



# Enhancement of replication of genetically engineered herpes simplex viruses by ionizing radiation: a new paradigm for destruction of therapeutically intractable tumors

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Human U-87 malignant glioma xenografts in mice were exposed to ionizing radiation, inoculated with a herpes simplex virus 1 mutant R3616 lacking both copies of the  $\gamma_134.5$  gene, or received both virus and radiation. Dual treatment caused a significantly greater reduction in volume or total regression of tumors than either radiation or infection alone. The significantly enhanced oncolytic

effects of the combined treatment correlate with two- to five-fold enhanced replication in irradiated tumor cells than in tumors receiving virus only. In addition, *in situ* hybridization with viral DNA probes showed that infected tumor cells were the dominant landscape of irradiated tumors and much less apparent in the nonirradiated tumors administered this virus.

**Keywords:** malignant gliomas; mouse models; attenuated herpesviruses

## Introduction

Malignant gliomas are the most common primary intracranial tumor and are largely incurable with conventional therapy. More than 90% of tumors recur within the initial tumor site.<sup>1,2</sup> Among novel approaches to treatment of malignant gliomas are genetically engineered viruses which themselves are cytotoxic or capable of delivering genes whose products render the cells susceptible to cytotoxic agents. Assessment of the capacity of herpes simplex viruses (HSV) for treatment of malignant gliomas has taken two approaches. The first set of recombinant viruses tested lacked one or more genes (eg thymidine kinase or ribonucleotide reductase) which reduced viral growth in nondividing cells.<sup>3–5</sup> The specificity of the cytotoxic effects of these mutant viruses for cancer cells was relatively modest. The second set of recombinant viruses tested centered on the deletion of both copies of the  $\gamma_134.5$  genes from the HSV-1 genome.<sup>3,6–11</sup> This gene encodes two functions: the capacity to replicate in the central nervous system cells maps throughout the gene whereas the capacity to block the phosphorylation of the  $\alpha$  subunit of the translation initiation factor eIF-2 and thereby prevent premature shutoff of protein synthesis maps in the 3' domain of the gene.<sup>12–15</sup> While  $\gamma_134.5$  mutants discriminate better between normal and malignant cells in terms of cytotoxicity, additional factors are required for total destruction of tumors in experimental animal models.<sup>12,16</sup>

Here we report that in a nude mouse xenograft model the tumoricidal effects of infection with mutant R3616 lacking both copies of the  $\gamma_134.5$  gene and ionizing radiation were significantly greater than those of R3616 or of radiation alone. Additionally, R3616 grows to higher titers in irradiated tumors than in nonirradiated tumors.

## Results

### *Effect of combined treatment with attenuated HSV-1 and ionizing radiation on tumor growth*

In the first two series of experiments, tumors produced in hind limb of mice by inoculation of 1 to 2 mm<sup>3</sup> tissue fragments of U-87MG cells and which have attained a volume of 200 mm<sup>3</sup> to 300 mm<sup>3</sup> were randomized into four groups as follows: (1) controls consisting of mice given no injection and mice injected with buffer solution; (2) mice administered on day 0 10  $\mu$ l ( $2 \times 10^7$  plaque forming units (p.f.u.)) of R3616 or buffer with a Hamilton syringe; (3) mice subjected to ionizing radiation and either not injected or injected with buffer solution; and (4) control mice or mice administered R3616 and subjected to irradiation for 20 Gy at 1 day after infection or mock infection and with 25 Gy at 2 days after infection or mock infection. Although large for conventional clinical radiation fractionation protocols, fraction sizes of 20–25 Gy are employed in stereotactic radiosurgery<sup>17–19</sup> and preliminary experiments suggest that smaller radiation doses will also be effective. The tumor mass was measured biweekly for 60 days or at least until the tumor mass reached 2000 mm<sup>3</sup>.<sup>18</sup> Fractional tumor volume was defined as tumor volume at the specified time-point divided by the initial tumor volume ( $V/V_0$ ). A tumor was

considered as having regressed if its volume was <10% of the original tumor volume.

