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A Novel Approach to Cancer Therapy Using an Oncolytic Herpes Virus to Package Amplicons Containing Cytokine Genes

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There are two promising herpes viral-based anticancer strategies: one involves replication-defective viruses to transfer therapeutic transgenes, and the other involves replication-conditional oncolytic viruses, which selectively infect and destroy cancer cells directly. This study examines a novel dual herpesvirus preparation, which combines the immunostimulatory effects of amplicon-mediated *IL2* expression with direct viral-induced oncolysis. The oncolytic virus G207 was used as the helper virus to package a herpes simplex virus (HSV)-amplicon vector carrying the gene *IL2* (HSV-IL2), yielding a single preparation with two complementary modes of action. *In vivo* comparison was carried out in a syngeneic squamous cell carcinoma flank tumor model. We directly injected established tumors with HSV-IL2, G207, G207 mixed with HSV-IL2, or G207-packaged HSV-amplicon carrying the *IL2* transgene (G207[IL2]). Significant inhibition of tumor growth was seen at 2 weeks in the G207[IL2]-treated tumors relative to controls (0.57 ± 0.44 cm^3 versus 39.45 ± 5.13 cm^3, $P < 0.00001$), HSV-IL2 (20.97 ± 4.60 cm^3), and the G207 group (7.71 ± 2.10 cm^3). This unique use of a replication-conditional, oncolytic virus to package a replication-incompetent amplicon vector demonstrates impressive efficacy *in vitro* and *in vivo*, and avoids the theoretical concerns of recombination with reversion to wild type.

Key Words: amplicon, herpes simplex virus type-1, interleukin-2, oncolytic virus

INTRODUCTION

Several paradigms of viral-based antineoplastic therapy are currently under investigation. Most of these approaches use replication-defective viruses as vectors to transfer therapeutic genes [1]. The replication-defective herpes simplex virus type-1 (HSV-1) amplicon vector is an efficient vehicle for gene transfer in human squamous cell carcinomas and other solid tumors [2–7]. The HSV-1 amplicon is a plasmid-based vector in which a transgene is directionally cloned into the HSV *PrPUC* backbone [8]. This plasmid is transfected into a cell that is then infected with a helper virus. The helper virus packages the plasmid thus producing the HSV-1 amplicon vector. It should be noted that the resultant product contains both HSV-1 amplicon and helper virus. The helper virus may have undesired effects and toxicity in gene transfer studies targeted at neoplastic processes. Traditionally, a replication-incompetent helper virus, namely D30EBA, has been used to produce HSV-1 amplicons [4,8,9]. Replication-conditional viruses, however, can also be used as helper viruses to create HSV-1 amplicon vectors [10]. Regardless of the helper virus used, HSV-1 amplicon vectors are promising vehicles for gene delivery.

Another approach to viral-based antineoplastic therapy uses replication-conditional viruses to target, infect, and lyse cancer cells [11–24]. These agents are genetically engineered to selectively infect malignant cells without causing disease in the host. Our laboratory has demonstrated therapeutic efficacy and safety of one such virus, G207, in animal models of several common epithelial malignancies [11,25]. Specifically, G207 lacks both copies of $\gamma_1 34.5$, a gene associated with neurovirulence. Additionally, the gene encoding viral ribonucleotide reductase (RR) is inactivated by an insertional mutation. This alteration confers selectivity for infecting rapidly dividing cells, as the virus requires this key enzyme of DNA synthesis to complete its life cycle [19]. The insertion of the *lacZ* marker gene allows infection to be

