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Delivery of herpesvirus and adenovirus to nude rat intracerebral tumors after osmotic blood–brain barrier disruption

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ABSTRACT **The delivery of viral vectors to the brain for treatment of intracerebral tumors is most commonly accomplished by stereotaxic inoculation directly into the tumor. However, the small volume of distribution by inoculation may limit the efficacy of viral therapy of large or disseminated tumors. We have investigated mechanisms to increase vector delivery to intracerebral xenografts of human LX-1 small-cell lung carcinoma tumors in the nude rat. The distribution of *Escherichia coli lacZ* transgene expression from primary viral infection was assessed after delivery of recombinant virus by intratumor inoculation or intracarotid infusion with or without osmotic disruption of the blood–brain barrier (BBB). These studies used replication-compromised herpes simplex virus type 1 (HSV; vector RH105) and replication-defective adenovirus (AdRSVlacZ), which represent two of the most commonly proposed viral vectors for tumor therapy. Transvascular delivery of both viruses to intracerebral tumor was demonstrated when administered intraarterially (i.a.) after osmotic BBB disruption ($n = 9$ for adenovirus; $n = 7$ for HSV), while no virus infection was apparent after i.a. administration without BBB modification ($n = 8$ for adenovirus; $n = 4$ for HSV). The thymidine kinase-negative HSV vector infected clumps of tumor cells as a result of its ability to replicate selectively in dividing cells. Osmotic BBB disruption in combination with i.a. administration of viral vectors may offer a method of global delivery to treat disseminated brain tumors.**

Gene therapy, using recombinant viral vectors, has recently gained prominence as a potential therapeutic modality for malignant gliomas and central nervous system (CNS) neoplasms (1, 2). One strategy is to target the herpes simplex virus thymidine kinase (HSV-tk) gene to tumor cells, rendering them sensitive to the antiviral agent ganciclovir (1–5). In most studies evaluating gene therapy of experimental brain tumors, the delivery of the recombinant viral vectors was accomplished by direct stereotaxic inoculation of the intracerebral tumor. However, brain tumors such as malignant gliomas and metastases are rarely localized to one brain region and often spread to other areas. The scheme of using retroviral vector packaging cell lines to transfer HSV-tk to brain tumor cells has recently been approved for use in human subjects following the encouraging results of preliminary *in vitro* and animal experiments. Antitumor activity has been observed, but gene delivery was observed only 5–25 cell diameters from the injection site.††

Our laboratory has been studying ways of facilitating greater access of therapeutic agents to brain and intracerebral neoplasms by temporary osmotic opening of the blood–brain barrier (BBB) (6). The BBB is a single layer of brain capillary endothelial cells that are bound together by tight junctions, which excludes entry of blood-borne molecules >180 Da and particularly large and complex biological molecules such as viruses from the brain (6, 7). Even the leaky barrier at the brain–tumor interface is relatively impermeable to such particles. Intraarterial (i.a.) administration of hypertonic mannitol leads to a reversible and transient opening of the BBB (6). We have previously demonstrated the feasibility of global delivery of a variety of agents including antibodies, chemotherapeutic drugs, and imaging agents throughout the cerebral hemisphere after osmotic modification of the BBB (6, 8). This technique may also be applicable for global delivery of viral particles (7, 9, 10).

In the present study, we have compared the ability of replication-compromised HSV (HSV-tk$^-$ RH105) (11, 12) and replication-defective adenovirus (AdRSVlacZ) (9, 13) to transfer the *Escherichia coli lacZ* gene into intracerebral tumors consisting of human small-cell lung carcinoma (LX-1) xenografted in nude rats. We tested the feasibility of using osmotic BBB disruption (BBBD) to increase vector delivery to brain tumors by comparing the effects of direct inoculation of the recombinant viruses to their intracarotid infusion with and without osmotic disruption of the BBB.

MATERIALS AND METHODS

Viral Vectors. The HSV mutant RH105 bears a mutation in the HSV-tk gene with the *lacZ* gene under control of the HSV IE3 promoter inserted into the HSV-tk locus (12). RH105 is replication-compromised in that it can replicate only in dividing cells (11). The titer of the HSV vector was determined to be 1×10^{10} plaque-forming units (pfu)/ml by plaque-forming assays on confluent monolayers of Vero African green monkey kidney cells (1).