The design of the third series of experiments was similar to the first two except that the tumor mass was inoculated on each of 3 successive days with  $2 \times 10^7$  p.f.u. of R3616 and irradiated 4 h after the second and third inoculation with 20 Gy and 25 Gy, respectively. The results of individual mice tumor volume are shown in Figures 1a–h. Figure 2 shows the average fractional tumor volume response for each group in pooled experiments 1 and 2 (a) and experiment 3 (b). The results were as follows:

- (1) All untreated tumors grew progressively to 2000 mm<sup>3</sup> and, in accordance with the animal use protocols approved by the University of Chicago, they were killed between days 21 and 30 after mock infection (Figures 1a and e).
- (2) In animals receiving radiation alone, the fractional tumor volume was  $2.42 \pm 0.78$  in the pooled group (experiments 1 and 2) and  $2.16 \pm 0.62$  in the third experiment at 60 days after initiation of treatment. Complete regression of tumor was observed in three

of 18 animals in the pooled experiments (Figure 1b) and none of 12 animals in the third experiment (Figure 1f).

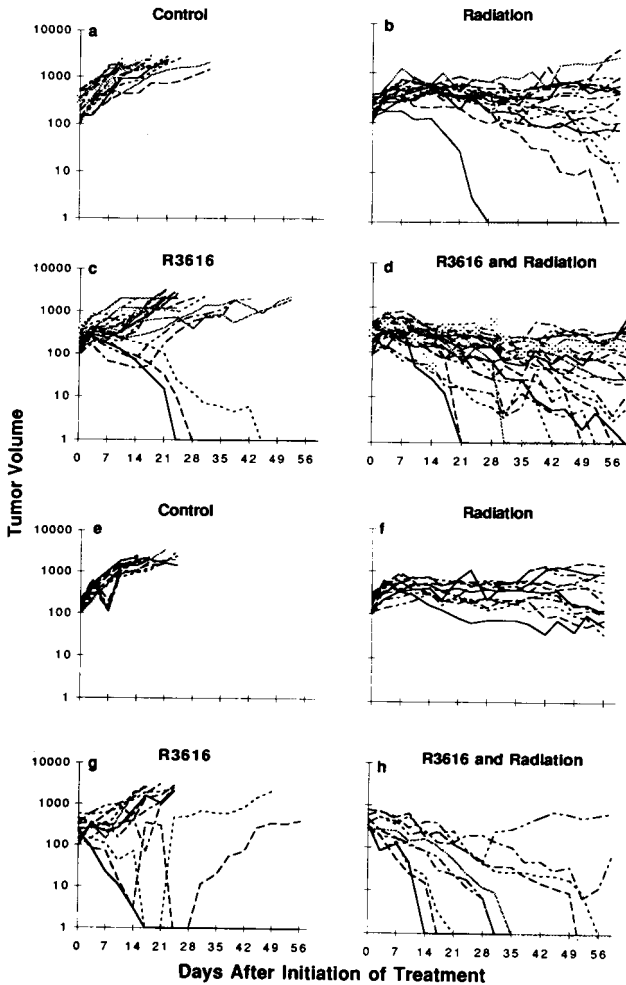
- (3) In animals injected with a single dose of  $2 \times 10^7$  p.f.u. of R3616, 18 of 21 tumors grew progressively to a volume of 2000 mm<sup>3</sup> by day 30 (Figure 1c). In the remaining three animals, the tumors were eradicated by day 60.
- (4) In animals receiving three daily consecutive injections of R3616 ( $2 \times 10^7$  p.f.u. per day) the tumors grew to a volume of 2000 mm<sup>3</sup> in 11 of 12 animals with complete tumor regression in the remaining animal (Figure 1g).
- (5) In animals inoculated with one dose of  $2 \times 10^7$  p.f.u. of R3616 followed by 45 Gy in two fractions, the fractional tumor volume was  $0.41 \pm 0.13$  mm<sup>3</sup>. Complete tumor regression was obtained in 13 of 23 animals by day 60 (Figure 1d).
- (6) Tumors treated with three injections of R3616 combined with 45 Gy had an average fractional tumor volume of  $0.19 \pm 0.16$ . Complete tumor regression was observed in nine of 10 mice (Figure 1h).

