Coadministration of a Herpes Simplex Virus-2–Based Oncolytic Virus and Cyclophosphamide Produces a Synergistic Antitumor Effect and Enhances Tumor-Specific Immune Responses

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Abstract

Despite their unique property of selective replication and propagation in tumor tissues, oncolytic viruses have had only limited antitumor effects in cancer patients. One of the major reasons is probably the host's immune defense mechanisms, which can restrict the ability of the virus to replicate and spread within tumors. The innate immune system, which can be rapidly activated during virus infection, likely plays a more pivotal antiviral role than does acquired immunity, as the antitumor effect of an oncolytic virus is mainly generated during the acute phase of virus replication. To exploit the potential of cyclophosphamide, a cancer chemotherapeutic drug that also inhibits innate immune responses, to enhance the activity of oncolytic viruses, we evaluated the effect of coadministration of this drug with a herpes simplex virus-2–based oncolytic virus (FusOn-H2) against Lewis lung carcinoma, which is only semipermissive to infection with FusOn-H2. This strategy synergistically enhanced the antitumor effect against lung carcinoma growing in mice. It also potentiated the ability of FusOn-H2 to induce tumor-specific immune responses. Together, our results suggest that coadministration of FusOn-H2 with cyclophosphamide would be a feasible way to enhance the antitumor effects of this oncolytic virus in future clinical trials. [Cancer Res 2007;67(16):7850–5]

Introduction

Several viruses, including herpes simplex virus (HSV), have been modified for oncolysis purposes (1–3). The antitumor effect of these oncolytic viruses derives from their ability to replicate and propagate in tumor tissues (4); however, the host's immune mechanisms can restrict viral replication, compromising the therapeutic potential of antitumor virotherapy. The innate immune system likely plays a more pivotal role than the acquired immunity in this respect (5), as the majority of antitumor activity by an oncolytic virus is generated during the acute phase of viral replication. Within hours after a natural virus infection, the innate immune system responses with activation of the complement cascade, cytokine release by infected epithelia and resident dendritic cells, and recruitment of cellular effectors, such as macrophages and natural killer cells, all of which cooperate to inhibit viral replication and spread within the infected tissues (6). Indeed, the crucial role of innate immunity in controlling virus infections, such as those due to HSV, is well documented (7–9). Consequently, it has been shown that strategies to block this arm of the immune system by coadministering cyclophosphamide, a well-known cancer drug and immunosuppressant, can significantly enhance the antitumor effects of a type I HSV (HSV-1)-derived oncolytic virus against brain tumors (5, 10, 11).

We recently constructed an oncolytic virus from type II HSV (HSV-2) that can selectively target tumor cells with an activated Ras signaling pathway (12, 13). Designated FusOn-H2, it has multiple antitumor mechanisms, including the induction of cell membrane fusion (syncytia formation) and apoptosis in tumor cells, in addition to its direct cytolytic effects (12). Importantly, tumor destruction by this virus induces tumor-specific immune responses to murine syngeneic tumors that otherwise are weakly immunogenic (14, 15).

Here, we investigated whether blocking the host's innate immunity with cyclophosphamide would enhance the antitumor activity of this HSV-2–based oncolytic virus in a nonbrain tumor model, Lewis lung carcinoma (LLC), which is significantly less sensitive than most xenografted human tumors to FusOn-H2 virotherapy and only marginally sensitive to cyclophosphamide. Our results show that, similar to findings in brain tumor models with the HSV-1–based oncolytic virus (11), coadministration of cyclophosphamide dramatically enhances the antitumor effect of FusOn-H2. This strategy also boosts the ability of virotherapy to induce tumor-specific immune responses.

Materials and Methods

Cell lines and viruses. African green monkey kidney (Vero) cells and LLC cells were obtained from the American Type Culture Collection. Both types of cells were cultured in DMEM containing 10% fetal bovine serum. FusOn-H2 is a mutant HSV-2, constructed by replacing the pharmacokinetic domain of the *ICP10* gene with the gene encoding green fluorescent protein; the details of this construct are described elsewhere (12). Virus stocks were prepared by releasing the virus from infected Vero cells with heparin followed by high-speed centrifugation as described (16).

