Experimental Therapy of Human Glioma by Means of a Genetically Engineered Virus Mutant

Robert L. Martuza,* Amy Malick, James M. Markert,†
Katherine L. Ruffner, Donald M. Coen

Malignant gliomas are the most common malignant brain tumors and are almost always fatal. A thymidine kinase–negative mutant of herpes simplex virus–1 (*dl*sptk) that is attenuated for neurovirulence was tested as a possible treatment for gliomas. In cell culture, *dl*sptk killed two long-term human glioma lines and three short-term human glioma cell populations. In nude mice with implanted subcutaneous and subrenal U87 human gliomas, intraneoplastic inoculation of *dl*sptk caused growth inhibition. In nude mice with intracranial U87 gliomas, intraneoplastic inoculation of *dl*sptk prolonged survival. Genetically engineered viruses such as *dl*sptk merit further evaluation as novel antineoplastic agents.

Gliomas are the most common primary tumors arising in the human brain (*1*). The most malignant glioma, the glioblastoma, represents 29% of all primary brain tumors or 5000 new cases per year in the United States (*2*). Despite surgery, chemotherapy, and radiotherapy, glioblastomas are almost always fatal, with a median survival rate of less than a year and a 5-year survival rate of 5.5% or less (*1–3*). After treatment, recurrent disease often occurs locally (*4*); systemic metastases are rare. Neurologic dysfunction and death are from local growth. No therapeutic modality has substantially changed the outcome of patients with glioblastoma (*2, 3*). Therefore, we are exploring a novel form of treatment: the use of a genetically engineered virus that will destroy glioma cells yet spare normal brain.

Malignant glioma cells are a dividing tumor cell population. In contrast, the surrounding normal brain is composed mostly of nondividing neurons and glia. Certain herpes simplex virus (HSV) mutants, including those that are deficient for the virus-encoded enzyme thymidine kinase (tk), can replicate in dividing cells but are severely impaired for replication in nondividing cells (*5, 6*) and for replication in the mammalian nervous system (*6–9*). We hypothesized that such an HSV mutant might replicate in gliomas yet spare normal brain so we tested the HSV-1 mutant *dl*sptk (*9*) because it completely lacks tk activity as a result of a 360–base pair deletion within the *tk* gene.

In cell culture, either the mutant (*dl*sptk) or its wild-type parent (KOS) was applied at multiplicities of infection (MOI) from 10^{-4} to 10^{1} in DME+ (Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics) onto a human glioma line (U87) and African Green monkey kidney (Vero) cells (*10, 11*). Cytopathic effects at 24 hours were proportional to the MOI and ranged from 1 to 5% of cells at MOI = 10^{-4} to 95% at MOI = 10. By day 9, >99% cytopathic effect was evident in U87 and Vero cells even for *dl*sptk at MOI = 10^{-4}. Thus, even the lowest inoculum of *dl*sptk sustained a spreading infection that destroyed the entire monolayer of U87 cells. We also tested *dl*sptk against a different human glioma line (T98G) at an MOI = 10^{1} and produced monolayer destruction within 4 days.

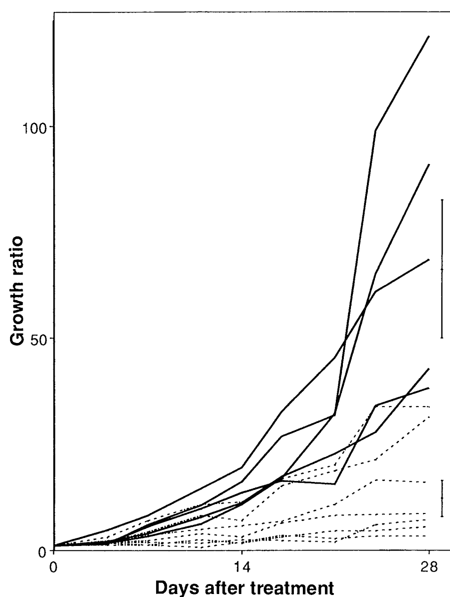

Fig. 1. Subcutaneous U87 tumors in nude mice were inoculated on day 0 with 5×10^{6} pfu of *dl*sptk and on day 14 with 1×10^{7} pfu of *dl*sptk (dotted lines). Control tumors (solid lines) were inoculated with an equal volume of DME+ alone on the same days. Tumors were measured twice weekly with calipers; volumes and growth ratios were calculated. We calculated the growth ratio by dividing the tumor volume on the day indicated by the volume of the same tumor on the day of the initial viral inoculation. Bars represent mean ± SE for each group.