Table 1 shows the number of complete tumor regressions obtained in each experiment. The results of pooled experiments 1 and 2 showed that the test for interaction between R3616 and radiation was significant ( $P = 0.003$ ) which implies an interaction between these two therapies.<sup>20</sup> The significant interaction between radiation and R3616 in the analysis of variance model implies that the effect of combined therapy is greater than the sum of each therapy alone. A similar analysis was done for experiment 3 which again showed a significant interaction between R3616 and radiation ( $P = 0.006$ ).

#### Viral replication in irradiated tumor cells

The significantly higher cure rates raised the question of whether the recombinant virus R3616 replicated differently in irradiated and nonirradiated tumors. To answer this question, tumors in groups of three were infected with  $2 \times 10^7$  p.f.u. of R3616 or infected with R3616 and received 45 Gy, excised on days 3, 5, 7 and 14 after infection and assayed for the quantity of virus in each tumor. The quantity of virus recovered from irradiated tumors was consistently higher than that recovered from unirradiated tumors on days 3, 5 and 7 days after infection (Figure 3a). Moreover, *in situ* hybridization studies of tumor sections shown in Figure 4 indicate that the number of infected cells in irradiated tumors was far greater and spread out more distantly from the needle track than that in nonirradiated tumors.

The carboxyl terminal domain of the protein encoded by  $\gamma_134.5$ , the gene deleted from R3616, is homologous to the corresponding domain of a conserved mammalian gene GADD34 (growth arrest and DNA damage gene 34).<sup>4,21</sup> GADD34 complements and substitutes for the  $\gamma_134.5$  gene to block the phosphorylation of eIF-2a.<sup>21</sup> Therefore the possibility existed that GADD34 induced following DNA damage by ionizing radiation complemented R3616 and allowed a higher level of viral replication.<sup>22</sup> To determine whether the effects of irradiation were specific for  $\gamma_134.5^-$  R3616, the experiment was repeated with R7020, another genetically engineered attenuated virus.<sup>22</sup> Figure 3b shows that the yields of R7020 from tumors were higher than those of R3616 as would be expected since R7020 is less attenuated. In



**Figure 1** Individual tumor volumes of experiments 1–3. Tumor volumes from experiments 1 and 2 (a–d). Mice were injected with  $2 \times 10^7$  p.f.u. of R3616 on day 0 and subjected to ionizing radiation at 20 Gy on day 1 and 25 Gy on day 2. Individual tumor volume measurements over time for each treatment group in experiment 3 (e–h). Experiment 3 was similar to Nos 1 and 2 except that virus was administered on days 0, 1 and 2.

