Cyclophosphamide Allows for *In vivo* Dose Reduction of a Potent Oncolytic Virus

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Abstract

The success of cancer virotherapy depends on its efficacy versus toxicity profile in human clinical trials. Progress towards clinical trials can be hampered by the relatively elevated doses of oncolytic viruses administered in animal models to achieve an anticancer effect and by the even higher doses required in humans to approximate an animal bioequivalent dose. Such elevated doses of injected viral proteins may also lead to undesirable toxicities and are also very difficult to produce in a biotechnological setting. We report that a relatively potent herpes simplex virus type 1 oncolytic virus (rQNestin34.5) produces 45% survivors at a dose of 3×10^4 plaque-forming units (pfu) in a 9-day-old mouse model of human glioma. Unlike our previous findings with less potent oncolytic viruses, though, the preadministration of cyclophosphamide did not enhance this survival or affect oncolytic virus tumor distribution and tumor volume. However, when oncolytic virus doses were reduced (3×10^3 and 3×10^2 pfu), cyclophosphamide significantly enhanced both animal survival and oncolytic virus tumor distribution and also reduced tumor volumes. These findings thus show that cyclophosphamide allows for dose reduction of doses of a relatively potent oncolytic virus, a finding with implications for the development of clinical trials.
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Introduction

Virotherapy represents a relatively novel modality for intractable cancers, such as malignant gliomas (1). A variety of different viruses and viral mutants have been described: each of these truly represents a different drug, with its own targeting and toxicity profiles, akin to drug therapy. In this regard, we have described a novel, genetically engineered recombinant of herpes simplex virus 1 (HSV-1), designated as rQNestin34.5, which displayed potent oncolytic effects while retaining a favorable safety profile in animal models (2).

In human clinical trials, favorable safety profiles have been reported with virotherapy agents (3–9). Nevertheless, it is not unreasonable to expect that a toxicity common to the majority of, if not all, virotherapy agents may be represented by the acute phases of the immune response leading to inflammation and secondary organ damage. Histologic, radiologic, and/or clinical symptomatologies for such responses have been reported in some trials (3, 7).

We have previously shown that the immunosuppressive and anticancer agent cyclophosphamide enhances the viral oncolytic effect by its relatively pleiotropic action on reducing systemic host responses to the initial phase of an oncolytic viral infection. These responses include the complement system (10, 11), neutralizing and innate humoral immunity (12), and, possibly, systemic peripheral blood mononuclear cells and their production of antiviral cytokines (13). In addition, within an oncolytic virus–injected brain tumor, preadministered cyclophosphamide reduces tumor-associated phagocytic activity and a global transcription profile associated with innate immune responses against pathogenic microorganisms.[1] Most of these studies were done with the HSV-1 oncolytic virus hrR3, although some were also done with the HSV-1 oncolytic viruses MGH1 (12) and MGH2.[2] For each of these, the addition of cyclophosphamide enhanced the anticancer action.

One relevant factor is that HSV-1 oncolysis has usually required administered doses of oncolytic virus of $>10^7$ to 10^8 plaque-forming units (pfu) in animal models. There are two important issues with such elevated doses: (*a*) they translate into large amounts of viral proteins, possibly increasing toxicity and undesirable side effects and (*b*) production of clinical grade virus for clinical trials requires a considerable scale-up, which is expensive and labor intensive (14). Instead, rQNestin34.5's (2) effects in animal trials required much lower doses (10^5 pfu) to achieve significant anticancer action.

Herein, we report that rQNestin34.5's action was not enhanced by cyclophosphamide even at a reduced dose of 3×10^4 pfu. However, when the dose of this relatively potent oncolytic virus was reduced by another order of magnitude, cyclophosphamide significantly enhanced viral oncolysis. This is relevant to the translation into clinical trials, suggesting the possibility of reduced dosing in humans with this oncolytic virus and the reduced need for extensive scale-up in the clinical grade production of this agent.

Materials and Methods

Viruses and cells. The rQNestin34.5 oncolytic virus was previously described (2). Briefly, it possesses deletions in the *UL39* gene encoding for the viral ribonucleotide reductase function and of both copies of the viral γ_1*34.5* gene. Within the deleted *UL39* locus, a transcriptional cassette was recombined that encompassed a reinserted copy of the γ_1*34.5* gene under control of the human nestin promoter/enhancer sequence. Nestin is overexpressed in malignant gliomas. In addition, a green fluorescent protein (GFP) transgene under control of a cytomegalovirus promoter was located immediately upstream of the nestin promoter/enhancer sequence. Human U87ΔEGFR glioma cells were obtained from Dr. Huang (Ludwig Institute, San Diego, CA) and are described in ref. (15). They were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units penicillin/mL, and 10 mg streptomycin/mL at 37°C in an atmosphere containing 5% CO_2.