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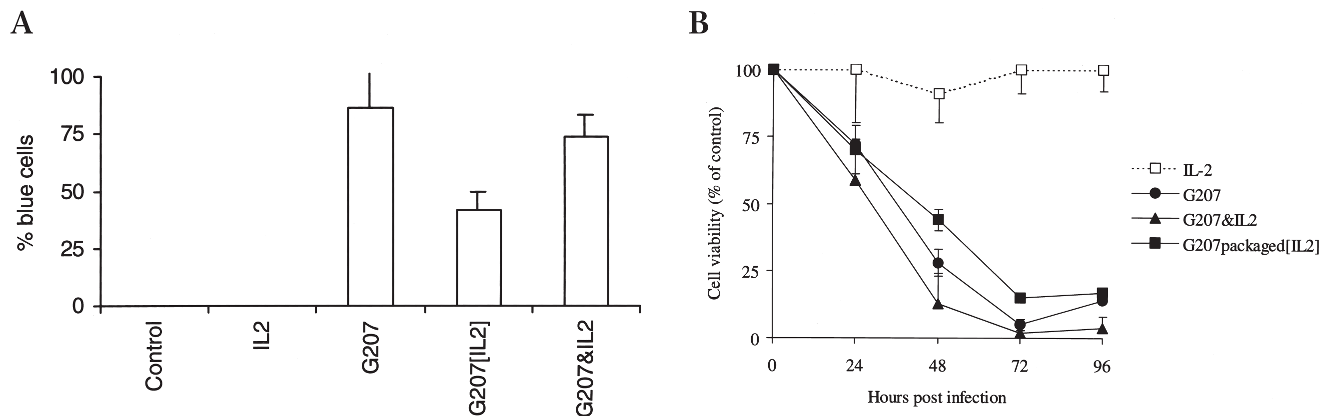

FIG. 1. *In vitro* infection efficiency and cytotoxicity of amplicon IL2, G207, G207-packaged amplicon IL2 (G207[IL2]), and G207 and amplicon IL2 (G207 & IL2) on SCCVII syngeneic cells. Both experiments were conducted with equivalent MOI (IL2 = 0.25 and G207 = 1.0). (A) Infection efficiency measured as the percentage of cells expressing the *lacZ* marker gene at 24 h after infection with the different viruses. (B) Cytotoxicity represented as cell viability by MTT assay of the different viruses over time compared with control. There is no statistical difference in efficacy of the different preparations of G207 (alone, mixed with IL2, or packaged with IL2).

tracked by histochemical analysis. The multiple mutations make reversion to wild type unlikely.

Here we evaluate the properties and efficacy of combining HSV-amplicon mediated gene transfer with viral oncolysis in a single viral preparation. We use the oncolytic virus G207 to package the herpes amplicon plasmid carrying the immunomodulatory transgene encoding interleukin-2 (*IL2*). This approach obviates the need for a defective packaging virus, which can be toxic and, when combined with an oncolytic virus, carries a theoretical risk of reversion to wild type. It is postulated that the high levels of IL2 produced in the tumor microenvironment by HSV-amplicon mediated gene transfer, in combination with the release of antigenic tumor cell components by selective G207 mediated cell lysis, would combine for a potent antitumor response.

RESULTS

Determination of Infection and Cell Lysis

To determine *in vitro* infection efficiency of G207[IL2], we carried out an assay for marker gene product production (β-gal) on SCC VII cells *in vitro*. Cells that turned blue upon exposure to X-gal solution were infected with G207 and were expressing the inserted viral gene *lacZ*. Uninfected cells and those transduced by HSV-IL2 did not express *lacZ*. All three G207 constructs demonstrated efficient transduction in the SCC VII cell line by 24 hours postinfection (Fig. 1A). The percentage of cells expressing *lacZ* was highest in wells infected by G207 alone (86 ± 26%). G207[IL2] also demonstrated efficient transduction in this cell line.

To determine comparative cytotoxic potential of the different viruses, SCC VII cells were infected *in vitro* at a multiplicity of infection (MOI) of 1.0. MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, a tetrazolium salt based colorimetric assay, was performed on identical plates over a 96-hour period. Cytotoxicity was measured as cell viability compared with control (Fig. 1B). Amplicon IL2 had no cytotoxic effect on the SCC VII cells. All three G207 constructs efficiently lysed the cells by 72 hours (cell viability of 15% or less). There was no statistical difference in *in vitro* cytotoxic potential of the parent virus or the packaged construct.

Demonstration of IL2 Production

We tested SCC VII cells infected with G207, HSV-IL2, and G207[IL2] for production of IL2 *in vitro*. G207 did not