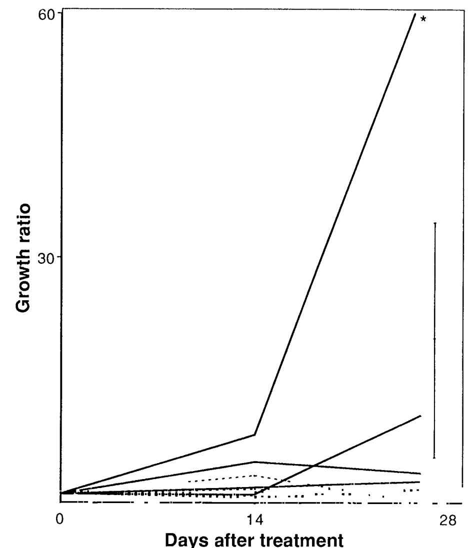

Fig. 2. U87 tumors grown in the subrenal capsule were inoculated on day 0 either with 2×10^{5} pfu of *dl*sptk (dotted lines) or with an equal volume of DME+ (solid lines). The line marked with an asterisk had a growth ratio of 62.3 on day 26. Bars represent mean ± SE for each group. Growth ratio was calculated as described in Fig. 1.

We established short-term glioma cultures by explanting three malignant human gliomas (one anaplastic astrocytoma and two glioblastomas obtained by surgery) in DME+ (*10*) and studied them at the second passage. In all three primary malignant gliomas, *dl*sptk (MOI = 10 or MOI = 1) had a cytopathic effect in a dose-dependent fashion. By day 4, >99% destruction was evident in all three cultures at both MOI tested.

To test the effects of *dl*sptk on human gliomas in vivo, we injected nude mice subcutaneously with 3.2×10^{6} U87 cells. Growing tumors (≥8 mm in diameter) were evident by week 5, at which time mice were divided equally into two groups. Six mice received an intraneoplastic inoculation of 5×10^{6} plaque-forming units (pfu) of *dl*sptk in 25 μl of DME+ and six controls received an inoculation of 25 μl of DME+ alone. After 2 weeks, all tumors were reinjected with twice the original inoculum of virus or medium (*12*). At day 28 (Fig. 1), virus-treated tumors were smaller than control tumors ($P < 0.001$, one-sided Wilcoxon rank test).

We next studied the effects of *dl*sptk on U87 cells grown in the subrenal capsule of the nude mouse, a site used for monitoring growth of other nervous system tumors (*13*). We implanted 1.5×10^{6} U87 cells in

R. L. Martuza, A. Malick, J. M. Markert, Molecular Neurogenetics Laboratory and Department of Surgery (Neurosurgery), Harvard Medical School, Massachusetts General Hospital–East, Charlestown, MA 02129.
K. L. Ruffner and D. M. Coen, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

*To whom correspondence should be addressed.
†Also of the Division of Neurosurgery, University of Michigan Medical Center, Ann Arbor, MI 48109.

the subrenal capsule of nine nude mice. After 10 days, the tumors were measured and inoculated with 2×10^5 pfu of *dl*sptk in 1 μl of DME+ or with 1 μl of DME+ alone. We reoperated on all mice 14 days and 26 days after inoculation to measure tumor size (*12*). At day 26 after inoculation (Fig. 2), virus-treated tumors were smaller than control tumors ($P < 0.01$, one-sided Wilcoxon rank test).

