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Short Communication

Toxicity and neuronal infection of a HSV-1 ICP34.5 mutant in nude mice

Todd M Lasner[1,2], Ruth Tal-Singer[2], Santosh Kesari[2,3], Virginia M-Y Lee[3], John Q Trojanowski[3] and Nigel W Fraser[2]

[1]The Division of Neurosurgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; [2]The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104; [3]The Department of Pathology & Laboratory Medicine, Division of Anatomic Pathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA

HSV-1 mutants in the RL-1 gene encoding the ICP34.5 protein have been demonstrated to have diminished neurovirulence in brain yet replicate as efficiently as parental virus in transformed tissue culture cells. Thus they have been proposed as candidates viruses for human brain tumor therapies. Evaluation of their replicative properties and pathogenesis within the nervous system has been limited. As most patients undergoing therapies for brain tumors are likely to be immunocompromised, it will be important to understand the pathogenesis of these viruses in immunocompromised hosts. To this end, the lateral ventricle of nude mice was injected with high (2.5×10^7 PFU), medium (10^5 PFU), or low dose (10^3 PFU) HSV-1 variant-1716, which has a deletion in the RL-1 gene. Ten of 10 mice died within 2–3 days following the high titer infection. Six of 19 animals with medium titer infection died within 9 days, and viral antigens were seen in ependymal cells as well as neurons within the brainstem and thalamus. Although only two of 19 animals became moribund 18 days after medium titer viral infection, many neocortical and hippocampal neurons were positive for HSV-1 antigens. However, plaque-purified viral isolates recovered from brain homogenates of these animals demonstrated no increase in pathogenicity. Nine of 20 animals died following low dose infection; six of these animals, from which tissue was analyzed, all had many HSV antigen-positive neurons in the neocortex and hippocampus. These data imply that if this type of virus is used for human brain tumor therapy immunosuppressed patients may suffer from significant viral pathogenesis outside the tumor.

Keywords: herpes simplex virus; ICP34.5; toxicity; nude mouse; replication; central nervous system

Introduction

Thompson and Stevens originally described the neuroattenuated phenotype of HSV-1 variants with mutations in the RL-1 gene encoding ICP34.5 (Thompson and Stevens, 1983). ICP34.5 deficient HSV-1 mutants are capable of replicating within certain dividing cell lines in tissue culture, but are growth-inhibited within post-mitotic neurons of the CNS, which suggests cell-state dependent viral replication (Brown *et al*, 1994; Chou *et al*, 1990, 1995; Chou and Roizman, 1994). Further, the toxicity of variant-1716 by intracranial (IC) inoculation in SCID mice has been reported, although viral replication was not evaluated in the brains of animals that died using immunohistochemical techniques or recovery of infectious virus (Valyi-Nagy *et al*, 1994). RL-1 viral variants have been used to treat mice bearing intracranial xenografts of human tumors by injection directly into tumors, causing a selective cytolytic infection of the tumor cells (Kesari *et al*, 1995; Markert *et al*, 1993). Focal intracerebral replication of variant-1716, which has a 759 base-pair deletion in RL-1 in a HSV-1 strain 17+ background (MacLean *et al*, 1991), has recently been demonstrated in the murine CNS, with viral antigen expression lasting for several weeks within neurons of tumor-bearing nude mice (Lasner *et al*, 1996). We therefore sought to evaluate the toxicity and replicative properties of variant-1716 with

Correspondence: NW Fraser
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intracerebral injection in nude mice using different inoculums by survival studies and analyses of the brains of these animals.

When non-tumor bearing nude mice were inoculated by intracerebral-intraventricular injection of 2.5×10^7 PFU of variant-1716 (Kesari *et al*, 1996), 10/10 nude mice died within 2–3 days (Table 1). Immunohistochemistry with a rabbit polyclonal antisera to viral capsid and core antigens (Adams *et al*. 1984) on the 6/6 animals from which tissue was recovered demonstrated foci of immunopositive cells in the ependyma and subependymal regions of the thalamus and brainstem (Figure 1a, b).