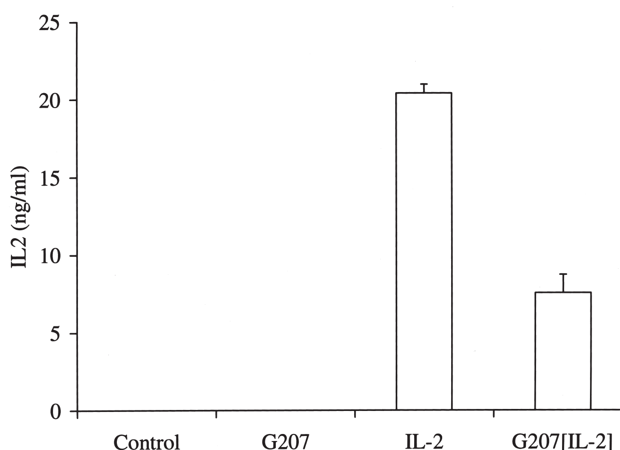

FIG. 2. *In vitro* IL2 production induced by G207, amplicon IL2, or G207 packaged with IL2 (G207[IL2]) in SCC VII. IL2 production quantified by ELISA in supernatants 24 h after infection with each virus. Both amplicon IL2 and G207[IL2] produce high levels of the cytokine, whereas G207 alone does not.

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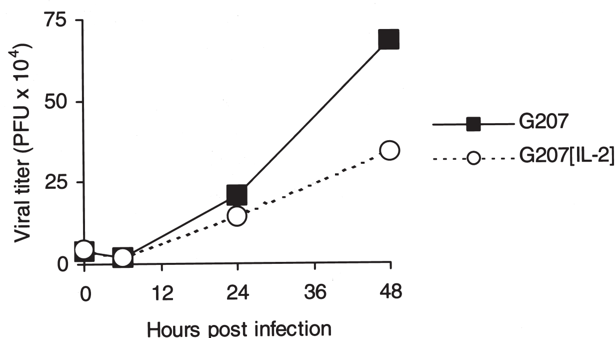


FIG. 3. *In vitro* viral growth curves for G207 and G207 packed with IL2 (G207[IL2]). We plated 5×10^5 SCC VII cells in 6-well plates and subsequently infected them with either virus at MOI 0.1. Cells and supernatant were collected by scraping at 0, 6, 24, and 48 h later and subjected to lysis and sonication. Titers were determined by standard Vero cell plaque assay. Both viruses demonstrate substantial replication in this animal cell line.

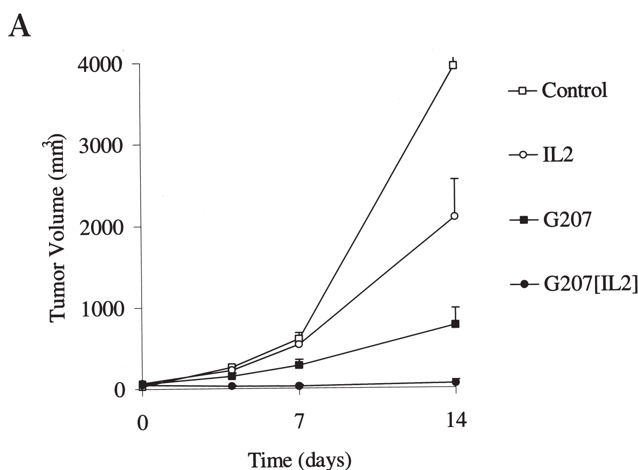
induce IL2 production in tumor cells, whereas HSV-IL2 and G207[IL2] induced nanogram quantities of the immune-stimulating protein (Fig. 2). The G207-packaged HSV-IL2 induced less IL2 production than the D30EBA-packaged HSV-IL2. These results, however, were not normalized to the number of remaining viable tumor cells, which may explain this finding. Specifically, the G207 helper virus present in the G207-packaged HSV-IL2 may have caused significant cell death and reduced the number of viable tumor cells available for gene transfer and IL2 production.

In Vitro Viral Growth Curves

To demonstrate viral replication in SCC VII cells, *in vitro* viral growth curves were constructed for G207 and G207[IL2]. SCC VII cells infected with each virus at a low MOI of 0.1 supported efficient viral replication (Fig. 3). At 0 hours, most of the virus was recovered (3.75×10^4 PFU). At 6 hours, viral recovery dropped for both constructs, suggesting active infection with viral uncoating and synthesis. By 48 hours, both viruses demonstrated significant replication within the SCC VII cell line.