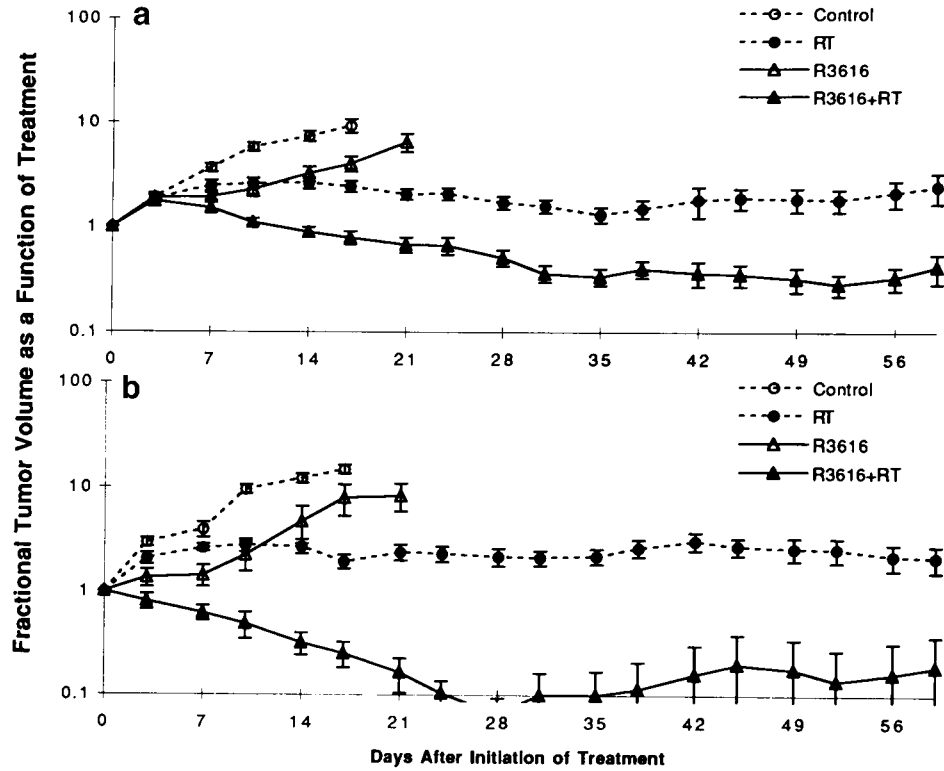


Figure 2 Average fractional tumor volumes as a function of treatment for experiments 1 to 3. (a) Experiments 1 and 2; (b) experiment 3.

Table 1 Complete U-87MG tumor regression based on treatment

Treatment	Experiments 1 and 2	Experiment 3
Control	0/17	0/12
Radiation	3/18	0/12
R3616	3/21	1/12
R3616 and radiation	13/23	9/10

addition, the yields of virus from irradiated tumors were higher than those from nonirradiated tumors. Thus, the capacity of tumor cells to support viral replication is augmented by ionizing radiation and is not specific for  $\gamma_{134.5^-}$  mutants.

### Discussion

The key discovery reported here is that the combined effects of ionizing radiation and infection with the R3616 recombinant virus were significantly greater than that of infection or ionizing irradiation alone ( $P < 0.006$ ). Malignant gliomas have been known to be resistant to radiation. While attenuated herpesviruses alone have not been tested in humans, the available data in experimental animals do not predict a high cure rate. For example, although nude mice are immunologically incapacitated, the capacity of R3616 to replicate and destroy cancer cells was restricted. Our studies with R3616 recombinant virus alone are consistent with the results in similar experimental systems, but with other genetically engineered

viruses. Specifically, infection alone produced few cures and the majority of infected tumors either grew more slowly or outpaced cell destruction.<sup>3-9</sup> Since these cells are susceptible to virus-induced cytopathic effects, the mechanisms responsible for the apparent resistance to virally mediated cytotoxicity *in vivo* are unclear.

The observed enhancement of viral replication in X-irradiated tumor may explain the interactive effects of the combined treatment. Although virus yields from irradiated tumors were only two- to five-fold higher, the results were reproducible. Moreover, the yields may underestimate the actual increase in the amounts of virus produced inasmuch as the distribution of infected cells exhibiting viral DNA by *in situ* hybridization was far greater in irradiated tumors (Figure 4).

Our studies raise two issues. The first is whether increasing the amount of virus could have the same effect as the combined radiation and infection. In the studies reported here injecting R3616 on 3 consecutive days showed no enhancement of tumor regression over a single injection. The cytotoxic effect appears to be limited by the amount of virus that infects tumor cells along the needle track. Irradiation may allow the virus to propagate further away from the needle track to allow for more complete regression of tumors.

The second issue concerns the mechanism by which ionizing radiation enhances viral replication. The hypothesis most attractive at this time is that: (1) ionizing radiation complements the defects resulting from deletions in the recombinant viruses by inducing cellular gene(s) encoding immediate-early products, cytokines and/or proteins involved in DNA repair, etc; (2) these proteins alter the metabolism and render glioma cells more susceptible to replication of viruses; and (3) the effect may

