Award Number: DAMD17-98-1-8490

TITLE: Herpes Virus Therapy of Prostate Cancer

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REPORT DATE: August 2001

TYPE OF REPORT: Final, Phase I

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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**REPORT DOCUMENTATION PAGE**

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13. ABSTRACT (Maximum 200 Words)

The development of replication-competent viral vectors has offered a distinct advantage for prostate cancer gene therapy because of viral multiplication and spread within the tumor. G207, a second generation, multi-mutated HSV-1 vector has been shown to be very effective against human prostate cancer cell lines in vitro and in vivo following both direct intranecoplastin inoculation as well as following systemic intravenous inoculation. Moreover this efficacy applies to both androgen-sensitive as well as androgen-insensitive tumors and for post-irradiation recurrences. We now demonstrate that in a rodent prostate cancer model, NV1023 was more effective against Tramp tumors than either G207 or G47 delta, a G207 derivative. Following intranecoplastin therapy, the survival of NV1023 treated mice was extended by 5 weeks as compared to mock mice. Moreover in a bilateral tumor model, the virus treated (left) tumor volume was reduced to approximately one third the size of mock tumors. The untreated (right) tumors also regressed as expected with the tumor volume reduced to half the size of mock tumors.

We have completed our studies of G207 toxicity following intraprostatic inoculation in animal models and demonstrated that G207 can be safely inoculated into the prostate. We have also demonstrated that sequential rather than simultaneous irradiation may be as beneficial for vectors such as G207 in treating prostate cancer.

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<td>Prostate Cancer</td>
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- Ionizing radiation does not alter the antitumor activity of herpes simplex virus vector G207 in subcutaneous models of human and murine prostate cancer. Jorgensen TJ, Katz S, Varghese S, Rabkin SD, Martuza RL. (Submitted for publication)
**INTRODUCTION:** Our goal has been to develop oncolytic herpes vectors to treat prostate cancer and to acquire the necessary data to advance toward clinical trials. The development of replication-competent viral vectors has offered a distinct advantage for prostate cancer gene therapy because of viral multiplication and spread within the tumor. Replication-competent mutants of HSV-1 have been shown to be highly effective against a variety of experimental tumor models. Oncolytic viral vectors based on engineered herpes simplex viruses offer unique advantages for prostate cancer therapy. They have minimal inherent immunogenicity and are much larger (153 kb) than those based on adenovirus or retrovirus backgrounds and thus can contain larger therapeutic gene elements. Safety features include well-identified genes for neurovirulence that can be deleted, and the availability of systemic anti-herpetic agents that can abort an unwanted infection. G207, a second generation, multi-mutated HSV-1 vector has been shown to be very effective against human prostate cancer cell lines in vitro and in vivo following both direct intraneoplastic inoculation as well as following systemic intravenous inoculation. Moreover this efficacy applies to both androgen-sensitive as well as androgen-insensitive tumors and for post-irradiation recurrences.

**BODY OF PROGRESS REPORT:**

**HYPOTHESIS 1:** When inoculated intraneoplastically into a murine prostate cancer in an immune competent syngeneic mouse, HSV will induce a cell-mediated anti-tumor response in both the primary inoculated tumor as well as in distant metastases, and will prevent or delay the development of prostate cancer.

As noted in our prior progress report, herpes cytopathic activity in rodent cells is known to be far less efficient than in human cells and this delayed our work in murine syngeneic models to some extent. Moreover, the double gene mutations of G207 further attenuated replication in rodent cells. In Tramp mice (transgenic mice carrying the SV40 large T antigen gene under the control of the rat probasin promoter so that they spontaneously develop prostate tumors, which progressively metastasize) we have begun to study other less attenuated HSV vectors. Tramp prostate cancer cell lines established from these mice grow well on syngeneic parental C57Bl/6 mice and thus provide for an immunocompetent model system to study both direct as well as immune mediated oncolytic effect. A direct comparison of the tumoricidal efficacy of G207, G47 delta and NV1023 was conducted using the Tramp prostate tumor model. Subcutaneous tumors generated by injecting 1 x 10^7 Tramp cells into the left flank of C57Bl/6 mice were allowed to grow until a diameter of 5-6 mm. At that time they were treated intratumorally twice, on days 0 and 3, with 1 x 10^7 pfu of either G207 or G47 delta or NV1023. Results, as shown in Fig. 1A, demonstrate that NV1023 was more effective against Tramp tumors than either G207 or G47 delta. Survival of the treated mice was monitored until the tumor grew to a diameter of 21 mm, at which time the mice were sacrificed. As shown in Fig. 1B, the survival of NV1023 treated mice was extended by 5 weeks as compared to mock mice.

![Figure 1A](image1a.png)

![Figure 1B](image1b.png)

G47 delta is derived from G207 and contains a 312 bp deletion in the US11 and ICP47 genes in addition to the deletions of both copies of ICP34.5 gene and a lacZ insertion inactivating the ICP6 gene. ICP47 gene product is associated with the down regulation of MHC class I molecules on the cell surface and therefore absence of this protein...
provides for enhanced immune recognition of tumors. NV1020 is an HSV1-HSV2 hybrid and retains one intact copy of the ICP34.5 gene. NV1023, derived from NV1020, has a deletion of the ICP47 gene similar to G47 delta. Since NV1023 has a deletion in ICP47 that renders an enhanced immunogenic effect, we tested the immune effect of NV1023 on contralateral Tramp tumors. For this, subcutaneous tumors were established on the left and right flanks of C57Bl/6 male mice by injecting 5 x 10^6 Tramp tumor cells on each flank. When the tumors reached 5-6 mm in diameter, the left tumor was treated with two inoculations of 1 x 10^7 pfu of NV1023. By twenty days after treatment, the virus treated (left) tumor volume was reduced to approximately one third the size of mock tumors. The untreated (right) tumors also regressed as expected with the tumor volume reduced to half the size of mock tumors (Fig. 3A and B).

![Graph 1](image1.png)  
**FIGURE 3A**  

![Graph 2](image2.png)  
**FIGURE 3B**

**TOXICITY OF INTRAPROSTATIC HSV (G207):** We have completed our studies of G207 toxicity following intraprostatic inoculation and demonstrated in Balb/C mice (supported through this grant) and confirmed these results in primates (supported through other funding) that G207 can be safely inoculated into the prostate. *A copy of this manuscript is enclosed which details these studies.*

**HYPOTHESIS 2:** As noted in the prior progress report, the initial funding letter noted that "This project was recommended for funding. The panel was intrigued with the possibility of using a mutated form of herpes virus as a novel therapy for prostate cancer but less enthusiastic about testing the IL-12 and GM-CSF vectors". Therefore, as noted in our prior progress report, we eliminated this hypothesis from the funded research of this initial grant and sought alternate funding for testing this hypothesis.

**HYPOTHESIS 3: HSV VECTORS WILL SENSITIZE PROSTATE CANCERS TO RADIATION INDUCED CELL-DEATH IN VIVO.**

It had been demonstrated at the time of this initial grant proposal that certain HSV mutants may be able to sensitize human brain tumor cells to radiation and that radiation could enhance viral replication. However, we could not confirm these results using prostate cancer cell lines in either athymic or in syngeneic animals. These findings are important in that they note previously unrecognized limitations on the work initially published by others and suggest that sequential rather than simultaneous irradiation may be as beneficial for vectors such as G207 in treating prostate cancer. *A copy of this manuscript is enclosed which details these studies.*
KEY RESEARCH ACCOMPLISHMENTS:

- We have demonstrated that a conditionally-replicating HSV vector can kill or inhibit the growth of transgenic murine prostate cancer tumors in syngeneic animals.

- We have demonstrated that G207 can effectively treat prostate cancers that have been previously irradiated but that simultaneous treatment with vector and irradiation is not synergistic.

- We have demonstrated prostate cancer killing can be made more effective by altering the genetic structure of the vector.

- We have demonstrated that the inoculation of one tumor can cause regression of a distant tumor, which in parallel studies by us has been demonstrated to be due to the induction of anti-tumor cell-mediated immunity.

- We have demonstrated in animal models that G207 can be safely inoculated into the prostate without toxicity forming the basis for a clinical trial for localized progressive prostate cancer following radiotherapy.

REPORTABLE OUTCOMES:


- Favorable peer review and awarding of Phase II grant to continue this work "Herpes Virus Therapy of Prostate Cancer" (Aug1, 2001-Aug 31, 2003).

CONCLUSIONS:

We have demonstrated that human prostate cancers grown in athymic mice can be killed by genetically engineered conditionally replicating herpes viral vector (G207). Moreover, this has been demonstrated for both androgen-sensitive and androgen-insensitive tumors and for tumors recurring following irradiation. We next demonstrated that direct intraprostatic inoculation of the same vector (G207) did not cause toxicity in mice or in non-human primates. Just as our pre-clinical data in brain tumors formed the basis for the first clinical trial of G207 in glioma, taken together, these two enclosed manuscripts detail the preclinical data that forms the basis for a possible clinical trial in patients with
locally recurrent prostate cancer following irradiation.

We next demonstrated that improvements in vector design can treat murine prostate cancer in a syngeneic system and that inoculation of one tumor can induce an immune response against a distant tumor. We have termed this an "in situ vaccine" effect and we are developing this as a potential treatment for metastatic prostate cancer.

REFERENCES


5. Todo T, Martuza RL, Rabkin SD, Johnson PA. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. PNAS 98:6396-6401, 2001

APPENDICES AND ATTACHMENTS:


- Ionizing radiation does not alter the antitumor activity of herpes simplex virus vector G207 in subcutaneous models of human and murine prostate cancer. Jorgensen TJ, Katz S, Varghese S, Rabkin SD, Martuza RL. (Submitted for publication)
Local and Systemic Therapy of Human Prostate Adenocarcinoma with the Conditionally Replicating Herpes Simplex Virus Vector G207

JONATHAN R. WALKER, KEVIN G. McGEAGH, PERIASAMY SUNDARESAN, TIMOTHY J. JORGENSEN, SAMUEL D. RABKIN, and ROBERT L. MARTUZA

ABSTRACT

Prostate adenocarcinoma is the most common nonskin malignancy in males and the second most common cause of cancer death in the United States (Landis et al., 1998). Initial treatments of surgery or radiotherapy may cause impotence and/or incontinence from neural damage (Eastham and Scardino, 1998; Porter et al., 1998). When extraprostatic or metastatic disease develops, castration or pharmaceutical androgen ablation is utilized (Catalona, 1994). Androgen-resistant recurrence indicates a poor prognosis and justifies experimental chemotherapy (Oh and Kantoff, 1998). G207 (Mineta et al., 1995; Yazaki et al., 1995) is a multimutated herpes simplex virus 1 (HSV) vector that replicates within cancer cells, causing cellular death; however, replication is limited in normal cells, including those of the nervous system. In vitro, G207 at a low multiplicity of infection (MOI of 0.01) is oncolytic for multiple human prostate cancer cell lines. In athymic mice, a single intrapleural inoculation of G207 completely eradicates >22% of established subcutaneous human prostate cancer tumors irrespective of hormonal responsiveness. Two intrapleural inoculations of G207 completely eradicated two of three recurrent previously irradiated tumors and two intravenous administration of G207 induced tumor regression in distant subcutaneous tumors and completely eradicated one-fourth of the tumors.