Inhibition of Tumor Growth

Established SCC VII tumors in C3HeJ mice were injected with various combinations of G207 and the HSV-1 amplicon carrying IL2. The greatest inhibition of tumor growth was seen in the group combining G207 with the HSV amplicon-IL2 (G207[IL2]; $P < 0.00001$; Fig. 4A). In this group, three of seven animals had a complete response in which tumors could no longer be palpated or seen on histological examination. It should be noted that there were two different combination groups evaluated. One in which G207 and the HSV-1 amplicon were prepared and injected separately (G207 & HSV-IL2), and one in which the G207 virus was used as the helper virus to package the HSV-1 amplicon vector (G207[IL2]). This second combination group used a technique that allowed a single preparation to contain both the oncolytic G207 virus and the HSV-1 amplicon vector. When a comparison was made between these two groups with respect to their efficacy in inhibiting tumor growth, no difference was seen (Fig. 4B).



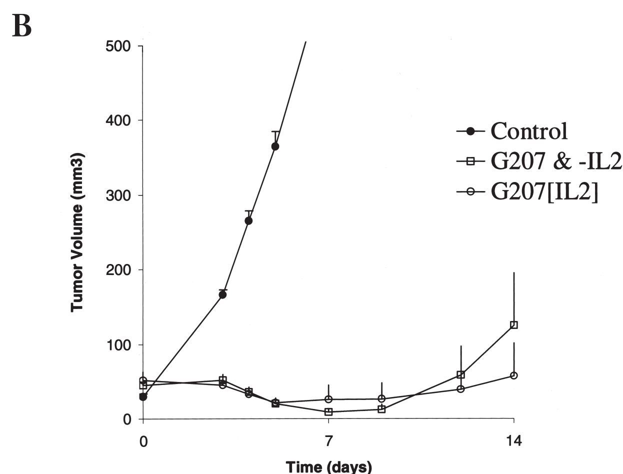


FIG. 4. Results of *in vivo* inhibition of the SCC VII flank tumors in a syngeneic tumor model (C3H/HeJ mice). (A) Graph showing tumor volume over time. Open boxes represent tumors injected with saline (Control); open circles represent the tumors injected with a single dose of 5×10^6 viral particles of HSV-1 amplicon carrying the *IL2* transgene (HSV-IL2); filled boxes represent the tumors injected with 1×10^7 viral particles of G207 on days 0, 3, and 6 [G207]; and filled circles represent the tumors injected with the G207 packaged HSV-1 amplicon-IL2 (G207[IL2]) on day 0 followed with 1×10^7 viral particles of G207 on days 3 and 6. (B) Graph showing tumor volume over time. Filled circles represent the tumors injected with saline (controls); open boxes represent the tumors injected with the G207 (1×10^7 viral particles) and HSV-1 amplicon–IL2 (5×10^6 viral particles) separately (G207 and HSV-IL2) on day 0 followed with 1×10^7 viral particles of G207 on days 3 and 6; and open circles represent the tumors injected with the G207-packaged HSV-1 amplicon–IL2 (G207[IL2]) on day 0 followed with 1×10^7 viral particles of G207 on days 3 and 6. Data plotted are the means ± standard errors of the mean.

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Determination of Cytokine Production and Infiltration of $CD4^+$ and $CD8^+$ Cells

IL2 production from SCC VII flank tumors was determined by sandwich enzyme immunoassay technique. Tumors that were injected with saline or the G207 virus alone had IL2 levels that were below detectable limits (Fig. 5). The various groups that had the HSV-1 amplicon carrying *IL2* as one of the components of the percutaneous injection all had significant levels of IL2, which exceeded 100 ng per gram of tumor tissue in one group. The G207-packaged HSV-IL2 induced greater IL2 production *in vivo* than the D30EBA-packaged HSV-IL2. These results are contrary to what one would expect from the *in vitro* data, in which the D30EBA-packaged HSV-IL2 yielded greater IL2 production. One explanation for this finding is that the human IL2 ELISA may not have been completely specific for human IL2, detecting both human and mouse IL2 produced as a result of the combination of G207-mediated tumor lysis and *IL2* transfer.

The proportion of $CD4^+$ and $CD8^+$ cells seen in the tumors is expressed as a percentage of lymphocytes or CD45-positive cells in Table 1. Injection of G207 alone resulted in a significant elevation in the percentage of $CD4^+$ and $CD8^+$ cells in the tumors. There also was a significant elevation of the percentage of $CD4^+$ and $CD8^+$ cells in both the HSV-amplicon-IL2 group and the G207[IL2] combination group.

