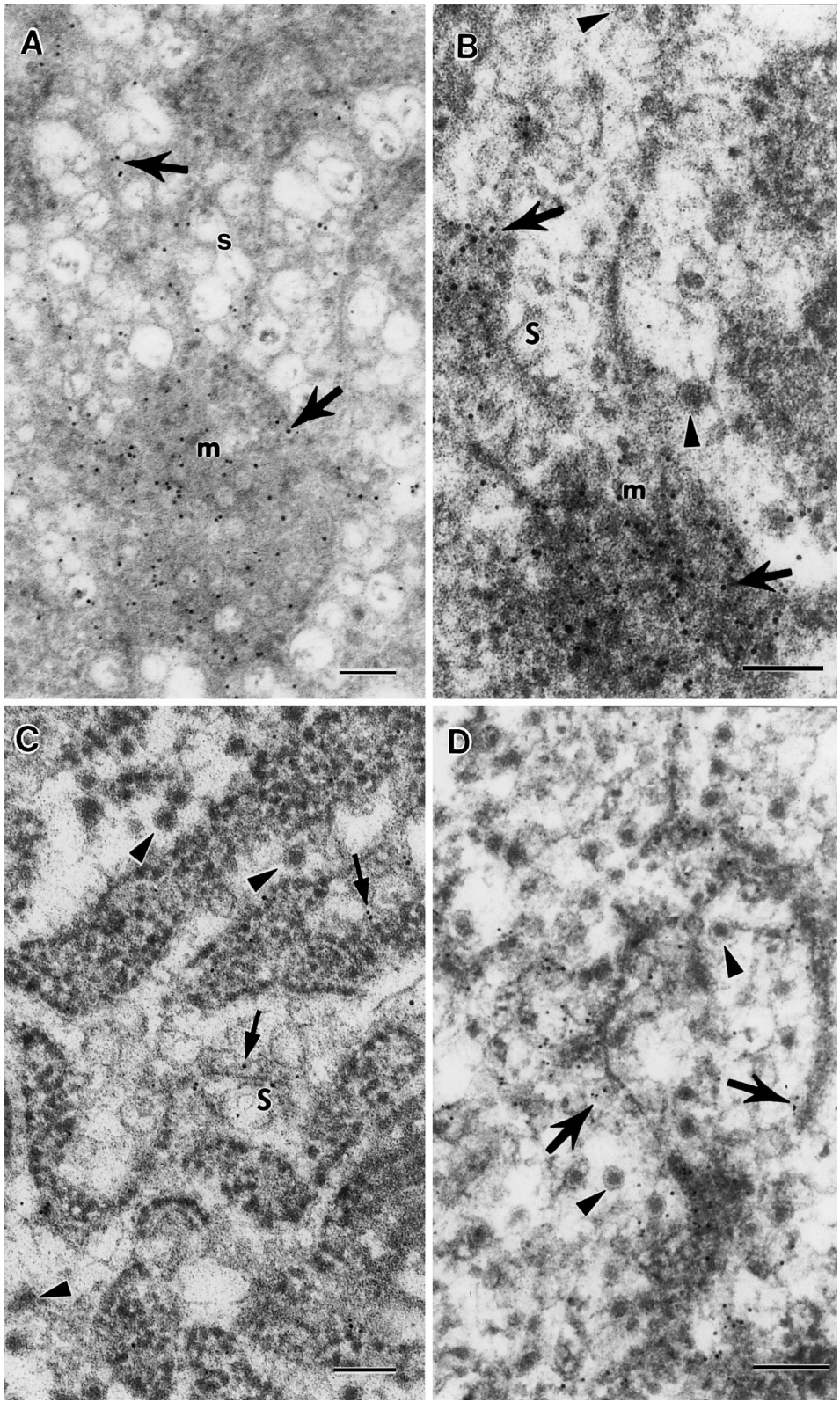
The Golgi apparatus seemed actively involved in the viral proliferation. However, it disappeared when vesiculation or vacuolization of hypertrophic rER occupied the whole cytoplasm of infected cells. MVS was more frequently found among the proliferating membranes in RP-2ms-infected neurons (Fig. 7A) than in RP-9-infected cells (Fig. 7B). Anti-NS3 antibodies were positively labeled on MVS as well as on rER membranes. Although SMS were found within the rER containing filaments and virus-like particles, no NS3 proteins were localized in the SMS in both RP-2ms and RP-9 infected cells (Figs. 7A, 7B).