The adenovirus mutant AdRSVlacZ contains a deletion in IE1 and a partial deletion in IE3 rendering it replication defective (9, 13). The *lacZ* gene has been inserted in IE1 under control of the Rous sarcoma virus promoter (9, 13). Freshly prepared virus stock, concentrated by gradient centrifugation in CsCl, was desalted by G50 Sephadex chromatography. A titer of 1.5×10^{12} particles per ml was determined by measuring absorbance at 260 nm. This has previously been shown to represent $1–5 \times 10^{10}$ pfu/ml by plaque assay (13).

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Abbreviations: CNS, central nervous system; HSV, herpes simplex virus; tk, thymidine kinase; BBB, blood–brain barrier; BBBD, BBB disruption; pfu, plaque-forming unit; i.a., intraarterial(ly).

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Table 1. Delivery of adenovirus and HSV to nude rat intracerebral tumors

No. of animals	Titer	Time of sacrifice	% tumor labeling	Normal brain labeling
		Adenovirus by direct inoculation		
4	1×10^9	2 days	None*	None*
2	1.2×10^9	4 days	8–10	1–2+
		HSV by direct inoculation		
3	2.4×10^7	2 days	10–30*	1–2+ (necrosis)
1	2.4×10^8	4 days	5–10*	1–2+ (necrosis)
3	2.4×10^8	4 days	30–50	2–5+ (necrosis)
1	2.4×10^8	1 day	60	3–5+ (necrosis)
		Adenovirus by BBBD		
1	2×10^{10}	4 days	1–2	3–5 glial cells
2	5×10^{10}	4 days	1–3	None
3	2×10^{11}	3–5 days	1–2	0–2 glial cells
1	5×10^{11}	1 day (died)	0	None
4	5×10^{11}	4 days	1–3	0–20 glial cells
		HSV by BBBD		
1	1×10^8	3 days	10–15	None
1	4×10^8	3 days	5–10	None
1	5×10^8	3 days	0*	None*
3	5×10^8	2–3 days	1–30	0–2 cells labeled
1	5×10^9	1 day (died)	10–15	None

In direct inoculation studies, staining was graded as follows: 1+, ≈10%; 2+, ≈20%; 3+, ≈30%; 4+, ≈40%; 5+, ≈50% of cells labeled. Titers are expressed as particle numbers for adenovirus and pfu for HSV.

*Animals analyzed by 5-bromo-3-chloro-3-indolyl β-D-galactoside histochemistry.

LX-1 Cells and Tumors. Lung carcinoma cell line LX-1 was suspended at a concentration of 9×10^4 cells per μl. Adult female nude rats (rnu/rnu) from the National Institutes of Health breeding colony (weighing 220–250 g) were anesthetized (ketamine, 50 mg/kg; xylazine, 2 mg/kg; i.p.) and inoculated stereotaxically with 10 μl of the cell suspension in the right caudate/putamen as described (14).

Virus Delivery by Direct Inoculation. Recombinant viral vectors were administered to nude rats, 6 days after tumor-cell inoculation by stereotaxic injection into the intracerebral tumor, using the same anesthesia and coordinates as described (14). Viral vectors (RH105, 2.4×10^8 pfu, $n = 8$; AdRSVlacZ, 1×10^9–10^{11} particles, $n = 6$) suspended in 24 μl of 0.9% (wt/vol) saline were delivered over a 20-min period.

Virus Delivery by BBBD. Six days after tumor implantation, nude rats were anesthetized with isoflurane inhalant (5% induction, 2% maintenance) in an air atmosphere, and mannitol (25% in H_2O) warmed to 37°C was infused cephalad via a catheter in the right external carotid artery, at a rate of 0.12 ml/sec using a constant flow pump (Harvard Apparatus) as described (14). Rats received either RH105 ($n = 7$) or AdRSVlacZ ($n = 9$) administered as an i.a. bolus at the dose indicated in Table 1 (1 ml over 1 min) immediately after the mannitol infusion. Control animals (sham modification) received normal saline (i.a.) instead of mannitol, followed by a similar amount of virus ($n = 4$ for RH105; $n = 8$ for AdRSVlacZ).

