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Treatment of Experimental Intracranial Murine Melanoma with a Neuroattenuated Herpes Simplex Virus 1 Mutant

BRUCE P. RANDAZZO,*,† SANTOSH KESARI,*,‡ RICHARD M. GESSER,*,§ DAVID ALSOP,¶ JOHN C. FORD,¶ S. MOIRA BROWN,‖ ALASDAIR MACLEAN,‖ and NIGEL W. FRASER*,[1]

*The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104; †Department of Dermatology, ‡School of Medicine, and ¶Department of Radiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104; §Children's Hospital, Division of Allergy, Immunology, and Infectious Diseases, Philadelphia, Pennsylvania 19104; and ‖MRC Virology Institute, Church Street, Glasgow, Scotland, United Kingdom

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Brain metastases occur commonly in the setting of a variety of human cancers. At present, such cases are invariably fatal and highlight a need for research on new therapies. We have developed a mouse brain tumor model utilizing the Harding–Passey melanoma cell line injected intracranially into C57Bl/6 mice. Tumors develop in 100% of the mice and can be detected by magnetic resonance imaging as early as 5 days post cell injection. Death from tumor progression occurs between 12 and 16 days post cell injection. Stereotactic injection of the neuroattenuated HSV-1 strain 1716 into brain tumors 5 or 10 days postinjection of the melanoma cells results in a statistically significant increase in the time to development of neurological symptoms and in complete tumor regression and the long-term survival of some treated animals. Moreover, viral titration studies and immunohistochemistry suggest that replication of this virus is restricted to tumor cells and does not occur in the surrounding brain tissue. These results suggest that HSV-1 mutant 1716 shows particular promise for use as a therapeutic agent for the treatment of brain tumors.

INTRODUCTION

It has been estimated that 70,000 cancer deaths occur each year in the United States with metastatic lesions to the CNS (Steck and Nicolson, 1993). This frequently results in severe and debilitating neurological complications, including headache, paralysis, seizures, and impaired cognition. Radiation and steroids are currently the principal therapies used; however, they are only palliative and frequently cause significant neuropsychological and endocrinological morbidity (Steck and Nicolson, 1993). Surgery is generally reserved for removal of solitary metastases and is often not curative (Feun *et al.*, 1982).

The use of viral therapy for the destruction of various experimental tumors has been attempted with parvovirus H-1, Newcastle disease virus, retroviral vectors containing drug susceptibility genes, and herpes simplex type 1 virus (HSV-1) (Dupressoir *et al.*, 1989; Markert *et al.*, 1992; Martuza *et al.*, 1991; Ram *et al.*, 1993; Reichard *et al.*, 1992; Takamiya *et al.*, 1993). The mechanisms by which viruses improve the outcome in experimental tumor systems are complex and poorly understood. Brain tumors represent a dividing cell population occurring within an essentially nondividing cell population of support cells and terminally differentiated neurons. Thus, in the context of brain tumor therapy, one rationale is to select a virus that replicates exclusively or preferentially in dividing cells. Such a virus may be capable of establishing a lytic infection exclusively in tumor cells within the CNS, ultimately destroying the tumors without infecting surrounding brain and without deleterious effects to the host.

Pioneering experiments with HSV showed a dose-dependent improvement in survival of nude mice bearing intracranial human gliomas following intratumoral therapy with mutant HSV-1 *dlsp*TK (Martuza *et al.*, 1991). This virus has a deletion in the viral thymidine kinase (TK) gene (Coen *et al.*, 1989) and exhibits a relatively neuroattenuated phenotype in mice (Tenser, 1991).

The terminal 1 kb of the long repeat region of the HSV-1 and HSV-2 genomes contains a gene (Ackermann *et al.*, 1986; Chou *et al.*, 1990; McGeoch *et al.*, 1991) that confers neurovirulence. Deletion or mutation of this gene (γ34.5) results in variants that grow as well as wild-type virus on dividing cells of many established cell lines, but show impaired replication on nondividing cells (Bolovan *et al.*, 1994; Chou *et al.*, 1990; McGeoch *et al.*, 1991). In mice, γ34.5 null mutants are incapable of replicating in the central nervous system and do not cause encephalitis (Chou *et al.*, 1990; Javier *et al.*, 1987; MacLean *et al.*, 1991; Taha *et al.*, 1990).

