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Rabies viruses infect primary cultures of murine, feline, and human microglia and astrocytes

Brief Report

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Summary. Recent studies have reported the detection of rabies viral antigens and virions in astrocytes and microglia of rabies-infected animals. As a first step toward understanding whether these glial cells may be involved in rabies virus replication, persistence, and/or pathogenesis, we explored their potential to be infected in vitro. Primary cultures of murine, feline, and human microglia and astrocytes were infected with several different rabies viruses: two unpassaged street virus isolates, a cell culture-adapted strain, and a mouse brain-passaged strain. Infection, as determined by immunofluorescence, was detected in 15 of the 16 (94%) virus-glial cell combinations. Replication of infectious virus, determined by infectivity assay, was detected in 7 of the 8 (88%) virus-cell combinations. These results show that astrocytes and microglia can be infected by rabies viruses, suggesting that they may have a potential role in disease, perhaps contributing to viral spread, persistence and/or neuronal dysfunction.

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Rabies viruses have been well documented to primarily infect neurons [19, 30, 33]. In addition, a number of studies have reported rabies viral antigens and virions in human [1, 13, 29] and murine astrocytes [10, 18, 23], and in murine ramified microglial cells [27], begging the question of the role of these cells in viral replication and persistence, as well as pathogenesis. Schneider [23]

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reported that glial cells of the pia mater appear to be involved in replication, and Matsumoto [18] indicated that astrocytes support virus replication in vivo. Tsiang also detected a minimal amount of virus when he infected a cloned mouse glioma cell line, but he did not detect virus when he attempted to infect primary glial cell cultures composed primarily of astrocytes [30]. Thus, it has not been clearly established whether rabies viruses productively infect microglia and astrocytes, leaving unanswered the question of whether these cells are susceptible to infection or simply absorb or phagocytose virus or cellular debris from infected neurons.

Evidence suggests that macrophages, which are related to microglial cells, and astrocytes play a critical role in many viral infections by supporting viral replication and sequestering viruses [2, 5, 6, 9, 11]. We have previously reported evidence for rabies virus replication and persistence in murine bone marrow-derived macrophages and in human macrophage-like cells [22]. It also has been shown that microglial cells and astrocytes infected by viruses, including vesicular stomatitis virus (VSV), may be involved in the disease process via secretion of cytokines and neurotoxins [3, 14, 32, 34], including nitric oxide (NO) [28]. In the present study, as an initial step toward evaluation of the potential involvement of these glial cells in rabies virus infections, we have directly examined the ability of different rabies virus strains and isolates to infect and replicate in primary cultures of microglia and astrocytes.

Four rabies viruses were used: 1) The Evelyn-Rokitnicki-Abelseth (ERA) strain, which had been adapted to tissue culture through multiple serial passages in CER cells [15]; 2) a street isolate (SRV) of bat origin [16] that had been serially passaged six times intracranially (ic) in mice; 3) an unpassaged isolate from a sheep brain; and 4) an unpassaged isolate from a skunk brain.

Murine, feline, and human glial cultures were used to examine the infection of glial cells from different species. Murine mixed glial cell cultures were prepared from brains of neonatal IRW mice by the method of Giulian and Baker [7] and incubated in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah) (RPMI-10), as previously described [17]. Microglia that were shaken off mixed glial cultures were incubated in a 50:50 mixture of RPMI-10 medium and conditioned RPMI-10 medium that had been harvested from one week old mixed glial cultures. Feline and human glial cultures were prepared in the same way with the exception that the cells were incubated in Dulbecco's MEM medium supplemented with 20% fetal calf serum. Mixed glial cultures were prepared from neonatal and adult cat brains, and human brain tissue derived from 17 week-old fetuses or adults during operative procedures for deep CNS lesions requiring the removal of healthy tissues [21]. Human fetal astrocyte cell cultures were prepared from mixed glial cultures as described [20].

Microglia and astrocytes were identified with specific reagents. Microglia were identified by their ability to endocytose fluorescent-labeled DiI-AcLDL [7] as well as by immunofluorescent staining with specific reagents including rat-anti-Mac-1 antibody for murine cells [22], rabbit anti-human ferritin

antibody for feline microglia [4], and anti-human macrophage antibody CD68 [8] for human microglia. Murine microglia were also identified by non-specific esterase staining and a phagocytosis assay [22]. Astrocytes of the three species were identified with rabbit anti-bovine glial fibrillary acidic protein (GFAP) antibody [4]. All glial cultures were free of fibroblasts as determined by staining with anti-human fibronectin antibody [20]. Rabies virus-infected cells were identified by double immunofluorescent labeling as previously described [22]. Because infected cells did not deteriorate during the time course of these experiments, microglia containing viral antigens were considered to be infected and not to have phagocytosed and sequestered infected cell debris.