To evaluate the efficacy of *dl*sptk in treating intracerebral gliomas, we stereotactically inoculated 20 nude mice in the right frontal lobe with 1.6×10^5 U87 cells, a cell inoculum that in a pilot study caused 100% mortality within 1.5 months. After 10 days, the animals were divided randomly into three groups. One group received 10^3 pfu of *dl*sptk, a second group received 10^5 pfu of *dl*sptk, and controls received DME+ alone (*12*). Inoculations were in 2 μl of DME+ at the stereotactic coordinates initially used to inject the U87 cells. By week 7, all six controls were dead. In contrast, three of seven (43%) of the 10^3 pfu group were alive ($P = 0.12$ versus controls, one-tailed Fisher exact test) and four of seven (57%) of the 10^5 pfu group were alive ($P < 0.05$ versus controls, one-tailed Fisher exact test). By week 14, all seven of the 10^3 pfu group were dead, but two of seven (29%) of the 10^5 pfu group were still alive. These two animals were still healthy and neurologically normal at week 19 (Fig. 3), at which time they were killed. The entire brain was then fixed, serially sectioned at 7-μm intervals, stained with hematoxylin and eosin, and microscopically examined (*14*). Some evidence of encephalitis with scattered foci of lymphocytic infiltration in the meninges and brain was noted, but no definite evidence of tumor could be found in either brain.

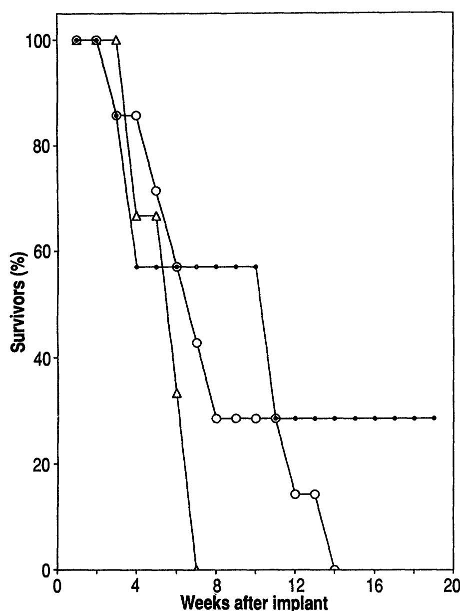

Fig. 3. Ten days after intracerebral injection of 1.6×10^5 U87 cells into each of 20 nude mice, the tumors were treated with either 10^3 pfu of *dl*sptk (open circles), 10^5 pfu of *dl*sptk (solid circles), or DME+ alone (triangles).

A concern for the therapeutic use of HSV against glioma is the potential for killing other dividing cells such as those of endothelium in brain vessels or at mucocutaneous sites after systemic spread. However, clinical studies indicate that even wild-type HSV-1 viruses generally neither spread far from the site of initial infection nor cause serious systemic disease in immune-competent individuals (*15*). Although mutants like *dl*sptk are relatively attenuated for neuropathogenicity (*6–9*), tk-deficient viruses have sometimes been associated with progressive disease in certain immunocompromised patients (*16, 17*). We do not know how pathogenic a pure population of a tk-negative virus would be in this setting; nevertheless, we sought to determine the sensitivity of *dl*sptk to antiviral drugs. This mutant is resistant to acyclovir (*9*), which requires tk for much of its antiviral activity. We therefore compared *dl*sptk and KOS in plaque reduction assays (*11*) for their sensitivities to vidarabine and foscarnet, which act without requiring viral tk activity and have been used to treat severe HSV infections (*17, 18*). We found that *dl*sptk was as sensitive to each of these drugs as its wild-type parent (Fig. 4).

Earlier investigators have used various wild-type or attenuated viruses to treat tumors in both animals and in humans (*19*). The proposed therapeutic mechanisms included, in some cases, direct cell killing by the virus, and, in others, the production of new antigens on the tumor cell surface to induce immunologic rejection. However, in all earlier studies, wild-type virus, passage-attenuated virus, or infected cell preparations were used. Genetically engineered viruses have not been previously tested.