## Materials and methods

### Cells and viruses

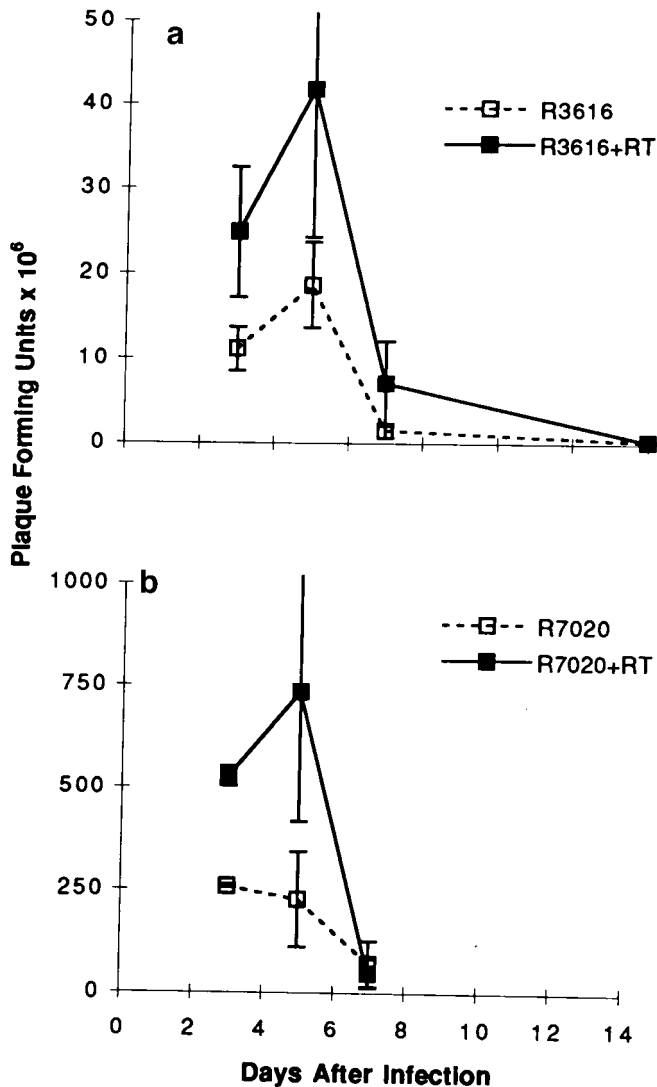
The African green monkey Vero cell line and human GBM cell line, U-87MG, were obtained from American Type Culture Collection (Rockville, MD, USA). The genetically engineered R3616 was derived from HSV-1(F), a prototype HSV-1 wild-type virus and lacks both copies of the  $\gamma_134.5$  and ORF P genes.<sup>3</sup> In the genetically engineered R7020 virus U<sub>1</sub>24, U<sub>1</sub>55 and U<sub>1</sub>56, and one set of inverted repeats each encoding one copy of the genes  $\alpha 0$ ,  $\gamma_134.5$ , ORF P and  $\alpha 4$ , was replaced by a DNA fragment derived from HSV-2 strain G encoding glycoproteins G, J, D and I.<sup>22</sup> Virus titers were determined on Vero cells as previously described.<sup>12,21</sup>

### Tumor regression studies

U-87MG cells in amounts of  $1 \times 10^7$  cells per mouse were suspended in sterile phosphate-buffered saline and injected subcutaneously into the right hind limb of a 5- to 6-week-old nude mouse and grown to 200 mm<sup>3</sup> (Fredrickson Cancer Research Institute, Bethesda, MD, USA). The tumor was then excised, cut into 1–2 mm<sup>3</sup> pieces which were then subcutaneously implanted into the right hind limb of mice, and allowed to grow to 200 mm<sup>3</sup>, and randomized into four groups as follows: (1) untreated controls and controls administered a buffer solution with a Hamilton syringe; (2) mice administered on day 0 10  $\mu$ l of R3616 or buffer with a Hamilton syringe; (3) mice subjected to ionizing radiation only; and (4) mice administered R3616 and subjected to irradiation. For irradiation mice were immobilized in lucite chambers and their whole body was shielded with lead except for the tumor bearing hind limb. This limb was irradiated with a Maxitron-250 X-ray generator (General Electric, Milwaukee, WI, USA; 191 cGy per min) for 20 Gy at 1 day after infection or mock infection and with 25 Gy at 2 days after infection or mock infection. The radiation doses were chosen to make apparent any interaction between radiation and virus. Although large for conventional clinical radiation fractionation protocols, fraction sizes of 20–25 Gy are employed in stereotactic radiosurgery.<sup>17–19</sup> The tumor mass was measured biweekly for 60 days or at least until the tumor mass reached 2000 mm<sup>3</sup>. Tumor volumes were calculated using the formula (length  $\times$  width  $\times$  height)/2 which is derived from the formula for an ellipsoid ( $\pi d^3$ )/6. Animal studies were done in accordance with a protocol approved by the Animal Resource Center at the University of Chicago. Fractional tumor volume is defined as tumor volume at the specified time-point divided by the initial tumor volume ( $V/V_0$ ). Animals with tumor volumes of 2000 mm<sup>3</sup> were killed.