OVERVIEW SUMMARY

G207 is a multimutated herpes simplex virus 1 vector that lacks both copies of the ICP34.5 gene and contains an insertion of the lacZ gene inactivating the ICP6 gene. G207 can replicate within cancer cells, causing cellular death; however, replication is limited in normal cells, including those of the nervous system. In vitro, at a low multiplicity of infection (MOI of 0.01), G207 is oncolytic for multiple human prostate cancer cell lines (LNCaP, DU-145, PC-3, and TSUPR-1). In athymic mice, a single intrapleural injection of G207 (2 x 10^7 PFU) into established subcutaneous human prostate cancer tumors caused a reduction in tumor volume (LNCaP, p < 0.05; DU-145, p < 0.01 versus controls) with complete eradication of >22% of tumors irrespective of hormonal responsiveness (LNCaP, 25%; DU-145, 22%). Two intrapleural injections of G207 (1 x 10^7 PFU) caused tumor volume reduction (p < 0.05 versus controls) and completely eradicated 66% of recurrent previously irradiated LNCaP tumors. Two intravenous doses of G207 (2 x 10^7 PFU) induced tumor regression in distant subcutaneous tumors (LNCaP, p < 0.001; DU-145, p < 0.05 versus controls) and completely eradicated one-fourth (LNCaP, 14%; and DU-145, 40%) of the tumors. G207 and related conditionally replicating HSV vectors warrant further evaluation for the treatment of local and metastatic prostate cancer.

INTRODUCTION

Various viral vectors have been utilized for antiprostate cancer therapy (Eastham et al., 1996; Rodriguez et al., 1997; Hall et al., 1998). However, delivery of viral particles to

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every cancer cell is a major limitation of this form of experimental therapy. Replication-competent vectors offer a potential advantage over replication-defective vectors owing to the possibility of viral multiplication within the tumor after viral delivery. The genetic modifications in G207 (deletion of both ICP34.5 genes and a lacZ insertion inactivating the ICP6 gene) permit replication within cancer cells but limit replication in normal cells, including those of the nervous system (Mineta et al., 1995; Yazaki et al., 1995). In athymic mouse models in which G207 has been studied, the antitumoral effect of G207 is primarily due to virally induced lysis of the infected cancer cells (Yazaki et al., 1995; Toda et al., 1998a). In immune-competent animals an additional benefit is due to the induction of an immune response to the tumor (Toda et al., 1998b, 1999). For example, studies have shown tumor regression of noninoculated established subcutaneous tumors in syngeneic bilateral tumor models after intraperitoneal injection of G207 into a distant tumor. This effect was not due to systemic viral spread, but to the induction of tumor-specific cytotoxic lymphocytes (Toda et al., 1999). This effect was lost when similar studies were done on immune-deficient animals.

G207 was originally developed to treat malignant brain tumors without harming surrounding normal brain cells (Mineta et al., 1995; Yazaki et al., 1995) and is now in a phase I clinical trial for recurrent malignant gliomas. More recently, G207 has been evaluated for the treatment of nonnervous system tumors (Toda et al., 1998a, 1999). Because G207 is nonneurovirulent and neural damage is the major morbidity associated with the treatment of prostate cancer, G207 provides an alternative therapeutic vector for prostate cancer.

MATERIALS AND METHODS

Cell lines and in vitro susceptibility testing

Human-derived prostate cancer cell lines included LNCaP (provided by E.P. Gelmann, Georgetown University Medical Center, Washington, D.C.), DU-145 and PC-3 (provided by the Lombardi Cancer Center, Georgetown University Medical Center), and TSUPR-1 (provided by W. Isaacs, James Buchanan Brady Urological Institute, Baltimore, MD). LNCaP was main-

![Graphs showing cell survival rates](image)

**FIG. 1.** G207 susceptibility of human-derived prostate cancer cell lines in vitro. Four human-derived prostate cancer cell lines (LNCaP, DU-145, PC-3, and TSUPR-1) were infected with G207 at MOIs of 1, 0.1, and 0.01. Each data point (mean of triplicate wells) is the percentage of surviving cells compared with the number of cells in control wells at each time point.
HSV G207 TREATMENT OF PROSTATE CANCER

tained in Iscove’s modified Eagle’s medium (IMEM; Bioflu-
oids, Rockville, MD) containing 5% calf serum (HyClone, Lo-
gan, UT), DU-145 and PC-3 were maintained in RPMI 1640
(Biofluids) containing 10% fetal calf serum (HyClone), and
TSUPR-1 was maintained in Dulbecco’s modified Eagle’s
medium (Biofluids) with 10% fetal calf serum. All cell lines
were maintained at 37°C in 5% CO₂ with penicillin and strep-
tomycin (Sigma, St. Louis, MO) added to the medium, and were
free of mycoplasma contamination. G207 (obtained from Neu-
roVir, Vancouver, BC, Canada) was titered on African green
monkey (Vero) cells (provided by D. Kniipe, Harvard Medical
School, Boston, MA). For in vitro susceptibility assays LNCAp,
DU-145, PC-3, or TSUPR-1 cells were plated in six-well dishes,
infected with G207 at different multiplicities of infection
(MOIs), and maintained in 1% heat-inactivated fetal calf serum
in buffered saline at 34.5°C. Cell counts were done in triplicate
using a ZM Coulter counter (Coulter, Miami, FL) for each MOI
on days 1, 2, and 3 postinfection, after washing with phosphate-
buffered saline (PBS) and detaching cells with trypsin–EDTA
(GIBCO-BRL, Grand Island, NY).

Subcutaneous tumor model and intraperitoneal
inoculation of subcutaneous tumors

Six- to 7-week-old male BALB/c nu/nu mice were obtained
from the National Cancer Institute (Rockville, MD). The
Georgetown University Animal Care and Use Committee
approved all animal procedures. The mice were anesthetized
with an intraperitoneal injection of a 0.25- to 0.30-ml solution
consisting of 84% bacteriostatic saline (Abbott Laboratories,
Chicago, IL), 10% sodium pentobarbital (1 mg/ml; Abbott Lab-
atories), and 6% ethyl alcohol or inhalation of 2–3 minimal
alveolar concentration of methoxyflurane (Schering-Plough
Animal Health, Union, NJ). Subcutaneous tumors were induced
by flank injection of 5 × 10⁶ LNCAp cells in 0.1 ml with an
equal volume of Matrigel (Collaborative Biochemical Products,
Bedford, MA), or 5 × 10⁶ DU-145 cells in 0.1 ml. Tumors were
measured by external caliper to within 0.1 mm, and volumes
were calculated (V = H × L × W) and recorded. Animals with
tumors less than 25 mm³ were excluded. Tumors were inocu-
lated intraperitoneally with G207 (2 × 10⁷ PFU in 20 μl) or
virus buffer (20 μl). Animals were sacrificed when the tumor
diameter was greater than 18 mm.

Intraperitoneal inoculation in irradiated
subcutaneous tumors

To study irradiated tumors, LNCAp tumors were established
subcutaneously as described above, and animals randomized.
Tumors received a total of 10, 20, 30, or 40 Gy fractionated
over a 5-day period, using a cesium-137 irradiator or mock
non-irradiated treatment. Tumors in the 10- and 20-Gy group
recurred after initial radiation treatment. Animals with recurrent
tumors were randomized into two new groups and the tumors
were either inoculated intraperitoneally with G207 (1 × 10⁷
PFU in 20 μl) or virus buffer (20 μl); repeat inoculation done
2 days later.

Intravenous inoculation of G207

LNCAp or DU-145 tumors were established subcutaneously
as described above, and randomized into two groups respec-
tively. Animals were inoculated by tail vein with either G207
(2 × 10⁷ PFU in 100 μl) or virus buffer (100 μl). This treat-
ment was repeated 4 days later.

Serum PSA determination

At the completion of subcutaneous LNCAp tumor treatment,
animals treated with either G207 (2 × 10⁷ PFU) or virus buffer
were sacrificed. Blood was obtained and centrifuged, and the

FIG. 2. The effect of intraperitoneal injection of G207 on subcutaneous human prostate tumor growth. (A) LNCAp and (B) DU-
145 subcutaneous tumors were established in BALB/c nu/nu mice. When the mean volume of LNCAp tumors (n = 8) and DU-
145 tumors (n = 1b) was 99 mm³ (range, 28–224 mm³) and 80 mm³ (range, 26–350 mm³), respectively, tumors received a single
intraperitoneal injection of G207 (2 × 10⁷ PFU) or virus buffer (day 0). G207-treated tumors showed a reduction in volume,
whereas buffer-treated tumors continued to grow (LNCAp, p < 0.05 versus controls on day 42; DU-145, p < 0.01 versus con-
trols on day 46). Complete eradication of 25 and 22% of the LNCAp and DU-145 tumors, respectively, was noted. (Bars represent
mean tumor volume ± the standard error of the mean.)
serum was collected. Serum was sent to Bayer (Tarrytown, NY) for determination of total prostate-specific antigen (PSA). (PSA testing was kindly done by G. Allard.)

RESULTS AND DISCUSSION

The sensitivity of four human-derived prostate cancer cell lines (LNCaP, DU-145, PC-3, and TSUPR-1) to G207 was evaluated. Effective cytotoxicity, with >90% cell destruction within 3 days, was noted for three of four cell lines (LNCaP, DU-145, and PC-3) at an MOI of 0.01 (Fig. 1). This contrasts with studies of other viral vectors that require MOIs > 1 to achieve the same level of oncolysis for prostate cancer cell lines (Eastham et al., 1996; Gotoh et al., 1997). It also represents a higher level of oncolysis at lower MOIs than indicated in prior studies by us using G207 against human nervous system tumor cell lines (Yazaki et al., 1995).

To study the effect of intraneoplastic G207 treatment on localized tumors, LNCaP and DU-145 were chosen for in vivo study since both cell lines had comparable in vitro oncolysis, while LNCaP is hormonally responsive and DU-145 is hormonally unresponsive (Tilley et al., 1990). Hormonally unresponsive tumors are generally more aggressive, with an increased rate of metastasis and poorer patient survival (Stephenson et al., 1992; Rembrink et al., 1997; Oh and Kantoff, 1998). Established subcutaneous LNCaP (n = 8) or DU-145 (n = 16) tumors in athymic mice were inoculated with either G207 (2 × 10^7 PFU) or virus buffer intraneoplastically. Control tumor volumes increased to the point that the animals had to be sacrificed. In contrast to the controls, G207 induced significant tumor regression of LNCaP (p < 0.05 versus controls on day 42, t test) and DU-145 tumors (p < 0.001 versus controls on day 46, t test) (Fig. 2A and B). Treatment with one inoculation of G207 (2 × 10^7 PFU) resulted in eradication of 25% of the LNCaP tumors and 22% of the DU-145 tumors. LNCaP tumors produce human PSA, which can be measured in the serum. Mean total serum PSA was significantly lower (0.18 ng/ml) in the G207-treated group than in the virus buffer-treated group (102 ng/ml) (p < 0.005 versus controls, t test).