A mutant HSV-1 called R3616, containing a 1000-bp deletion in γ34.5, with an LD_{50} (minimum dose of virus that kills 50% of infected animals) that is at least 3×10^3 fold greater than wild-type F strain virus from which it was derived (Chou *et al.*, 1990), has been shown to im-

[1] To whom reprint requests should be addressed.

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prove the survival of nude mice bearing intracranial human gliomas (Markert *et al.*, 1993). In the work presented here, we have utilized an HSV-1 strain 17 mutant virus called 1716 that has a 759-bp deletion in γ34.5 (MacLean *et al.*, 1991). This mutant has an LD_{50} following intracranial inoculation that is approximately 10^6-fold greater than the wild-type 17^+ virus from which it was derived (MacLean *et al.*, 1991).

The majority of published work on HSV-1-based viral therapy of brain tumors has utilized xenografts of cell lines derived from primary human brain tumors in immunoincompetent animals (Markert *et al.*, 1992, 1993; Martuza *et al.*, 1991). We wanted to establish a model with which to study the viability of HSV-1-based therapy of a metastatic tumor, since clinically these are significantly more common than primary brain tumors (Steck and Nicolson, 1993). In addition, we felt that it was important to study viral therapy in the context of an immunocompetent animal model, since specific host immune responses undoubtedly play a role in the outcome of therapeutic viral infection.

Melanoma is a prevalent malignancy. Cerebral metastases occur in up to 75% of patients with metastatic disease and are among the most common causes of death (Amer *et al.*, 1978; Budman *et al.*, 1978; delaMonte *et al.*, 1983; Einhorn *et al.*, 1974; Patel *et al.*, 1978).

The Harding–Passey (H–P) melanoma cell line, derived from an outbred mouse, is tumorigenic in C57Bl/6 mice (Bleehen, 1974; Castillo *et al.*, 1982; Garcia-Borron *et al.*, 1985; Lopez-Ballester *et al.*, 1991; Schwabe *et al.*, 1990; Vicente *et al.*, 1987). Using this cell line to form intracranial tumors in C57Bl/6 mice, we have established a straightforward, clinically relevant, reproducible model in which to study the complex host–tumor–virus interactions that occur during viral therapy. This work expands the spectrum of malignancies amenable to HSV-1-based viral therapy to a common metastatic tumor and shows that viral therapy is viable in a host with an intact immune system. While extrapolation from mouse to man is always difficult, this report shows that the neuroattenuated HSV-1 mutant 1716 is a safe and effective therapeutic agent for intracranial melanoma.

MATERIALS AND METHODS

Animals

Female C57Bl/6 mice (4 to 6 weeks old, weighing approximately 20 g) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Tumor cells

S91 Cloudman melanoma cells (Yasamura *et al.*, 1966) were obtained from the ATCC (Rockville, MD). B16 melanoma cells (Fidler, 1973) and Harding–Passey melanoma cells (Bleehen, 1974; Castillo *et al.*, 1982; Garcia-Borron *et al.*, 1985; Lopez-Ballester *et al.*, 1991; Schwabe *et al.*, 1990; Vicente *et al.*, 1987) were a generous gift from Dorothee Herlyn (Wistar Institute, Philadelphia, PA). Cells were grown in plastic flasks in AUTO-POW medium containing penicillin, streptomycin, and 5% calf serum. When originally obtained, all cell lines were grown and then frozen in 95% calf serum/5% DMSO so that all experiments could be initiated with cells of a similar passage number. On the day of intracranial injection, cells in subconfluent monolayer culture were passaged with 0.25% trypsin solution in EDTA, washed ×1 in cell culture medium, resuspended at the appropriate concentration in medium without serum, and held on ice.

Intracranial tumor production

Mice were anesthetized with ketamine/xylazine (87 mg/kg ketamine/13 mg/kg xylazine im). The head was cleansed with 70% EtOH. A small midline incision was made in the skin of the head exposing the skull. Stereotactic injection of tumor cell suspensions was performed using a small animal stereotactic apparatus (Kopf Instruments, Tujunga, CA). Injections were done with a Hamilton syringe through a disposable 28-gauge needle. The needle was positioned at a point 2 mm caudal of the bregma and 1 mm left of midline. Using a separate 27-gauge needle with a shield that limits the length of the needle exposed to 0.5 mm, the skull was breached at the appropriate coordinates. The injection needle was advanced through the hole in the skull to a depth of 2 mm from the skull surface and then extracted 0.5 mm to create a potential space. Cells (5×10^4) in a total volume of 2 μl were injected over 1 min. Following the injection, the needle was left in place for 3 min and then slowly withdrawn. The skin was sutured closed.

