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Short Communication

Infection and establishment of latency in the dog brain after direct inoculation of a nonpathogenic strain of herpes simplex virus-1

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A number of diseases affecting the CNS occur in the dog and can be used as models for gene therapy in a large brain. HSV-1 has several potential advantages as a vector to transfer genes into the CNS. However, the ability of HSV-1 to infect CNS cells varies among species and no information was available for the dog. When the nonpathogenic 1716 strain of HSV-1 was injected into the brains of normal dogs it established a latent infection without signs of pathology. Thus, it appears to be suitable as a vector for therapeutic, or marker genes, in this species. *Journal of NeuroVirology* (2001) **7,** 149–154.

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Herpes simplex virus-1 (HSV-1) is a human herpesvirus that naturally causes lytic and latent infections over a wide host range of animals (Roizman and Sears, 1995). The initial infection occurs in epithelial and nerve cells at the point of initial contact with the virus. An immediate lytic infection results in early cell death, local edema, and blister formation. After infecting nerve termini in the region of the acute infection, some of the virus particles travel by fast retrograde axonal transport to the ganglion associated with the site of infection and then into the brain, where they establish a latent infection in susceptible hosts. Latency is established when the viral genes are not expressed and the genome becomes coated with nucleosomes, assuming a chromatin conformation (reviewed in Fraser *et al*, 1991). In contrast to the high level of viral gene expression during the replication phase, transcription during latency occurs from only one region of the viral genome, the latency associated transcript (LAT) region (Stroop *et al*, 1984; Deatly *et al*, 1987; Stevens *et al*, 1987). Several species of LAT RNAs have been identified but no translation product has been found (reviewed in Fraser *et al*, 1991; Roizman and Sears, 1995).

HSV-1 has several potential advantages as a vector to transfer genes into the central nervous system (CNS) (Glorioso *et al*, 1997). The genome is large and can potentially accommodate multiple foreign transcription units. The virus naturally infects nervous system cells, and the viral genome is maintained in a nonintegrated state for the lifetime of an infected individual (Fraser *et al*, 1991). Experimentally, HSV-1 vectors have been used to transfer reporter genes (Ho and Mocarski, 1989) and therapeutic genes (Wolfe *et al*, 1992) as anti-tumor agents (Martuza *et al*, 1991; Randazzo *et al*, 1995; Kesari *et al*, 1995) and as track tracing probes since they cross multiple synapses (Card *et al*, 1993). Since the HSV-1 LATs are found in latently infected humans and experimental animals apparently for life, the LAT promoter has been used for long-term expression of foreign genes in neurons (Ho and Mocarski, 1989; Dobson *et al*, 1990; Wolfe *et al*, 1992; Goins *et al*, 1994; Deshmane *et al*, 1995; Huang *et al*, 1997; Berthomme *et al*, 2000; Zhu *et al*, 2000).

One of the major requirements of HSV-1 mediated gene transfer is to overcome pathogenic effects induced by the acute phase replication of the virus. When wild-type HSV-1 (strain 17+)

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virus is injected intraocularly, or directly into the brain parenchyma of nude mice, it causes a fatal encephalitis from only 10 plaque-forming units. In contrast, the mutant strain 1716 has a 759-bp deletion in the gamma 34.5 gene resulting in an LD_{50} one million times greater than the 17+ virus (MacLean *et al*, 1991; Wolfe *et al*, 1996). The 1716 virus expresses LAT at similar levels as the wild-type virus; thus, it may be useful as a nonpathogenic vector. In addition, 1716 replicates in and kills human melanoma and teratocarcinoma cells transplanted into mice without affecting adjacent normal tissues, including in the brain (Randazzo *et al*, 1995; Kesari *et al*, 1995). Thus it also may be a useful anti-tumor agent in other species. Recently, phase 1 clinical trials have been performed on humans using HSV vectors without deleterious effects (Markert *et al*, 2000; Rampling *et al*, 2000).

A number of naturally occurring diseases affecting the CNS have been described in the dog, including single gene inherited disorders (Patterson *et al*, 1988; Haskins *et al*, 1997; Ostrander *et al*, 2000) and naturally occurring CNS tumors that are refractory to other treatments (Summers *et al*, 1995). These are excellent models for testing HSV-mediated gene therapy strategies in a large brain, but the susceptibility of the dog brain to infection with HSV-1 was unknown: whether HSV-1 would infect dog cells, whether latency would be established, and whether the LAT sequence would be expressed.

