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WEST NILE VIRUS INFECTION OF PRIMARY MOUSE NEURONAL AND NEUROGLIAL CELLS: THE ROLE OF ASTROCYTES IN CHRONIC INFECTION

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Abstract. Primary cultures of embryonic murine neurons and newborn mouse astrocytes were inoculated with West Nile virus (WNV) strain NY385-99 to compare the pathogenesis of WNV infection in these types of CNS cells. Two different outcomes were observed. WNV infection in the neurons was rapidly progressive and destructive; within 5 days, all of the neurons were destroyed through apoptosis. WNV infection in the astrocytes evolved more slowly and did not seem to be highly lethal to the cells. The infected astrocytes continued to produce infectious virus ($10^{4.6}$–$10^{6.5}$ PFU/mL) for 114 days, in a permissive, persistent infection. During this period, WNV antigen could be shown in the cytoplasm of the infected astrocytes by immunocytochemical assay, transmission electron microscopy of ultrathin sections, and in the cell culture medium by complement fixation test. Our results with this *in vitro* experimental murine cell model indicate that astrocytes can develop chronic or persistent infection with WNV, suggesting that these cells may play a role in the maintenance of WNV in the CNS.

INTRODUCTION

In vitro models of viral infection, using primary neuron cultures, can differentiate cell injury caused directly by virus from cell injury caused by the immune response.[1] Primary cultures of embryonic or neonatal mouse and rat neuronal cells have been used as models to study infection with neurotropic viruses such as polio, herpes simplex type 1, Japanese encephalitis, rabies, and Sindbis viruses.[2–7] Other *in vitro* studies have shown that West Nile virus (WNV) can infect neuroblastoma cells, neurons, astrocytes, and oligodendrocytes in culture,[8–11] although *in vivo* animal experiments have only shown WNV infection of neurons.[12–15] Recently, Penn and others[16] described fatal encephalitis in an immunocompromised patient with immunohistochemical and polymerase chain reaction (PCR) evidence of WNV infection of neurons and astrocytes.

Pogodina and others[17] and Xiao and others[12] reported that WNV could be recovered from the brains of rhesus monkeys and hamsters for several months after experimental infection. The persistence of WNV-reactive IgM antibodies in sera of convalescent, confirmed human cases of West Nile encephalitis for 18 months or more after the initial infection suggests that the virus may persist in some humans as well.[18] The mechanism by which WNV can be maintained in the CNS for prolonged periods is unknown. To address this question, we established primary cultures of mouse neurons and astrocytes and infected both cell types with WNV. This paper describes our results, which suggest that astrocytes may play a role in chronic WNV infection of the CNS.

MATERIALS AND METHODS

Virus. The virus used in this study was WNV strain NY385-99, which was originally isolated from a dead bird in New York City during the 1999 West Nile virus outbreak.[12] The virus had two prior passages in Vero cells.

Animals. The animals used to initiate the primary cell cultures were outbreed ICR strain mice, obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the University of Texas Medical Branch. All work with the infected animals was carried out in animal biosafety level 3 facilities.

Preparation of primary mouse neuron and glial cultures. Primary cultures of neurons were prepared from mouse embryos by the modified protocol of Brewer.[19] Briefly, a pregnant (16–18 days gestation) mouse was killed under halothane anesthesia. The uterus with embryos was immediately extracted under sterile conditions and placed in a 100-mm Petri dish with cold, sterile Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY), supplemented with 1.0 mmol/L sodium pyruvate (Sigma, St. Louis, MO) and 10 mmol/L HEPES (Gibco), pH 7.4. Brains were removed from the embryos, and individual cells were isolated by trituration in cold HBSS. After allowing non-dispersed tissue to settle for 3–5 minutes, the supernatant was centrifuged for 5 minutes at 100*g*. The cell pellet was resuspended in neurobasal medium (Gibco), supplemented with 0.5 mmol/L Glutamax (Gibco), 25 μmol/L glutamate (Sigma), and B27 (2 mL of a 50× concentrate; Gibco). The cells were plated onto LABORATORY-TEC chamber slides (Nunc, Naperville, IL) or 12.5-cm^2 plastic flasks (Falcon, Franklin Lakes, NJ) at a concentration of ~2×10^6 cells/mL in neurobasal medium. The slides and culture flasks were treated previously with a 6.25 μg/mL solution (0.16 mL/cm^2 surface area) of cold poly-L-ornithine overnight. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere, and the medium was changed twice weekly.