We first examined the ability of rabies viruses to infect and replicate in neonatal murine glial cultures that were greater than 95% microglia. Infection of cells with either the tissue culture-adapted ERA strain, or mouse brain-adapted virus resulted in substantial virus replication, evident by 48 h postinfection and increasing in titer throughout the course of the study (up to 192 h) (Fig. 1A). The infected cells remained phagocytic, but appeared to phagocytose fewer Fluorescebrite (Polysciences, Inc., Warrington, PA) beads per cell than uninfected cells (Fig. 2A). Infected cells also were detected by double immunofluorescent staining (Fig. 2B and 2C). Unpassaged street viruses also infected murine microglia (Table 1). In addition, double immunofluorescent staining revealed that the ERA strain infected astrocytes present in the mixed glial cultures (Table 1 and Fig. 3).

We next examined the ability of rabies viruses to infect and replicate in feline glial cells. Both the neonatal and adult cultures were greater than 80%

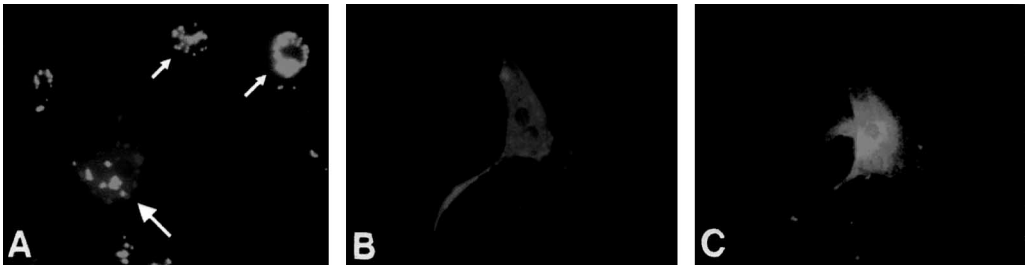

Fig. 1. ERA strain and mouse brain-passaged street rabies virus (SRV) replication in murine, feline, and human glial cells. **A** Murine neonatal microglial cells infected with ERA or SRV. Virus titers were compared with the half-life of ERA and SRV in media alone. **B** Feline adult microglia infected with ERA or SRV, and neonatal microglial cells infected with SRV. **C** Human adult microglial cells infected with ERA or SRV, and fetal astrocytes infected with ERA. Replication data represents the geometric mean of two independent experiments, excluding a single experiment for SRV replication in adult human microglial cells. Cells were infected at a multiplicity of infection of 0.1. Murine experiments were done in 6-well, 35 mm plates (Corning, Corning, NY). At various timepoints, 0.2 ml of media representing 10% of the culture volume was harvested and 0.2 ml of fresh RPMI-10 was added to the cultures. Experiments with feline and human cells were done in Linbro TC-24 plates (Flow Laboratories, MacLean, VA): entire cultures were harvested. Infectious virus was assayed on CER cells using a fluorescent focus assay [24]