Note: We recognize the memory of Dr. Keiro Ikeda who, over 5 years ago, suggested to the senior author the possibility that cyclophosphamide would allow for very small doses of an oncolytic virus to be injected.

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[1] G. Fulci and E.A. Chiocca, unpublished data.

[2] Unpublished data.

Animal studies. Nude (*nu/nu*) mice were obtained from Charles River Laboratories (Wilmington, MA) or from the breeding facility at the Ohio State University. Brain tumors were initiated by stereotactic injection of 2×10^5 cells into the right frontal lobe (2 mm lateral and 1 mm anterior to the bregma at a depth of 3 mm). Seven days after tumor implantation, animals were randomly divided and cyclophosphamide (Bristol-Myers Squibb Co., Princeton, NJ), or saline was administered i.p. at a dose of 300 mg/kg. Two days later, the rQNestin34.5 virus was inoculated into the brain tumor using the same stereotactic coordinates previously used for the tumor cell injections. The dose of virus is indicated in each legend, and the volume of injectate was constant (3 μL). In a small group of animals, intracardiac perfusion fixation with 4% paraformaldehyde was done to harvest brains. Frozen sections were sliced to a thickness of 20 μm before staining with H&E or measurements of green fluorescence. All animal studies were done in accordance with guidelines issued by Ohio State University Subcommittee on Animal Care. Viral inoculation and care of animals harboring viruses were done in approved BL2 viral vector rooms.

Results and Discussion

We sought to determine if rQNestin 34.5's action against a human U87ΔEGFR glioma xenograft in athymic mouse brains was enhanced by preadministration of cyclophosphamide. We determined that a dose of 300 mg/kg i.p. led to immunosuppressive effects equivalent to the 80 to 100 mg/kg dose used in rodents (10–13). For the study, we selected the following end point assays: (*a*) qualitative estimates of tumor volume by histology, (*b*) qualitative estimate of the distribution of oncolytic virus–mediated gene distribution by GFP, and (*c*) measurement of animal survival. Figure 1*A* to *C* shows that inoculation of rQNestin34.5 at a dose of 3×10^4 pfu in an established glioma xenograft was *not* enhanced by cyclophosphamide, as measured by all three assays. Because an important mechanism of cyclophosphamide's action within a neoplasm may be associated with a reduction in the number of