Primary mixed glial cultures were prepared as described previously,[20] with slight modification. Briefly, brains of anesthetized 2-day-old mouse pups were removed and disrupted by mechanical trituration in sterile Dulbecco phosphate-buffered solution (D-PBS) supplemented with 0.6% D-

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glucose (Sigma). After allowing non-dissociated tissues to settle for 3–5 minutes, the cell supernatant was centrifuged for 5 minutes at 100*g*. The pellet was resuspended in Dulbecco modified Eagle's medium/nutrient mixture F-12 Ham (Sigma), supplemented with 10% fetal bovine serum (FBS), 0.2 mmol/L glutamine (Sigma), 0.6% D-glucose, 3 mmol/L sodium bicarbonate (Sigma), and 0.5 mg/mL penicillin-streptomycin (Gibco), henceforth referred to as "glial medium." The dissociated cells were placed into 12.5- or 75-cm^2 plastic culture flasks at a concentration of ~2×10^6 in glial medium. The culture flasks were treated previously with a 6.25 μg/mL solution (0.16 mL/cm^2 surface area) of cold poly-L-ornithine (MW 30,000–70,000) overnight. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere, and the medium was changed twice weekly. These cultures usually reached confluence within 10–14 days.

Primary astrocyte cultures. The procedures for preparation of primary mouse astrocytes cultures were adapted from a previously described protocol.[21] Briefly, 10–14 days after plating of the glial cells, flasks containing confluent cells were rinsed in glial medium and mechanically shaken at 250 rpm for 18 hours in an incubator at 37°C. After shaking, the supernatant was composed mainly of oligodendrocytes and microglias that had detached during shaking. The glial medium was replaced onto a predominantly astrocyte monolayer. After 3 days, the flask-adherent astrocytes were trypsinized, collected, and plated onto LABORATORY-TEC chamber slides or 12.5-cm^2 flasks at a concentration of ~2×10^6/mL in glial medium. The culture flasks were treated previously with a 6.25 μg/mL solution of cold poly-L-ornithine overnight, as before. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere; and after the first 12 days of cultivation, one half of the medium was changed twice weekly.

Virus infection and titration. Primary cell cultures were inoculated with 0.1 mL of a suspension of WNV strain 385-99 containing ~$10^{3.0}$ plaque forming units (PFUs) of virus. At various times (days) after inoculation, medium (100 μL) from the neuron and astrocyte cell cultures was removed and frozen at −80°C for subsequent titration. The neuron cultures were sampled daily until they showed > 75% cytopathic effect (CPE). The astrocyte cultures were sampled less frequently but were maintained for up to 114 days after infection. For virus titration, serial 10-fold dilutions of the medium from the respective cultures were made from 10^{-1} to 10^{-6} in phosphate-buffered saline (PBS), pH 7.4, containing 25% heat inactivated (56°C for 30 minutes) FBS. Virus titrations were done in 24-well microplate cultures of Vero cells, using 2 wells per dilution, as described before.[22] Plaques were counted 4 days after inoculation; virus titers were calculated as the number of PFU per milliliter of cell culture medium.

Serologic tests. Hemagglutination (HA) and complement fixation (CF) tests were also done on the cell culture medium to detect the level of WNV antigen. CF tests were done by a standard microtechnique,[23] using two full units of guinea pig complement. For the HA tests, the neuron and astrocyte culture fluids were first extracted by the acetone method (unpublished data).