Six of 19 mice died 6–9 days following intraventricular injection with 1×10^5 PFU of variant-1716 (Table 1: row 2). Viral antigens were detected focally within the ependyma and subependymal regions in 4/4 animals from which tissue was recovered (Figure 1c, d). These observations were similar to those with the higher titer infection. Two of the 13 surviving animals (M1, M2) had continuous seizures and were emaciated at 18 days post-infection (PI). Immunohistochemistry demonstrated many HSV-1 antigen positive neurons in the superficial cortex and hippocampus (Figure 2a–c), whereas the ependymal cells were denuded (not shown). To determine whether virus recovered from M1 and M2 represented a new, more virulent variant, brain homogenate (10 μl aliquots from 1000 μl homogenate; Kesari *et al*, 1995) from M1 and M2 was injected intraventricularly into two nude mice each (see Figure 4). One of these four animals (M2D19) became emaciated and had continuous seizures 19 days after infection. Extensive viral protein expression was detected within the cerebral cortex, hippocampus, and thalamus, similar to observations from the M1 and M2 animals (data not shown).

Experiments were then performed to determine whether serial passage of plaque-purified virus through nude mice selects for a more lethal viral phenotype. To this end, brain homogenates from the M1 and M2 mice were subjected to viral plaque purification, and 1×10^5 PFU of plaque purified virus (D18M1, D18M2) were injected intraventricularly into two nude mice (n=4). One of these animals died at 21 days PI (D18M2D21). As seen in the M1 and M2 mice, many HSV-1 antigen positive neurons were detected within the superficial cortex, hippocampus, and thalamus (Figure 2d).

To determine if virus purified from M1 and M2 represented more virulent variants, a brain homogenate of M2D21 was subjected to plaque purifica-

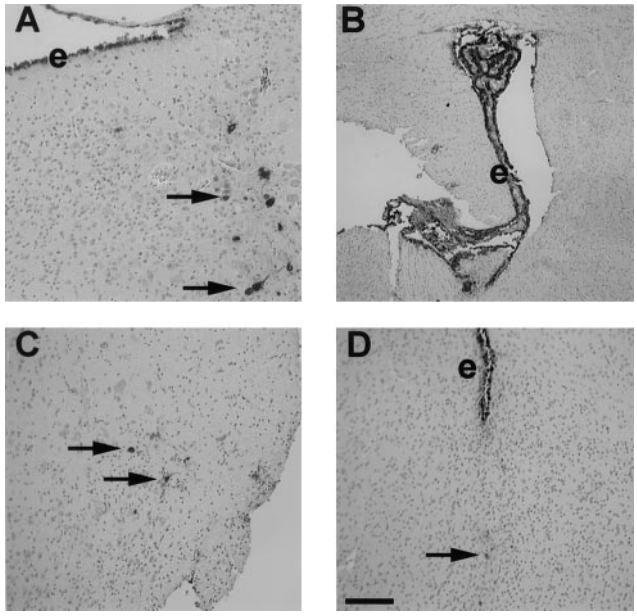

Figure 1 Replication of variant-1716 in the brains of nude mice that died. Nude mice were inoculated into the right lateral ventricle with 2.5×10^7 (**A,B**) and 1×10^5 (**C,D**) PFU of variant-1716. These animals died 2 days (**A,B**) and 7 days (**C,D**) after the infection. Immunohistochemistry for HSV capsid and core antigens demonstrates viral replication within the brainstem (**A,C**: arrows), ependyma (e) of the fourth ventricle (**A**), lateral ventricles (**B**), and third ventricle (**D**), and thalamus (**D**: arrow). Bar = 30 μm (**A,C**), 80 μm (**B**), 50 μm (**D**).

Table 1 Survival of nude mice following intracranial infection with virus.