Discussion

The subcellular localizations of viral proteins in the JEV-induced membranes indicated that most of the proliferated membranes [6, 7, 18, 19] were involved directly in the morphogenesis of JEV particles in neuronal cells. The neurotropism of JEV starts by approach of viral particles to an unknown receptor on the neuronal cell membrane followed by viral entry into the cells. Mutants on the E protein at E/aa 138 from Glu to Lys in RP-2ms strain reduced its neurotropism in cultured neuronal cells for both human and murine models [4, 8, 18, 24, 26]. Envelope protein of attenuated RP-2ms strain of JEV did not bind to a cellular 57 kDa-protein derived from BHK-21 cells [4]. Similar to the previous in vivo results [3, 4], our present data indicated that the infection of RP-2ms strain to neuroblastoma NT2 cell showed higher intracellular titer than virulent RP-9 strain (Fig. 1). However, when detected by immunocytochemistry, less infection rate of the cells was observed by RP-2ms strain in the cultured system [4, 29]. Furthermore, the present study confirmed lower infection rate of RP-2ms strain than RP-9 or wild type strain of JEV in BHK-21 cells as well as in suckling mice brain.

A specific structure, dense body of membrane vesicle structure (MVS), has been identified in cultured neuronal cells but not in BHK-21 cell [29] after JEV infections. MVS was mostly found abundant in cells infected with RP-2ms strain of

Fig. 5. Immunoelectron microscopy of JEV-induced proliferation of membranes in the cerebrum infected by RP-2ms and RP-9 JEV strains. Virus-induced whirl-like membranous structures derived from endoplasmic reticulum (*er*) were frequently found in RP-2ms-infected pyramidal neuron cells. These new membranes were NS3 protein-positive as labeled by colloidal gold particles (arrows) (**A**). Similar whirl structures (*er*) were also found but less frequently in RP-9 strain infected cells (**B**). Viral particles were found in the swollen rER structure, which showed NS3-gold particles on the membrane (arrowheads), in both RP-2ms and RP-9 infected-JEV strains (**C, D**). Structures of SMS (*s*) were seen in the rER. **A, B** $\times$ 112 100, bar: 0.1 μm; **C, D** $\times$ 65 000, bar: 0.1 μm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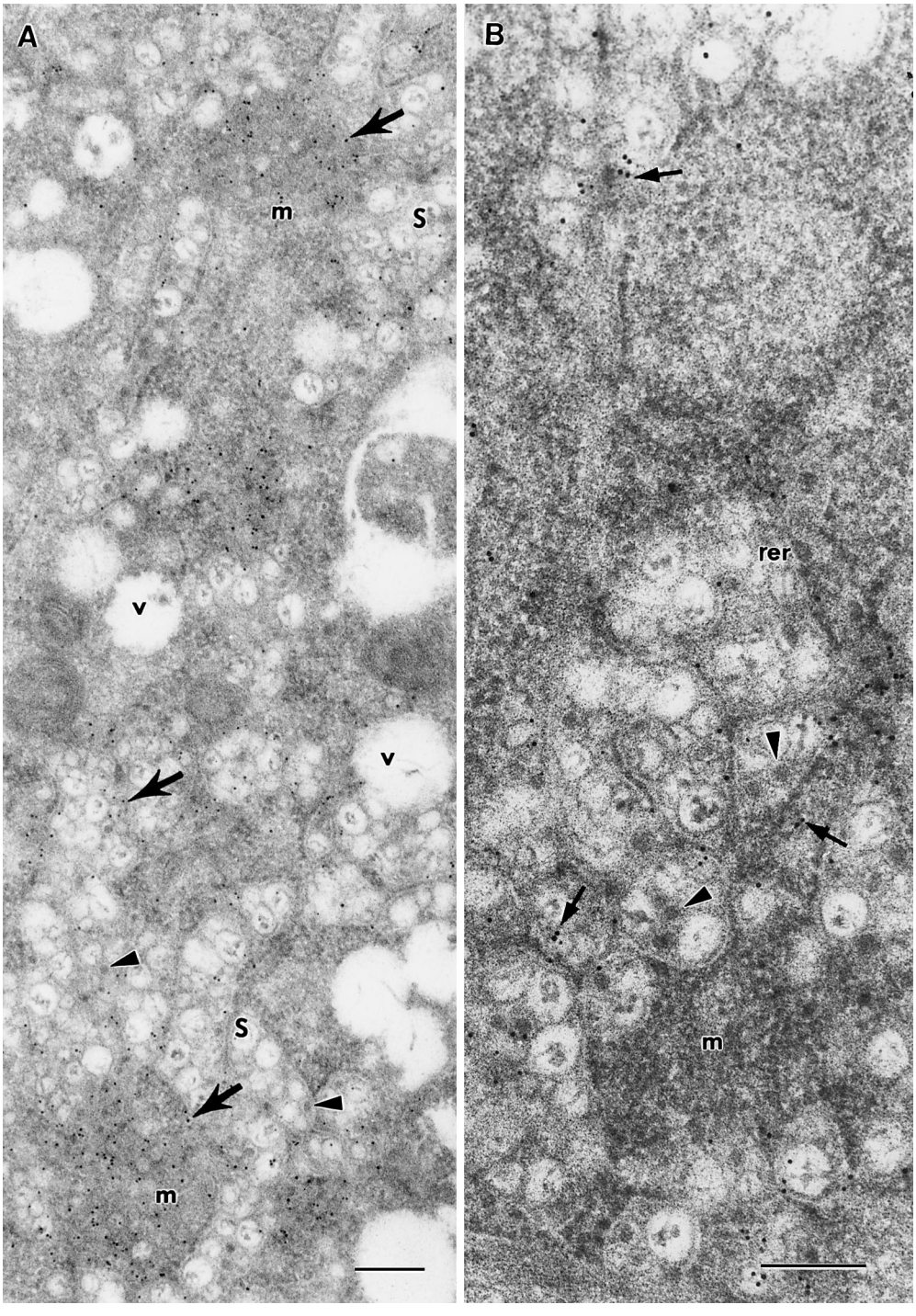
JEV in our previous [29] and present observations. According to our preliminary observations, JEV-induced apoptosis [13] may seldom occur in the mouse brains inoculated by JEV (unpubl. data). Thus, MVS could not be the structure indicating apoptosis. Strong NS3 and E protein-positive reactions in both MVS and proliferated membranes imply that intracellular trapping mechanism may explain that higher intracellular virus titers was obtained with the RP-2ms infection. Although the structure of MVS was also occationally observed in cells infected by RP-9 strain (Fig. 7B), most cases were found in RP-2ms-infected neuronal cells (Fig. 7A). The attenuation in mutant strains may result from the slow entry of virus particles, probably caused by mutated E, PrM or NS3 proteins. Alternatively, the attenuation may also result from the storage or trapping of the viral precursor proteins in MVS, and subsequently delaying the release of viral particles.