Immunocytochemical analysis. Immunocytochemistry (ICC) was done by the immunoperoxidase system, using a commercial kit (R.T.U. VectaStain; Vector Laboratories, Burlingame, CA). WNV-infected and control cells, grown in 12.5-mL flasks coated with poly-L-ornithine, were fixed with 4% paraformaldehyde in D-PBS for 60 minutes and were permeabilized with 0.5% Triton X-100 for 10 minutes at room temperature. Endogenous peroxidase activity was removed by incubation in 3% H_2O_2 for 30 minutes followed by several washes with D-PBS. Non-specific reactions were blocked for 60 minutes with 10% normal horse serum (Sigma) in D-PBS, followed by incubation with the avidin/biotin blocking system (Vector Laboratories) for 15 minutes. Primary antibody was added and incubated for 60 minutes at room temperature or overnight at ~5°C. The following antibodies were used: anti-WNV monoclonal antibody H5-46 at 1:30 dilution, Sigma anti-neurofilaments at 1:200 dilution, and Sigma anti-glial fibrillary acidic protein (GFAP) at 1:150 dilution. After washing, the cells were incubated with biotinylated secondary antibody, ABC reagent, and peroxidase substrate reagent (VIP or DAB; Vector Laboratories) and were developed according to the manufacturer's instructions. Control experiments, using WNV-infected cells without the primary antibodies, did not show non-specific staining. Immunoperoxidase labeling was examined, using a BX-70 Olympus microscope equipped with a DP-II digital camera (Olympus America, Melville, NY).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end–labeling assay. At various time-points, the cells were fixed in 10% formalin for 24 hours and kept in 75% ethanol at 4°C. The fixed cells were scraped off and diluted in 150 μL PBS after being washed twice in PBS. The cell samples were put onto microscopic slides by cytocentrifugation with the Shandon Cytospin 4 (Thermo Electron Corp., Waltham, MA) at 1,000 rpm for 10 minutes. These slides were dried at room temperature for 30 minutes and post-fixed in precooled ethanol:acetic acid (2:1 vol/vol) for 5 minutes. After two final washes in PBS, excess liquid was carefully removed. An equilibration buffer was added directly to the slides and incubated for 10 seconds at room temperature. After gently tapping off excess liquid and carefully blotting around the section, terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) reaction mixture (Serologicals Corp., Norcross, GA) containing fluorescein isothiocyanate-labeled dUTP and terminal deoxynucleotidyl transferase was added immediately, followed by incubation at 37°C for 1 hour in a moist dark chamber. These cytologic slides were transferred to a coplin jar containing work strength stop/wash buffer and incubated for 10 minutes at room temperature, after it was agitated for 15 seconds. Excess liquid was carefully removed, and mounting medium containing 0.5–1.0 μg/mL propidium iodide was added. The slide was covered with a glass coverslip and viewed under a fluorescent microscope (Olympus BX51), using standard fluorescein excitation and emission filters.

Transmission electron microscopy. Immediately after removal of the medium, cell monolayers (infected and non-infected) were fixed in a mixture of 1.25% formaldehyde and 2.5% glutaraldehyde in 0.05 mol/L cacodylate buffer, pH 7.3, with 0.03% trinitrophenol and 0.03% $CaCl_2$, as described previously.[24] After primary fixation, monolayers were washed in 0.1 mol/L cacodylate buffer. The cells were scraped off the plastic, pelleted by light centrifugation (1000 rpm for 5 minutes) in buffer, and post-fixed in 1% OsO_4 in the same buffer. Cells were stained *en bloc* with 1% uranyl acetate in 0.1 mol/L maleate buffer, pH 5.0, dehydrated in ethanol, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were prepared using a Reichert/Leica Ultracut S ul-