Virus	*Titer (PFU)*	*Location*	*# of dead animals /total # infected*	*Day of death PI (# of animals)*
1716HT	2.5×10^7	ventricle	10/10	2(6):3(4)
1716MT	1×10^5	ventricle	8/19	6(4):7:9:18(2)
1716MT	1×10^5	caudate	9/20	8(2):14(3):16:22(3)
D18M2D21PP3	1×10^5	ventricle	4/20	13:20(2):42
1716LT	1×10^3	ventricle	9/20	14(3):15(2):18(1):20(1):21(1):27(1)
vehicle	0	ventricle	0/4	none
1716HTUV	0	ventricle	0/5	none
17+	1×10^5	ventricle	5/5	2(3), 3(2)

Variant-1716 (rows 1, 2, 3, 5), plaque-purified isolates from animals which died following variant-1716 infection (D18M2D21PP3: row 4), vehicle without virus (row 6), UV inactivated high titer 1716 (1716HTUV: row 7), and parental wild-type strain 17+ (row 8) were injected intracranially into nude mice. Mortality is reported in number of animals dead (column 4) and days PI (column 5). Note the toxicity from variant-1716 (rows 1, 2, 5), the similar phenotype of plaque-purified isolates to variant-1716 (rows 2, 4), and the similar mortality from intraventricular and caudate injections (rows 3, 4).

tion (M2D21PP3), and 1×10^5 PFU were injected intracranially into 20 nude mice. Four of these animals died: one animal at 13 days PI and 42 days PI, and two animals at 20 days PI (Table 1: M2D21PP3, row 4). Multiple HSV positive neurons were observed by immunostaining in all 3 of the brains from which tissue was salvaged, as seen in M1 and M2 (data not shown). Thus we did not isolate a more virulent or pathogenic phenotype of HSV-1 variant-1716 by serial passage of the virus through nude mice.

To determine whether the pathogenic phenotype of variant-1716 resulted from contamination with the parental strain 17^+ (Brown *et al*, 1973), viral DNA was isolated from plaque-purified isolates (D18M1, D18M2) (Pignatti *et al*, 1979), and polymerase chain reaction (PCR) was performed using primers for ICP27 (a gene present in variant 1716 and wild type strain 17^+) and the ICP34.5 gene (which is present in strain 17^+ but is deleted in variant-1716). Viral DNA was isolated and purified using phenol extractions. Reactions were performed in 25 μl volumes containing 10 ng of purified virion DNA, 200 μM each deoxynucleoside triphosphate (Pharmacia), 1 μM of each primer, 2.5 U of Taq Polymerase with PCR Buffer A (Fisher). The primer pair used for amplification of the ICP34.5 gene produces a 135 bp PCR product as described (Perng *et al*, 1996). Primers for amplification of the ICP27 gene were as described in Tal-Singer *et al*. (1997), yielding a 260 bp product. Cycling reactions were performed with a Perkin Elmer (Norwalk, Conn) thermal cycler. After one cycle of 4 min denaturation at 94°C, cycles were as follows: (i) 1 min denaturation at 94°C, (ii) annealing at 60°C for 1 min, (iii) extension for 2 min at 72°C. The final cycle was terminated with a 7 min extension at 72°C. Amplification was carried out for 40 cycles. PCR products were visualized on 2.5% agarose gels stained with ethidium bromide. As expected, ICP27 DNA was detected in all viral samples (Figure 3). In contrast ICP34.5 DNA was detected only in strain 17+ samples. This experiment was repeated three times using independent samples with similar results (data not shown). Therefore, the M1 and M2 mice died from infection with virus lacking the ICP34.5 gene. Taken together, our results indicate that in a small subpopulation of nude mice, variant-1716 can cause massive intracerebral infection and death.