Quantification of *in vitro* and *in vivo* virus replication. For *in vitro* assay of virus replication, LLC cells seeded into 24-well plates were infected with FusOn-H2 at either 1 or 10 plaque-forming unit (pfu)/cell for 2 h. The cells were then washed once with serum-free medium to remove unadsorbed and uninternalized viruses. The infected cells were then cultured in normal medium with or without the addition of 3 μmol/L activated form of cyclophosphamide (4-hydroxy cyclophosphamide). Cyclophosphamide activation was done *in vitro* using a procedure described by Connors et al. (17). The liver microsomes used for the activation were prepared from hepatocytes obtained from C57BL mice using the method described by Grover and Sims (18). Cells were harvested at 4, 24, and 48 h after infection and then subjected to sonication to release virus. The viruses were titrated on Vero cells by a plaque assay.

For quantification of virus replication *in vivo*, tumor nodules were aseptically explanted at different times after virus administration. Tumor

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tissues were homogenized and then sonicated to release viruses. Virus titration was done by the same assay as used for the *in vitro* experiment.

***In vivo* animal experiments.** Immune-competent female C57/BL6 mice (4–6 weeks old) were purchased from The Jackson Laboratory. All *in vivo* experimental protocols were approved by Baylor College of Medicine Institutional Animal Care and Use Committee.

Freshly harvested LLC cells (3×10^5) were injected into the right flanks of C57/BL6 mice, which were then randomly divided into four groups. Nine days after tumor cell implantation (i.e., immediately before virotherapy), when tumor diameters had reached the approximate size of 3 to 5 mm, mice received an intratumor injection of either PBS or 1×10^7 pfu of FusOn-H2 in a volume of 50 μL or i.p. injection of cyclophosphamide (300 mg/kg body weight). Another group of mice received the combined treatment, in which mice were injected i.p. with 300 mg/kg body weight of cyclophosphamide at 48 h before virotherapy. Four mice from each group were killed on days 2, 4, and 7 after virotherapy for quantification of virus replication *in vivo* and histologic examination. The remaining mice were kept for 3 weeks, during which time the growth of tumors was monitored weekly after virus administration by measuring two perpendicular tumor diameters with a caliper. Tumor volume was calculated by the following formula: tumor volume (mm^3) = [length (mm)] × [width (mm)]^2 × 0.52.

To detect antitumor immune responses, we euthanized the animals at 14 days after treatment and collected spleens for the preparation of splenocytes that were used to determine the frequency and cytolytic activity of tumor-specific CTLs as described below. For histologic examination of tumor specimens, mice were euthanized at different times after treatment and sections of tumor were fixed in 10% neutral-buffered formalin for 24 h, dehydrated in serial ethanol, and embedded in paraffin. Sections (5 μm thick) were prepared, mounted on slides, and stained with H&E.

Chromium-51 release assay. A ^{51}Cr release assay was used to determine the cytotoxic activity of tumor-specific CTLs prepared from spleens collected 14 days after virotherapy. Initially, the splenocytes were prepared from minced spleens, and the cells were dispersed with a syringe plunger. The cell suspension was then filtered through cell strainers, and erythrocytes were lysed with an 0.83% ammonium chloride lysis solution. The splenocytes were then restimulated *in vitro* for 5 days supplemented by recombinant murine interleukin-2 (BD Biosciences). The target tumor cells (LLC) or a syngeneic control cells (B16 murine melanoma cells) were resuspended and labeled by adding radioactive sodium chromate (Amersham). The cell suspension was incubated for 45 min at 37°C before being added to 96-well, U-bottomed plates. Purified splenocytes were then added as effector cells to the 96-well plate at various E:T cell ratios (80:1, 40:1, 20:1, and 10:1) in a final volume of 200 μL/well and incubated at 37°C for 4 h. RPMI 1640 served as a negative control. Supernatants (100 μL) were harvested, and ^{51}Cr release was measured by a scintillation counter (Beckman). Spontaneous release was measured in wells containing target cells alone. Triton X-100 was used to lyse the target cells for maximal release. The percentage of specific lysis was calculated by the following formula: (experimental release cpm − spontaneous release cpm / maximum release cpm − spontaneous release cpm) × 100 = percentage specific lysis.

Enzyme-linked immunospot assay. Ninety-six–well filter plates for high-throughput separations (Millipore) were precoated with anti-IFN-γ monoclonal antibody (BD Biosciences) and incubated overnight at 4°C. The plates were blocked for 1 h at 37°C. Purified splenocytes were then dispensed at a predetermined density into duplicate wells and stimulated with irradiated LLC cells or the control B16 tumor cells. After incubation at 37°C for 18 to 24 h followed by washing, biotinylated anti-IFN-γ antibody (BD Biosciences) was added to each well and the plates were incubated for another 1 h at 37°C. A streptavidin-alkaline phosphatase conjugate was added to the wells, and after 1 h of further incubation, the chromogenic alkaline phosphatase substrate was added. The colorimetric reaction was terminated within 5 to 20 min by washing with tap water. After drying, the spots were counted.