Because patients who choose radiation therapy as their primary treatment may develop a localized recurrence of prostate cancer the effectiveness of G207 on previously irradiated localized tumors was studied. Subcutaneous LNCaP (n = 15) tumors in athymic mice were initially irradiated. All tumors not irradiated (0 Gy) progressed until all animals had to be sacrificed. All tumors treated with 40 Gy and 33% of tumors treated with 30 Gy showed complete tumor eradication without recurrence. In contrast, all tumors treated with 10 or 20 Gy showed transient tumor volume reduction followed by regrowth. These 10- and 20-Gy radiation failures were then grouped together and randomized to receive either intraneoplastic injection of G207 (1 × 10^7 PFU) or virus buffer with treatment repeated 2 days later. All buffer-treated tumors continued to grow, whereas

![Image](image-url)

FIG. 3. The effect of radiation and G207 on subcutaneous LNCaP tumor growth. Subcutaneous LNCaP tumors were established in BALB/c nu-nu mice. When the mean tumor volume for LNCaP tumors (n = 15) was 348 mm^3 (range, 120–693 mm^3), tumors were randomized into five groups. Radiation was fractionated in equal doses over 5 days for a total dose of 10, 20, 30, or 40 Gy. Radiation treatment was noted to cause an initial reduction in tumor volume at all treatment doses when compared with 0 Gy. Tumor eradication was noted in 100% of the 40 Gy-treated tumors, and in 33% of the 30 Gy-treated tumors. All of these animals were then excluded from further studies. After the initial reduction in volume, all tumors in the 10- and 20-Gy treatment groups showed an increase in tumor volume. These tumors (n = 6) were then randomized to receive either G207 (1 × 10^7 PFU) intraneoplastically, followed 2 days later by a second injection, or two injections of virus buffer. G207-treated tumors had a marked reduction in tumor volume (p < 0.05 versus control on day 86) and tumor eradication was noted in 60% of the G207-treated tumors. (Bars represent mean tumor volume ± the standard error of the mean.)
two intrapneoplastic treatments with G207 caused tumor eradication in 66% of these previously irradiated tumors \((p < 0.05\) versus controls on day 86, *t* test) (Fig. 3). While this total eradication rate is higher than that of Fig. 2, it should be noted that two additional variables must be considered. First, the animals in Fig. 3 received two doses of virus whereas those in Fig. 2 received only one dose. Second, experiments in other tumor systems have shown enhanced antitumor activity when radiation therapy is combined with oncolytic herpes simplex virus (HSV) therapy (Advani *et al.*, 1998). When this is done concomitantly increased viral growth has been documented. An effect seen after temporal spacing could be due to the interactions of apoptotic pathways, injury to tumor vasculature, or other mechanisms worthy of additional study. Whatever the mechanism, it is important to note that prior exposure to radiation did not negatively impact the effectiveness of G207. Thus further preclinical studies are warranted to consider viral oncolytic therapy for patients with recurrent prostate cancer after radiotherapy.

To test the possible use of intravenous delivery of G207 in the treatment of distant metastatic tumors, a preliminary study with subcutaneous DU-145 tumors was performed. DU-145 tumors \((n = 4)\) were established subcutaneously in athymic mice and either G207 \((2 \times 10^7\) PFU) or virus buffer was administered by tail vein inoculation. Animals were sacrificed on day 4, and tumors were removed and stained for β-galactosidase (Mineta *et al.*, 1994). Diffuse areas of LacZ expression were noted in the tumors of G207-treated animals but none was noted in the controls (data not shown). Therefore, LNCaP \((n = 12)\) or DU-145 \((n = 12)\) tumors were established subcutaneously in athymic mice. These mice were randomized and treated with either virus buffer or G207 \((2 \times 10^7\) PFU) by tail vein inoculation. The treatment was repeated 4 days later. G207 caused significant tumor regression or growth inhibition versus virus buffer for both LNCaP \((p < 0.001\) versus controls on day 38, *t* test) and DU-145 \((p < 0.05\) versus controls on day 26, *t* test) (Fig. 4). Although inhibition or regression of tumor growth was observed with the dose regimen used, tumor recurrence was noted in the majority of the animals. Complete tumor eradication occurred overall in one-fourth of the tumors (one of seven LNCaP and two of five DU-145) treated with the two-dose regimen described.

This study has shown that intravenous delivery of G207 can induce tumor growth inhibition, regression, and eradication of distant prostate cancers. Others have demonstrated that localized vascular delivery of replicating HSV vectors is effective in the treatment of experimental brain tumors after intracarotid delivery (Rainov *et al.*, 1998) and in experimental hepatic tumors after hepatic artery delivery (Y. Fong, personal communication, 1999). A word of caution is in order since studies of wild-type and certain attenuated mutant strains of HSV have documented that intravenous administration can produce toxicity of spinal cord, adrenal glands, and liver (Hill *et al.*, 1986; Irie *et al.*, 1998). However, thus far, studies have shown that intravenous delivery of 107 PFU of G207 has been nontoxic in BALB/c mice (W. Hunter and P. Sundaresan, personal communication, 1999). Nonetheless, additional preclinical toxicity studies after both intravenous and intraprostatic G207 inoculation are warranted.

The mutations engineered into G207 are important for the significant reduction in neurovirulence after viral infection. G207 has been shown to be nonneurotoxic at 107 PFU after di-
rect inoculation into the brain of mice (BALB/c and AJ) (Mineta et al., 1995), the sciatic nerve of mice (G. Mashour, personal communication, 1998), or into the brain of nonhuman primates (Aotus) (Mineta et al., 1995). Further, G207 is currently in a phase I clinical trial for the treatment of recurrent malignant glioma and, as of this writing, 3 \times 10^6 PFU has been inoculated intracranially into humans without the production of encephalitis or neural toxicity. Current techniques allow for well-localized stereotactic delivery of a vector to the prostate (Holm et al., 1983). G207 could allow for the in situ ablation of prostate cancer cells in a "nerve-sparing" fashion, limiting the incontinence and impotence often associated with radiotherapy or surgery.

In conclusion, G207 may be valuable for treating various stages of prostate cancer including localized, hormonally resistant, previously irradiated, and metastatic tumors. Further improvement of these results may be possible simply by altering the dosing regimen as noted above. In addition, vector modifications providing improved viral replication, or the use of specific promoters, or the expression of cytokines and other anti-cancer genes, are worthy of development. Preclinical studies of possible toxicity after intravenous and intraprostatic inoculation will be necessary in order to consider this form of therapy for human use.

ACKNOWLEDGMENTS

We thank J.T. Newsome and A. Murray for their technical assistance with the project and veterinary support; P. Walsh, J. Lynch, E. Gelmann, and M. Lippman for inspiration and advice; G. Allard (Bayer Corp.) for the PSA determination; and P. Johnson (Neurovir, Inc.) for providing G207.

This work was supported in part by grants from CaPcURE and from the Department of Defense to R.L.M. (PRCP-97 IDEA).

S.D.R. and R.L.M. are consultants to Neurovir, Inc., which has a license from Georgetown University for G207.

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Received for publication March 15, 1999; accepted after revi-
sion June 17, 1999.
Preclinical Safety Evaluation of G207, a Replication-Competent Herpes Simplex Virus Type 1, Inoculated Intraprostatically in Mice and Nonhuman Primates

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ABSTRACT

G207, a replication-competent herpes simplex virus type 1 (HSV-1) virus, has been previously shown to be effective against human prostate cancer xenografts in mice. This study assesses its safety in the prostate of two animal models known for their sensitivity to HSV-1. BALB/c mice were injected intraprostatically with either HSV-1 G207 or strain F and observed for 5 months. None of the G207-injected animals exhibited any clinical signs of disease or died. However, 50% of strain F-injected mice displayed sluggish, hunched behavior and died by day 13. Histopathologically, the G207-injected prostates were normal whereas strain F-injected prostates showed epithelial flattening, sloughing, and stromal edema. Four Aotus nancymae monkeys were also injected with G207 intraprostatically and observed short term (up to 21 days) and long term (56 days). Safety was assessed on the basis of clinical observations, viral biodistribution, virus shedding, and histopathology. None of the injected monkeys displayed evidence of clinical disease, shedding of infectious virus, or spread of the virus into other organs. Except for minor histological changes unrelated to the study, no significant abnormalities were observed. These results demonstrate that G207 can be safely inoculated into the prostate and should be considered for human trials for the treatment of prostate cancer.

OVERVIEW SUMMARY

G207, an attenuated replication-competent herpesvirus, offers distinct advantages for treating prostate cancer: it efficiently kills human prostate tumors in mice and, on the basis of safety studies in human brain and peripheral nerves in mice, it spares adjacent nervous system tissues. As part of the preclinical testing of G207 for treating prostate cancer, this study evaluated its safety in the prostates of BALB/c mice and Aotus nancymae. Results from morbidity, viral shedding, PCR-based detection of dissemination in various tissues, and histopathology of major tissues suggest that the virus can be safely inoculated into the prostate. Thus, the safety of G207 in prostate, coupled with its effectiveness in destroying human prostate cancers and its lack of neurovirulence, provides a strong basis for a human phase I clinical trial as a "nerve-sparing" method of treating prostate cancer.

INTRODUCTION

Prostate cancer is the leading cause of cancer death among older men in Western countries and ranks second to lung cancer in cancer mortality (Woolf, 1995). The incidence of prostate cancer has increased steadily in the last two decades and is the most frequently diagnosed noncutaneous neoplasm. The availability of prostate-specific antigen (PSA) as a tumor marker has profoundly enhanced the early detection and diagnosis of clinically significant prostate cancer (Potosky et al.,

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1995). Nevertheless, substantial controversy surrounds the most appropriate treatment.

Early-stage, organ-confined tumor is usually treated by radical prostatectomy or radiation therapy (external beam or brachytherapy). Locally advanced cancers or metastatic disease, with lymph node, lung, or skeletal bone involvement, are treated by ablative androgen therapy and/or radiation therapy. However, with time, androgen-independent disease resistant to the standard therapeutic regimen may arise (Dorkin and Neal, 1997). Moreover, present treatment strategies (radical prostatectomy, external beam irradiation, and brachytherapy) may be associated with risks of urinary incontinence, proctitis, or erectile dysfunction.

Many features unique to prostate cancer make it an ideal candidate for gene therapy; prostate cancer is relatively slow growing, the gland is easily accessible, and a circulating marker, PSA, is available for early detection and for monitoring the treatment response. A serious limitation of gene therapy protocols is the lack of efficient delivery of the vector to every cancer cell (Peng and Russell, 1999). Until recently, various gene therapy studies against prostate cancer have utilized replication-deficient adenoviruses (Eastham et al., 1996; Ko et al., 1996; Herman et al., 1999; Lu et al., 1999; Nasu et al., 1999). The development of replication-competent viral vectors (adeno, herpes, and vaccinia viruses) has offered a distinct advantage of viral multiplication and spread within the entire tumor. Replication-competent adenoviruses CN706 (Rodriguez et al., 1997), and CV764 and CV787 (Yu et al., 1999a,b), constructed with specificity to the prostate have been shown to be effective against prostate cancer cells and tumor xenografts. However, both adenovirus and vaccinia virus vectors elicit strong host immune responses that may limit their efficacy. This shortfall may be overcome by the use of herpesvirus vectors. Attenuated replication-competent mutants of herpes simplex virus type 1 (HSV-1) have been shown to be highly effective against a variety of experimental tumor models (Martuza et al., 1991; Boviatisis et al., 1994; Mineta et al., 1995; Yazaki et al., 1997; Andreansky et al., 1996; Brandt et al., 1997; Kramm et al., 1997; Pyles et al., 1997; Randazzo et al., 1997; McKie et al., 1998; Yoon et al., 1998; Lambright et al., 1999; Toyozumi et al., 1999).