Animals that received adenovirus were sacrificed 2–5 days after virus administration with the majority sacrificed at 4 days (Table 1). Animals receiving HSV were sacrificed at 2 days after vector administration, corresponding to periods of peak transgene expression, as determined by preliminary studies (Table 1) and published data (13, 15).

Immunohistochemistry. After sacrifice, the rats were perfusion-fixed prior to serial sectioning at 100 μm (in the coronal plane) with a Vibratome (Oxford). Histochemical detection of β-galactosidase was initially used as described (14), but immunocytochemical detection gave more consistent results. Tissue sections were immunocytochemically labeled with purified polyclonal (rabbit) antibodies to *E. coli* β-galactosidase (Cappel) as described (16). The extent of transgene expression in tumor was determined by the ratio of the number of immunoreactive cells to the total number of tumor cells in randomly chosen, thionin counterstained coronal sections of tumor-bearing brain. The extent of gene transfer to normal brain and brain around tumor was estimated by a 1–5 grading system (1+, ≈10%; 2+, ≈20%; 3+, ≈30%, 4+, ≈40%; 5+, ≈50% of cells labeled).

RESULTS

***lacZ* Transgene Expression After Delivery of Virus to Rat Brain by Direct Inoculation.** Both AdRSVlacZ and RH105 infected the tumor and normal brain around tumor after direct intratumoral inoculation (Figs. 1 and 2*A*) The extent of adenovirus-mediated gene expression was considerably less

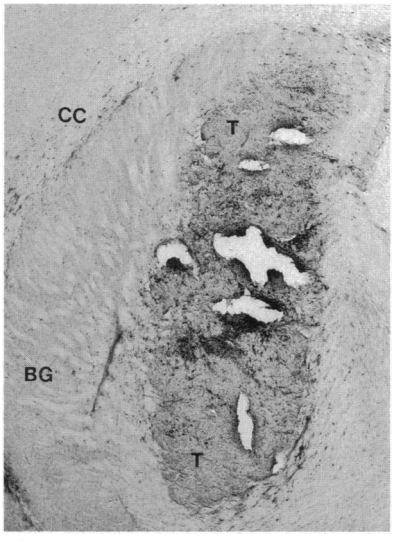

FIG. 1. Coronal section through tumor-bearing region of rat brain labeled for β-galactosidase 4 days after inoculation of AdRSVLacZ (1.2×10^9 particles). About 10% of tumor cells are labeled for the transgene product, which is dispersed as clumps in the tumor xenograft. T, tumor; BG, basal ganglia; CC, corpus callosum. (×17.)

than that seen with the HSV vector RH105, despite the use of 100-fold higher adenoviral titers (Table 1). After adenovirus inoculation, the extent of *lacZ* gene expression within the tumor varied between 5 and 10% of the total tumor cell mass, with the stained cells being in dispersed clumps scattered over a wide area of the tumor xenograft (Fig. 1). Parenchymal cells expressing β-galactosidase reactivity could be detected in cells with the morphological appearance of astrocytes extending along the corpus callosum (Fig. 1). No cytopathic effects were noted. The tumor cells expressing the *lacZ* gene product (Table 1) in the xenograft did not show evidence of oncolysis, and there was no evidence of inflammation, cell death, or cavitation in brain parenchyma adjacent to tumor or along the corpus callosum.

Tumors that received tk^- HSV by direct inoculation, in contrast, frequently demonstrated necrotic centers after 4 days (Fig. 2*A*). Intense β-galactosidase reactivity was observed in tumor cells that were immediately adjacent to these necrotic regions (Fig. 2*A*). Approximately 50–60% of the tumor xenograft was immunoreactive for the β-galactosidase transgene product. The parenchymal cells adjacent to the tumor were also infected, with immunoreactivity detected primarily within neurons (Fig. 2*B*). Infection could also be traced in neurons along the corpus callosum (Fig. 2*A*), with no astrocytic profiles being apparent as noted with the adenoviral vector (Fig. 1). Tissue damage was evident in normal brain with necrosis evident along the corpus callosum (Fig. 2*A*).