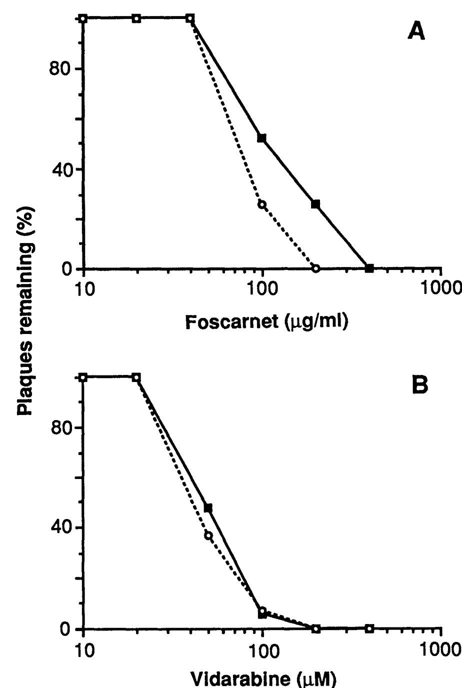

Fig. 4. The effects of (**A**) foscarnet and (**B**) vidarabine were tested against *dl*sptk (○) and its wild-type parent, KOS, (■) in a plaque reduction assay.

We have demonstrated that *dl*sptk is capable of destroying human glioblastoma cells both in cell culture and in nude mice. Further genetic alterations of this virus might be used to increase specificity, to further decrease neuropathogenicity, or to provide alternative mechanisms of tumor cell modulation or killing. Experiments to test the efficacy of *dl*sptk in the presence of an immune response to HSV will also be important. Our study suggests that genetically altered viruses are worthy of further exploration as a means of therapy for some tumors, such as malignant human gliomas, that are resistant to currently available treatments.

REFERENCES AND NOTES

1. B. S. Schoenberg, in *Oncology of the Nervous System*, M. D. Walker, Ed. (Nijhoff, Boston, 1983), pp. 1–30; A. L. Levin, G. E. Sheline, P. H. Gutin, in *Cancer: Principles and Practice of Oncology*, V. T. DeVita, S. Hellman, S. A. Rosenberg, Eds. (Lippincott, Philadelphia, ed. 3, 1989), vol. 2, pp. 1557–1611.
2. M. S. Mahaley, Jr., C. Mettlin, N. Natarajan, E. R. Laws, Jr., B. B. Peace, *J. Neurosurg.* **71**, 826, (1989).
3. W. R. Shapiro *et al.*, *ibid.*, p. 1; Y. Onoyama *et al.*, *Am. J. Roentgenol.* **126**, 481, (1976); O. M. Salazar, P. Rubin, M. L. Feldstein, R. Pizzutiello, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1733 (1979); M. D. Walker *et al.*, *N. Engl. J. Med.* **303**, 1323 (1980); C. Daumas-Duport, B. Scheithauer, J. O'Fallon, P. Kelly, *Cancer* **62**, 2152 (1988); T. S. Kim, A. L. Halliday, E. T. Hedley-Whyte, K. Convery, *J. Neurosurg.*, in press.
4. F. H. Hochberg and A. Pruitt, *Neurology* **30**, 907 (1980).
5. A. T. Jamieson, G. A. Gentry, J. H. Subak-Sharpe, *J. Gen. Virol.* **24**, 465 (1974).
6. H. J. Field and P. Wildy, *J. Hyg.* **8**, 267 (1978).
7. R. B. Tenser, R. L. Miller, F. Rapp, *Science* **205**, 915 (197
8. H. J. Field and G. Darby, *Antimicrob. Agents Chemother.* **17**, 209 (1980).
9. D. M. Coen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4736 (1989). Two additional advantages of *dl*sptk are (i) that it cannot revert spontaneously to the tk+ phenotype and (ii) that its deletion lies outside the UL24 gene and thus should not be impaired for UL24 function in replication in glioma cells.
10. Human glioma lines U87 and T98G cells were obtained from the American Tissue Cell Collection (ATCC, Camden, NJ). Three primary human malignant glioma cultures were started from explants obtained from surgical specimens after consent was obtained under the guidelines of the Massachusetts General Hospital Subcommittee on Human Studies. Vero (African Green monkey kidney) cells were obtained from P. Schaffer. Viruses were grown and titered on Vero cells as previously described (*11*) and are recorded as plaque-forming units per specified volume.