G207 is a second-generation, modified HSV-1 vector that was initially developed as a therapeutic agent for brain tumors with the specific aim of targeting cancer cells but not normal cells within the brain. The virus has been genetically altered, through deletion of both copies of the γ34.5 (RL1) gene and an Escherichia coli lacZ insertion inactivating the ICP6 gene, in order to permit replication within cancer cells but not in normal cells (Mineta et al., 1995). Moreover, the virus is hypersensitive to the antiviral drugs ganciclovir and acyclovir. G207 has been shown to be effective against many different types of tumors, including malignant glioma, meningioma and neuroblastoma, gastric cancer, cancers of the prostate, bladder, breast, colon, pancreas, ovaries, and head and neck (Mineta et al., 1995; Yazaki et al., 1995; Toda et al., 1998; Chahlavi et al., 1999; Lee et al., 1999; Walker et al., 1999; Bennett et al., 2000; Coukos et al., 2000; Oyama et al., 2000).

The significance of using G207 in treating human prostate cancer in vivo is based on the following two observations: (1) the virus demonstrates effective oncolysis of human prostate cancer cell lines in vitro and in vivo tumor models established in mouse (Walker et al., 1999); and (2) safety evaluation of the virus injected intracerebrally in HSV-1-sensitive nonhuman primates (Aotus nancymae) and via intracerebral, intracerebroventricular, intravenous, or intrahepatic routes in mice demonstrated no HSV-associated neural or general pathology or disease (Hunter et al., 1999; Sundaresan et al., 2000; Todo et al., 2000). G207 was also demonstrated to be safe in humans, in a phase I clinical trial of patients with malignant gliomas (Markert et al., 2000). Direct stereotactic intratumoral inoculation of up to 3 × 10^9 PFU was tolerated without the production of encephalitis or other limiting toxicity. Furthermore, it has been shown that injection of G207 into healthy sciatic nerves in BALB/c mice caused no neurological sequelae (Mashour et al., 2001). These studies suggest that G207 may be a promising agent for prostate cancer therapy by destroying tumor cells without harming adjacent nerves. Targeted delivery of G207 or other replicating viral vectors is currently possible because of the availability of precise stereotactic injection of a vector into the prostate (Holm et al., 1983).

This study was aimed at evaluating the safety of G207 injected intraprostatically in mice and New World owl monkeys (Aotus nancymae). The results, based on observations of morbidity, mortality, biodistribution (based on polymerase chain reaction [PCR] detection of viral DNA), and histopathology, suggest that the virus can be safely inoculated into the prostate. When considered in conjunction with prior studies of efficacy in treating prostate cancer (Walker et al., 1999), the current study strongly suggests that G207 be considered in human trials as a nerve-sparing treatment for prostate cancer.

**MATERIALS AND METHODS**

**Viruses**

G207 (Mineta et al., 1995) was obtained from NeuroVir (Vancouver, BC, Canada), and viral stocks were prepared as described previously (Todo et al., 2000). HSV-1 strain F was provided by B. Roizman (University of Chicago, Chicago, IL). Viruses were titered on Vero (African Green monkey kidney) cells as previously described (Miyatake et al., 1997).

**Animals**

Six- to 7-week-old BALB/c male mice were obtained from the National Cancer Institute (Frederick, MD). New World owl monkeys (Aotus nancymae) were obtained through the Veterinary Resources Program, National Center for Research Resources, National Institutes of Health (Poolesville, MD). The six monkeys used for the study were between 4 and 8 years of age and weighed approximately 840–1264 g. Monkeys T348 and T442 had received two intracerebral (left frontal lobe) inoculations of G207 (Hunter et al., 1999) 2 and 3 years prior to the start of this study whereas T635 and T659 were naïve for known exposure to HSV. Two other preexposed (prior intracerebral inoculation) animals, T317 and T344 (Hunter et al., 1999), were used as prostate-noninjected controls. All animal studies were conducted at Georgetown University under the review and approval of the Georgetown Animal Care and Use.
G207 SAFETY IN MOUSE AND MONKEY PROSTATE


Intraprostatic injection of mice and monkeys

Mice were anesthetized via inhalation of methoxyfluorane (Schering-Plough Animal Health Corporation, Union, NJ). The abdominal hair was shaved and the skin was disinfected with povidone-iodine (Betadine) and 70% alcohol. A midline skin incision was made in the lower abdomen and the abdominal wall was opened between the rectus muscles. The urinary bladder and seminal vesicles were identified and retracted so as to expose the dorsolateral prostate. Twenty microliters of G207 (1 × 10⁷ PFU), strain F (1 × 10⁵ PFU), or virus buffer consisting of sterile 10% glycerol in phosphate-buffered saline (mock) was injected with the aid of a dissecting microscope into the left lobe of the prostate, using a 27-gauge needle. A bleeding beneath the prostate capsule confirmed the injection. The abdominal contents were repositioned, the fascia was closed with absorbable sutures, and the skin was reaproximated with wound clips.

Monkeys were sedated with tiletamine plus zolazepam (Tela-zol, 4 mg/kg, A.H. Robbins, Richmond, VA). A 24-gauge angiogast was placed in the lateral saphenous vein and anesthesia was induced with a bolus of propofol (Diprivan, 40–60 mg; Astra Zeneca, Wilmington, DE). Monkey maintenance anesthesia was with an intravenous drip infusion (50–100 ml/kg/hr) of propofol. A linear midline incision was placed in the lower abdominal region to expose the urogenital organs. G207 (1 × 10⁷ PFU) in 20 μl was injected with a 27-gauge needle into the left lobe of the prostate. The body wall was closed with Vicryl sutures in a simple interrupted pattern and skin was closed using a subcuticular pattern.

Clinical monitoring

After inoculation of the viruses into the prostate, the animals were closely monitored for any behavioral changes. The monkeys underwent regular physical examinations that included periodic temperature and body weight determinations and neurological assessment. Blood analysis, including serum clinical chemistries and complete blood cell counts, was done at 7- or 14-day intervals for the short-term and long-term animals, respectively. Serum anti-HSV-1 antibody for each of the monkeys was determined pre- and postinjection, using an enzyme-linked immunosorbent assay (ELISA) by BioReliance Corporation (Rockville, MD). The animals were killed at designated time points: the short-term monkeys T348 and T635 on days 14 and 21, respectively, and long-term monkeys T442 and T659 on day 56. For each animal, all tissues, including the prostate (approximately 0.7 × 0.25 cm in size per lobe), were divided into three pieces: one each for PCR, histopathology, and frozen storage.

Virus shedding studies

Blood, cerebrospinal fluid (CSF), urine, and mucosal swabs of urethral, rectal, buccal, and conjunctival sites were collected on days 7, 14, and 21 for the short-term monkeys and on days 14, 28, and 56 for the long-term monkeys. The sterile sample swab tip was placed in 2 ml of Multi-Microbe medium (M-4; Micro Test, Snellville, GA), snap frozen, and stored at −80°C.

Shedding of infectious virus in each sample was determined by culturing the samples over a monolayer of Vero cells (conducted by NeuroVir). Vero cells plated in a 6-well plate at a density of 2 × 10⁵ cells/well were incubated for 4 hr at 37°C to allow cell attachment. Sample was then added at 1:2 or 1:10 dilution in 1 ml and allowed to incubate overnight to aid viral adsorption. Another 1 ml of growth medium containing 10% fetal calf serum in Dulbecco’s minimum essential medium was added and the cells were observed daily for 7 days for cytopathic effect.

Biodistribution studies by PCR analysis

Tissue distribution studies were conducted by Bioreliance, using a standardized real-time (TaqMan) PCR assay. Detection of HSV-1 DNA polymerase sequences was done with total DNA isolated from tissues collected at necropsy. The assay was consistently linear between 10 and 10⁵ template copies and detects 100 copies of template in the presence of 1 μg of monkey genomic DNA.

Histopathology

The samples for histopathology were sent to Pathological Associates International (PAI, Frederick, MD) for analysis. Tissues obtained at biopsy were fixed in formalin, embedded in paraffin, sectioned on a sliding microtome (8 μm thick), and stained with hematoxylin and cosin. The histopathology slides were blinded and reviewed independently by two board-certified pathologists: one of the authors (P.N.) and the other from PAI.

RESULTS

The intraprostatic safety of G207 was tested in two different HSV-susceptible animal models: mice (BALB/c) and non-human primates, Aotus nancymae.

Intraprostatic injection of HSV in BALB/c mice

Clinical observations and survival. BALB/c mice received intraprostatic inoculation of G207 (1 × 10⁷ PFU), wild-type strain F (1 × 10⁶ PFU), or mock (n = 8, 8, and 14, respectively). Two animals from the mock-injected group died within the 48-hr post-operative period and these deaths were ascribed to anesthesia and surgery. The surviving animals were observed daily for the first 14 days and thereafter periodically for any changes in behavior or activity. There was no obvious change in activity or behavior in any of the G207- or mock-injected animals. All eight of the G207-injected animals survived until 5 months postinjection, when they were killed. Only the HSV-1 wild-type strain F-injected mice exhibited any clinical changes. Their behavior was sluggish in the first few days postinjection and four of eight animals also had hunched posture. All of these hunched animals died by day 13 (Fig. 1).
Histopathology. Prostate tissues were obtained at necropsy and histological analysis was performed by hematoxylin and eosin staining (Table 1 and Fig. 2). None of the G207- or mock-injected BALB/c mice showed any abnormal pathology (Fig. 2, top), whereas all the strain F-injected mice exhibited varying degrees of epithelial sloughing and stromal edema in the prostate tissue (Fig. 2, bottom). In one injected mouse (F-4) from the strain F group, only minimal prostate was detected and in another mouse (F-1), no prostate tissue could be identified although normal fat was observed (Table 1).

Intraprostatic injection of G207 in nonhuman primate (Aotus nancymae)

The G207 safety study in Aotus nancymae consisted of a short-term arm and a long-term arm, with two animals per arm. Each arm consisted of one previously inoculated animal (T348 and T442) and one naive animal (T635 and T659). The short-term animals, T348 and T635, were sacrificed on days 14 and 21, respectively, after intraprostatic injection of G207 while the long-term animals, T442 and T659, were sacrificed on day 56. Two monkeys, T317 and T344, included as prostate-noninjected controls, were sacrificed to compare the normal histology of prostates in these animals. Because of the known toxicity of the wild-type strain F in Aotus (Meignier et al., 1990; Hunter et al., 1999) and the demonstrated toxicity of intraprostatic injection of strain F in BALB/c mice (Fig. 1), no strain F virus was inoculated into monkeys.

Clinical observations and survival. Clinical monitoring, including activity and behavior checks, was performed on a daily basis. Body weight and temperature were measured at regular intervals until the time of sacrifice. There was no significant change in body weight or temperature during the course of the study, in any of the monkeys. None of the monkeys exhibited any altered behavior or died during the period of the study, and they were killed at the designated time points.

Blood was collected at 7- or 14-day intervals and analyzed for complete blood count (CBC), differential count, and serum chemistry (Table 2). The study subjects showed some variations from the normal range calculated from the precollection values of the four prostate-injected (T348, T442, T635, and T659) and the two prostate-noninjected (T317 and T344) animals. The prostate-injected monkeys were also tested for the presence of HSV antibody by ELISA from serum samples obtained pre- and postintraprostatic injection with G207 (Table 3). T348 and T442 had a positive titer both prior to and after the intraprostatic injection. Monkey T659 was naive at the onset of the study and, as expected, seroconverted after G207 injection into the prostate.