LacZ Transgene Expression Following Viral Delivery by BBBD. Transvascular delivery of both viruses to intracerebral tumor was achieved when the recombinant vectors were administered i.a. after osmotic BBBD (Figs. 3*A* and 4). No viral infection or β-galactosidase transgene product could be detected in tumor or normal brain around tumor of sham-disrupted animals (where saline was used in lieu of mannitol). These control experiments indicate that the brain capillary endothelium is not sufficiently compromised, even at the "leaky" brain–tumor interface, to allow transvascular entry of either viral vector after i.a. delivery.

Adenoviral infection could be detected in the tumor xenograft (Table 1) after transvascular delivery with osmotic BBBD, as evidenced by β-galactosidase staining (Fig. 3). Only 2–3% of tumor cells were immunoreactive 4 days after BBBD (Fig. 3*A*), with the infected cells organized as isolated clusters within the tumor (Fig. 3*B*). Although no labeling of parenchymal cells could be detected at the tumor–brain interface, immunoreactive cells were found scattered throughout the osmotically disrupted cerebral hemisphere. A few brain regions, such as the hippocampal dentate gyrus (Fig. 3 *C* and *D*), the thalamus, and the perisagittal cortex, showed the highest density of stained cells. The immunolabeled parenchymal cells in the disrupted cerebral hemisphere appeared to be primarily astrocytes, and the neurons did not appear to be labeled. There was no evidence of necrosis in immunostained regions of the tumor, at the brain–tumor interface, or along the corpus callosum (Fig. 3*A*).

HSV infection following BBBD, in contrast, was confined essentially to the tumor xenograft (Fig. 4). The immunolabeled cells in the tumor accounted for 20–30% of the tumor cell mass. The β-galactosidase staining was organized in clumps, and areas of necrosis were often evident at the centers of the labeled tumor cell clusters. No labeling could be detected in normal brain parenchyma of the disrupted cerebral hemisphere distant from the tumor, although in a few animals some neurons at the tumor–brain interface were stained for β-galactosidase. In contrast to results of the direct inoculation studies, there was no necrosis of the brain parenchyma along the corpus callosum or any other region of the disrupted cerebral hemisphere. The only necrosis observed was confined selectively to the tumor xenograft.