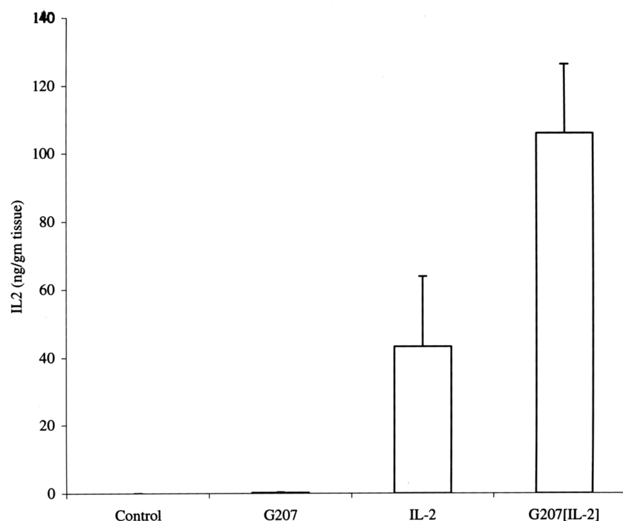

FIG. 5. *In vivo* quantification of IL2 production in SCC VII flank tumors 24 h after direct injection of equivalent titers of G207, HSV-1 amplicon IL2 (HSV-IL2), or G207-packaged HSV-1 amplicon IL2 (G207[IL2]). The graph shows the level of IL2 measured by ELISA in tumor cell lysates 24 h after injection of control (saline); G207, 1×10^7 viral particles of G207; HSV-IL2, 5×10^6 viral particles of HSV-1 amplicon carrying the *IL2* (HS-IL2); and G207[IL2], HSV-1 amplicon-IL2 (5×10^6 viral particles) packaged by G207 (1×10^7 viral particles). Data plotted are the means ± standard errors of the mean.

DISCUSSION

Most viral-based antineoplastic therapies currently in clinical trial use replication-defective viruses as vehicles for gene transfer [1]. Although oncolytic viral therapies offer a promising alternative, few studies have combined the immunostimulatory effects of virally mediated cytokine gene transfer with an oncolytic virus such as G207. In a prior study [10], the replication-conditional oncolytic virus G207 was used to package amplicons carrying *IL12*. The antitumor effect of this preparation in a mouse colon carcinoma model was demonstrated. Amplicons carrying GM-CSF packaged in a similar fashion using G207 as the helper virus failed to show efficacy in suppressing tumor growth [10]. Here we produced HSV-1 amplicons carrying the *IL2* transgene using G207 as the helper virus. This unique combination offers a treatment paradigm that causes tumor cell lysis, resulting in the release of antigenic tumor cell fragments, along with the production of high levels of cytokine in the tumor microenvironment. The data presented here support this as an effective antineoplastic treatment in a solid tumor model.

TABLE 1: Quantification of $CD4^+$ and $CD8^+$ cells in SCC VII flank tumors in a syngeneic tumor model (C3H/HeJ mice)

	% CD4 Positive	% CD8 Positive
Control	2.49	1.08
G207	11.9	17.4
Amplicon IL2	3.08	2.91
G207[IL2]	11.6	25.9

Shown is the percentage of CD45-positive cells recovered from tumor cell suspensions on day 10 that are $CD4^+$ or $CD8^+$ for the various experimental groups. Control groups were injected with saline; the G207 group was injected with 1×10^7 viral particles of G207 on days 0, 3, and 6; the IL2 group was injected on day 0 with 5×10^6 viral particles of HSV-1 amplicon carrying the *IL2* transgene (amplicon IL2); and the G207[IL2] group was injected with 1×10^7 viral particles of *G207* and 5×10^6 viral particles of HSV-1 amplicon carrying *IL2* (amplicon IL2) on day 0 and 1×10^7 viral particles of G207 days 3 and 6.