Statistical analysis. All data were normally distributed, and Student's *t* test (two tailed) or one-way ANOVA was used to determine the statistical significance ($P < 0.05$) of various comparisons. The results are reported as means ± SDs.

Results

Replication of oncolytic HSVs *in vitro* in LLC cells in the presence or absence of cyclophosphamide. We initially examined the permissiveness of LLC cells to HSV-FusOn-H2 at 1 or 10 pfu/cell, generally considered a very high dose for HSV vectors. The cells were harvested at different times after infection, and the virus yield was determined by titration. The results showed that, although FusOn-H2 could replicate in the murine tumor cells (Fig. 1), the virus yield (up to 8×10^3 pfu/mL) was >2 logs lower than that obtained from human cancer cells (12, 13). Thus, compared with human tumor cells, LLC cells are only semi-permissive to FusOn-H2 replication. We viewed this property as advantageous because any synergistic antitumor effect produced by the combined therapy would be readily apparent.

We also conducted an *in vitro* experiment to determine if the presence of cyclophosphamide in the culture medium alters the replication of FusOn-H2. As cyclophosphamide per se is an inactive prodrug that requires bioactivation by liver cytochrome *P*450 to convert it to the active 4-hydroxy metabolite, it was initially activated *in vitro* with mouse liver microsomes as described (17). LLC cells were infected with FusOn-H2 and then incubated with medium alone or with medium containing 3 μmol/L of the activated cyclophosphamide, a concentration not found to interfere with cell viability or growth (11). Virus replication was examined at different times after infection. The results showed that the presence of the activated cyclophosphamide in the medium does not significantly alter FusOn-H2 replication in LLC cells by comparison with findings in wells that lacked the drug (Fig. 1).

Replication of FusOn-H2 within established LLCs with or without coadministration of cyclophosphamide. To assess the effect of cyclophosphamide on the *in vivo* growth of FusOn-H2 within established LLCs, we inoculated the right flanks of immune-competent C57/BL6 mice with 3×10^5 freshly harvested LLC cells. When tumors became palpable, half of the mice received i.p. injection of cyclophosphamide at a dose of 300 mg/kg body weight. Forty-eight hours later, mice with or without receiving

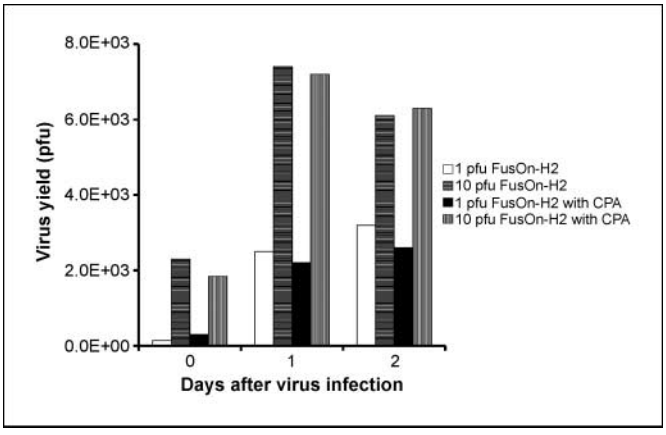

Figure 1. Virus replication in LLC cells *in vitro* with or without cyclophosphamide (*CPA*) addition. LLC cells seeded into 24-well plates were infected with FusOn-H2 at either 1 or 10 pfu/cell. The infected cells were then cultured in medium with or without the activated form of cyclophosphamide at a concentration of 3 μmol/L. Cells were harvested at the indicated times after infection, and the virus yield was determined by plaque assay. There was no statistically significant difference in virus yield with or without cyclophosphamide addition at any time point.