Two normal male dogs from our MPS VII carrier colony were used which were approximately six months old and weighed about 15 kg. The animals had received a normal regimen of immunizations. All work was approved by the institutional IACUC and all procedures conformed to AALAS and NIH standards. The dogs were anesthetized and the head was cleansed with 70% EtOH and chlorhexidine scrub. The head was placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) and prepared for surgery under standard sterile conditions. The calvarium was exposed and three holes were drilled in the skull over the right cerebral hemisphere. Using a Hamilton syringe with a 30-gauge needle, a total of 5 μl of virus suspension was injected into each of the three tracks approximately 2 cm lateral of the midline and 1 cm apart in a rostral-caudal direction starting from bregma (Figure 1A). The virus stock of 1×10^7 pfu/ml HSV-1 in 1716 was produced as previously described (Randazzo *et al*, 1995). The injections were initiated at a depth of 2 cm and 1 μl of virus was injected at 2-mm intervals along the needle track as the needle was withdrawn, at a rate of 1 min per inoculation.

The dogs were examined and observed daily for clinical signs of infection, encephalitis, or other indicators of neurological abnormalities. The dogs tolerated the surgery and injections very well, and showed no significant signs of encephalitis or other illness attributable to the virus inoculation. The incisions healed unremarkably, with no suppuration or other evidence of infection. One dog had an elevated temperature of 104°F (normal in the dog is 101–102.5°F) at one time point 2 days after surgery but was otherwise within the normal range. Both dogs showed a mild tendency to circle to the right (the incision side) for approximately 1 week after the surgery. Cessation of this behavior coincided with wound healing. One dog had an absent right menace response in the hours following the surgery, but the response resumed the following day. The neurological signs of circling and the lost menace response were transient and are probably sequelae to the surgical procedure in dogs. Thus these signs were unlikely to be due to any effect of the virus.

Four weeks after inoculation the animals were killed and the brains were divided into pieces for sectioning and in situ hybridizations. The dogs were tranquilized with 2 ml oxymorphone and 1 ml valium intravenously, then injected with an overdose of barbiturate (Euthanol solution). The brains were removed immediately, grossly sliced into eight transverse (coronal) slabs and fixed 24 h in 4% paraformaldehyde and 1× PBS solution at pH 7.3. After fixation, specimens were stored in 70% EtOH at 4°C. The brains were sliced into transverse slabs approximately 5-mm thick spanning the injection sites. The slabs were divided into quadrants which separated the uninjected (Figure 1B, A and C) from the injected (Figure 1B, B and D) sides. The sections were further cut in half coronally to fit into the embedding cassettes, paraffin embedded, and sectioned by standard RNase-free procedures. Paraffin removal was performed using xylene (5 min), 95%, 70%, and 50% EtOH for 5 min each and then PBS for 5 min. The slides were placed in 0.2 N HCl for 20 min, at room temperature, washed in ddH_2O, incubated in proteinase K solution (10 mM Tris, pH 7.4, 2 mM $CaCl_2$, 1 μg/mL proteinase K, preheated to 37°C) for 15 minutes, and dehydrated by reversing the hydration procedure.

The LAT probe was prepared as described (Kesari *et al*, 1995) using a ^{35}S-dCTP. The hybridization solution consisted of 2× SSC, 45% formamide (deionized), 10 mM Tris pH 7.4, EDTA, 1× Denhardt's, 10% dextran sulfate, 10% salmon sperm DNA, and ddH_2O. The labelled probe was concentrated to >100,000 cpm, calibrated by scintillation counting. Probe was applied to each section and allowed to hybridize for 24 h. The posthybridization washes were performed to minimize background signal in the development step. The cover slips were soaked off the slides and washed in a solution of 10mM Tris, pH 7.4, 1 mM EDTA, and 45% formamide for 30 min at 50°C. The slides were then soaked for 2 h in SSC solution and dehydrated in 0.3 M ammonium acetate and 70%, then 95%, EtOH for 5 min each time. The slides were dipped into photographic emulsion and allowed to dry overnight. The slides were developed for 4 days, then the emulsion was dipped 3 min in