To determine whether this lethal phenotype resulted from intraventricular injection of virus by spread of the virus through CSF pathways, 1×10^5 PFU of variant-1716 was injected into the caudate nucleus of 20 nude mice. Nine of the animals died between 8 and 22 days PI (Table 1: row 3). Many

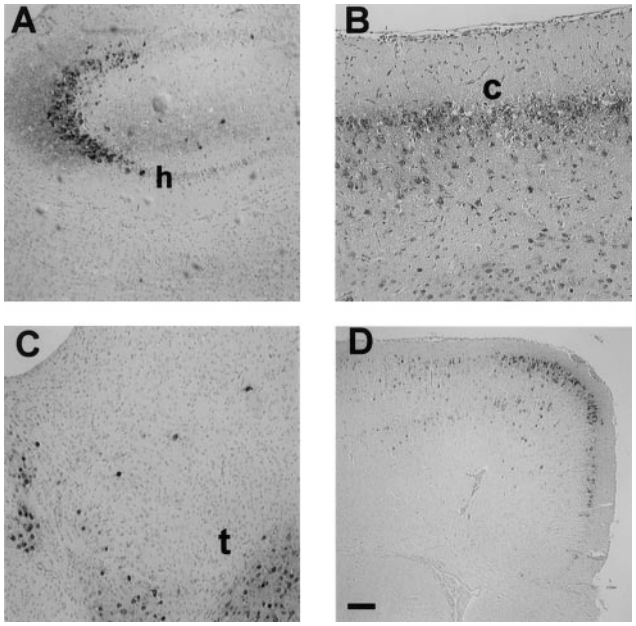

Figure 2 Replication of variant-1716 in the brains of nude mice that died several weeks after viral inoculation. Nude mice were inoculated into the right lateral ventricle with 1×10^5 PFU of variant-1716, and two mice died 18 days after the infection (M1, M2) (**A,B,C**). Immunohistochemistry for HSV antigens demonstrates viral replication within the hippocampus (**A**: h), cerebral cortex (**B**: c), and thalamus (**C**: t) of M2, which was similar to finding from M1 (not shown). Following homogenization of 1/2 of the brain, plaque purified isolates of recovered virus were injected IC into four nude mice. One of four animals died at 21 days PI. Viral antigens were seen in the superficial cortex (**D**), in addition to the hippocampus and thalamus (not shown). Bar = 40 μm (**A,C**), 20 μm (**B**), 80 μm (**D**).

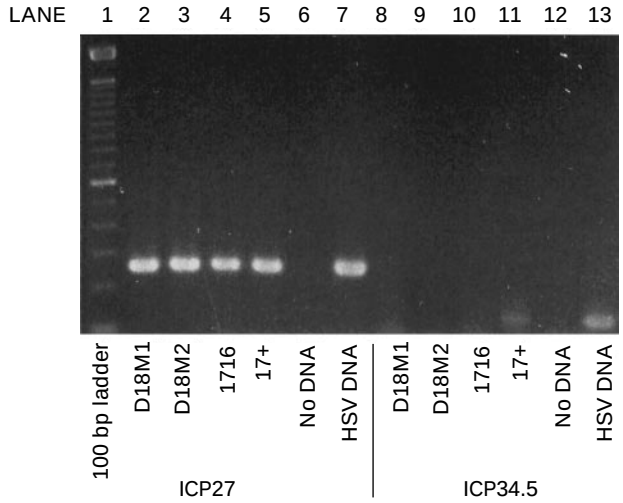

Figure 3 Analysis of recovered virus by PCR. Negative controls lacking template DNA (lanes 6 and 12) were included in each set of experiments. Purified HSV-1 DNA from previous isolation was used as a positive control (lanes 7 and 13). Purified input viral DNA: 17+ (lanes 5 and 11) and variant-1716 (lanes 4 and 10) were used to confirm ICP 34.5 phenotype. Plaque purified isolates from two animals that died 18 days after intracranial inoculation of variant-1716 D18M1 (lanes 2 and 8) and D18M2 (lanes 3 and 9) demonstrate the gene encoding ICP27 but not ICP34.5.