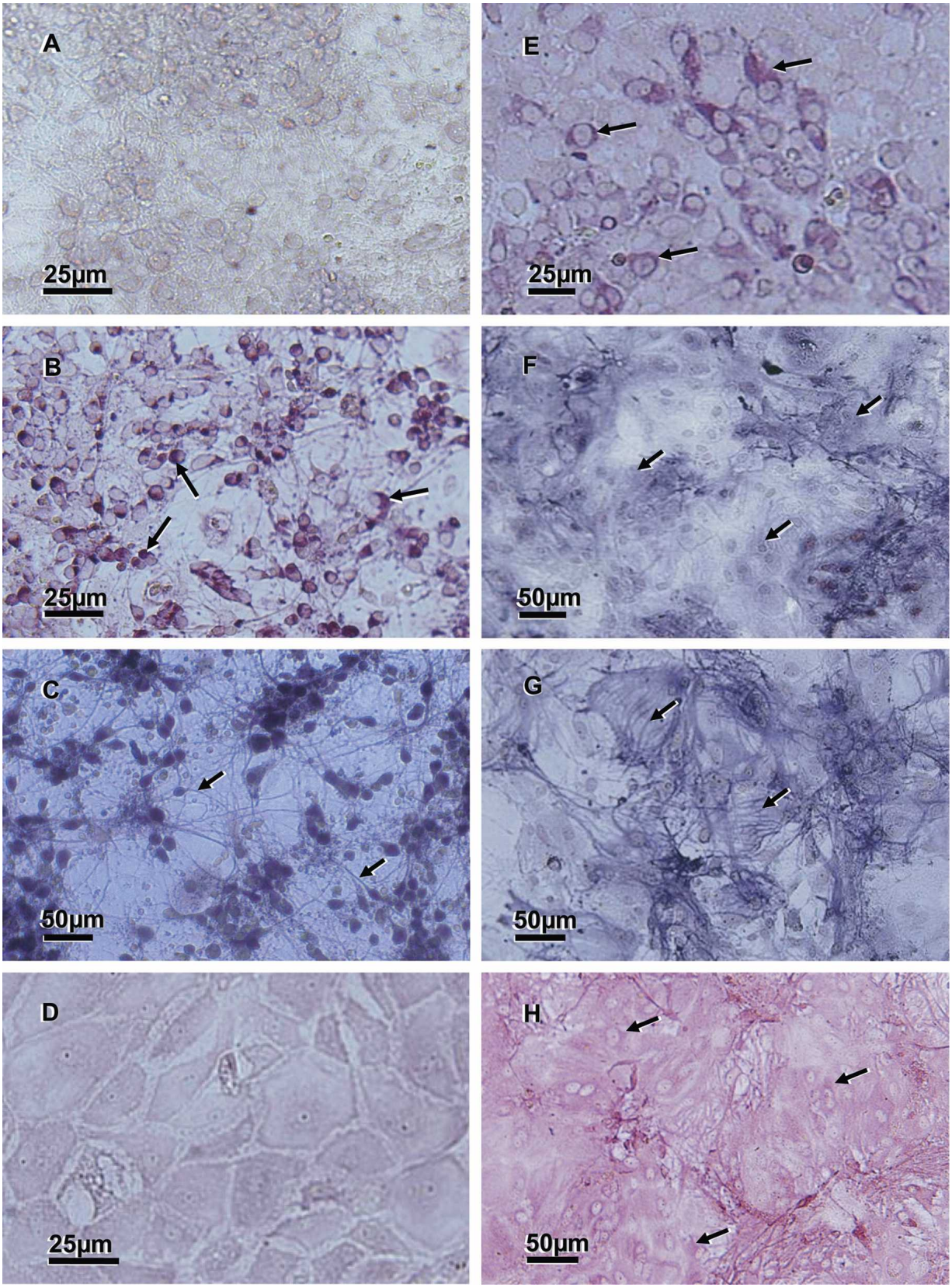

FIGURE 1. Immunocytochemical assays on cultures of mouse embryonic neurons and mouse newborn astrocytes for WNV antigen (red staining/VIP substrate) and cell protein markers (dark-gray/DAB-Nickel). **A,** Uninfected neuron culture. **B,** WNV-infected neurons. Arrows point to cells expressing WNV antigen. **C,** Infected neuron expressing neurofilaments (arrows). **D,** Uninfected astrocyte culture. **E,** Early WNV-infected astrocytes (2 days). Arrows point to cells expressing WNV antigen. **F,** Uninfected; and **G,** Astrocytes 50 days after infection expressing (arrow) GFAP, showing characteristic cellular intermediate filaments. Observe morphology change of infected astrocytes. **H,** Infected cells with typical astrocytes morphology expressing WNV antigens near the cellular nucleus (arrows) 50 days after infection.

tramicrotome (Leica Microsystems, Bannockburn, IL). Sections were stained with 2% aqueous uranyl acetate and 0.4% lead citrate and were examined in a Philips 201 or Philips CM-100 electron microscopes of 60 kV (Philips Electron Optics, Eindhoven, Netherlands).