11. D. M. Coen, H. E. Fleming, Jr., L. K. Leslie, M. J. Retondo, *J. Virol.* **53**, 477 (1985).
12. Animal studies were done in accordance with guidelines for animal care by the Massachusetts General Hospital Subcommittee on Animal Care. Female nude mice (NCr/Sed nu/nu) were obtained from the Edwin L. Steele Laboratory for Radiation Biology at the Massachusetts General Hospital. Nude mice were anesthetized with 0.3 ml (intraperitoneal) of Avertin (2,2,2-tribromoethanol, 99%, and *tert*-amyl alcohol, 99%, Aldrich Chemical Co., Inc., Milwaukee, WI). Animals dying within 48 hours of any procedure were considered perioperative deaths and were excluded from analysis. Also excluded was one animal in the subcutaneous series in which the inoculum went into the subcutaneous space and not intraneoplastic. Procedures involving viruses were in accordance with the guidelines of the Harvard Office of Biological Safety. Viral inoculation and subsequent care of animals harboring a virus was done in an approved designated viral vector room. Statistical analyses were performed by D. Schoenfeld, Massachusetts General Hospital Biostatistics Center.
13. A. Medhkour, M. Van Roey, R. A. Sobel, H. J. Fingert, R. L. Martuza, *J. Neurosurg.* **71**, 545 (1989); J. K. Lee, T. S. Kim, E. Z. Chiocca, A. Medhkour, R. L. Martuza, *Neurosurgery* **26**, 598 (1990).
14. L. G. Luna, *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology* (McGraw-Hill, New York, ed. 3, 1968).
15. L. Corey and P. G. Spear, *N. Engl. J. Med.* **314**, 749 (1986).
16. K. S. Erlich *et al.*, *ibid.* **320**, 293 (1989).
17. S. L. Sacks *et al.*, *Ann. Intern. Med.* **111**, 893 (1989).
18. M. S. Hirsch and R. T. Schooley, *N. Engl. J. Med.* **309**, 963 (1983); *ibid.*, p. 1034; M. M. Youle *et al.*, *Lancet* **ii**, 341 (1988); P. A. Chatis, C. H. Miller, L. E. Schrager, L. S. Crumpacker, *N. Engl. J. Med.* **320**, 297 (1989); K. S. Erlich *et al.*, *Ann. Intern. Med.* **110**, 710 (1989).
19. H. Kobayashi, *Adv. Cancer Res.* **30**, 279 (1979); F. C. Austin and C. W. Boone, *ibid.*, p. 301; C. Sauter *et al.*, in *Current Chemotherapy: Proceedings of the Tenth International Congress of Chemotherapy*, W. Siegenthaler and R. Lüthy, Eds. (American Society for Microbiology, Washington, DC, 1978), pp. 1112–1114; W. A. Cassel, D. R. Murray, H. S. Phillips, *Cancer* **52**, 856 (1983); K. H. Burdick and W. A. Hawk, *ibid.* **17**, 708 (1964); H. E. Pearson and D. L. Lagerborg, *Proc. Soc. Exp. Biol. Med.* **92**, 551 (1956); M. Sigel, A. Bernstein, L. P. Pope, *J. Immunol.* **75**, 386 (1955); A. E. Moore, *Prog. Exp. Tumor Res.* **1**, 411 (1960); C. M. Southam, *Trans. N.Y. Acad. Sci.* **22**, 657 (1960).
20. We thank D. Schoenfeld, Massachusetts General Hospital Biostatistics Center, for help with statistical analysis of the data; J. P. Vonsattel for neuropathological analysis of the specimens; E. Messner for technical assistance; X. O. Breakefield, L. B. Jacoby, and M. Hirsch for critical advice in manuscript preparation; and the Massachusetts General Hospital Neurosurgery Service for providing tumor specimens. Supported by grants from NF Inc. (Massachusetts Bay Area) to R.L.M. and from the National Institutes of Health (NS24279 to R.L.M.; RO1-AI26126, SO7RRO5381, and PO1AI2410 to D.M.C.).