HSV-1 positive neurons were detected by immunostaining in all 7 of the animals from which tissue could be analyzed, and the ependymal cells did not express viral antigens (data not shown). Thus, fatal viral replication is not exclusive to intraventricular inoculation with variant-1716. Additionally, nine of 20 nude mice died between 14 and 27 days PI following low titer infection with variant-1716 (10^3 PFU) (Table 1: row 5). All six of the animals from which tissue was salvaged demonstrated many HSV positive neurons in the neocortex and hippocampus (data not shown). Thus, fatal infection with variant-1716 may be seen regardless of inoculation site.

Additionally, virus may spread via the subarachnoid space due to reflux along the needle tract thereby infecting cells at sites distant from the initial inoculation. Thus, neurons that are distant from the intended inoculation may become infected. In support of this hypothesis, our data suggest that the location of IC injection is not critical to the occasional fatal neuronal infection associated with variant-1716.

Although previous studies have confirmed the neuroattenuated phenotype of HSV-1 variants with mutations in RL-1 *versus* wild type virus, an evaluation of the replicative properties of these viruses within the CNS has not been published. In studies which have evaluated the treatment of experimental brain tumors with ICP34.5 mutated HSV-1 variants, dead animals were not evaluated for the presence of persistent viral infection within either the brains or tumors (Chambers *et al*, 1995; Kesari *et al*, 1995; Markert *et al*, 1993; Mineta *et al*, 1995). Thus, it is possible that viral replication within the brains contributed to the death of some of these animals.

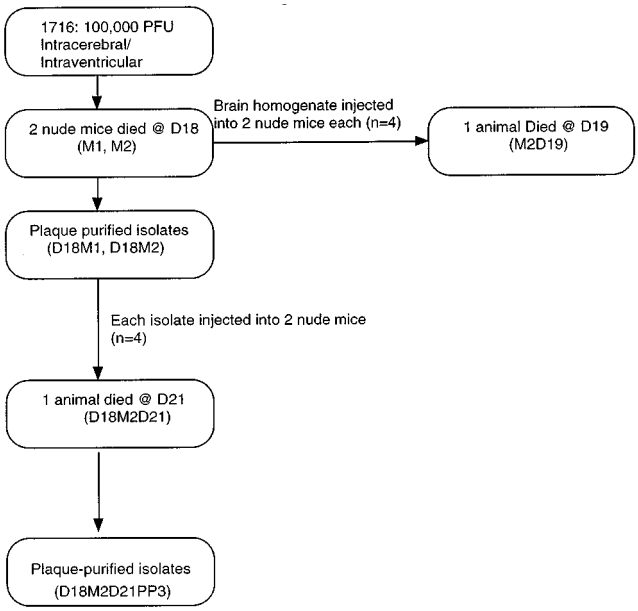

Figure 4 History of animals and viral isolates undergoing serial passaging through nude mice.

It is possible that the earlier studies did not detect the occasional virulence of variant-1716 because of the small sample size studied. Twenty mice were used in the present study to assess the virulence of variant-1716; whereas eight animals were used to assess the toxicity of variant-1716 in severe combined immunodeficiency (SCID) mice (Valyi-Nagy *et al*, 1994), and the number of mice used to assess the toxicity of R3616 (a strain F ICP34.5 mutant) was not reported (Chou *et al*, 1990). As massive intracerebral viral antigen expression was seen in less than 50% of the animals, eight animals may not be an adequate sample to evaluate this uncommon toxic effect. Further, there may be strain-specific differences in neuronal susceptibility to variant-1716 between nude and SCID mice.

The toxicity of variant-1716 prior to 10 days PI may be related to viral replication within ependymal cells, causing obstruction of flow of the CSF due to sloughing of virally infected ependymal cells, or replication of virus within critical regions in the adjacent brainstem or hypothalamus (Lasner *et al*, 1996). In control experiments, mice were inoculated with ultraviolet-light (UV) inactivated high titer variant-1716 or virus free media. All animals survived (Table 1), indicating that viral replication is required for the toxic effect of variant-1716. Thus, a direct toxic effect from inactivated virus is not sufficient to kill nude mice.