A concern of the combination of the G207 virus with HSV-1 amplicon mediated cytokine production is the theoretical possibility of recombination of the G207 virus with the helper virus used to produce the HSV-1 amplicon and resultant reversion to wild-type HSV-1 virus. The two viral preparations reported here use two different helper viruses to package the amplicons. The first uses the D30EBA virus as the packaging virus [4,8,9]. This virus has a deletion in the IE3 gene and as a result is replication incompetent. The amplicon preparation produced by this helper virus contains both amplicons as well as the D30EBA helper virus. When this is used in combination with the G207 virus, a theoretical risk of recombination between the D30EBA helper virus and the G207 virus and reversion to the wild type exists. The other viral preparation using the replication-competent, multimutated G207 virus as the packaging virus, however, averts this risk. In

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this preparation, the G207 virus is used as the helper virus to package the amplicons and thus yields a preparation which contains the amplicon plasmids within the HSV-1 envelope, provided by G207, as well as the oncolytic G207 virus. This packaging process provides an HSV-1 amplicon and an oncolytic virus in a single preparation and thus avoids the chance of wild-type reversion. Additionally, as helper-free virus systems are developed, this chance for recombination and reversion to wild type will also be negated [26–31]. Finally, novel approaches that avoid the potential for wild-type reversion, such as the use of oncolytic viruses (which themselves encode cytokine transgenes), may also be possible.

It is interesting to note from the *in vivo* data that treatment of flank tumors with the oncolytic G207 virus alone resulted in a significant increase in the percentage of both $CD4^+$ and $CD8^+$ cells within the tumor. This suggests that G207-mediated effects on flank tumors result in an immune response characterized by an increase in immune effector cells. Additionally, in other preclinical studies, a distant bystander effect has been seen in tumors distant from tumors treated with the oncolytic virus G207 [11,32]. An explanation for this distant bystander effect postulates that G207-mediated tumor lysis results in an antitumor immune response which suppresses tumors distant to the treated tumor. A distant bystander effect was lost when an athymic mouse model was used, suggesting that a T-cell component is responsible for the G207-mediated distant bystander effect [32]. These observations support an antitumor immune response related to the effects of G207 on tumor cells. An approach that attempts to augment the G207-mediated antitumor immune response to increase the antitumor efficacy of this therapy naturally follows. In our report, the use of HSV-1 amplicon mediated gene transfer of *IL2* to bolster the effects of the G207 virus resulted in substantial augmentation of the inhibition of tumor growth. This antitumor effect was greater than the effect of either G207 or HSV-1 amplicon mediated gene transfer of *IL2* alone. It is hypothesized that the local production of cytokines in the microenvironment where tumor cells undergo G207-mediated cell lysis yields a potent antitumor immune response. The characteristics of this antitumor response remain undetermined. Possible mechanisms by which viral-mediated tumor cell lysis and cytokine expression combine to cause a significant increase in antitumor response may be related to the immunomodulatory effects of HSV-1 infection and oncolysis on immune effector cells and/or antigen presenting cells.

Although this study evaluated the use of a single cytokine, IL2, with an oncolytic virus, other cytokines and immunoregulatory products may have potent effects in augmenting the effects of oncolytic viruses on tumors. As previously mentioned, IL12 and GM-CSF have been studied in combination with G207 [10]. In this study, only IL12 was shown to augment the antitumor effects of G207. Local production of IL12A or IL2 results in a myriad of effects on the local immune response to tumor and tumor antigen presentation. IL12 stimulates the production of interferon-γ, the proliferation of natural killer (NK) cells, the proliferation of T cells, the cytolytic activity of $CD8^+$ T cells, and induction of $CD4^+$ T cells to differentiate into T helper type 1 (Th1) cells [33–36]. IL2, in contrast, stimulates the production of interferon-γ, IL-4, and TNF, the proliferation of activated T cells, and the cytolytic activity of T cells [37,38]. Although the plethora of effects mediated by these two cytokines shows significant overlap, studies combining IL2 and IL12 have shown synergy, suggesting activation of different components of immune response [39,40]. The data presented here, as well as the data reported previously [10], support the efficacy of either of these two cytokines (IL2 or IL12) in augmenting the antitumor immune response when combined with G207 [10]. Maximizing the synergy between G207-mediated tumor cell lysis and cytokines, chemotactic cytokines, costimulatory molecules, and other immunomodulatory agents awaits further study.

Our study provides evidence that the combination of the multi-mutated, oncolytic, replication-competent HSV-1 virus (G207 combined with the HSV-1 amplicon vector carrying an immunoregulatory transgene) has *in vivo* antitumor activity against squamous cell carcinoma tumors. This combination may be a useful agent in the treatment of solid human malignancies and these data encourage human trials using combined immunologically based gene therapy with oncolytic viruses in the treatment of human head and neck cancer.