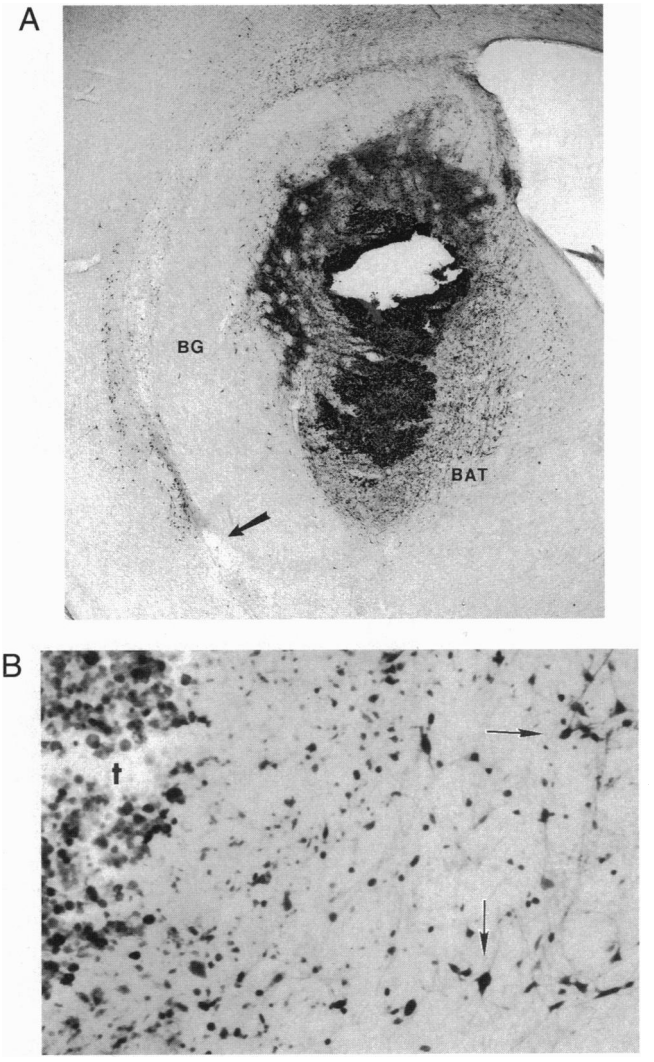

FIG. 2. Coronal section through tumor-bearing region of rat brain labeled for β-galactosidase 4 days after inoculation with HSV RH105 (2.4×10^8 pfu). (*A*) Labeling can be seen within tumor cells in the xenograft (≈50% stained), parenchymal cells in the brain around tumor (BAT), and within neurons along the corpus callosum. Some necrosis is apparent along the corpus callosum (arrow). BG, basal ganglia. (×15.) (*B*) Immunolabeling within tumor cells and primarily within neurons in brain parenchyma (arrows) at the brain–tumor interface. t, Tumor. (×60.)

Mortality Studies. No mortality was observed in the animals that received the recombinant viral vectors (RH105, $n = 8$; AdRSVlacZ, $n = 6$) by direct stereotaxic inoculation. Histological analysis of brain was performed on all injected animals (Table 1).

Of the 13 animals in the control group (sham modification) that received AdRSVlacZ i.a. after saline administration, 8 survived (38.5% mortality) and were used for histological analysis. All four controls (sham modification with saline) administered RH105 survived (0% mortality). Of a total of 16 tumor-bearing nude rats that received AdRSVlacZ by BBBD, 7 died after this procedure (mortality rate, 44%). The brains of 11 survivors were processed for histology as described. Nine animals were administered RH105 by BBBD, 3 of which died (mortality rate, 33%). Histological analysis was performed on the 6 survivors and on 1 animal that died 24 hr after virus administration.

DISCUSSION

Gene therapy using recombinant viral vectors holds considerable promise as an approach for delivery of oncolytic mole-