To determine whether animals that survived infection with variant-1716 had persistent viral antigen infection, five animals were sacrificed 116 days after intracaudate injection of virus. Immunohistochemical analysis of the brains of all five animals demonstrated focal viral antigen expression within the superficial cortex, as previously described in the brains of tumor-bearing nude mice (Lasner *et al*, 1996). Although healthy animals that failed to succumb to infection with variant-1716 had persistent viral infection, the degree of neuronal infection was less than that seen in animals that died. Thus 1716 forms persistent infection which is lethal only in a fraction of animals. Plaque purification of virus from moribund animals suggests that this lethality is not caused by selection for a more virulent variant.

Variant-1716 and other ICP34.5 deletion mutant viruses have previously been shown to establish widespread latency and no morbidity following IC inoculation in immunocompetent mice, confirming infection of a large number of cells after IC infection (Markovitz *et al*, 1997). HSV-antigen staining is absent in these immunocompetent animals after 7 days PI Kesari *et al*, 1996). Thus, our observation of HSV-1 antigen expression in addition to the recovery of infectious virus from the brains of

animals that were moribund confirm viral protein synthesis in our present study.

It is also possible that the injected virus underwent a mutation in a gene other than ICP34.5 in the nude mice to form a more virulent phenotype. However, following plaque-purification and injection of 1×10^5 PFU of these isolates intracranially in nude mice, there was no rapid mortality or neurological morbidity and the lethality of the recovered virus was similar to that seen with variant-1716. For example, when 1×10^5 PFU of wild-type strain 17+ were injected intraventricularly into five nude mice, all of the animals died within 3 days (Table 1: row 8). It is thus unlikely that a new viral genotype was formed *in vivo*, but extensive analysis of the viral genome would be required to evaluate this possibility. However, by Southern blot analysis, there were no large insertions or deletions in the viral genome (data not shown). Additionally, the PCR data confirmed that reversion of the RL-1 deletion did not occur.

A mutant virus which appears to have spontaneously overcome the ICP34.5 block to viral replication has been recently described by He *et al*. (1997), the properties of this virus, Sup-1, are not readily explained by a second mutation in Us11 as was shown by Mohr and Gluzman (1996) for some suppressor mutations, thus an alternate, as yet undefined, mechanism of gain of virulence is proposed.

The present study demonstrates that toxicity can result from the intracerebral injection of HSV-1 variants with mutations in the ICP34.5 gene, and that this toxicity is related to viral replication within the mammalian nervous system. The underlying mechanism for virus 1716 to replicate and kill a small percentage of mice is not clear. A virus with a neurovirulent phenotype could not be plaque purified from dead animals suggesting a genetic mutation had not occurred. The fact that virus killed only immuno compromised but not immune competant animals (Kesari *et al*, 1996) and the relatively long time to death is reminiscent of the data obtained for replication crippled viruses such as dlSPTK and in1814 (Valyi Nagy *et al*, 1992; 1994). However the low mortality is different from that of other replication crippled viruses, or represents a virus more crippled than any previously examined. Because HSV mutant viruses are being considered for both gene transfer and tumor destruction in humans, this viral toxicity needs to be evaluated.

In a subpopulation of nude mice inoculated intracranially with variant-1716, there can be a fatal viral neuronal infection. The use of an immunocompromised host to study the toxicity of viral vectors may provide a model for evaluating the full replicative properties of candidate viruses for human brain tumor therapy in certain clinical situations. Further, since many patients receiving treatment for brain tumors also may be immunosuppressed by treatment with corticosteroids, our nude mouse data suggest that an immunocompromised host may allow for a smoldering infection to propagate into a life threatening process. Thus, it will be important to continue to use such animals in assessing the toxicity of candidate neuroattenuated HSV mutants prior to consideration in clinical trials.

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