Virus

To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV strains *in*1814, 1716, *dlsp*TK, or wild-type 17^+. Virus was concentrated from the culture and titrated by plaque assay as previously described (Spivack and Fraser, 1987). All viral stocks were stored frozen in viral culture medium (AUTO-POW medium containing penicillin and streptomycin) at −70° and thawed rapidly just prior to use.

Viral inoculation

Mice were anesthetized im with ketamine/xylazine, and the head was cleansed with 70% EtOH. Using a Hamilton syringe with a 30-gauge disposable needle, the appropriate amount of virus was injected, in a volume of 2 μl, through a midline incision at the same stereotactic coordinates used for tumor cell injection. The injection was performed over 1 min, and following the injection the needle was left in place for 3 min and then slowly

withdrawn. The amount of 1716 used in all experiments (5×10^5 PFU/mouse) was the maximum attainable, given the constraints of the volume of injection and the titer of virus stock that could be produced.

Magnetic resonance imaging

Mice were imaged using a 1.9-tesla 30-cm-bore animal MRI system located in the Hospital of the University of Pennsylvania MRI facility. Animals were anesthetized with ketamine/xylazine (87 mg/kg ketamine/13 mg/kg xylazine im). Subsequently, each animal was injected with 10 units of Gd (DTPA) via a tail vein. The animal was immobilized within a Plexiglass RF coil and imaged.

Titration of virus from tumor and brain

Mice were sacrificed by lethal injection of anesthesia. Brains with or without *in situ* tumors were removed aseptically, snap frozen in liquid nitrogen, and stored at −70°. Each tissue sample was rapidly thawed in a 37° water bath, and the tissue was homogenized in viral culture medium at a 10% w/v ratio using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3000 *g* for 10 min at 4°. The supernatant of each tissue homogenate was diluted logarithmically in medium, and the viral titer of each was determined by plaque assay on BHK cells (Spivack and Fraser, 1987).

Immunohistochemistry

HSV-infected cells were detected by an indirect avidin–biotin immunoperoxidase method (Vectastain ABC kit, Vector Labs, Burlingame, CA) as specified by the manufacturer with slight modification. Briefly, tissue sections were deparaffinized, rehydrated, quenched in peroxide (H_2O_2), and blocked in 3.5% goat serum (Sigma Chemical Co., St. Louis, MO). Tissue sections were incubated overnight at 4° with the primary antibody, a rabbit antiserum to HSV-1, which was raised to infected cell lysate and thus detects both structural and nonstructural HSV antigens (Dako Corp., Carpinteria, CA), used at a dilution of 1:1000. Next, the tissues were incubated at room temperature with biotinylated goat anti-rabbit IgG, the avidin–biotin horseradish peroxidase complex, and finally AEC substrate. Sections were counterstained with hematoxylin and examined under the light microscope. As a control for the specificity of immunostaining, tissues were processed as above, except that nonimmune rabbit serum was substituted for the primary HSV-1 antiserum.

Statistics

Standard deviation and Student's *t* test, two-sample assuming unequal variances, were calculated using Microsoft Excel (Redmond, WA) on an Apple Macintosh computer (Cupertino, CA).

TABLE 1

Relative Susceptibility of Melanoma Cells to Lysis by HSV-1

	Cell type			
Virus	Cloudman S91	Harding–Passey	BHK	B-16
*in*1814	10^4	10^4	10^3	$>10^8$
*dlsp*TK	10^3	10^4	10^3	$>10^8$
1716	10^4	10^4	10^3	$>10^8$
17^+ (wild type)	10^3	10^3	10^2	$>10^8$

Note. Cells were plated in 24-well tissue culture plates at a density of 5×10^4 cells/well. The viruses were diluted logarithmically and cell monolayers were infected in triplicate. After 72 hr of culture, the highest dilution of virus at which complete destruction of the monolayer still occurred was recorded for each virus–cell combination. Data are expressed as the number of PFU of virus obtained for each virus–cell combination.