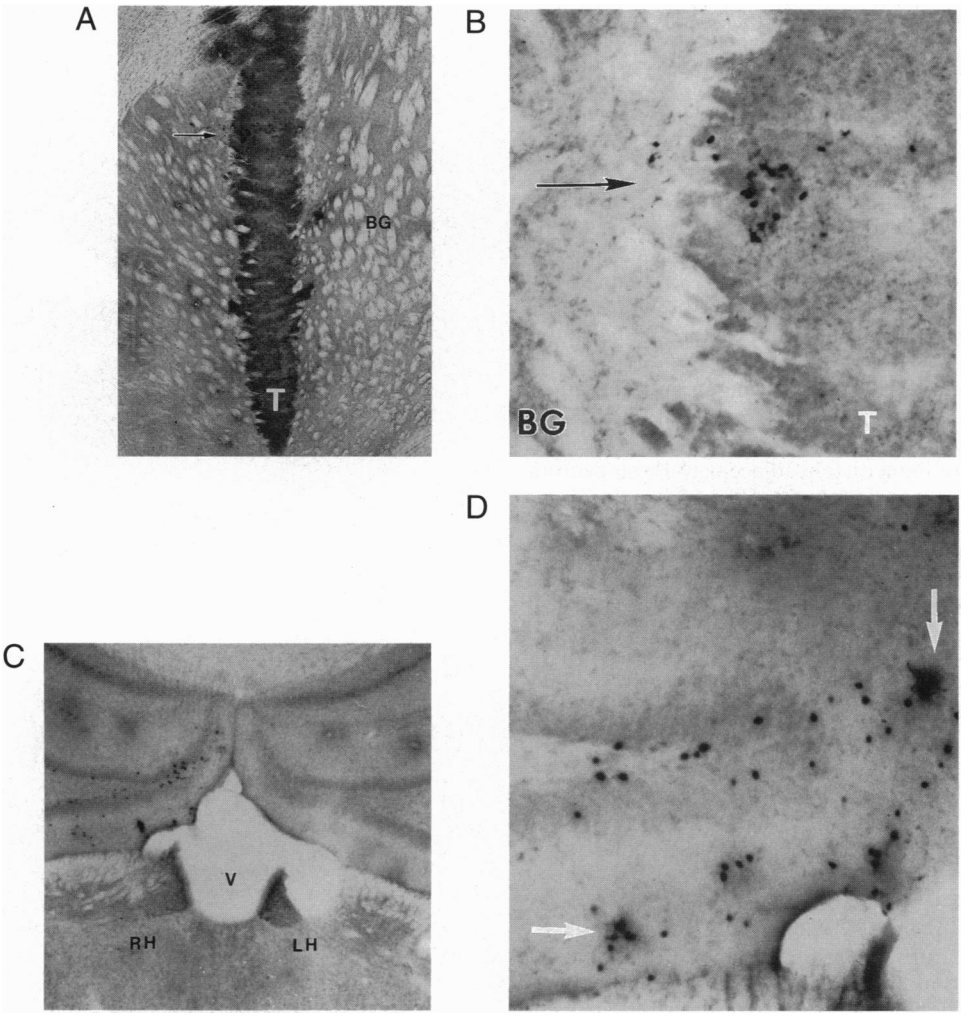

FIG. 3. Coronal section through tumor-bearing region of rat brain stained for β-galactosidase after BBBD administration of AdRSVLacZ (5×10^{11} particles). (*A*) A very small region of the tumor (2–3%) is positive for β-galactosidase (arrow). (×15.) (*B*) This higher magnification through the tumor shows discrete immunostaining within a clump of tumor cells (arrow). (×60.) (*C*) Low-power photomicrograph through the hippocampal and thalamic regions, showing labeling of parenchymal cells in the disrupted right hemisphere (RH) and not in the nondisrupted left hemisphere (LH). V, ventricle at midline. (×15.) (*D*) Glial profiles can be identified in the hippocampal dentate gyrus (arrows). (×60.)

cules to CNS neoplasms (1–4). There is, nevertheless, a critical need for more global access of the viral vector to brain and intracerebral neoplasms. In the present report, we have evaluated the efficacy of BBBD for delivery of viral vectors as gene transfer vehicles to intracerebral human lung tumor cells in a nude rat model. The results demonstrate that the blood–tumor barrier does not normally allow entry of either adenovirus or HSV to the intracerebral tumor. Transient disruption of the barrier, however, facilitates access of both vectors. These encouraging preliminary results suggest a potential role for BBB modification in conjunction with intravascular administration of viral vectors as a means of facilitating a more global delivery of these agents in the treatment of disseminated brain tumors.

Striking differences in β-galactosidase staining in brain around tumor and brain distant to tumor may be due to differential distribution of the two vectors following BBBD. The HSV vector is larger than adenovirus and consequently may be excluded from normal brain on the basis of size. Increased intracranial fluid pressure from the tumor mass may further serve to exclude the larger HSV vector from the normal brain. Another possibility may relate to the cell-type partiality of gene expression seen with these two vectors. We have found that AdRSVlacZ results in preferential transgene expression in astrocytes, whereas RH105 infection appears to be preferentially confined to neurons. We have also shown that neuronal infection with RH105 can occur after BBBD in normal rodent brain (10). In tumor-bearing animals, the combination of alterations in pressure dynamics from the tumor and associated increased brain water, the large size of the HSV virion, and the cell-type specificity for gene expression may limit RH105 infection strictly to the tumor.

The ability of the RH105 HSV vector to replicate within dividing tumor cells and its spread by lysogenic cell death should result in greater viral titers in the tumor with time (11), in contrast to the replication-defective adenovirus AdRSV-lacZ. The low level of tumor transduction observed with adenovirus in this and other studies may still be sufficient to effect cell killing using the HSV-tk gene to confer susceptibility to ganciclovir (1–4). The transfer of toxic metabolites from the transduced to the uninfected tumor cells could result in eventual destruction of a significant population of noninfected cells by the "bystander effect" (17). Ross *et al.* (5) have also reported significant changes in tumor doubling times after direct inoculation of a tk-expressing adenovirus into intracranial gliomas. While the precise mechanism for this growth retardation by the "bystander effect" is currently unknown, some studies have suggested a role for the immune system in mediating this effect (18, 19).