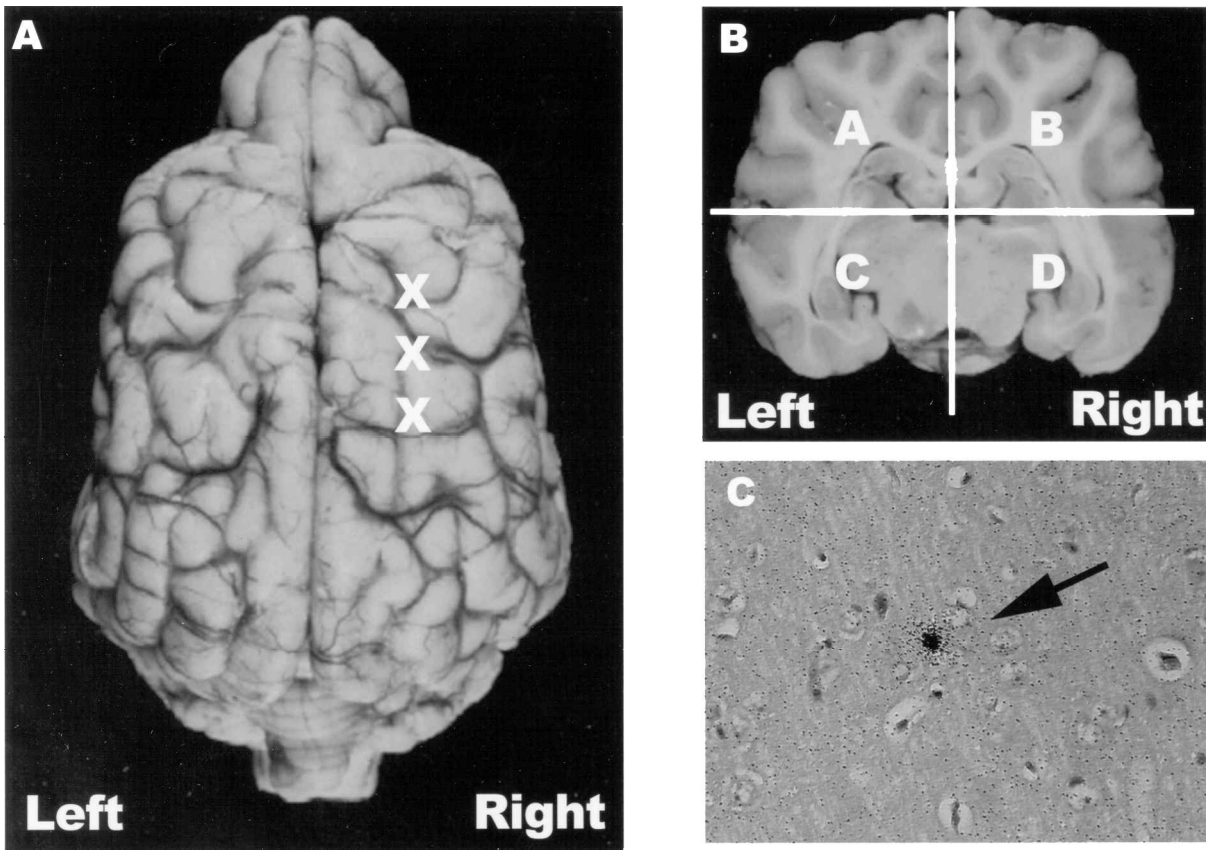

Figure 1 (**A**) Dorsal view of a dog brain showing the approximate sites (X) of viral inoculation into the right cerebral hemisphere. (**B**) Transverse section of the dog brain at the level of the midbrain, which were divided into quadrants separating the uninjected left side (a and c) from the injected right side (b and d). (**C**) Example of a positive LAT hybridization signal in a section of dog brain. Note the silver aggregation concentrated over the nucleus of the cell and diffusing outward.

developer and 3 min in fixer solutions for signal enhancement and to minimize background. The slides were examined microscopically and scored for positive signals.

The injection sites in dog M771 were in quadrant b (Figure 1B) and in dog M765 the virus was injected into quadrants b and d (Figure 1B). Each section was 5-μm thick and every tenth section was hybridized to obtain data at 50-μm intervals, the approximate diameter of an average neuron. Therefore, the positives probably represent single infected neurons rather than several adjacent positives from successive slices of a single neuron. Cells were scored positive only if the signal was concentrated in the center over the nucleus and diffused outward in a symmetrical pattern (Figure 1C). The positives were similar to those seen when 1716 virus was injected into the mouse brain (Deshmane *et al*, 1995; Wolfe *et al*, 1996). Sections from latently infected mouse brains were hybridized as positive controls and uninfected negative-control brains never showed an equivalent appearance. Uneven or asymmetrical aggregates of silver granules were not counted, nor were symmetrical positives of the correct density of granules which were smaller than expected relative to the nucleus size or which were located on a tissue margins. Since a strict scoring system was used, cells with weaker signals were not scored positive and the enumeration probably undercounted the number of positive cells.