METHODS

Cell culture. The SCC VII cell line was a gift from Ditmar Seeman (University of Florida). Cells were grown in minimal essential media supplemented with 10% fetal calf serum (FCS) under standard cell culture conditions.

Viruses. We cloned human *IL2* directionally into HSV *PrPUC*, containing the HSV-1 immediate early 4/5 promoter, as described [3,4,7,8]. A thymidine kinase negative variant of the D30EBA helper virus was prepared by growth on RR1 cells, which were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L), 10% FCSA, 1% penicillin/streptomycin, and 400 μg/ml bioactive geneticin (G418; Gibco BRL, Gaithersberg, MD). To package amplicons, RR1 cells were transfected by adding Lipofectin (Gibco BRL), waiting 5 min, and then adding the amplicon DNA solution drop-wise. Approximately 20 h after transfection, the D30EBA helper virus was added at a multiplicity of infection (MOI) of 0.2. After 1 h, additional medium containing 5% FCS was applied and amplicon virus stocks were harvested 48 h later. The amplicon to helper virus ratio was determined by titering each component separately. The helper virus was titered by standard plaque assay on RR1 cells and the HSV-1 amplicon vector carrying *IL2* (HSV-IL2) was titered by slot blot assay as described [4]. The amplicon titer used in this experiment was 2×10^8 amplicons per ml and the ratio of amplicon to helper virus (D30EBA) was 1:2.3.

The G207 virus was a gift from Robert L. Martuza and Samuel D. Rabkin (Georgetown University Medical Center). The G207 virus was constructed as previously described by inserting the *lacZ* marker gene into the ICP6 sequence of the $\gamma_1$34.5-deleted HSV-1 mutant R3616 [16,17]. G207 was propagated in African green monkey kidney cells (Vero cells), which were

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maintained in DMEM supplemented with 5% FCS, at 34°C. Forty-eight hours after exposure to an MOI of 0.02, cells were subjected to freeze thaw lysis and sonication to harvest virus. Following centrifugation (300*g* for 10 min at 4°C) supernatants were titered by standard plaque assay on Vero cells and stored at –80°C.

The G207 packaged amplicon vector was prepared in similar fashion to the HSV-1 vector described above, except G207 was used as the helper virus in place of D30 EBA. Briefly, Vero cells (in place of RR1 cells) were transfected with amplicon DNA carrying *IL2* 5 min after exposure to Lipofectin (Gibco BRL). After 20 h, the G207 virus was added to achieve an MOI of 0.2, followed 1 h later by addition of media containing 5% FCS. The G207 packaged HSV-amplicon carrying the *IL2* transgene preparation (G207[IL2]) was harvested at 48 h. The amplicon-IL2 to helper virus (G207) ratio was determined by titering each component separately as described above [4]. The amplicon titer used in this experiment was 8×10^6 amplicons per ml and the ratio of amplicon to helper virus (D30EBA) was 1:6.3.

In vitro. SCC VII murine squamous cell carcinoma cells were plated in 96-well, flat-bottomed plates at a density of 5×10^3 cells per well. After 12 h, wells were infected with HSV-IL2 (MOI 0.25), G207 (MOI 1.0), G207, and HSV IL2 (MOI 1.0 and 0.25) and G207[IL2] (MOI 1.0[0.25]). All assays were performed in triplicate and control wells were treated with medium alone. To measure infection efficiency, X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) staining was carried out on one plate 24 h following infection, using a described technique [5]. Infection efficiency was determined by counting blue cells and dividing by the total cell count.

To measure cytotoxic effects of the different viruses, MTT (Thiazolyl blue, Sigma) colorimetric assay was performed on identical plates 24, 48, 72, and 96 h following infection. Viable cells convert this tetrazolium salt to an aqueous-insoluble purple compound by means of functional mitochondrial dehydrogenase. The product is then dissolved in DMSO and measured on a standard plate reader at 550 nm. Results are recorded as ratio of abs_{550} of samples to controls.

To quantify *in vitro* IL2 production, SCC VII cells were plated in 12-well plates at 5×10^5 cells per well. After 12 h, triplicate wells were exposed to G207 (MOI 1.0), HSV-IL2 (MOI 0.25) or G207[IL2] (MOI 1.0/0.25) for 20 min, followed by two washes with medium. After 24 h, supernatants were harvested and centrifuged at 2000 rpm for 5 min. Subsequently, IL2 levels in the supernatant were quantified by human IL2 enzyme linked immunosorbent assay (ELISA) as described [5].