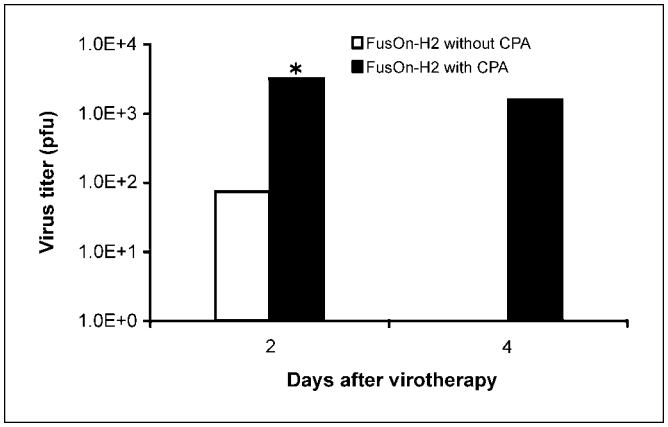

Figure 2. Comparison of virus replication in tumor tissues from mice with or without coadministration of cyclophosphamide. Tumor-bearing mice were injected with FusOn-H2 alone (intratumorally) or in combination with cyclophosphamide (i.p. 48 h in advance). Tumor nodules were explanted at days 2 and 4 after virus administration, and the virus yield was determined by plaque assay. *, $P < 0.005$, compared with FusOn-H2 alone.

cyclophosphamide were injected intratumorally with FusOn-H2 at a dose of 1×10^7 pfu. Mice were killed 2 and 4 days ($n = 4$ mice per time point) after virus injection, and the tumors were explanted, minced, and homogenized to release the virus. Virus within the tumor tissues was quantified by plaque assay on Vero cell monolayers.

In the absence of cyclophosphamide, oncolytic virus was completely cleared from the tumor tissues between days 2 and 4 after intratumoral administration. I.p. administration of cyclophosphamide 48 h before virus inoculation significantly increased virus replication on day 2 ($P < 0.01$; Fig. 2). It also extended the duration of virus replication within tumors so that virus titers measured on day 4 were nearly identical to those on day 2, indicating robust virus replication at the later time point (Fig. 2). Thus, although cyclophosphamide failed to enhance FusON-H2 replication in cultured LLC cells (Fig. 1), its coadministration with the oncolytic virus significantly enhanced and prolonged virus replication in tumor tissues.

Coadministration of cyclophosphamide can significantly improve the therapeutic effect of FusOn-H2. We next tested whether the ability of cyclophosphamide to enhance and prolong virus replication in tumor cells could be exploited to improve the antitumor effect of virotherapy. Tumor-bearing mice received a single injection of either PBS, FusOn-H2 (intratumorally), or cyclophosphamide (i.p.) or were given both agents as described in Fig. 2. The tumors were measured twice weekly and tumor volumes were calculated as described in Materials and Methods. Although FusOn-H2 given alone showed some antitumor activity (Fig. 3), its effect was clearly inferior to results obtained against human tumor xenografts (12, 13), as might be expected from the decreased permissiveness of LLC cells to virus infection, and was not significantly different from PBS controls. Administration of cyclophosphamide alone produced an antitumor effect similar to that of virotherapy and not statistically significant by comparison with the PBS-treated group either. However, coadministration of FusOn-H2 with cyclophosphamide increased the antitumor effect dramatically as shown by tumor reductions to barely detectable sizes ($P < 0.005$ for comparisons on days 20, 24, and 28; Fig. 3). Most importantly, the antitumor effect persisted until the end of the experiment, although the therapeutic regimen was given only once.

Histologic findings after coadministration of FusOn-H2 with cyclophosphamide. To examine the histology of tumors treated with the different regimens, we prepared sections from the tumor explants described above and stained them with H&E. In the mice treated with FusOn-H2 alone, infiltration of both lymphoid cells and monocytic cells was readily apparent in tumors taken at any time point after virus injection (Fig. 4). However, in mice treated with FusOn-H2 plus cyclophosphamide, only infiltrating lymphoid cells could be seen, as cyclophosphamide does not seem to appreciably affect lymphocyte function (11). The tumor cells often showed distinct morphologic changes. In samples taken on day 2 after the combined treatment, for example, there was evidence of either tumor cell degeneration and/or necrosis with prominent vacuolar cytoplasm. On day 4 after virotherapy, necrotic tumor cells were easily seen across the tumor section. On day 7, the sections from mice receiving FusOn-H2 alone showed largely intact tumor structures and an abundance of healthy tumor cells, with occasional infiltration of lymphoid cells. By contrast, in the mice treated with Fuson-H2 plus cyclophosphamide, the majority of tumor cells were gone by day 7, with the remaining tumor cells interspersed with infiltrating lymphoid cells and having a blurred appearance and in distinct structure (Fig. 4). These results indicate that cyclophosphamide effectively inhibits the infiltration of FusOn-H2–treated tumors by innate monocytic cells while sparing lymphoid cells. The combined cytotoxic effect of virotherapy and cyclophosphamide was quite profound, leading to the eventual destruction of the vast majority of tumor cells.