Virus shedding in Aotus. To determine whether intraprostatic injection would lead to virus shedding, urine, serum, and swabs from various mucosal regions (buccal, conjunctiva, urethra, and rectum) were tested for the presence of infectious virus by plaque assay. No virus was detected in any of these samples (data not shown). We attempted to collect semen samples by electroejaculation but were unsuccessful in most cases except for T442, from which semen sample was obtained on days 0 and 14, and no virus was detected from these samples. CSF was collected at necropsy from the long-term monkeys only and was negative for infectious virus.


**Table 1. Histology of Prostate Tissue from BALB/c Mice Inoculated Intraprostatically with HSV**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Survival day (D or S)</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-2</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-3</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-4</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-5</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-6</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-7</td>
<td>158 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-8</td>
<td>158 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-9</td>
<td>158 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-10</td>
<td>158 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>M-11</td>
<td>158 (S)</td>
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</tr>
<tr>
<td>M-12</td>
<td>158 (S)</td>
<td>Normal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Survival day (D or S)</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>G-2</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>G-3</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>G-4</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
<tr>
<td>G-5</td>
<td>151 (S)</td>
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<td>G-6</td>
<td>151 (S)</td>
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<td>G-7</td>
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<tr>
<td>G-8</td>
<td>151 (S)</td>
<td>Normal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Survival day (D or S)</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-1</td>
<td>158 (S)</td>
<td>No prostate detectable; normal fat</td>
</tr>
<tr>
<td>F-2</td>
<td>158 (S)</td>
<td>Stromal edema, some epithelial flattening</td>
</tr>
<tr>
<td>F-3</td>
<td>13 (D)</td>
<td>Sloughed epithelial cells, stromal edema</td>
</tr>
<tr>
<td>F-4</td>
<td>13 (D)</td>
<td>Minimum prostate detected with sloughed epithelium and stromal edema</td>
</tr>
<tr>
<td>F-5</td>
<td>3 (D)</td>
<td>Epithelial (focal) flattening and sloughing</td>
</tr>
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<td>F-6</td>
<td>10 (D)</td>
<td>Epithelial sloughing and stromal edema</td>
</tr>
<tr>
<td>F-7</td>
<td>120 (D)</td>
<td>NA</td>
</tr>
<tr>
<td>F-8</td>
<td>152 (D)</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Histology of prostate tissue from BALB/c mice inoculated intraprostatically with HSV. Mock mice were injected with 20 μl of virus buffer; G207 mice and strain F mice received either 1 × 10^7 or 1 × 10^6 PFU, respectively, in a 20-μl volume. Tissues were collected at necropsy, fixed in formalin, and paraffin embedded. Sections were stained with hematoxylin and eosin. D, death; S, sacrifice; NA, tissue not available (F-7 and F-8 died secondary to cagemate trauma).

**Virus biodistribution in Aotus.** To determine the distribution of intraprostatically injected G207 to other organs, tissue samples from necropsy were analyzed by real-time quantitative PCR for HSV DNA polymerase sequences. High copy numbers of HSV DNA were detected in the brain (temporal and frontal lobe) and spinal cord of the preexposed (intracerebrally injected) animals, T348 and T442 (Table 4). One long-term animal, T659, had quantifiable but low copy numbers of HSV DNA present in the local retroperitoneal lymph node (12 copies), spleen (28 copies), and urethra (penile, 66 copies). Nonquantifiable amounts (1–10 copies) of DNA were detected in a few tissues, including spinal cord (T348), retroperitoneal ganglia (T659), retroperitoneal lymph node (T348 and T635), adrenals (T635), spleen (T442), and prostate (T659).

**Histopathology of Aotus.** At necropsy, the major tissues from each of the monkeys were weighed and dissected to check for gross morphology. No significant abnormalities were seen in any of the organs in these animals, except for the prostate of T659, which exhibited surgery-related scarring in the left lobe (injection site). Histopathological examination revealed no abnormalities in the prostate of T635 and T442 (Fig. 3 and Table 5), or in the erectile tissues of T348, T442, and T635. Prostate tissue was not identifiable in the formalin-fixed wet tissues from T348 and T659, although prostates with normal morphology were seen in these monkeys at the time of necropsy. Most likely, for T348 and T659, the majority of the prostate (approximately 0.7 × 0.25 cm in size per lobe) was used for PCR and only periprostatic tissue was found in the formalin-fixed sample. These adjuvant tissues were found to have normal histology. However, the possibility that G207 could destroy some normal prostate tissue cannot be excluded.

All animals, including the prostate-noninjected T317 and T344 monkeys, exhibited interstitial inflammation in the kidney, and abnormalities in lung (mild vascular congestion) and liver (Kupffer cell hemosiderin pigmentation, prominent lipofuscin in hepatocyte, centroacinar dissociation, degeneration of hepatocytes) (Table 5). These changes are common observations in *Aotus nancymae* (Todo et al., 2000).

**DISCUSSION**

Previous studies from our laboratory have demonstrated that G207 is effective in killing human prostate cancer cells (Walker et al., 1999). Both androgen-dependent (LNCaP) and androgen-independent (DU-145, PC-3, and TSU-PR-1) cell lines were sensitive to G207 (Walker et al., 1999). This observation is important in light of the common development of androgen-refractory disease during the natural course of prostate cancer progression. In addition, we have also shown that subcutaneous human prostate cancer tumors generated in athymic or immunocompetent mice respond efficiently to G207 administered either intratumorally or intravenously (Walker et al., 1999; Jorgensen et al., 2001). Thus, G207 would be an ideal vector candidate to be used in viral therapy studies to treat prostate cancer.

As part of the preclinical testing of G207, the current study was aimed at assessing the safety of intraprostatic injection of G207 in two animal models known for their sensitivity to HSV-1: BALB/c mice and a nonhuman primate, *Aotus nancymae*. We have demonstrated that direct injection of G207 into the brain of these animals or via intracerebroventricular, intravenous, and intrahepatic routes in BALB/c mice did not result in any toxicity or complications (Hunter et al., 1999; Sundaresan et al., 2000; Todo et al., 2000). G207 dose of up to 3 × 10^7 PFU in mice and up to 1 × 10^6 PFU in monkeys could be administered safely whereas 1 × 10^6 PFU of the wild-type strain F virus resulted in pathology in these animals. Extensive histopathological and PCR studies did not reveal any evidence
Table 2. Blood Analysis of Aotus nancymae inoculated intraprostatically with G207

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Preoperation average</th>
<th>T348 (PI day 7)</th>
<th>T635 (PI day 14)</th>
<th>T442 (PI day 14)</th>
<th>T659 (PI day 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mg/dl</td>
<td>93-165</td>
<td>159</td>
<td>162</td>
<td>93</td>
<td>184</td>
</tr>
<tr>
<td>Urea nitrogen, mg/dl</td>
<td>15-23</td>
<td>21</td>
<td>18</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.9-1.1</td>
<td>1.2</td>
<td>0.9</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Total protein, g/dl</td>
<td>7.7-8.3</td>
<td>9</td>
<td>6.9</td>
<td>8.4</td>
<td>8.6</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>4.8-5.2</td>
<td>5.4</td>
<td>3.3</td>
<td>4.5</td>
<td>4.8</td>
</tr>
<tr>
<td>Bilirubin, total: mg/dl</td>
<td>0-3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Alkaline phosphatase, units/liter</td>
<td>207-335</td>
<td>306</td>
<td>588</td>
<td>339</td>
<td>248</td>
</tr>
<tr>
<td>ALT (SGPT), units/liter</td>
<td>69-139</td>
<td>195</td>
<td>106</td>
<td>36</td>
<td>90</td>
</tr>
<tr>
<td>AST (SGOT), units/liter</td>
<td>110-144</td>
<td>432</td>
<td>138</td>
<td>141</td>
<td>134</td>
</tr>
<tr>
<td>GGTP, units/liter</td>
<td>6.6-15.4</td>
<td>54</td>
<td>24</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>89.4-106.6</td>
<td>156</td>
<td>99</td>
<td>144</td>
<td>142</td>
</tr>
<tr>
<td>Calcium, mg/dl</td>
<td>8.8-9.2</td>
<td>12</td>
<td>9.6</td>
<td>10.2</td>
<td>11</td>
</tr>
<tr>
<td>Phosphorus, mg/dl</td>
<td>3.2-6.8</td>
<td>5.7</td>
<td>3.9</td>
<td>3.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Na. mEq/liter</td>
<td>148-150</td>
<td>160</td>
<td>144</td>
<td>160</td>
<td>144</td>
</tr>
<tr>
<td>K. mEq/liter</td>
<td>4.5-5.5</td>
<td>6</td>
<td>5.5</td>
<td>5.4</td>
<td>5.6</td>
</tr>
<tr>
<td>BUN/Cre. ratio</td>
<td>16-26</td>
<td>18</td>
<td>20</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>Globulin, g/dl</td>
<td>2.6-3.4</td>
<td>3.6</td>
<td>3.6</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>15-19</td>
<td>15.5</td>
<td>17.6</td>
<td>ND</td>
<td>15.5</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>46-58</td>
<td>46.4</td>
<td>51.2</td>
<td>ND</td>
<td>48.2</td>
</tr>
<tr>
<td>WBC</td>
<td>6.2-7.8</td>
<td>15.3</td>
<td>7</td>
<td>ND</td>
<td>7.1</td>
</tr>
<tr>
<td>RBC</td>
<td>6.4-7.6</td>
<td>5.82</td>
<td>7.07</td>
<td>ND</td>
<td>5.92</td>
</tr>
<tr>
<td>Bands</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Polymorphonuclear leukocytes</td>
<td>22-54</td>
<td>16</td>
<td>55</td>
<td>ND</td>
<td>26</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>38-72</td>
<td>82</td>
<td>42</td>
<td>ND</td>
<td>73</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1.2-4.8</td>
<td>2</td>
<td>2</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.7-2.7</td>
<td>0</td>
<td>1</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: PI, Postinjection; ALT, alanine transaminase (SGPT, serum glutamic-pyruvic transaminase); AST, aspartate aminotransferase (SGOT, serum glutamic-oxaloacetic transaminase); GGTP, γ-glutamyltranspeptidase; BUN, blood urea nitrogen; Cre, creatinine; WBC, white blood cell; RBC, red blood cell; ND, not determined, due to clotting.

*Blood analysis of Aotus nancymae inoculated intraprostatically with G207 virus (1 × 10⁷ PFU). Data shown are for day 7 (T348) and day 14 (T442, T635, and T659) postinoculation. Preoperation values are the average values of the four prostate-injected (T348, T635, T442, and T659) and two noninjected (T317 and T344) monkeys.

Safety of intraprostatic G207 in Aotus and mice was assessed on the basis of animal survival, viral biodistribution, virus shedding, and histopathology. The survival data indicate that none of the injected mice or monkeys succumbed to G207. This was in contrast to strain F-injected mice, in which half died within 13 days postinjection despite the strain F dose being 1 log lower than the G207 dose. It is interesting to note that 50% of mice died after intraprostatic inoculation with 1 × 10⁶ PFU of strain F as compared with 1 × 10⁵ PFU after intracerebral inoculation or 1 × 10⁴ PFU after intracerebroventricular inoculation (data from Sundaresan et al., 2000).