RESULTS

Neuron cultures. Primary cultures of embryonic neurons infected with WNV began to show CPE within 2 days after inoculation (Figure 1B). The CPE increased in intensity, and by the fifth day after infection (pi), the cell monolayer was completely destroyed. The CPE was characterized by fragmentation of the cytoplasmic axons, cell shrinkage, and features of apoptosis (Figure 2) and detachment of the cell monolayer. HA antigens were detected in the neuron cell culture fluid on the fourth day pi; endpoint antigen titers ranged from 1:4 to 1:64 in different culture flasks. Neuron cell culture medium also gave a positive reaction in CF tests from the second day onward (Table 1). ICC and IFA examinations of the infected neurons were also positive; antigens were detected mainly in the cytoplasm but were also observed in cytoplasmic projections of the infected neurons (Figure 1C).

Maximum WNV titers in culture fluids of the infected neurons were ~10^7 PFU/mL by plaque assay in Vero cells. Electron microscopy of the infected neurons revealed viral particles within vesicles in the cytoplasm and large vacuoles filled with the structures associated with WNV replication (Figure 3A).

Astrocyte cultures. Primary astrocyte cultures also showed CPE; however, morphologic alterations of the cells were not observed until the eighth day pi. Contrary to the CPE observed in the neuron cultures, the progress of viral CPE in the astrocyte cultures was slow and gradually increased until day 30 pi, when it appeared to stabilize. During this period, CF tests done on the spent culture medium gave consistently positive results for WNV antigen (Table 1). Two thirds of medium in the astrocyte cultures was changed once or twice a week, but the infected cells continued to show CPE for at least 114 days pi (Figure 4). Unlike the infected neurons, the infected astrocytes became elongated in shape, with large cytoplasmic prolongations, before death (Figure 1E). In CF

TABLE 1

Presence of WNV antigen, as detected by HA and CF tests, in spent media from primary cultures of mouse neurons and astrocytes after infection with WNV

Culture	Day of infection	HA test result	CF test result
Neurons	2	+	+
	3	+	+
	4	+	+
	5	–	+
Astrocytes	2	–	+
	12	–	+
	30	–	+
	32	–	+
	41	–	+
	64	–	+
	66	–	+
	69	–	+
	75	–	+
	76	–	–
	109	–	–
	111	–	–
	114	–	–

+, WNV antigen detected; –, antigen not detected.

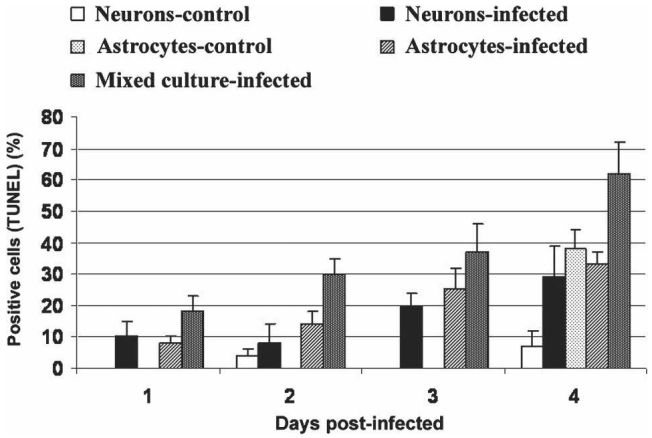

FIGURE 2. Apoptosis of neurons and astrocytes infected with WNV. Neuron control, uninfected neuron culture; neuron-infected, neuron culture infected with WNV; astrocytes control, uninfected astrocyte culture; astrocytes infected, astrocyte culture infected with WNV; mixed culture infected, mixture of neurons and astrocytes infected with WNV.