Positive cells were found in greatest concentration near the sites of inoculation, but were also found scattered throughout the parenchyma and in the contralateral side of the brain from the inoculation sites (Table 1). A total of 87 positive cells in 61 slides analyzed were counted in the injected quadrant in dog M771. In dog M765, 220 positive cells were seen in 136 slides, most of which were in quadrant b, but positives were also seen on the ipsilateral side in quadrant d, and the contralateral side in quadrant a. Cells expressing viral LAT sequences were present in many brain sections (Table 1) and some clusters of positive cells were seen. The greatest concentrations of latent virus were found within specific sections (Table 1), e.g., region b3 of dog M765 had 13, 10, and 24 positive cells in sections 5, 8, and 19, respectively. These sections matched up anatomically with the drill holes in the calvarium, thus the areas of greatest concentration were near the inoculation sites. The finding of LAT in the contralateral side

Table 1 LAT positive cells in dog brains 29 days after HSV-1-1716 inoculation

Dog	*Region*	*Total rostral-caudal distance (μm)*	*No. sections*	*No. LAT-positive cells**	*Average no. LAT-positive cells per section*
M765	b1	750	13	15	1.2
	b2	700	14	18	1.3
	b3	1200	20	80	4.0
	b4	1250	23	60	2.6
	b5	750	12	10	0.8
	d3	750	13	7	0.5
	d4	750	12	14	1.2
	a3	700	12	5	0.4
	a4	600	10	11	1.1
M771	b1	600	11	24	2.2
	b2	600	9	6	0.7
	b3	700	13	16	1.2
	b4	350	5	13	2.6
	b5	750	12	5	0.4
	b6	500	6	4	0.7
	b7	450	6	19	3.2

*Known LAT-positive and -negative mouse brains were used as hybridization controls.

of the brain was expected based on previous studies in the mouse (Kesari *et al*, 1995; Wolfe *et al*, 1996; Zhu *et al*, 2000). The virus is known to travel by fast retrograde transport through connected tracts in the brain and the LAT found contralateral to the injection sites may correspond to sites connected across the brain by these tracts.

The brains were examined for replicating virus after the animals were killed at 29 days postinoculation. Sections adjacent to those used for LAT hybridization were assayed for the presence of viral proteins by immunohistochemistry to look for replicating virus. None of the sections of dog brain were positive for antibodies to acute phase proteins (not shown), nor was any evidence for replicating virus found in homogenates of brain tissue (Table 2). This is consistent with HSV infection and latency in the mouse, where no evidence of replicating virus is found after about 10 days and latency is well established by 1 month. To look for evidence of replicating virus being shed after infection, serum samples were taken at 1, 4, 10, 14, and 29 days after inoculation and swabs were taken from the mucosal surface of the eyes at 2 and 4 weeks. The samples were stored at −70°C, then all of them were tested at the same time for replicating virus using the CV-1 cell plaque assay. No plaques were found at any time point, indicating that replicating virus was not being shed in the secretions examined (Table 2).

Table 2 Assay for replicating HSV in dog tissues after 1716 injection

		Cytopathic effect	
Tissue	*No. days p.i.*	*Dog M771*	*Dog M765*
Neg control	NA	Neg	Neg
Pos control	NA	Pos	Pos
Serum	Pre-injection	Neg	Neg
	1	Neg	Neg
	4	Neg	Neg
	10	Neg	Neg
	14	Neg	Neg
	29	Neg	Neg
Eye mucosa	Pre-injection	Neg	Neg
	14	Neg	Neg
	29	Neg	Neg
Brainstem	Postmortem	Neg	Neg
Hindbrain	Postmortem	Neg	Neg
Forebrain	Postmortem	Neg	Neg
Cerebellum	Postmortem	Neg	Neg
Trigemminal ganglion	Postmortem	Neg	Neg

These data show that HSV-1 strain 1716 is infectious, becomes latent, and expresses LAT in the normal dog brain after intracerebral inoculation. The 1716 strain of HSV-1 we used has been shown to be safe when it was inoculated intrathecally in MPS VII mice, which are more susceptible than normal animals (Wolfe *et al*, 1996). The virus appears to be safe for use in the dog brain because direct injection did not cause the dogs to become acutely sick, nor was any evidence of viral replication found. Viral LAT sequences were expressed at 1 month postinoculation, which indicated that the virus was present in a latent form. This suggests that it may be feasible to extend HSV-mediated gene transfer and anti-tumor studies from rodents to dog models. Because LAT is expressed in the dog CNS cells, its promoter should be useful for driving foreign gene expression, as has been demonstrated in mouse studies (Wolfe *et al*, 1992; Zhu *et al*, 2000).

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