No monkeys were injected intraprostatically with the parental strain F, but we have shown previously that these monkeys are exquisitely sensitive to HSV-1 strain F, with en-
TABLE 3. Serum Anti-HSV-1 Antibody Titer in Aotus nancymae

<table>
<thead>
<tr>
<th></th>
<th>Short term</th>
<th>Long term</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T348</td>
<td>T635</td>
</tr>
<tr>
<td>Preinjection (day -60)</td>
<td>2.36</td>
<td>0</td>
</tr>
<tr>
<td>Postinjection (sacrifice day)</td>
<td>1.58</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Serum anti-HSV-1 antibody titer in Aotus nancymae before and after intraprostatic injection with G207 virus (1 × 10³ PFU). Determination of titer was performed by enzyme-linked immunosorbent assay (ELISA). Values shown represent absorbance at OD₉₅₀. ND, Not done.

encephalitis developing within 5 days of an intracerebral (1 × 10³ PFU) inoculation (Hunter et al., 1999). On the other hand, G207-injected monkeys in this study displayed no abnormalities on physical or neurological examination. Minor variations in blood chemistry values were observed and were considered insignificant to the study since none of these changes were consistently observed in all the injected animals. Some of these changes (abnormal glucose, ALT, AST, GGTP levels) were observed in the subjects even before prostate injection with G207 (data not shown). The Aotus monkeys used in this study were immunologically uncompromised on the basis of normal CBC values obtained prior to the study and no significant change was observed during the study. In response to G207 injection, the naive animal, T659, seroconverted for HSV-1 antibody and the preexposed (intracerebral) animals, T348 and T442, continued to generate anti-HSV-1 antibodies.

In ascertaining the safety of G207, which is a replication-competent virus, potential shedding of the virus was monitored by assaying for infectious virus in blood, CSF, urine, semen, and various mucosal (urethral, rectal, buccal, and conjunctival) sites. Extensive analysis on days 7, 14, and 21 for short-term monkeys and on days 14, 28, and 56 for long-term monkeys demonstrated the absence of any infectious virus. This observation is important in light of the finding that continuous virus shedding occurs throughout the period of survival in owl monkeys infected with wild-type HSV-1 (Meignier et al., 1990). In addition, tissue distribution of the virus was assessed by PCR detection of HSV-1 DNA polymerase sequences from various tissues obtained at necropsy (day 14 or 21 for short-term monkeys, T348 and T635, and day 56 for long-term monkeys, T442 and T659). None of the monkeys tested had PCR-detectable sequences in prostate (except T659) or the neighboring tissues such as testis, adrenals, and urethra (except T659 with 66 copies) at the end of the study period, suggesting a lack of prolonged viral replication or latency in the normal urogenital tissues. A significant copy number was detected, however, in the brain and especially in the injection site (left frontal lobe) of the two monkeys (T348 and T442) that had been previously inoculated with G207. Since the two naive monkeys, T635 and T659, did not contain detectable HSV-1 sequences at this site, it would indicate that the origin of the viral sequences detected in T348 and T442 is from the prior intracerebral inoculation and is not due to dissemination from the prostate injection.

The lack of viral spread from prostate to distant tissues is further supported by the histopathological findings of various tissues obtained at necropsy in both mice and Aotus monkeys. All the G207-injected mice had normal prostate histology whereas the prostates from strain F-injected mice displayed varying degrees of epithelial sloughing and stromal edema. Most of the monkey tissues examined, including the prostates of T442 and T635, were found to have normal histology. All

TABLE 4. Biodistribution Analysis of G207 Injected into the Prostate of Aotus nancymae

<table>
<thead>
<tr>
<th>Prior intracerebral G207 inoculation:</th>
<th>T348</th>
<th>T635</th>
<th>T442</th>
<th>T659</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postinoculation day:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>460</td>
<td>Neg.</td>
<td>NQ</td>
<td>Neg.</td>
</tr>
<tr>
<td>Frontal lobe (left)</td>
<td>140</td>
<td>NBR</td>
<td>1400</td>
<td>Neg.</td>
</tr>
<tr>
<td>Frontal lobe (right)</td>
<td>Neg.</td>
<td>NBR</td>
<td>14</td>
<td>Neg.</td>
</tr>
<tr>
<td>Spinal cord (cervical)</td>
<td>NQ</td>
<td>ND</td>
<td>ND</td>
<td>NQ</td>
</tr>
<tr>
<td>Spinal cord (thoracic and lumbar)</td>
<td>ND</td>
<td>NBR</td>
<td>310</td>
<td>NBR</td>
</tr>
<tr>
<td>Retroperitoneal ganglia</td>
<td>NBR</td>
<td>Neg.</td>
<td>NQ</td>
<td>12</td>
</tr>
<tr>
<td>Retroperitoneal lymph node</td>
<td>NQ</td>
<td>NQ</td>
<td>Neg.</td>
<td>NBR</td>
</tr>
<tr>
<td>Adrenals</td>
<td>Neg.</td>
<td>NQ</td>
<td>Neg.</td>
<td>NBR</td>
</tr>
<tr>
<td>Spleen</td>
<td>Neg.</td>
<td>Neg.</td>
<td>NQ</td>
<td>28</td>
</tr>
<tr>
<td>Testis</td>
<td>Neg.</td>
<td>Neg.</td>
<td>NBR</td>
<td>Neg.</td>
</tr>
<tr>
<td>Prostate</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>NQ</td>
</tr>
<tr>
<td>Urethra (penile)</td>
<td>Neg.</td>
<td>NBR</td>
<td>Neg.</td>
<td>66</td>
</tr>
</tbody>
</table>

Abbreviations: Neg., Negative; NQ, not quantifiable (1–10 copies); NBR, not biologically relevant (less than 1 copy); ND, not done.

*aNumbers represent copy number.
*BRadioimmunoassay analysis of G207 (1 × 10³ PFU) injected into the prostate of Aotus nancymae, using a standardized real-time (TaqMan) PCR assay for the detection of HSV-1 DNA polymerase sequences from total DNA from tissues collected at necropsy. The assay was consistently linear between 10 and 10⁸ template copies and detects 100 copies of template in the presence of 1 µg of monkey genomic DNA.
<table>
<thead>
<tr>
<th>Prior intracerebral</th>
<th>G207 inoculation:</th>
<th>Postinoculation day:</th>
<th>Short-term injected</th>
<th>Long-term injected</th>
<th>Prostate noninjected</th>
</tr>
</thead>
<tbody>
<tr>
<td>T348</td>
<td>Yes</td>
<td>Day 14</td>
<td>T635</td>
<td>T442</td>
<td>T317</td>
</tr>
<tr>
<td>G207</td>
<td>No</td>
<td>Day 21</td>
<td></td>
<td>T659</td>
<td>T344</td>
</tr>
<tr>
<td>Brain</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Retropertoneal lymph node</td>
<td>Hemorrhage, mild</td>
<td>Hemorrhage, mild</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td>Lung</td>
<td>Congestion, mild</td>
<td>Congestion, mild</td>
<td>Congestion, mild</td>
<td>Congestion, mild</td>
<td>Congestion, mild</td>
</tr>
<tr>
<td>Heart</td>
<td>ND</td>
<td>ND</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td>Liver</td>
<td>KC-pigmentation,</td>
<td>KC-pigmentation,</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Hepato-pigmentation,</td>
<td>Hepato-pigmentation,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Centro-dissoc./degen.</td>
<td>Centro-dissoc./degen.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Islet cell hyperplasia, mild</td>
<td>ND</td>
<td>Inflammation, mild</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Kidney</td>
<td>Inflammation, mild</td>
<td>Inflammation, mild</td>
<td>Normal</td>
<td>Inflammation, mild</td>
<td>Inflammation, mild</td>
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<tr>
<td>Adrenals</td>
<td>ND</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Testis/vas deferens</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Prostate</td>
<td>NA</td>
<td>Normal</td>
<td>Normal</td>
<td>Tubular degeneration</td>
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<tr>
<td>Erectile tissue</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>NA</td>
<td>Normal</td>
</tr>
<tr>
<td>Periurethral lymph node</td>
<td>ND</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bladder</td>
<td>Normal</td>
<td>Hemorrhage, mild</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rectum</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pelvic fat</td>
<td>Normal</td>
<td>ND</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>ND</td>
<td>ND</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Abbreviations: ND, Not determined; KC, Kupffer cell; NA, not available; Centro-dissoc., centroacinar dissociation; degen., degeneration.

*Histopathology of tissues obtained at necropsy from Aotus nancymae. T348, T635, T442, and T659 were injected with G207 (1 × 10⁷ PFU) intraprostatically; T317 and T344 were not injected intraprostatically. Tissues were fixed in formalin and paraffin embedded. Sections were stained with hematoxylin and eosin.*
the Aotus monkeys exhibited changes in kidney, liver, and lung, which seemed unrelated to the study, as this characteristic was found to be common in this species (Todo et al., 2000). Both of the prostate-noninjected monkeys, T317 and T344, also had these histological features. The absence of histological changes, even in tissues containing PCR-detectable viral DNA sequences, supports the safety of G207 injection into the prostate. Taken together, these results demonstrates that G207, a conditionally replication-competent virus, effective against human prostate tumor xenografts in mice, is a safe vector in both mice and nonhuman primates after intraprostatic inoculation.

Whether immunosuppression, such as after chemotherapy, would alter the spread of virus is unknown. However, two earlier studies conducted with G207 may address this indirectly. We conducted a phase I clinical trial of G207 in patients with malignant glioma (Markert et al., 2000). Some of these patients were receiving dexamethasone, an immunosuppressive drug, and no encephalitis or other HSV-1-associated disease was detected at doses up to $3 \times 10^9$ PFU of G207. In a mouse syngeneic tumor model, short-term administration of dexamethasone did not affect the efficacy of G207 treatment or the titers of infectious G207 isolated from tumors measured up to 5 days postinjection (Todo et al., 1999b).

One concern about using G207 in treating human prostate cancer is whether reactivation of wild-type HSV-1 can occur from dorsal root ganglia and cause disease in individuals who are latently infected with HSV. Although this has not been tested directly after intraprostatic inoculation with G207, two studies suggest that reactivation might be unlikely. We have previously shown that superinfection with $10^7$ PFU of G207 into the brains of BALB/c mice in which prior KOS strain infection was established did not lead to detectable reactivation of infectious virus or disease (Sundaresan et al., 2000). Also, Wang et al. (1997) have shown that intracerebral infection with hrR3 strain of HSV-1 did not reactivate wild-type KOS virus in adult rats that were latently infected with KOS by corneal scarification or intracerebral injection.