RESULTS

In our initial studies, we wanted to make a straightforward *in vitro* determination of the relative abilities of HSV-1 wild-type and mutant viruses to lyse various murine melanoma cells. We also wanted to compare how efficiently these melanoma cells were lysed by HSV-1 relative to BHK cells, which is a standard cell line used to propagate and titer HSV-1. The genotypes and phenotypes of 1716 and *dlsp*TK are given in the Introduction. HSV-1 mutant *in*1814 possesses a 12-bp insertion in the αTIF gene that affects only the transactivating activity of the molecule (Ace *et al.*, 1989).

As demonstrated in Table 1, complete lysis of melanoma and BHK cell monolayers in this crude assay required approximately 1 log greater amounts of mutant virus than wild-type 17^+, but is quite efficient. Approximately 1 log greater amounts of mutant or wild-type virus were required to completely lyse Cloudman S-91 and H–P murine melanoma cell monolayers compared to that required for BHK cells. Interestingly, B-16 was completely resistant to lysis by all of the mutant viruses tested and wild-type 17^+ virus.

The capacity of each melanoma cell line to produce intracranial tumors was then evaluated. For each cell line, 10 C57Bl/6 mice were injected stereotactically with 5×10^4 cells in the right cerebral hemisphere. Mice were observed daily and sacrificed when they appeared moribund (limb paralysis, severe hunching with bradykinesia, extreme cachexia) or after 6 weeks if they remained asymptomatic. Each brain was fixed, sectioned, stained, and examined histologically for tumor. Both H–P and B-16 formed intracranial tumors in 10 of 10 C57Bl/6 mice, while Cloudman S-91 formed a tumor in only 1 of 10 mice.

We decided to proceed with the H–P model since these cells were susceptible to lysis by the relevant HSV-1 mutants and formed brain tumors efficiently.

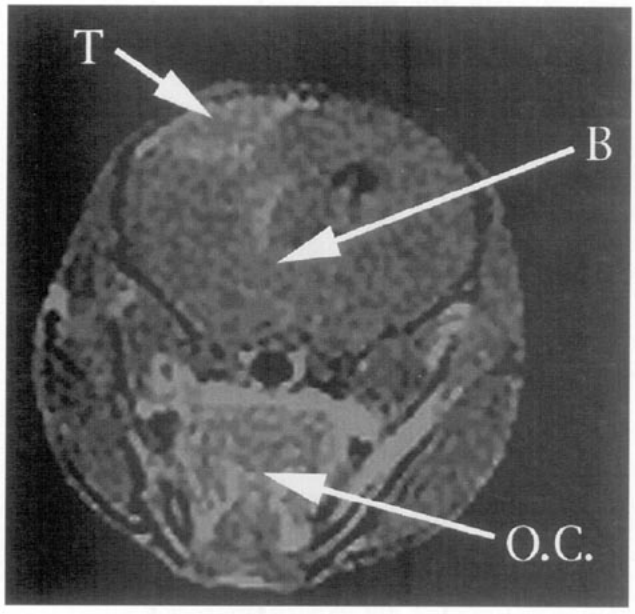

FIG. 1. Gadolinium-enhanced T1 weighted MRI of intracranial melanoma. C57Bl/6 mice were injected with 5×10^4 H–P cells. Seven days later, MRI was performed with a 9-cm-bore 1.9-T magnet after the intravenous injection of 10 units of Gd (DTPA). In this figure the tumor is marked with the letter T, brain with the letter B. O.C., oral cavity.

A technical advantage of this system is that the presence of a brain tumor can be verified by magnetic resonance imaging (MRI) (see Fig. 1) prior to treatment or simply by observation of a pigmented area on the skull overlying the tumor site, generally by 5 days post cell injection. The tumors progressed to a size that caused the mice to become moribund from neurologic symptoms in approximately 2 weeks (see Fig. 2).

As shown in Fig. 2a, stereotactic injection of HSV-1 mutant 1716 into brain tumors 10 days after establishment resulted in a statistically significant increase in the length of time elapsed until the mice become moribund [$P(T \leq t)$ one-tail: 1.016×10^{-4}]. However, no long-term survivors were obtained. When viral therapy was performed 5 days after tumor establishment (Fig. 2b), significant improvement in outcome was again seen in the treatment group [$P(T \leq t)$ one-tail: 7.707×10^{-3}], and 2/10 treated mice were cured. One long-term survivor was sacrificed on Day 39 post viral infection. Microscopic examination of serial sections of the brain did not reveal any residual tumor (data not shown). The second animal is still alive and asymptomatic at greater than 150 days post-treatment. Treated animals that became moribund showed progression of their brain tumors upon examination of tissue sections (data not shown). Intracranial injection of an equivalent amount of 1716 did not cause any morbidity or mortality in control non-tumor-bearing C57Bl/6 mice ($n = 5$; data not shown).