Our previous studies have demonstrated that E proteins could be incorporated in the vacuoles of infected C6/36 and Vero cells for infection of dengue type 2-virus [27]. Although JEV particles were present in the cisternae of nuclear envelope of infected cells (Fig. 3A), weak or negative intracellular E protein-labelings would not convince that E protein had been assembled into virus particles during this stage of infection. Alternatively, it is possible that the E protein, which was already in virions, has not matured properly yet to expose the incorrect epitopes, which can be recognized by the MAbs used in the study. Since the positively stained immuno-gold particles on the viral particles attached to the cell surfaces, those E proteins located within the cell should be labeled at the same time as well as that on virus particles on the cell surface. Thus, the weak or negative results obtained in our studies could possibly be true. In addition, the negative E protein labeling on the rER and cell membrane may also suggest that E protein is not expressed or accumulated on the cell surfaces during JEV assembly. Somewhere else, such as the MVS, serves as an important reservoir for E protein accumulation [29], suggesting MVS might be the major place where virus morphogenesis occurs. Nonetheless, it will be interesting to find how and where the E proteins are exactly incorporated into JEV particles.

Fig. 6. Immunoelectron microscopy of JEV-induced proliferation of membranes in the cerebrum infected by RP-2ms strain. Dense MVS bodies (*m*) were NS3-positive as indicated by immuno-gold particles (arrowheads). The SMS (*S*) were free in the cytoplasm and showed NS3-negative (**A**). The NS3-positive (arrows) in the MVS (*m*) showing multivesicular components. MVS was connected to the membranes of rER, which were also NS3-positive. SMS with filamentous component (*S*) were seen within the rER cisternae (**A**). The viral particles in the rER cisternae and close to the NS3-positive (arrows) MVS bodies (*m*), on the contrary, were NS3-negative (**B**). JEV particles (arrowhead) along with SMS (*S*) were found in the swollen rER, which was surrounded by ribosomal granules at the cytoplasmic surfaces. Note the filamentous materials revealed in the SMS (**C**). The viral particles released from the membrane-bound rER were found in the cytosol. Residues of rER membranes with NS3-gold labelings (arrows) were seen in the cytoplasm (**D**). *S* SMS; *m* membrane vesicle structure; arrows: NS3-gold labelings; arrowheads: viral particles; **A** × 63 200, bar: 0.15 μm; **B** × 104 300, bar: 0.1 μm, **C** × 66 500, bar: 0.15 μm; **D** × 68 600, bar: 0.15 μm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