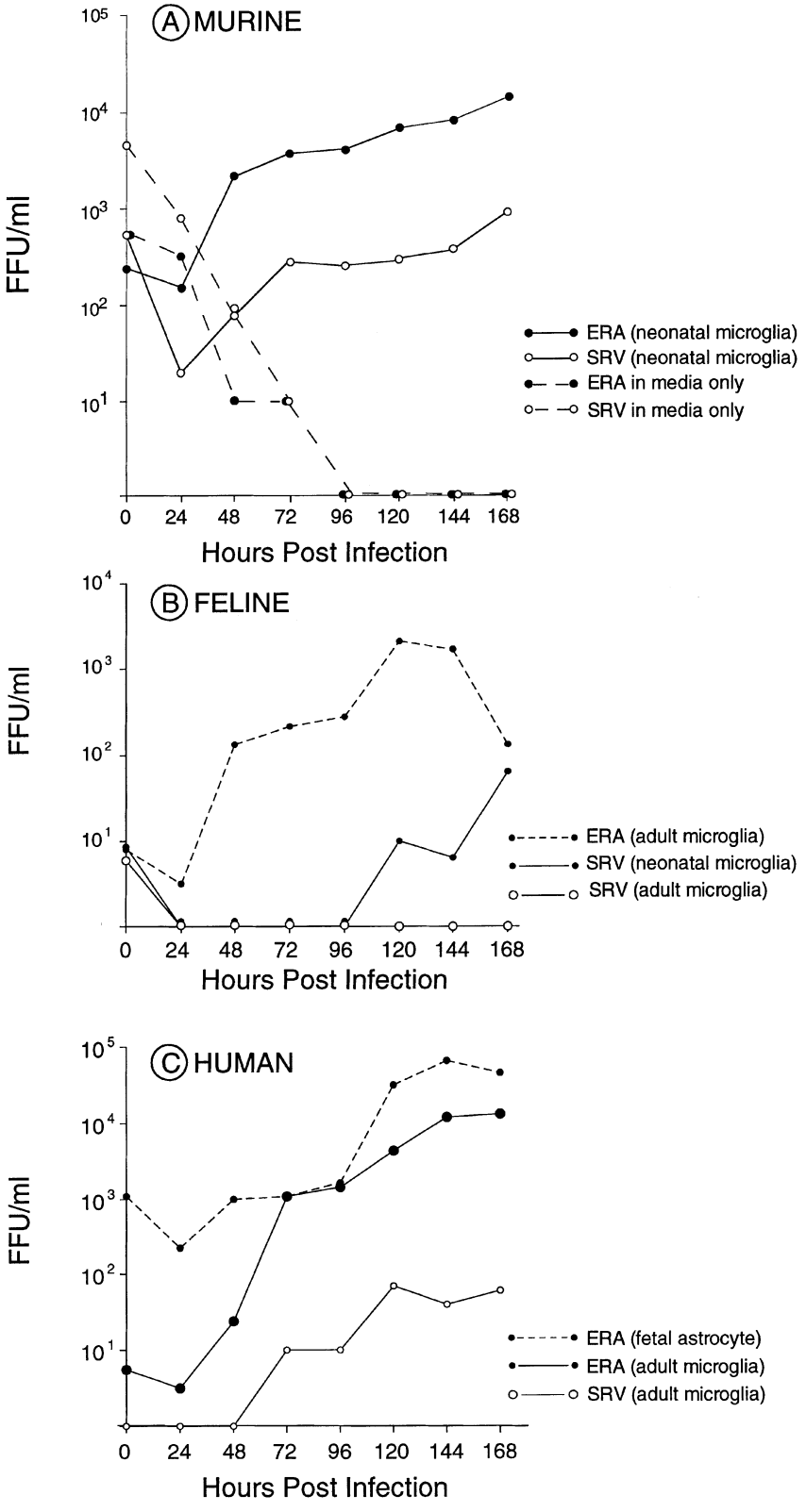
A MURINE
FFU/ml
10^5
10^4
10^3
10^2
10^1
0 24 48 72 96 120 144 168
Hours Post Infection
ERA (neonatal microglia)
SRV (neonatal microglia)
ERA in media only
SRV in media only
B FELINE
FFU/ml
10^4
10^3
10^2
10^1
0 24 48 72 96 120 144 168
Hours Post Infection
ERA (adult microglia)
SRV (neonatal microglia)
SRV (adult microglia)
C HUMAN
FFU/ml
10^5
10^4
10^3
10^2
10^1
0 24 48 72 96 120 144 168
Hours Post Infection
ERA (fetal astrocyte)
ERA (adult microglia)
SRV (adult microglia)

Table 1. Infection of murine, feline and human glial cells with rabies viruses[a]

SPECIES	PRIMARY GLIAL CELL	VIRUSES			
		Tissue culture adapted ERA	Mouse adapted bat SRV	UNPASSAGED BRAIN HOMOGENATE	
				Skunk	Sheep
MURINE	NEONATAL ASTROCYTE	+			
	NEONATAL MICROGLIA	⊕	⊕	+	+
FELINE	ADULT ASTROCYTE	+			
	ADULT MICROGLIA	⊕	−		
	NEONATAL MICROGLIA		⊕		
HUMAN	FETAL ASTROCYTE	⊕	+	+	+
	ADULT ASTROCYTE	+			
	ADULT MICROGLIA	⊕	⊕		

[a] + Detection of rabies virus fluorescent antigens in double fluorescent stained cells
⊕ Replication of infectious virus
− No infection
Experiment not done

microglia. The ERA strain of virus replicated well in adult microglia, with a greater than 100-fold increase in virus titer between 24 and 120 h. The mouse brain-passaged isolate replicated in neonatal microglia (greater than 10-fold increase in titer), but replication was not detected until 120 h after infection. In contrast, this isolate did not infect adult feline microglia (Fig. 1B), a situation that may have been due to the state of activation and/or differentiation of the cells [22]. Using double immunofluorescent staining, it also was determined that the ERA strain infected the few astrocytes that were present in the microglia cultures (Table 1).