Having demonstrated the ability of HSV-1 mutant 1716 to improve the outcome of intracranial melanoma-bearing mice and induce tumor regression, we initiated experiments designed to show if replication of 1716 was restricted to tumor cells as desired. Thus, we quantified the replication of 1716 in tumor by direct titration of infectious virus and compared this to titration data from non-tumor-bearing mouse brain for 1716 and the nonneuroattenuated parental virus 17^+. For 1716 the maximum possible input dose (5×10^5 PFU) was used. To allow reasonable survival time, an input dose of wild-type 17^+ of 10^3 PFU was used. For each point on the graph (including time 0, immediately postinoculation) the data represent the number of PFU of virus that was recovered from tissue homogenates. The presence of a tumor was easily confirmed in each mouse by visual observation of a pigmented mass at the time of brain removal.

As shown in Fig. 3, the titer of wild-type 17^+ from non-tumor-bearing brain increased exponentially over time as expected, reflecting efficient replication. No mice infected with this dose of 17^+ survived post Day 4. In contrast, the titer of 1716 recovered from non-tumor-bearing brain decayed over time, and infectious 1716 could no longer be isolated by Day 3 postinoculation. However, when 1716 was injected into brain tumors, significant replication occurred, as evidenced by recovery of an amount of infectious 1716 on Day 1 postinoculation that is more than 2 logs greater than that recovered at time 0 and substantially greater than the original input amount. Under these conditions, infectious 1716 could still be

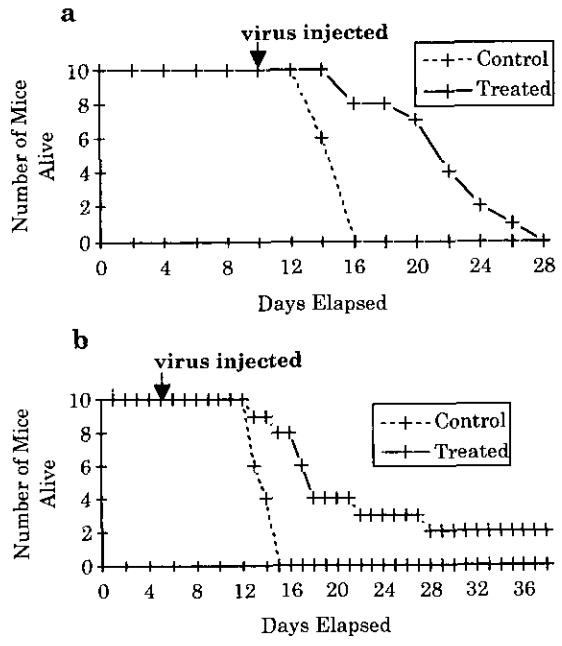

FIG. 2. Outcome experiment. C57Bl/6 mice were injected stereotactically in the right cerebral hemisphere with 5×10^4 Harding–Passey melanoma cells. After 10 days (a) or 5 days (b), 5×10^5 PFU of HSV 1716 was injected at the same stereotactic coordinates. The number of days elapsed between injection of tumor cells and time mice became moribund is shown on the x axis. Control mice were injected with an equal volume of viral culture medium at the appropriate time.