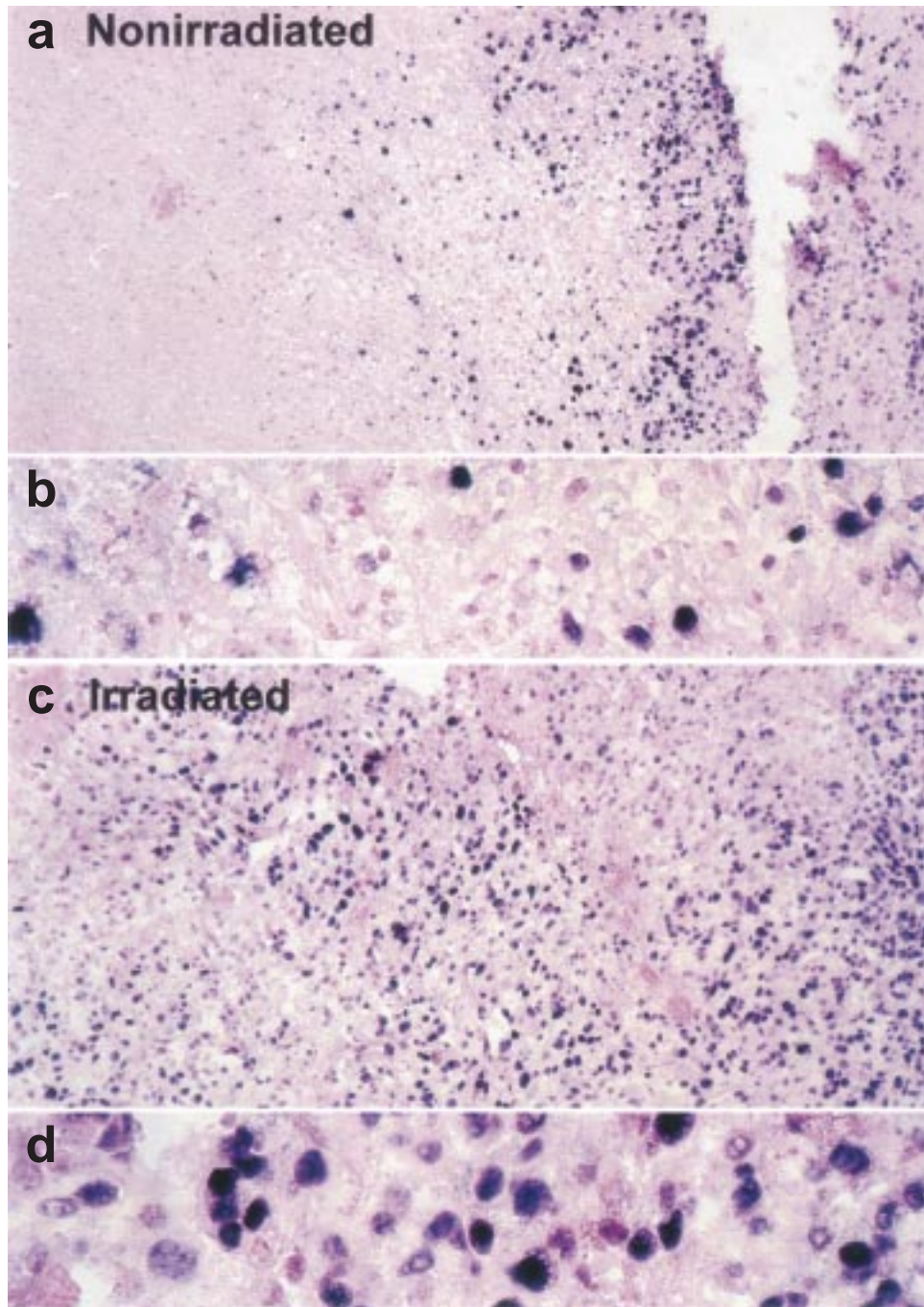
### Statistical analysis

The changes in tumor volume were analyzed by modeling time as a regression variable for each animal.<sup>20,23</sup> The percentage change of tumor volume from baseline as a function of time was calculated using a linear regression model without intercept. The slopes from the regression of the percentage change of tumor volume over time were then analyzed in a variance model that included the effect of radiation alone, R3616 alone, and the combination of radiation and R3616.



**Figure 3** Recovery of R3616 (a) or R7020 (b) virus from tumor tissue. The treatment protocol described in the text was identical to that used in experiments 1 and 2. The tumors were then harvested and assayed for virus 3, 5, 7 and 14 days after viral infection. The error bars show  $\pm 1$  standard deviation.

be transient in that once these proteins cease to function or turn over, the cells again become resistant to viral infection. If this hypothesis is tenable, our observation could lead to two practical applications. The first would require identification and cloning into the genetically engineered viral genome factors that render the cells more amenable to destruction as a consequence of viral replication. The second and currently more practical approach is to define the experimental conditions that would result in sustained synthesis of these factors by repeated irradiation after specific time intervals. The advantage of combining radiation and genetically engineered HSV irradiation stems is that ionizing radiation can be targeted to a confined target volume leading to a restricted environment in which the virus replicates at an enhanced rate.