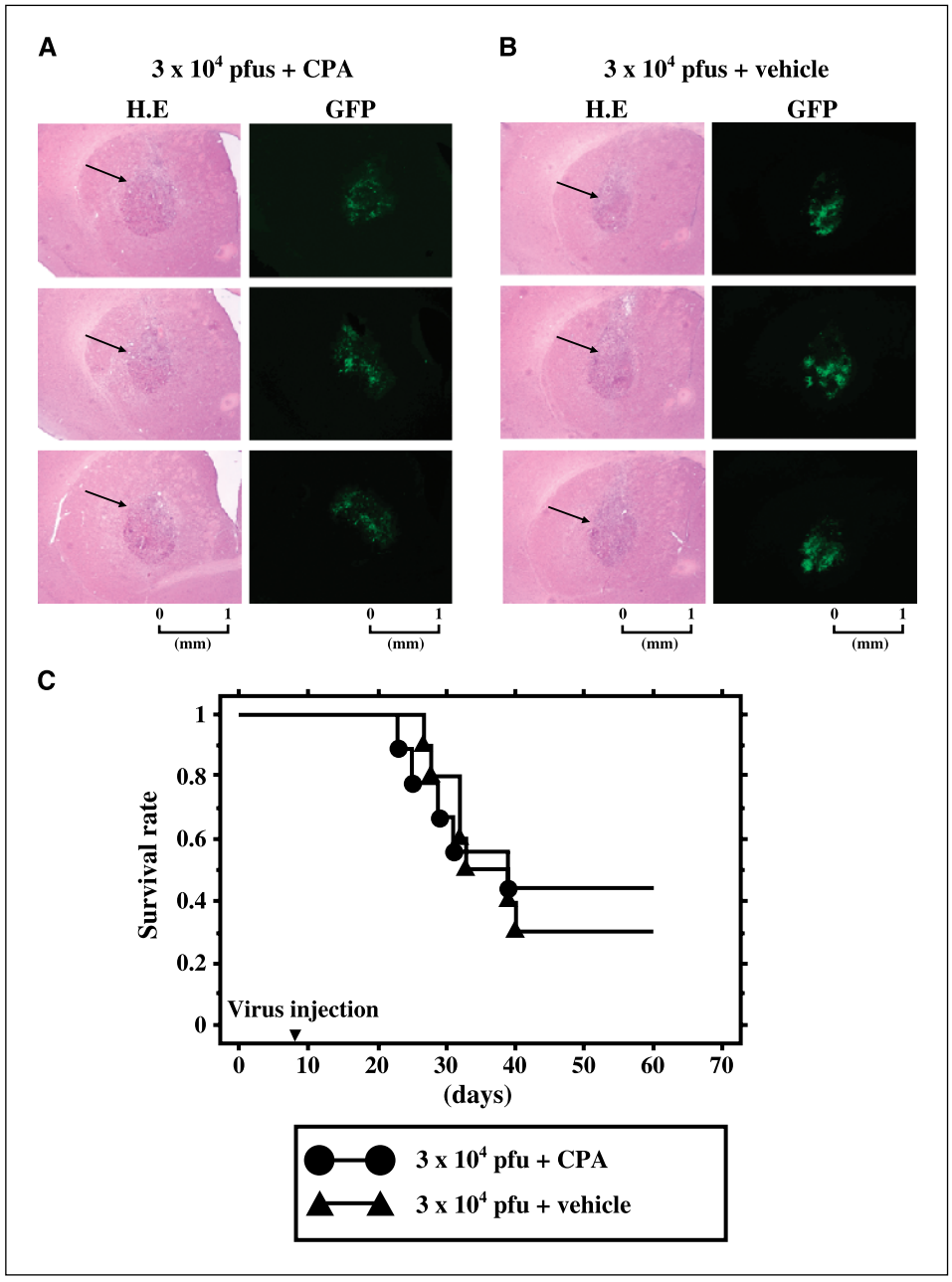

Figure 1. Comparison of rQNestin34.5 effects at a dose of 3×10^4 pfu as a function of preadministered cyclophosphamide (*CPA*). Human U87ΔEGFR glioma cells (2×10^5) were inoculated into the brain of athymic mice on day 0. Cyclophosphamide (*A*) or saline (*B*) were administered i.p. on day 7. rQNestin34.5 was injected at a dose of 3×10^4 pfu into tumors on day 9 (*C, arrows*). A group of animals ($n = 3$) was sacrificed on day 11 for histologic (*H.E*) and oncolytic virus distribution (*GFP*) assays. Representative H&E (*H.E*)–stained oncolytic virus–injected tumors within brains of animals pretreated with cyclophosphamide (*A*) or vehicle (*B*). Tumor (*arrows*) volumes are approximately similar. GFP gene expression for the adjacent section provides an estimate of the distribution of oncolytic virus–mediated gene expression as a function of cyclophosphamide. *C,* Kaplan-Meier survival curves are depicted for both treatments ($n = 10$ per group).