Paielli et al. (2000) reported on the safety evaluation of a replication-competent adenovirus (Ad5-CD/TKrep) administered intraprostatically in C57BL/6 mice and showed that the virus persisted in the mouse urogenital tract (prostate and testis) and liver up to 28 days postinjection but with minimal toxicity (Paielli et al., 2000). Virus persistence in the male urogenital tract was not associated with germ line transmission of the virus. However, persistence in the liver was associated with sustained but minimal inflammation of liver and gall bladder, which became acute with prodruk (5-flourouracil plus ganciclovir) therapy. In a phase I clinical trial, administration of replication-deficient adenovirus (AdV/RSV-tk) into the prostate of humans resulted in some hepatic toxicity after ganciclovir (GCV) treatment (Herman et al., 1999). The results from our study, demonstrating that intraprostatic administration of G207 does not lead to any complications in the liver of mice and nonhuman primates, suggest that use of G207 might prove to be a safe alternative for viral and/or gene therapy of prostate cancer. In particular, it might be initially appropriate for locally recurrent prostate cancer after radiation therapy since surgery has a higher complication rate and a higher incidence of neural damage in those patients who were previously irradiated. G207 has been shown to be efficacious in treating human prostate cancer occurring in animal models after radiation therapy (Walker et al., 1999). In addition, G207 was found also to act in some settings as an in situ cancer vaccine by inducing systemic antitumor immunity (Todo et al., 1999; Todo et al., 1999a).

The issue of whether G207 can replicate in normal human prostate tissue is not conclusively addressed in this study and destruction of normal prostate tissue by G207 could potentially occur. Nevertheless, the destruction of normal prostate tissue would likely be clinically tolerated, as it is different from surgical removal, so long as the tumor is destroyed and the adjoining nervous tissue is spared. Most of the side effects, such as incontinence or erectile dysfunction, observed with current treatment protocols for prostate cancer, arise from nerve tissue damage. This can be mitigated with the use of G207, which is nonpathogenic to nerves, and thus offers an advantage for the treatment of prostate cancer. The current study demonstrating safety of intraprostatically injected G207 in mice and nonhuman primates, and previous data of G207 safety in murine peripheral nerves and in the brain of humans, provide a strong basis for a phase I trial of G207 for prostate cancer as a "nerve-sparing" vector.

ACKNOWLEDGMENTS

This study was supported by grants to R.L.M. from the Association for the Cure of Cancer of the Prostate (CapCure) and the United States Department of Defense (Army Prostate Cancer Research Program) and from NeuroVir, Inc. We also acknowledge the contributions of Jonathan Walker, M.D., and Ali Sajadi, M.D. (residents of the Department of Urology, Georgetown University Hospital, Washington, D.C.), and Laura Rutten-Call (Veterinary Technician, Division of Comparative Medicine, Georgetown University Medical Center, Washington, D.C.). S.D.R. and R.L.M. are consultants to NeuroVir, Inc., which has a license from Georgetown University to commercialize G207.

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G207 SAFETY IN MOUSE AND MONKEY PROSTATE


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Received for publication December 15, 2000; accepted after revision March 16, 2001.
Ionizing Radiation Does Not Alter the Antitumor Activity of
Herpes Simplex Virus Vector G207 in Subcutaneous Tumor Models of
Human and Murine Prostate Cancer

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Sanford Katz1
Ellen K. Wittmack1
Susan Varghese2,4
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RUNNING TITLE: Radiation and viral therapy for prostate cancer
KEY WORDS: Herpes virus, gene therapy, prostate cancer, radiotherapy

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ABSTRACT

Viral gene therapy against malignant tumors holds great promise for tumors that are susceptible to the oncolytic activity of viruses. One advantage of oncolytic viral therapy is that it can potentially be combined with other therapies, such as radiotherapy, to obtain an enhanced tumor response. In the case of prostate cancer, herpes simplex virus (HSV) mediated therapies have been shown to be highly effective in animal models; however, studies of the efficacy of combined viral and radiation therapy have not yet been reported. In this study, we have combined G207, a multimutated HSV type 1 vector, with external beam radiation therapy of prostate tumors grown subcutaneously in mice. We examined both the human LNCaP tumor in athymic mice, and the mouse transgenic TRAMP tumor in either athymic mice or its syngeneic host, C57BL/6 mice. Virus was delivered either intravenously, in the case of LNCaP, or intratumorally, in the case of TRAMP. We found that individually, either G207 or radiation was effective in delaying tumor growth in these models. However, delivering the treatments simultaneously did not produce an enhanced effect.

NONSTANDARD ABBREVIATIONS: None
INTRODUCTION

The use of replication-competent viruses for tumor therapy is a promising strategy that has progressed to early clinical trials (1-5). Replication-competent or conditionally-replicating herpes simplex virus type 1 (HSV-1) vectors have been generated by mutating genes involved in viral DNA synthesis and/or virulence in order to target viral replication and toxicity to tumor cells (6, 7). G207 is a multimutated HSV-1 vector that lacks both copies of the ICP34.5 gene, a gene required for neurovirulence, and contains an insertion of the lacZ gene inactivating the ICP6 gene, encoding the large subunit of ribonucleotide reductase (8). Studies in both human xenograft and syngeneic mouse tumor models have demonstrated the value of HSV based therapies in terms of both growth inhibition and cures (9). These findings await confirmation in human clinical trials.

One advantage of viral gene therapy is that it can potentially be combined with other therapies to obtain an enhanced tumor response. In fact, recent reports about combination viral/radiotherapy and viral/chemotherapy in glioma and head and neck cancer animal models suggest that this may be a viable approach (10-15). Human prostate tumor cells are particularly sensitivity to HSV-1 vectors (16), and this has lead to experimental treatment strategies that deliver mutated viruses by either intratumoral or intravenous injection. However, studies on the efficacy of combined viral and radiotherapy have not been reported. In this study, we assess the ability of radiation to affect the activity of G207 against prostate cancer. G207 treatment was combined with external beam radiation therapy of prostate cancer grown subcutaneously in mice. We used both the LNCaP human tumor in athymic mice, and the transgenic TRAMP mouse
tumor in either athymic mice or its syngeneic host, C57BL/6 mice. Virus was delivered either intravenously, in the case of LNCaP, or intratumorally, in the case of TRAMP. We found that G207 and radiation were each effective in producing growth delay in these models, but simultaneously delivering the treatments did not produce an enhanced effect.
MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human prostate cancer cell line LNCaP (Georgetown University Medical Center, Lombardi Cancer Center Tissue Culture Shared Resource, Washington, DC) was maintained in RPMI 1640 (Biofluids) containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). The TRAMP-C2 mouse prostate cancer cell line (17) was grown in DMEM high glucose (DMEM-HG, Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Life Technologies, Inc.), 5% Nu-serum IV (Collaborative Biomedical Products), and $10^{-8}$ M dihydrotestosterone (Sigma Chemical Co., St Louis, MO). Penicillin-streptomycin (Mediatech) and L-glutamine (Mediatech) were added to each culture and the cells were maintained at 37°C with 5% CO$_2$. Both cell lines were confirmed to be free of mycoplasma contamination.

Preparation and Injection of Cells into Animals

Four to six week old C57BL/6 black or NCRNU athymic male mice were obtained from Harlan Laboratories (Indianapolis, IN), or Taconic Laboratories (Germantown, NY), respectively. All animals were quarantined for one week before the study and allowed access to food and water ad libitum. The animals were anesthetized by intraperitoneal injection of 0.15-0.2 ml of 10% sodium pentobarbital (Abbott Laboratories, North Chicago, IL) in bacteriostatic saline (0.9% sodium chloride; Abbott Laboratories) with
6% ethyl alcohol. C57BL/6 mice were shaved in the rump before injections. 1x10^7 cells in 0.1 ml were injected subcutaneously in the sacral region of each animal to induce tumors in the TRAMP experiments. For the LNCaP experiments, the cells (1x10^7) were first mixed with an equal volume of Matrigel (Collaborative Biochemical Products, Bedford, MA) and then injected. Tumors were measured twice weekly by calipers to within 0.5 mm, and volumes were calculated (V=HxLxW) and recorded. Animals were randomized into treatment groups once their tumor size was in the range of 100-320 mm^3. After tumor cell injection, LNCaP tumors took 5-6 weeks to grow to treatment size, whereas TRAMP tumors grew in 1-3 weeks. In the LNCaP experiment, animals were given G207 (2 x 10^7 pfu) by tail vein injection on days 0 and 4, and irradiated on days 1-5. In both TRAMP experiments, animals were given G207 (2 x 10^7 pfu) intratumorally on days 0, 3, and 6, and irradiated daily on days 1-5. On days with both virus injection and irradiation, the virus was given to the animals before the irradiation occurred. The animal procedures described here were approved by the Georgetown University Animal Care and Use Committee.

Irradiation of Prostate Tumors LNCaP and TRAMP

A ^{137}Cs Shepherd Mark I irradiator was used to irradiate the tumors in the sacral region of the animal. The mice were restrained in clear plastic holders with a lead cover, which contains a port through which the radiation can enter to irradiate the tumor. The holders were placed behind a lead wall, which shields the mouse’s body, exposing the
irradiation port of the holder above the edge of the wall. Four animals were irradiated simultaneously. Doses to the tumor under this geometry were confirmed using a phantom mouse and thermoluminescent dosimetry. In the LNCaP experiment, the tumors were given 10 Gy fractionated over days 1-5 (i.e. 2 Gy/day). In the TRAMP experiments, the tumors were given a total of 20 Gy over days 1-5 (i.e. 4 Gy/day).

Clonogenic Cell Survival Curves

Logarithmically growing cells were harvested and seeded into tissue culture flasks at various cell numbers depending upon the radiation dose which the flask was to receive (i.e. more cells for higher doses), so that the final number of survivors in each flask would be similar. After allowing time for attachment, the flasks were irradiated to various doses and returned to the incubator for two weeks. The flasks were stained to reveal colonies produced from the clones of surviving cells and counted. The fraction of survivors relative to the original number of cells seeded was calculated and then normalized to the zero-dose plating efficiency to determine the surviving fraction at each dose. The data were fitted to the single-hit multitarget curve model (18).

RESULTS

Human LNCaP Tumor Treatment

LNCaP is a commonly used hormone-responsive human prostate cell line that grows well, albeit slowly, as subcutaneous tumors in athymic mice (19). LNCaP tumor
cells are particularly sensitive to G207, both in vitro and in vivo (16). The LNCaP tumor is wild-type for p53 and secretes prostate specific antigen (PSA) (19). Its cellular radioresponses have been characterized for both clonogenic survival and apoptosis (20-23). Because the LNCaP/athymic mouse xenograft model has been prevalent and important in prostate cancer research (24), we chose this as one of our models for studying combined radiation/G207 effects.

We previously found LNCaP tumors to be highly sensitive to intratumorally and intravenously injected G207 (16). Even a single intratumoral injection of G207 (2 X 10^7 pfu) caused a major reduction in tumor volumes with complete eradication of 25% of the tumors. This viral response was too great for combined radiation/viral studies, where partial responses for each agent are needed to assess potential interactions. Even when the viral titres were lowered to 10^5 pfu significant cures ensued (data not shown). For this reason, we decided to employ intravenous treatment with G207, which we knew gave a less robust treatment response (16). We employed two intravenous injections spread over four days, and combined it with five daily fractions of radiation, starting on the day after the first viral treatment.

Radiation therapy was effective at inhibiting tumor growth (Fig. 1), however, the irradiated tumors grew back relatively quickly. In contrast, volumes of G207-treated tumors were markedly reduced and regrowth occurred much more slowly than for irradiated tumors (Fig. 1). Nevertheless, by 40 days all of the tumors had started to grow back. For the combined radiation and G207 treatment, tumors regressed slightly faster than for G207 alone, but ultimately reached the same minimum volume -- again on day 40
-- at which point they began to regrow at a rate that was indistinguishable from G207
treatment alone (Fig 1). Nadir tumor volumes were statistically significantly different at p
< 0.05 between all treatment groups, except for radiation plus G207 versus G207 alone,
which were not significantly different from each other. There were no cures in any of the
groups.