**Figure 4** Photographs of *in situ* hybridizations with viral DNA probes in tumors harvested 7 days after infection. (a) Nonirradiated tumor, low magnification; (b) same, high magnification; (c) irradiated tumor, low magnification; (d) same, high magnification. The needle tracks are visible in (a) and (c).

*In situ* hybridization of HSV DNA in U-87 Gg xenografts  
U-87MG tumors were grown to  $\geq 200 \text{ mm}^3$  in the nude mouse hind limb and were injected with  $2 \times 10^7$  p.f.u. on day 0 followed by irradiation with 20 Gy on day 1 and with 25 Gy on day 2. Tumors were removed 7 days after infection, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, mounted on to Teflon-coated target slides (Ventana Medical Systems, Tuscon, AZ, USA), baked at  $60^\circ\text{C}$  for 1 h, cleared in xylene and

hydrated through a descending alcohol series. The HSV DNA *in situ* assays were done on two to three tumors per treatment group in a Ventana Gen automated immunohistochemistry and *in situ* system using a biotinylated probe prepared by nick translating two HSV genomic clones, an 8 kb fragment from HSV-1 and a 16 kb fragment from HSV-2 (Enzo Diagnostics, Farmingdale, NY, USA). The automated *in situ* system used avidin-AP as a detection system and nitroblue tetrazolium as a substrate

producing a blue-black reaction product in HSV-infected cells. The slides were counterstained with nuclear fast red, cleared and coverslipped.

#### Harvesting of U-87MG xenografts for virus titrations

Tumors were each harvested aseptically at specified times after infection in 1 ml of medium 199V and homogenized for 20 s using the Polytron tissue homogenizer (Kinematica, Switzerland). The homogenate was then sonicated three times for 15 s each. Before freezing at  $-80^{\circ}\text{C}$ , 1 ml of sterile skim milk was added to the suspension to stabilize the virus. Virus titers were obtained previously.<sup>12,21</sup>

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