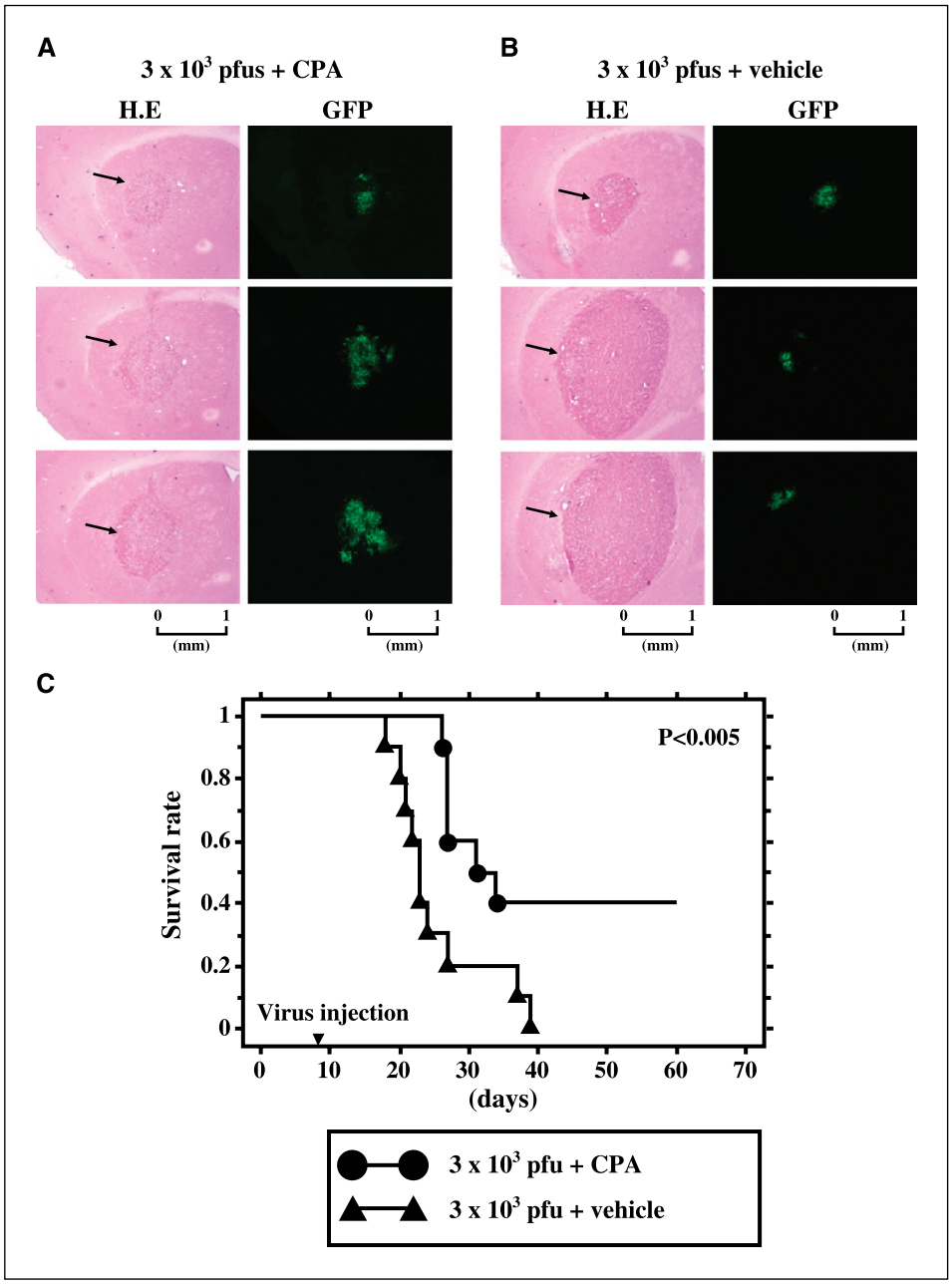

Figure 2. Comparison of rQNestin34.5 effects at a dose of 3×10^3 pfu as a function of preadministered cyclophosphamide (*CPA*). Human U87ΔEGFR glioma cells (2×10^5) were inoculated into the brain of athymic mice on day 0. Cyclophosphamide (*A*) or saline (*B*) were administered i.p. on day 7. rQNestin34.5 was injected at a dose of 3×10^3 pfu into tumors on day 9 (*C, arrows*). A group of animals ($n = 3$ per group) was sacrificed on day 11 for histologic (*A*) and oncolytic virus distribution assays (*B*). Representative H&E (*H.E*) stain and GFP gene expression for the oncolytic virus–injected tumor (*arrows*) within brains of animals pretreated with cyclophosphamide (*A*) or vehicle (*B*). *C,* Kaplan-Meier survival curves are depicted for both treatments, showing a statistically significant increase in survival for the cyclophosphamide-pretreated group ($n = 10$ per group, $P < 0.005$, Wilcoxon log-rank).