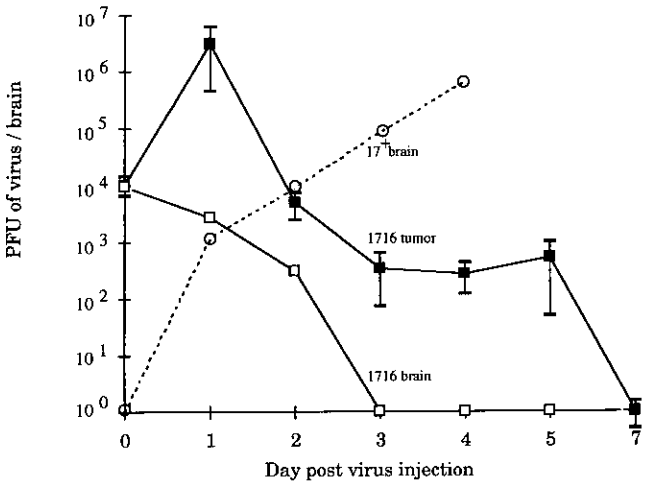

FIG. 3. Titration of virus from tumor and brain after infection. To investigate the extent of 1716 replication in brain tumors, C57Bl/6 mice were injected with Harding–Passey melanoma cells right of midline. Seven days later, each mouse was infected with 5×10^5 PFU of 1716 at the same stereotactic coordinates. At the times indicated (including Day 0), mice were sacrificed, and the brains were frozen in LN_2 and stored at −70°. Specimens were thawed rapidly and homogenized, and viral titration was performed in triplicate on BHK cells (■). These data represent the means of four mice at each time point ± standard deviation. To establish the growth characteristic of 1716 and wild-type 17^+ in brains without tumors, mice were injected intracranially with either 5×10^5 PFU 1716 (□) or 1×10^3 PFU of 17^+ (○). Mice were sacrificed at the times shown and processed as described above. Each point is the mean of two mice.

isolated from tumor-bearing mice on Day 5 postinoculation, but not on Day 7.

In the next series of experiments we used immunohistochemistry to investigate the specificity of 1716 replication at the cellular level. Figure 4a shows a representative section from a control animal, which did not receive virus. No immunohistochemical staining was seen with anti-HSV-1 in tumor or brain. Sections from tumor-bearing mice infected with 1716 are shown in Figs. 4b–d. Figure 4b is a low-power view of a section from a mouse 3 days post 1716 infection. HSV-1 antigen staining is localized to the periphery of the tumor mass. Figure 4c is a low-power view of a section from a mouse 6 days post 1716 infection. A significant number of melanoma cells within tumor were positive for HSV-1 antigen both within the large tumor mass and in smaller projections of tumor along the needle track. Figure 4d is a higher power view from another mouse 6 days post 1716 infection and again shows significant HSV-1 antigen staining within tumor, including smaller projections of tumor dispersed within brain. Moreover, in tumor-bearing mice treated with 1716, no HSV-1 antigen staining was seen in brain tissue adjacent to tumor or in any other areas of brain in all sections examined. In addition, no histologic evidence of encephalitis was seen in any 1716-treated mice at any time (Figs. 4b–d, and additional data not shown). Figure 4e shows a high-power view of a representative immunohistochemical section from a mouse in which tumor was infected with wild-type 17^+. In contrast to the findings with 1716, tumor-bearing mice infected with wild-type 17^+ virus exhibited multiple focal areas of HSV-1 immunohistochemical staining both within tumor and in surrounding and distant brain as well. As shown in Fig. 4e, there are a large number of neurons that stain with anti-HSV-1. A significant encephalitis characterized by perivascular polymorphonuclear leukocytes and extravasation of red blood cells is seen in areas of this and other sections examined. In control experiments, no immunohistochemical staining was seen with anti-HSV-1 in virally infected brain tumor sections subjected to the full immunohistochemical protocol with normal rabbit serum substituted for the primary anti-HSV-1 antibody (data not shown).

DISCUSSION

Our initial investigations were aimed at establishing a model system in which to test the viability of HSV-1 mutants as therapeutic agents for brain tumors. We chose melanoma because it is a common human malignancy with an incidence that is rising dramatically, that frequently metastasizes to brain, and for which no effective therapies exist (Balch *et al.*, 1983).

We examined three murine melanoma cell lines regarding their ability to be lysed efficiently by HSV-1 *in vitro* and to form intracranial tumors in C57Bl/6 mice. The Cloudman S1 melanoma arose in a C × DBA F1 mouse and has been reported to be tumorigenic in allogeneic C57Bl mice (Yasamura *et al.*, 1966). However, we were able to generate an intracerebral tumor in only 1 of 10 mice with these cells. The Harding–Passey melanoma cell line arose in an outbred mouse, and thus no syngeneic mouse strain exists. These cells have been reported to be tumorigenic in C57Bl/6 mice (Bleehen, 1974; Castillo *et al.*, 1982; Garcia-Borron *et al.*, 1985; Lopez-Ballester *et al.*, 1991; Schwabe *et al.*, 1990; Vicente *et al.*, 1987) and were so in our hands. The B-16 melanoma cell line arose in C57Bl/6 mice and is extremely tumorigenic (Fidler, 1973).