The current generation of HSV vectors retain an unacceptably high level of neurotoxicity. Infection of the brain after

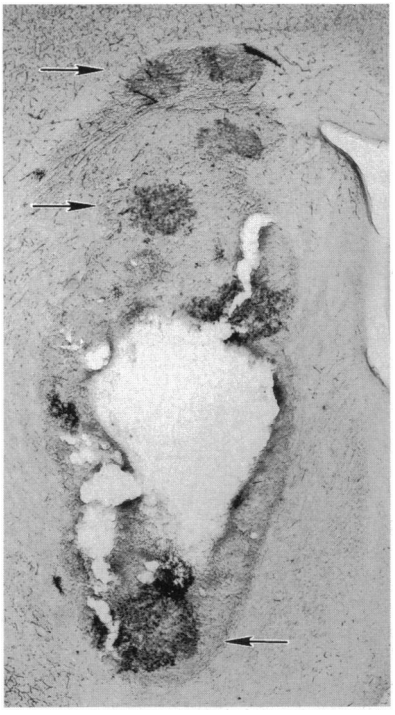

FIG. 4. Coronal section through tumor-bearing region of rat brain stained for β-galactosidase 3 days after BBBD administration of RH105 (5×10^8 pfu). About 20–30% of tumor cells can be seen to be immunoreactive for β-galactosidase in clumps (arrows). There is no labeling of brain parenchyma adjacent to the tumor or along the corpus callosum. The only necrosis observed is confined to the tumor xenograft. (×17.)

direct inoculation of RH105 was associated with necrosis at the inoculation site and along the corpus callosum. In contrast, no histological evidence of neurotoxicity was observed after adenoviral inoculation at the titers used. Other investigators also have reported on cytotoxic effects with replication-defective HSV vectors (15, 20). The precise cause for HSV toxicity is unclear. Mutation of the viral ribonucleotide reductase (RR1) gene reduces toxicity 10^6-fold (21). RR^--HSV mutants, such as hrR3 (1), hold promise as antitumor agents because their HSV-tk gene is intact, allowing the use of antiviral agents such as ganciclovir for oncolysis. This vector/drug combination has been shown to be effective in a rat model of glioma (1); BBBD delivery of this vector in our intracerebral tumor model remains to be evaluated.

Mortality was observed when the RH105 virus was delivered by BBBD. Similar toxic effects were not observed in the sham-disrupted animals that received equivalent amounts of i.a. HSV, but mortality was observed in sham-disrupted animals that received the adenovirus. More recent BBBD experiments with RR^- HSV mutant hrR3 (10) have revealed no procedure-related deaths with i.a. delivered virus. The relative contributions of CNS and/or systemic toxicity in the mortality observed in the present studies are unclear. Detailed histological studies to determine the precise underlying pathology and the extent of both systemic and CNS viral toxicity associated with the use of these vectors remain to be done. Our previous studies, however, have demonstrated BBBD to be a safe and effective procedure in humans, and clinical trials of enhanced chemotherapy delivery have already demonstrated the efficacy and safety of osmotic BBBD in a variety of malignant brain tumors including glioma, lymphoma, germ cell tumors, and primitive neuroectodermal tumors (6, 22).

Our results demonstrate that both adenovirus and HSV may have utility for tumor therapy when delivered with BBBD. The tk^- HSV vector appeared to spare normal brain after barrier opening, while infecting a larger percentage of the tumor, probably as a result of its ability to replicate in tumor cells. Adenoviral-mediated gene expression appeared to be more limited within the tumor, probably reflecting the fact that it is replication defective. Adenovirus-mediated gene delivery was noted throughout the disrupted cerebral hemisphere and may therefore also be effective against disseminated tumor cells. The necrosis observed in normal brain tissue after direct inoculation of RH105 may limit the applicability of inoculations for tumor gene therapy. In contrast, the absence of infectivity and necrosis in brain around and distant to tumor following the transvascular administration of the HSV vector, the increased number of β-galactosidase-labeled tumor cells, and the selective oncolysis noted with BBBD delivery imply major potential for the recombinant HSV vector for antitumor therapy when administered by BBBD. These results may provide a mechanism to extend the promising results of virus/drug combination therapy of small rodent brain tumors to the large and disseminated tumors found in human patients.

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