Antitumor immunity after coadministration of cyclophosphamide and FusOn-H2. We previously showed that FusOn-H2 induces measurable antitumor immune responses to weakly immunogenic tumors in at least two syngeneic murine tumor models (14, 15). LLC cells are considered weakly immunogenic in syngeneic C57/BL6 mice (19) but are on the other hand

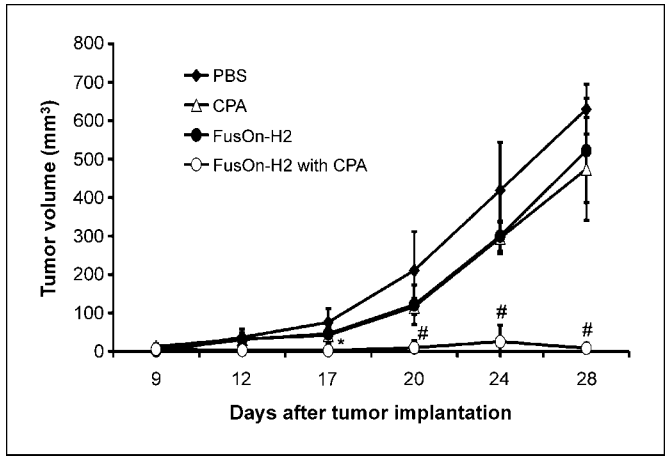

Figure 3. Antitumor effect of FusOn-H2 *in vivo* with or without coadministration of cyclophosphamide. Mice bearing LLC on the right flank received monotherapy or combined treatment with FusOn-H2 and cyclophosphamide or were injected with PBS as a control ($n = 8$ mice per group). Tumor growth was monitored weekly for 3 wks and tumor volume was plotted against time (days) after tumor implantation. *, $P < 0.01$ versus FusOn-H2 or cyclophosphamide alone; #, $P < 0.005$ versus FusOn-H2 or cyclophosphamide alone. Differences between the groups treated with either FusOn-H2 or cyclophosphamide and the PBS control group were not significantly different.

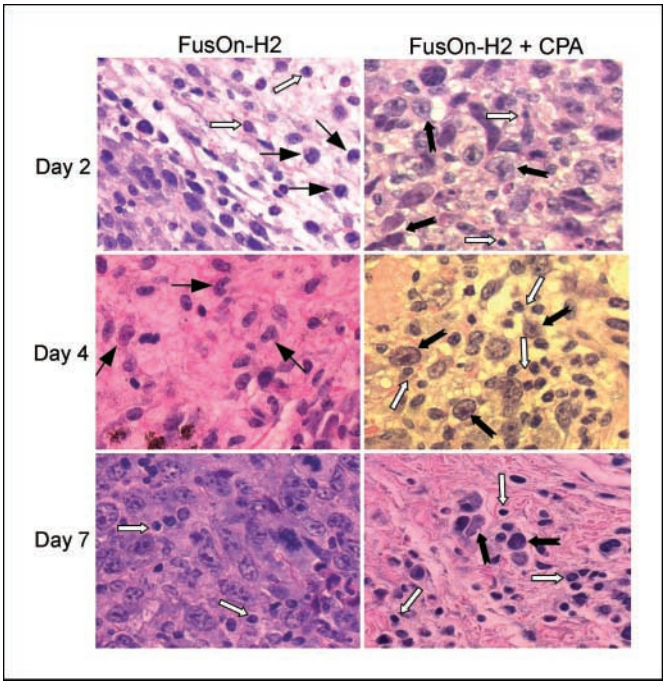

Figure 4. Tumor histology after single- or combined-agent therapy. Tumor nodules were explanted on days 2, 4, and 7 after the start of therapeutic injections. One typical field within H&E-stained tumor sections from mice treated with FusOn-H2 alone or in combination with cyclophosphamide is presented at each time point. The infiltrating lymphoid cells (⇨), monocytic cells (➔), and LLC cells (➡) are labeled. Some of the indicated tumor cells show degeneration and/or necrosis with prominent vacuolar cytoplasm. Original magnification, ×400.

significantly less permissive to infection of FusOn-H2 than the other two tumors we previously studied. To determine if coadministration of cyclophosphamide would enhance the ability of the virus to induce tumor-specific immune responses against LLC cells, we conducted an experiment in which mice were euthanized on day 14 after the initiation of therapy and spleens were harvested for assays of tumor-specific cellular immune responses by measuring both the cytolytic activity of splenocyte tumor cells and quantifying the frequency of tumor-specific CTLs by enzyme-linked immunospot (ELISPOT) assay.