There is no doubt that H–P cells are tumorigenic in C57Bl/6 mice and that spontaneous rejection/regression of tumors does not occur. In our hands, tumor take is 100%, and all untreated tumors progress and ultimately cause death. The tumorigenic potential of H–P cells in C57Bl/6 mice is not limited to the intracerebral site, as we could generate subcutaneous tumor in 10 of 10 mice as well (Randazzo *et al.*, unpublished observation).

During our initial *in vitro* screening of murine melanoma cell lines, we were surprised to find that B-16 was completely resistant to lysis by all of the HSV-1 isolates tested. The host range of HSV-1 is extremely broad (Roizman, 1990), and thus it is likely that B-16 is unusual in its resistance to HSV-1 lysis and not that this is indicative

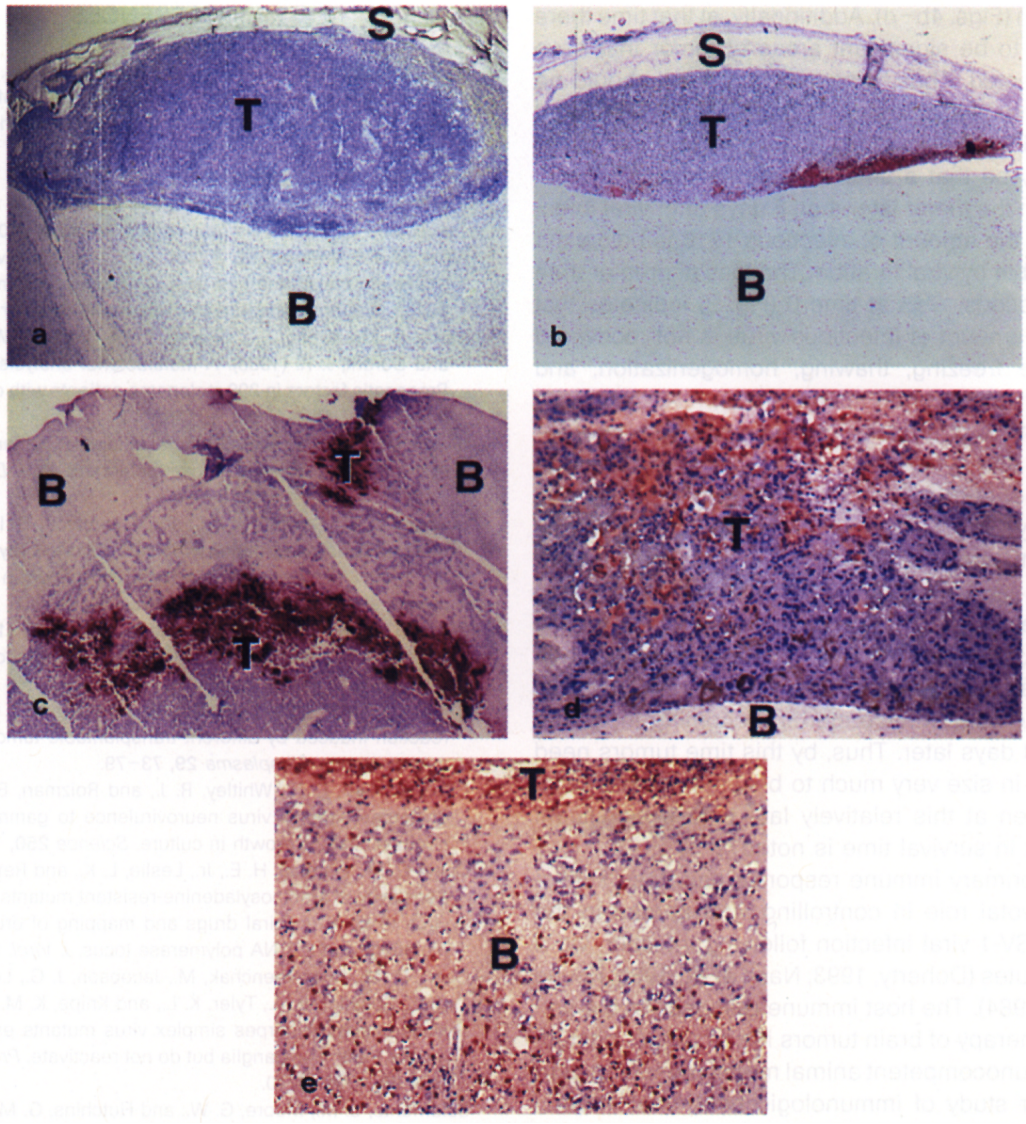

FIG. 4. Immunohistochemical staining of intracerebral melanoma for HSV-1 antigen following viral therapy. C57Bl/6 mice were injected stereotactically with 5×10^4 H–P cells. For the section in a mice were injected with 2 μl of viral culture medium at the same stereotactic coordinates 7 days after tumor cell injection and were sacrificed 3 days later. For the sections in b–d mice were injected with 5×10^5 PFU of HSV 1716 at the same stereotactic coordinates 7 days after tumor cell injection. At 3 (b) and 6 (c and d) days post-viral-infection, mice were sacrificed. For the section in e mice were injected with 1×10^3 PFU of HSV 17^+ 7 days after tumor cell injection. At 3 days post viral infection, mice were sacrificed. Following sacrifice, the heads were fixed and decalcified, and paraffin sections were made and reacted with polyclonal anti-HSV antibody. Immunoperoxidase was performed using an AEC substrate. In these sections decalcified skull stains very pale and is marked with the letter S, brain stains pale blue and is marked with the letter B, tumor stains darker blue and is marked with the letter T, and HSV antigen stains red.

of a general resistance of melanoma to HSV-1 lysis. In fact, 26 of 26 human melanoma cell lines we have examined to date are efficiently lysed by 1716 and 17^+ *in vitro* (Randazzo *et al.*, unpublished observation).

The restriction of 1716 replication to tumor tissue as judged by immunohistochemistry is striking. To date, we have examined multiple sections from 12 different mice (Figs. 4b–d and data not shown) and have never seen HSV-1 antigen staining in brain outside of tumor cells. Moreover, we do not see any histologic evidence of encephalitis during the acute phase of viral therapy. The lack of encephalitis was also noted by Markert *et al.* in a system utilizing HSV-1 R3616 to treat human glioma in nude mice (Markert *et al.*, 1993).