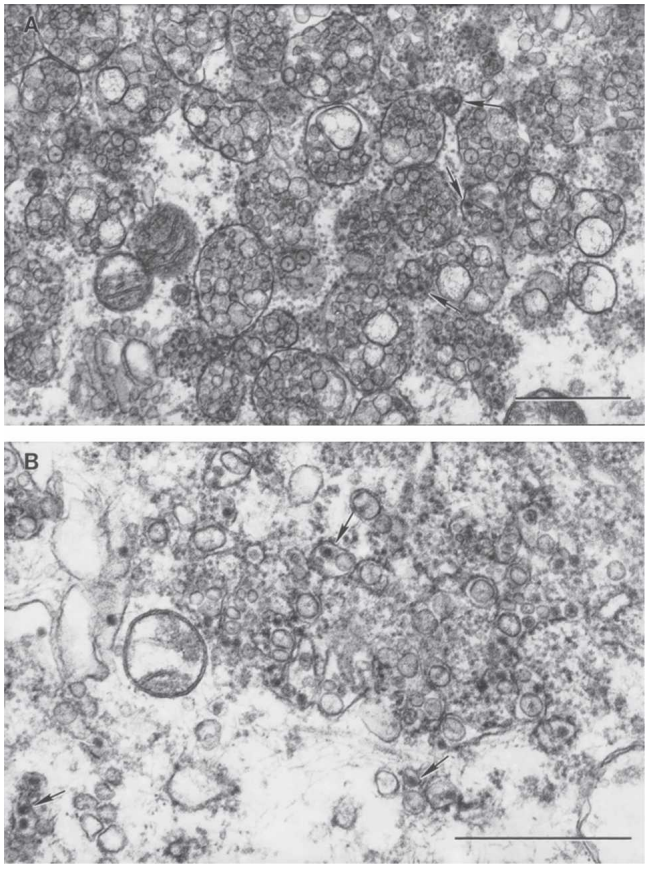

FIGURE 3. Electron micrograph of WNV particles (arrows) and structures associated with WNV replication inside of the cytoplasmic vesicles and vacuoles in cultured neurons (**A**) and astrocytes (**B**). Bars = 0.5 μm.

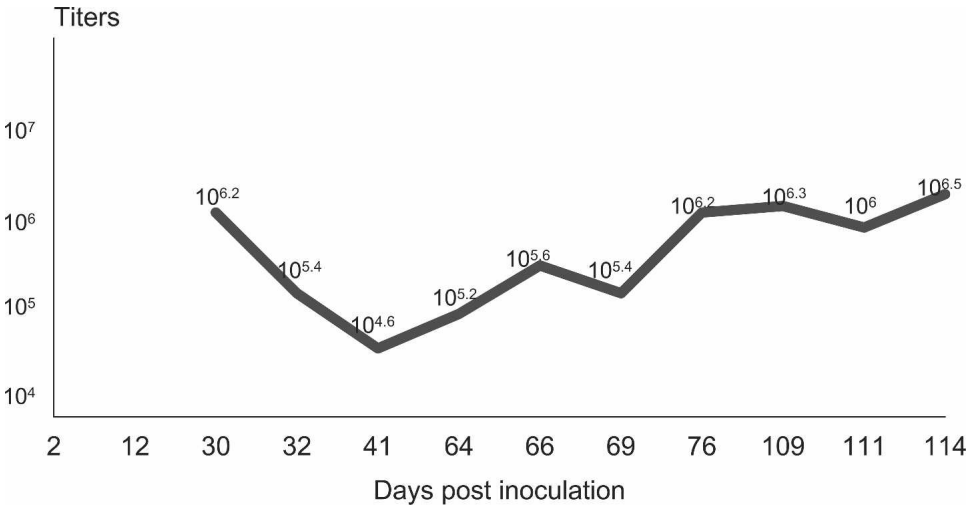

FIGURE 4. WNV titers in culture fluid from infected astrocytes. Samples of fluid were removed from the culture at various intervals (days) after initial virus infection. Virus titers expressed as $\log_{10}$ PFU/mL of medium.

tests, the presence of WNV antigen was detected in the astrocyte cell culture medium for ~75 days pi (Table 1). Interestingly, HA tests on the same samples of culture medium were consistently negative, suggesting that the infected astrocytes expressed less WNV hemagglutinating antigen than the infected neurons. This was confirmed by titrations of culture fluid from the infected astrocytes; WNV titers in the astrocyte culture fluids varied from ~$10^{4.6}$ to $10^{6.5}$ PFU/mL over the 114-day period (Figure 4). ICC assays done on the infected astrocytes were also positive; WNV antigen was found within the cytoplasm of infected cells (Figure 1E). In ultrathin sections of infected astrocyte cultures as well as in neurons, virions were found inside the intracytoplasmic vesicles (Figure 3B). GFAP antibody assays were performed on uninfected and infected astrocyte cultures, and microfilaments expressing GFAP were easily stained, especially in infected astrocytes (Figure 1F and G), confirming that cells in cultures were in fact astrocytes.