**Murine TRAMP Tumor Treatment**

TRAMP represents a relatively new animal model for prostate cancer research (17, 25). In this syngeneic mouse model, transgenic mice carry the SV40 large tumor antigen
linked to a prostate specific promoter. Expression of the transgene in prostate tissue
caused tumors to arise *in situ* at about 8 weeks of age, and these tumors resembled
naturally arising prostate cancer. Several tumor cell lines have been established in tissue
culture from the prostate tumors of TRAMP mice. These tumor lines no longer express
large T antigen (17).

TRAMP tumor cell lines grow very well subcutaneously in either athymic mice or
in the syngeneic parental mice, C57BL/6, from which the TRAMP transgenic mice were
derived. This feature of the model provided major advantages, since G207 treatment of
murine tumors in syngeneic mice leads to a potent antitumor immune response (26, 27).
The TRAMP tumor model allows us to examine the effect of the immune system on both
treatment strategies and their combination by comparing efficacy in immunocompromised
and immunocompetent mice.
The TRAMP model system is not as well characterized for radiation responses as LNCaP; however, it is known to be wild-type for p53, like LNCaP (17). We were able to confirm the p53 phenotype by showing radiation induction of the p53-transcriptionally-activated p21WAF1/CIP1 protein in both LNCaP and TRAMP cells in vitro (data not shown). Preliminary studies with subcutaneous TRAMP tumors suggested that they were twice as resistant to radiation therapy compared to LNCaP. This was consistent with in vitro clonogenic cell survival curve analysis (Fig. 2), suggesting that the tumor resistance of TRAMP was an intrinsic property of the cells, and not due to possible tumor physiology differences. Hypoxia, for example, has been reported to affect tumor radioresponses in other prostate cancer animal models (28). Due to the greater radioresistance of TRAMP tumors, 20 Gy was used for the therapy dose, rather than the 10 Gy that was used for LNCaP.

Mouse tumor cells, in general, are less susceptible to G207 replication and cytotoxicity than human tumor cells. Growth curves for untreated TRAMP tumors in athymic mice showed that they grow twice as fast as LNCaP tumors, and the delay produced by G207 was much less than that for LNCaP (Fig. 3). TRAMP tumors treated with radiation therapy alone produced about twice the growth delay of G207 alone. Combined radiation and G207 produced no greater delay than radiation therapy alone.

In order to assess the possible influence of an intact immune system in the TRAMP response to G207 and radiation, the TRAMP tumor experiment was also conducted in C57BL/6 mice (Fig. 4). Compared to the athymic mouse host, G207 was much more effective in inhibiting TRAMP tumor growth in C57BL/6 mice (Fig. 3 vs.
Fig. 4). In fact, the G207 delay was now greater than the delay for radiation alone. This increased efficacy of G207 in syngeneic mouse model is consistent with results obtained with CT26 colon carcinoma in BALB/c mice, N18 neuroblastoma in A/J mice, and M3 melanoma in DBA/2 mice (26, 27), suggesting that immune responses contribute to the antitumor activity of G207.

In contrast to the G207 response, tumor growth with radiation alone was similar in athymic and C57BL/6 mice, suggesting that the immune system was not affecting TRAMP tumor regression caused by radiotherapy. Although tumor radioresponses are largely thought to be driven by intrinsic properties of tumors(29), recently it has been reported that T-cells and natural killer cells may promote radiotherapy tumor regression in some tumors(30). Nevertheless, in our model the presence or absence of a thymus did not affect tumor radioresponse, and the combined radiation and G207 treatment produced no greater tumor delay than G207 therapy alone, in either host. Also, an interaction between G207 and radiation treatment was not seen for the TRAMP tumor regardless of the host strain.

DISCUSSION

It has been reported that the efficacy of therapeutic HSV-1 R3616 against subcutaneous and intracranial human U87 malignant glioma xenografts in athymic mice is significantly enhanced by radiation (10, 13). Similar findings were reported for HSV-1 R7020 against subcutaneous human head and neck cancer cell line, SQ20b (11). In the current study, G207, another therapeutic herpes vector is evaluated for combination
therapy against prostate cancer. *When the combination of G207 and radiation did not enhance efficacy in the TRAMP syngeneic tumor model, we hypothesized that this might be due to species differences or the effect of cellular immunity. Therefore, we examined a human xenograft in athymic mice, as was the case in the Weichselbaum studies (10, 11, 13). In neither a human nor a mouse tumor model system was there a benefit from combining radiation with G207.*

*In our experiments, radiation treatments were fractionated over five consecutive days (i.e. experimental days 1-5). We find that this fractionation regimen is amenable to a wide variety of subcutaneous tumor models, regardless of differences in tumor growth rates or intrinsic cellular radiosensitivities, and it allows easier cross comparisons between the radioresponses of tumors since the number and timing of fractions is always constant. For intratumoral injections, we used three injections, timed to be before (day 0), during (day 3), and after (day 6) radiation therapy, in order to help ensure that we would cover all possible temporal sensitivity windows for radiation/virus interactions. For intravenous injections we injected on day 0 and day 4, because we had previously shown that this injection regimen worked well with LNCaP tumors(16). It was not our intention to directly duplicate the protocols Weichselbaum's group because different experimental regimens were used in each of their three publications(10, 11, 13), and no particular protocol appeared to be critical. Also, because radiation treatments with different doses and fractionation schemes was seen to enhance the antitumor activity of both R7020 and R3616 in glioma and carcinoma, we did not expect that it would be necessary to exactly imitate a particular treatment regimen in order to see an effect with*
G207. Furthermore, Weichselbaum and coworkers did not directly compare the effect of different time delays of irradiation post-infection; however, in the one study where different delays were used, there were no indications that a shorter time (4 hr) was better than a longer delay (24 hr). Therefore, we do not believe that the failure of radiation and G207 to act synergistically or additively in our prostate tumor models can be attributed merely to minor temporal differences between virus and irradiation treatments. Rather, it is more likely that differences in tumor type and biology play a role in the phenomenon reported by Weichselbaum and coworkers.

Despite the fact that the combination of G207 and radiation did not enhance the tumor response over the most effective individual therapy, there are several findings that are important for designing tumor therapy. The TRAMP tumor maintained the same radiosensitivity in either the athymic or immunocompetent host. This was expected, since immune responses are not thought to be a major factor in tumor radioresponses (30). G207, however, worked worse than radiation in athymic mice, but better than radiation in C57BL/6 mice. This illustrates the dual factors contributing to the viral antitumor response -- direct oncolytic activity and induction of tumor-specific immune responses.

Although radiation did not sensitize the tumors to virus, it also did not decrease the efficacy of the virus either. These results combined with our earlier published results (16), indicating that tumors that recurred following radiation therapy remained sensitive to virus, suggest that G207 therapy might be a useful therapy for tumors that recur in the radiation field. Also, since localized radiotherapy would not result in systemic immunosuppression, it is less likely than chemotherapy to interfere with subsequent
immune-mediated tumor cell killing by G207. Along these lines, intravenous administration of herpes virus following irradiation might have the added benefit of targeting metastases, that local radiation therapy alone cannot reach. Conversely, these results suggest that little benefit is derived from delivering both treatments simultaneously. Since the radiation adds little to the cytotoxicity of prostate tumors produced by the virus alone, delivering radiation at the time of viral treatment might even preclude later use of radiotherapy to treat recurring local disease. These results taken together with our earlier findings that recurring irradiated tumors remained fully sensitive to G207 (16) suggest that sequential, rather than simultaneous, treatment with radiation followed by G207 may produce the best results in terms of delaying tumor regrowth and prolonging survival.

The discrepancy between these findings in prostate, and those previously reported with glioma and head and neck cancer, may reflect more than simple tumor type differences. R3613 and R7020 are more virulent viruses that replicate better than G207. If, as the previous investigators suggest, the enhanced effect is due to increased viral replication in irradiated cells (10) -- possibly as the result of host cell DNA damage (31) -- then viruses with a high replication capacity might be better able to capitalize on this. Alternatively, the relatively high sensitivity of prostate tumors to G207 herpes virus (16) may mean that they are already fully sensitized to the cytotoxic effects of the virus, and irradiation cannot further enhance killing by virus. Regardless, our findings suggest that tumor sensitization to herpes viral therapy by radiation is not a universal phenomenon,
and that both viral and tissue specific factors may be strong determinants of the effect, and warrant further study.

ACKNOWLEDGEMENTS

This study was supported by grants from the Association for the Cure of Cancer of the Prostate (CaP Cure) and the United States Department of Defense DAMD17-98-1-8490 (Army Prostate Cancer Research Program). S.D.R. and R.L.M. are consultants to MediGene Inc., which has a license from Georgetown University for G207.

REFERENCES


LEGENDS

Figure 1: Mean relative volumes of LNCaP prostate tumors in athymic male mice. The mean relative tumor volumes, compared to the first day of treatment, of tumor-bearing mice treated with virus buffer (Controls), G207, irradiation (RT), or both (RT + G207). Irradiated animals were treated for Days 1-5 of the experiment with 2-Gy daily fractions for a total dose of 10 Gy. Virus-treated animals were given virus at $2 \times 10^7$ pfu by tail vein injection on Days 0 and 4 of the experiment. Each point represents the mean tumor volume plus/minus 1 S.E.M. In some cases where individual animals were sacrificed due to large tumor burden, the tumor size at the time of sacrifice was used for subsequent calculations of mean tumor volume.

Figure 2: Radiation survival curves for TRAMP and LNCaP cells. Points represent the mean of four independent experiments, each performed on different days; bars represent the standard deviation of the means. The data were fitted to the single-hit multitarget survival model and $D_0$ and $D_q$ parameters, respectively, measured in Gy, for each cell line which were as follows: Tramp, 1.763 and 1.219; LNCaP, 1.015 and 1.631.

Figure 3: Mean relative volumes of TRAMP prostate tumors in athymic male mice. The mean relative tumor volumes, compared to the first day of treatment, of tumor-bearing mice treated with virus buffer (Controls), G207, irradiation (RT), or both (RT + G207). Irradiated animals were treated for Days 1-5 of the experiment with 4-Gy daily
fractions for a total dose of 20 Gy. Virus-treated animals were given virus at $2 \times 10^7$ pfu by intratumoral injections on Days 0, 3, and 6 of the experiment. Each point represents the mean tumor volume plus/minus 1 S.E.M. In some cases where individual animals were sacrificed due to large tumor burden, the tumor size at the time of sacrifice was used for subsequent calculations of mean tumor volume.

Figure 4: Mean relative volumes of TRAMP prostate tumors in male C57BL/6 mice.

The mean relative tumor volumes, compared to the first day of treatment, of tumor-bearing mice treated with virus buffer (Controls), G207, irradiation (RT), or both (RT + G207). Irradiated animals were treated for Days 1-5 of the experiment with 4-Gy daily fractions for a total dose of 20 Gy. Virus-treated animals were given virus at $2 \times 10^7$ pfu by intratumoral injections on Days 0, 3 and 6 of the experiment. Each point represents the mean tumor volume plus/minus 1 S.E.M. In some cases where individual animals were sacrificed due to large tumor burden, the tumor size at the time of sacrifice was used for subsequent calculations of mean tumor volume.