tumor-associated antiviral phagocytic cells,[3] we reasoned that a potent oncolytic virus, such as rQNestin34.5, may be escaping such host responses and that lower doses of the virus may unmask the enhancement of viral oncolysis. In fact, as the dose was decreased to 3×10^3 pfu (Fig. 2*A-C*) or even to 3×10^2 pfu (Fig. 3*A*), a significant enhancement of viral oncolysis by cyclophosphamide was now measured by all three assays. These results indicated that cyclophosphamide allowed for dose reduction of this highly potent oncolytic virus. In fact, long-term survival of approximately the same percentage (40-50%) of animals was observed with 3×10^5 pfu of rQNestin34.5 as with 3×10^3 pfu with cyclophosphamide (compare Fig. 3*B* with Fig. 2*C*). Cyclophosphamide thus allowed for a dose reduction of the oncolytic virus by at least two orders of magnitude. Although this study was conducted with an HSV-1-based oncolytic virus, we are still evaluating if cyclophosphamide also enhances the oncolytic effect of other oncolytic viruses (such as replicating adenoviruses). In addition, published data shows that cyclophosphamide's effect on HSV-1 base oncolytic viruses occurs *in vivo* but not *in vitro* (13). We do possess evidence of effects of cyclophosphamide on innate immune mechanisms responsible for antiviral host defenses,[4] which are operative in athymic animals. The effect of cyclophosphamide is best observed when administered before oncolytic virus administration, and the drug also enhances brain tumor infection and oncolysis with systemic administration of the oncolytic virus (10–13). Cyclophosphamide alone does not result in an effective antitumor effect (10–13).

[3] G. Fulci and E.A. Chiocca, unpublished data.

[4] G. Fulci and E.A. Chiocca, submitted for publication.

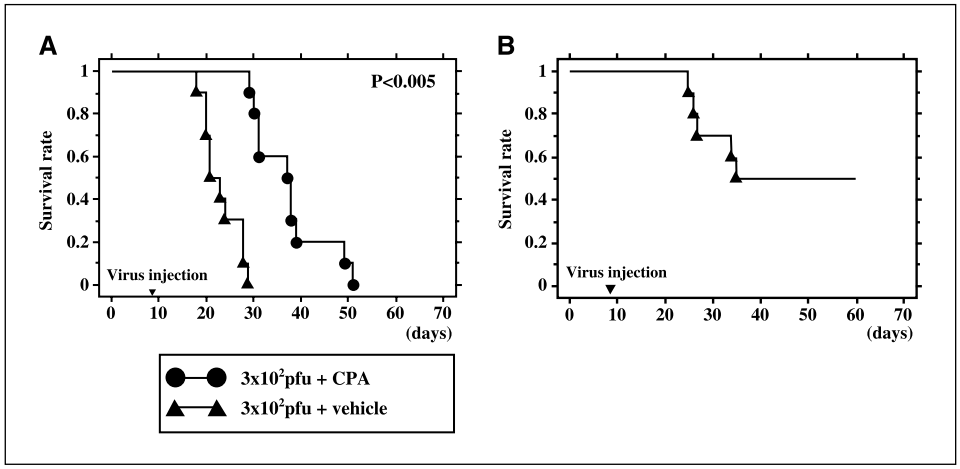

Figure 3. Effect of other doses of rQNestin 34.5. *A,* mice were treated with cyclophosphamide (*CPA*) or saline, and 2 days later, 3×10^2 pfu of rQNestin34.5 were administered, using the same protocol as described in Figs. 1 and 2. There was a statistically significant increase in survival for the cyclophosphamide-pretreated group ($n = 10$ per group, $P < 0.005$, Wilcoxon log-rank). *B,* 3×10^5 pfu of rQnestin34.5 produced approximately the same percentage of survivors as 3×10^3 pfu of oncolytic virus with cyclophosphamide (see Fig. 2).

The production of oncolytic viruses, such as HSV-1, for clinical trials requires a considerable scale-up effort in terms of labor, supplies, and quantity of materials. In fact, the human phase I trials with G207 were stopped at a dose of 3×10^9 pfu without reaching a maximum tolerated dose (MTD) because of the inability to obtain higher stocks of oncolytic virus (5). Similarly, a phase I trial of the oncolytic virus ONYX-015 was also stopped at a dose of 10^{10} pfu due to the lack of higher titer virus before an MTD could be determined (3). Therefore, the demonstration of bioequivalence at a lower titer of an oncolytic virus would represent significant progress with regard to process development. Cyclophosphamide clearly represents an *in vivo* adjunct that allows the attainment of bioequivalence at a lower titer by almost two orders of magnitude in the studied animal model. In addition, the reduction of injected viral proteins, which could produce toxic effects per se, is likely to increase the safety and therapeutic index of this treatment.

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