We were unable to find evidence of significant replication of 1716 in brain by titration, and these results are similar to those found by others examining γ34.5 null HSV-1 mutants (Bolovan *et al.*, 1994; MacLean *et al.*, 1991). While these data do not rule out the possibility of low-level replication of 1716 in brain, they suggest that 1716 replication is restricted to tumor cells in this system.

Direct comparison of the titration data with immunohistochemistry is difficult. By immunohistochemistry, HSV-1 antigen staining is seen in areas of tumor on Day 6 post

1716 infection (Figs. 4b–d). Additionally, at that time there still appears to be significant areas of tumor that have not been infected. Thus, it seems that there should be areas of tumor still permissive for 1716 replication at that time and that replication should continue into these areas. It may be that a smoldering infection is still occurring within the tumor later than 6 days after viral infection, but that the amount of infectious 1716 is below the level detectable by viral titration. The loss of greater than 1 log of infectious 1716 at time 0 (Fig. 3) indicates that a significant amount of infectious virus is not recovered following the freezing, thawing, homogenization, and centrifugation of the brain specimens.

Because of the aggressive take and growth rate of H–P tumors in this system, we were able to cure only a limited number of mice, and only when viral therapy was initiated early (Day 5) after implantation of tumor cells. At Day 5 the tumors are quite small, and we suspect that some of the early deaths in the treatment group may have been technical failures in which the injected virus missed the tumor, was essentially unable to replicate in brain, and was inactivated. By Day 10 tumors were large, and untreated control mice started becoming moribund as soon as 3 days later. Thus, by this time tumors need not increase in size very much to become symptomatic. However, even at this relatively late time a significant improvement in survival time is noted.

The host primary immune response has been shown to play a pivotal role in controlling viral spread in the context of HSV-1 viral infection following infection via a number of routes (Doherty, 1993; Nash *et al.*, 1985; Rouse and Lopez, 1984). The host immune response in the setting of viral therapy of brain tumors is completely unstudied. The immunocompetent animal model presented here will allow for study of immunologic host–viral interactions that are critical to determining the general applicability of viral therapy. Our inability to recover infectious virus 7 days after tumor infection is likely due in part to host antiviral immune responses, be they specific or nonspecific, and we are presently working to investigate this.

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Note added in proof. Subsequent to submission of our manuscript Chambers *et al.* (PNAS, 92:1411, 1995) reported on the efficacy of a γ34.5 null HSV-1 mutant in a human glioma-SCID mouse xenograft brain tumor system.

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