To demonstrate that G207[IL2] is capable of replicating in SCC VII cells, a viral growth curve was constructed and compared with one established for native G207. We plated 5×10^5 cells in 6-well plates. After 12 h, wells were infected with G207 or G207[IL2] at an MOI of 1.0 (based on G207 titer). Cells and supernatant were collected by scraping at 0, 6, 24, and 48 h from successive wells and subjected to three cycles of freeze-thaw lysis, sonication, and centrifugation at 2000 rpm for 10 min at 4°C. Resultant titers were determined by standard plaque assay on confluent Vero cells.

In vivo. Individual flank tumors were established in 50 C3HeJ mice by injecting 5×10^5 SCC VII tumor cells subcutaneously. When the tumor nodules became palpable (about 5 mm in diameter), animals were randomized to receive treatment with percutaneous, intratumoral injections of virus in various combinations. The groups were as follows: controls (n = 10) received injections of normal saline on days 0, 3, and 6; G207 (n = 10) received 1×10^7 PFU of G207 on days 0, 3, and 6; HSV-IL2 (n = 10) received 5×10^6 BFU of HSV-IL2 on day 0; G207 & HSV-IL2 (n = 10) received 1×10^7 PFU of G207 and 5×10^6 BFU HSV-IL2 on day 0 followed by 1×10^7 PFU of G207 on days 3 and 6; and G207[IL2] (n = 10) received 1×10^7 PFU of G207 and 5×10^6 BFU of HSV-IL2 in a single preparation on day 0 followed by 1×10^7 PFU of G207 on days 3 and 6. The animals were examined daily and their weights and tumor volumes were recorded three times per week. Tumor volume was assessed by assuming the shape to be that of a prolate spheroid with semi-axes "a" and "b" being one-half of the two maximal dimensions, and volume equal to $4/3\pi ab^2$ [41].

Determination of cytokine production and infiltration of $CD4^+$ and $CD8^+$ cells. Flank tumors were established in 50 C3HeJ mice by injecting 5×10^5 SCCVII tumor cells subcutaneously. When the tumor nodules became palpable (about 5 mm in diameter), animals were randomized to receive treatment with percutaneous, intratumoral injections of virus in various combinations. The groups were as follows: controls (n = 10) received injections of normal saline on days 0, 3, and 6; G207 (n = 10) received 1×10^7 PFU of G207 on days 0, 3, and 6; HSV-IL2 (n = 10) received 5×10^6 BFU of HSV-IL2 on day 0; G207 & HSV-IL2 (n = 10) received 1×10^7 PFU of G207 and 5×10^6 BFU HSV-IL2 on day 0 followed by 1×10^7 PFU of G207 on days 3 and 6; and G207[IL2] (n = 10) received 1×10^7 PFU of G207 and 5×10^6 BFU of HSV-IL2 in a single preparation on day 0 followed by 1×10^7 PFU of G207 on days 3 and 6. Twenty-four hours following the final treatment, tumors were harvested and cell lysates were prepared from the tumor tissue and analyzed for IL2 levels by ELISA as described [5]. The animals were euthanized on day 10 and tumors were harvested. Tumor cell suspensions were prepared by mincing the tumors and digesting them in collagenase (0.1%) for 45 min at 37°C and then washing them in PBS. Cells were then stained with anti-murine CD4 (Pharmagen, San Diego, CA; 1:100) and anti-murine CD8 (Pharmagen, San Diego, CA; 1:100) monoclonal antibodies. A FITC-labeled secondary antibody (Pharmagen, San Diego, CA; 1:100) was then used and the cells were also stained with a PE-labeled anti-murine CD45 monoclonal antibody (Pharmagen, San Diego, CA; 1:100). Flow cytometric analysis was carried out and the percentage of CD45 positive cells that were either $CD4^+$ or $CD8^+$ was calculated. The percentage of lymphocytes in the tumors that were $CD4^+$ or $CD8^+$ was calculated by dividing the number of cells that were either $CD4^+$ or $CD8^+$, respectively, by the number that were positive for CD45.

Statistical analysis. All data are expressed as means ± standard error of the mean. Comparisons between groups were made using a two-tailed Student's t-test.

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