3 October 1990; accepted 25 February 1991

Identification of a Peptide Specific for *Aplysia* Sensory Neurons by PCR-Based Differential Screening

JEAN-FRANÇOIS BRUNET, ELI SHAPIRO, SHARON A. FOSTER, ERIC R. KANDEL, YUICHI IINO

In order to identify genes specific for the sensory neurons of *Aplysia*, a miniaturized differential screening method based on the polymerase chain reaction and applicable to small amounts of tissue was used. One messenger RNA was isolated that is expressed in every mechanoreceptor sensory cluster of the *Aplysia* central nervous system. This messenger RNA encodes a peptide that seems to function as an inhibitory cotransmitter. The peptide selectively inhibits certain postsynaptic cells but not others and thereby allows the sensory neurons to achieve target-specific synaptic actions.

Each of the two symmetrical pleural ganglia of *Aplysia californica* contains a homogeneous cluster of about 200 medium-sized neurons, which are primary mechanoreceptors and which have receptive fields that include the tail, the foot, the parapodium, and the body walls of the animal (*1*). These cells make up the afferent pathway of the monosynaptic component of the tail withdrawal reflex, which, like the gill and siphon withdrawal reflex (*2*), shows both short-term and long-term behavioral plasticity (*3*). The two homogeneous clusters have been useful for the biochemical study of presynaptic changes underlying short-term and long-term facilitation of the sensory to motor cell synapse (*4*). To explore further the characteristics of this synapse, we have sought to identify molecules specific to the sensory neurons. As a first step, we looked for transcripts differentially expressed between the pleural sensory cells and another identified neuron R2, the excitatory motor neuron to the mucous cells in the foot (*5*). Toward this end we devised a method for differential screening of cDNA libraries which matches the best documented sensitivity of conventional methods, yet requires only a small amount of tissue such as single neurons or clusters of cells.

Howard Hughes Medical Institute and Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, 722 West 168 Street, New York, NY 10032.

We amplified whole populations of cDNA in vitro by using the polymerase chain reaction (PCR) methodology (*6*). The first strand of cDNA was synthesized from total RNA by priming with an oligo dT–containing primer-adapter (T primer). After removal of the T primer, a tail of dC was added and the second strand synthesis was primed with an oligo dG–containing primer-adapter (G primer). This second strand, now flanked with two distinct and known sequences, was amplified by PCR with the T and G primers (7). A whole population of heterogeneous sequences can thus be coamplified. This in vitro amplified cDNA is inserted into a vector to generate a library (*8*). In addition, the in vitro amplified cDNA can also be labeled and used as a total cDNA probe (*9*). Thus, we could overcome the limitation of starting material for both library and probe.

We used this technique to isolate genes differentially expressed between pleural sensory neuron clusters (positive) and the motor neuron R2 (negative). In the first round of differential screening, we selected nine clones (SCR2-A to SCR2-I) that hybridized with the sensory neuron cluster probe but not with the R2 probe. Cross-hybridization revealed that they were all copies from a single mRNA species, hereafter designated PSC1 (pleural sensory cluster 1). We next performed a Northern blot on RNA derived from two pleural sensory clusters (*10*) and obtained a band of 0.7 kb using SCR2-I as a probe. No detectable signal was apparent on an equivalent amount of RNA derived from R2, thus verifying the differential expression of PSC1 (Fig. 1). When we used SCR2-I as a probe on two other independently generated libraries, we assessed the frequency of this clone at about 1 to 3% of the library. Moreover, this method allowed us to detect much rarer messages at an abundance of 0.03%, which is 100-fold below that of PSC1.

Fig. 1. Differential expression of PSC-1 in sensory cells and the motoneuron R2. (**Upper panel**) The insert from clone SCR2-I was used as a probe on 1.5 μg each of total RNA from R2 (R2), sensory clusters (SC), and the central nervous system (CNS). The hybridization was in 5× standard saline citrate (SSC), 50% formamide 5× Denhardt solution, 0.1% SDS, and salmon sperm DNA (100 μg/ml). The blot was washed for 20 min each in 2× SSC plus 0.1% SDS and 0.2× SSC plus 0.1% SDS. It was exposed for 12 hours at −70°C with an intensifying screen. (**Lower panel**) Same blot as in the upper panel rehybridized with cDNA encoding F4, a ubiquitous polyadenylated ribosomal RNA. Exposed for 3 hours.