Fig. 2. Identification of infected murine microglial cells by phagocytosis and double immunofluorescent labeling 72 h postinfection with ERA strain virus. **A** Cells were incubated with Fluorescebrite beads for 1 h at 37 °C followed by extensive washing, fixation, and immunofluorescent staining with anti-rabies antibody. The large arrow indicates an infected cell; smaller arrows indicate uninfected cells. **B** Anti-rabies antibody, **C** anti-Mac-1 antibody double immunofluorescent staining of an infected microglial cell

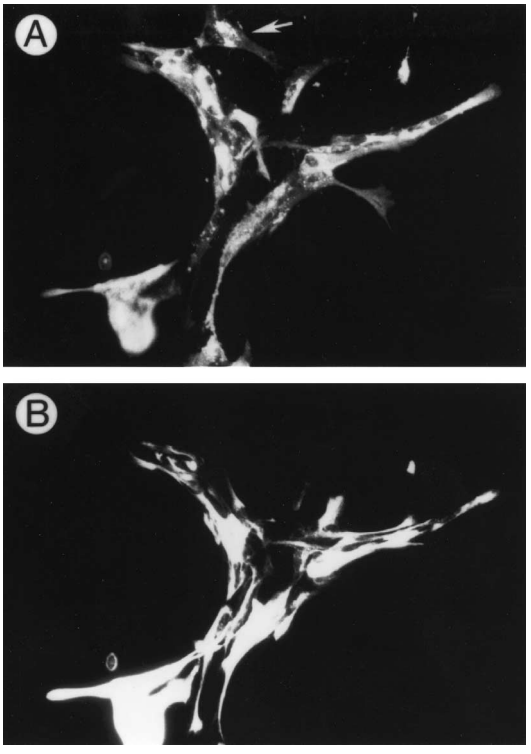

Fig. 3. 72 h postinfection double immunofluorescent labeling with anti-rabies virus (**A**) and anti-GFAP (**B**) antibodies of ERA strain virus-infected murine astrocytes. The arrow identifies a cell that was infected, but did not stain with GFAP

Lastly, we examined the ability of the viruses to infect and replicate in cultures of human glial cells. The ERA strain and the mouse-passaged virus replicated well in adult glial cultures that were greater than 80% microglia, with a greater than 1000-fold and a greater than 10-fold increase in titer, respectively, between 24 and 168 h postinfection (Fig. 1C). The ERA strain also replicated well in fetal glial cultures that were greater than 98% astrocytes, with a greater than 100-fold increase in viral titer between 24 and 144 h postinfection (Fig. 1C). Double immunofluorescent staining revealed that unpassaged street virus isolates and the mouse passage isolate also infected the human fetal astrocytes (Table 1).

In summary, primary glial cell cultures from three different species were infected with several different rabies virus strains and isolates in 15 of the 16 (94%) virus-glial cell combinations tested. In addition, productive viral replication was detected in glial cells of all three species in 7 of the 8 (88%) combinations tested. The fact that mouse-passaged and unpassaged street viruses infected and replicated in these glial cells suggests that this was not a virus-cell passage-adaptation laboratory phenomenon. Furthermore, the viral replication in primary microglia and astrocytes suggests that the previous detection of rabies viral antigens and virions within these cells may have been infectious virus rather than phagocytosed debris or absorbed virus from infected neurons [27]. Natural anatomical relations and cellular interactions within the central nervous system are known to be important for rabies virus infection of

glial cells in vivo [1], possibly making infection of relatively pure cultures of primary glial cells difficult. This situation may explain why others failed to detect rabies challenge virus strain (CVS) antigens in cultures of primary neonatal murine astrocytes 18 to 72 h postinoculation [30].

We can only hypothesize on the role of glial cells in rabies virus infections. Our data suggest that microglial cells and astrocytes support viral replication independent of neuronal infection, possibly contributing to viral spread or persistence of virus at the site of exposure in infections of extended incubation periods [25]. Glial cells may also be involved in rabies virus pathogenesis by affecting the physiological health of neurons via the release of cytokines or neurotoxins. Support for this involvement has been shown recently in rabies-infected mice and rats. In these animals, increased levels of inducible nitric oxide synthase (iNOS), which generates NO in microglial cells and astrocytes [26], relate to or directly correlate with the neuronal dysfunction that underpins clinical rabies disease [12, 31]. Clearly, the infection of glial cells by rabies viruses and their potential role in viral replication, persistence or pathogenesis is intriguing and merits further investigation.

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