Neuronal and astrocytic apoptosis induced by WNV. As shown in Figure 2, a small percentage of the infected cells started to show apoptosis 1 day pi; this became more apparent 2–3 days after infection, particularly in the astrocyte groups and the mixed culture group. By the fourth day pi, nearly 30% of the remaining cells in each cell population exhibited apoptosis by TUNEL stain. Interestingly, on all the days examined, the culture containing a mixture of neurons and glial cells showed a much higher percentage of apoptotic cells, suggesting that there might be a synergic effect in terms of severity of cellular injuries caused by WNV infection, because of some intercellular communication yet to be determined.

DISCUSSION

WNV infects a wide variety of vertebrate and invertebrate cell lines *in vitro*, but relatively little attention has been given to WNV infection of primary cell cultures of the CNS, despite the fact that WNV is a significant cause of encephalitis in humans.[25,26] Relatively few *in vivo* or *in vitro* studies have been published of neuronal infection with WNV.[1,8,9,11,12,16] In these experiments, we used embryonic neurons and astrocytes of newborn mice to study the nature of WNV infection in these CNS cells.

WNV infection in the primary neuron cultures rapidly produced CPE and cell death, indicating that neurons are a target cell for the virus in the CNS. Neuronal cultures infected with WNV began to show signs of viral pathology within 2 days pi, and by the fifth day pi, the entire cell monolayer was destroyed. Acute infection of neurons was accompanied by the release of high titers of virus into the culture medium. By electron microscopy, the neurons showed morphologic changes indicative of cell injury, and by ICC, an increasing number of apoptotic cells were observed as the infection progressed (Figure 2).

In contrast, WNV infection of astrocytes evolved more slowly, and some of the cells showed no evidence of infection. Mild CPE was first observed 8 days pi; it slowly increased until day 30 pi, when it seemed to stabilize. WNV infection of the astrocytes was not completely lethal to the cells, because cells exhibiting CPE were successfully cultured for up to 114 days, at which time the experiments were terminated. During this period, the cells continued to produce infectious virus (Figure 4), and WNV antigen could be detected in the cells by ICC (Figure 1H) and CF assays.

Our results confirm earlier work by Shrestha and others[11]; these authors reported that WNV has the capacity to replicate in several different CNS cells derived from embryonic stem cells. These authors also observed that WNV preferentially infects neurons but that afterward, it infects microglia and astrocytes. Although WNV-infected astrocytes have not been described *in vivo*, our results with primary murine astrocyte cell cultures suggests that these cells could be involved in chronic or persistent WNV infection in the CNS. In contrast to the neurons that were rapidly destroyed by the WNV virus infection, the astrocytes seemed to be more permissive to the infection and persistent WNV infection was established in culture.

Persistent infection of the CNS with WNV has been shown before in experimental animal studies. Xiao and others[12] reported the isolation of WNV from the brains of apparently healthy, convalescent hamsters for up to 53 days after initial

infection with the virus. Pogodina and others[17] reported similar results in convalescent rhesus monkeys. The latter investigators could recover WNV from brains of some monkeys for up to 5.5 months after experimental infection. Pogodina and others[17] also showed that persistent WNV infection in the monkeys resulted in neuron destruction and replacement by glial cells, especially astrocytes. Unfortunately, these authors did not determine whether the astrocytes were also infected with the virus.

It is still uncertain whether astrocytes behave *in vivo,* as shown with our *in vitro* model. However, the aforementioned results indicate that astrocytes can be infected and could be the source of persistent WNV in humans, as have been recently suggested by Penn and others.[16] Indeed, these authors reported the detection of WNV antigens by ICC and viral RNA by RT-PCR in neurons and glial cells of an encephalitis patient who died > 100 days after his initial WNV infection. Unfortunately, the type of glial cells that become infected in the aforementioned case was not described in the article. However, our results tend to support their hypothesis.

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