The results show that, without coadministration of cyclophosphamide, a single intratumoral injection of FusOn-H2 induced only a slight level of CTL activity against LLC cells, whereas coadministration of cyclophosphamide significantly enhanced the cytotoxic activity of splenocytes against LLC cells ($P < 0.05$; Fig. 5*A*). The specificity of the lysis was confirmed by the lack of cytolysis by the same splenocytes against syngeneic B16 melanoma cells (Fig. 5*B*). The ELISPOT assay revealed that cyclophosphamide coadministration also led to the generation of a 4-fold higher frequency of CTLs among splenocytes than in the group treated with FusOn-H2 alone ($P < 0.005$; Fig. 6). Thus, combined treatment with cyclophosphamide and FusOn-H2 can boost the capacity of the virus to induce antitumor immune responses, in addition to directly increasing its oncolytic activity.

Discussion

Unlike gene-based forms of cancer therapy, oncolytic viruses kill tumor cells directly through selective replication/cytolysis and consequent spread to surrounding tumor tissues. These properties represent a major advantage over the inherent limitations of gene delivery and the resultant restrictions on tumor cell eradication by this method. Nonetheless, convincing evidence of the clinical antitumor efficacy of oncolytic viruses remains to be obtained. Part of the difficulty is probably due to *in vivo* factors that impede the activity of oncolytic viruses, and efforts to overcome these obstacles will likely improve the overall antitumor efficacy of virotherapy. For example, unlike cultured tumor cells, the growing tumor tissues are composed of connective tissues and stroma cells that may prevent the spread of the virus. Consequently, coinjection of an oncolytic HSV with bacterial collagenase has been shown to degrade fibrillar collagen within the extracellular matrix, resulting in a significant improvement in the distribution of viruses within the tumor (20). Another important factor that can compromise the ability of an oncolytic virus to lyse tumor cells is the host's innate antiviral immune mechanisms. Unlike the adaptive immune responses of T and B lymphocytes, which require preexposure to viral antigens and thus may take days or even weeks to develop fully, the host can mount an innate immune response against viruses and other invading pathogens almost instantly. The resultant innate immune cells infiltrate the infected tissue within hours, and the release of cytokines, such as IFNs, during HSV infections may even be more rapid (21). Thus, the vigor of the host's innate antiviral immunity is thought to be a central determinant of the antitumor effect of any oncolytic virus. Indeed, recent studies by Fulci et al. (11) clearly show that a blockade of host innate immunity with cyclophosphamide can significantly enhance the replication of a HSV-1–based oncolytic virus in brain tumors, thus enhancing its antitumor efficacy.

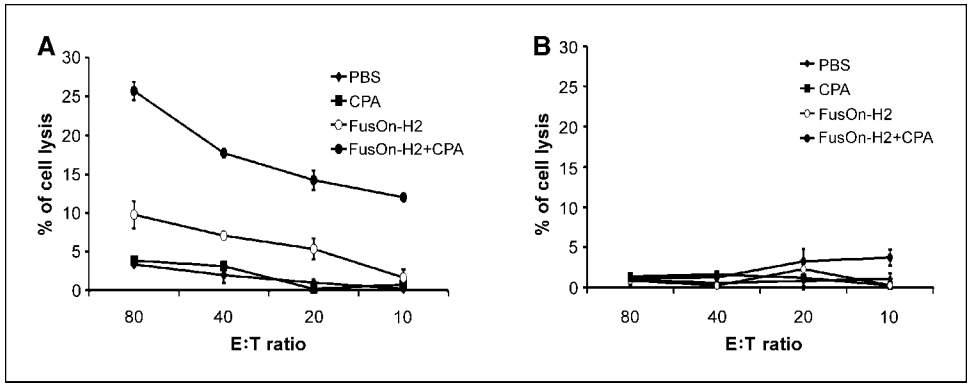

Figure 5. Tumor-specific cytotoxic activity. Tumors were established and treated as described in the legend to Fig. 3 ($n = 5$ mice per group). Mice were killed at 14 d after injection, and their spleens were explanted. The splenocytes were prepared for ^{51}Cr release assay of cytotoxicity against LLC cells (*A*) and syngeneic B16 melanoma cells (*B*). There was a significant difference between the cytotoxic activity of splenocytes against LLC cells from mice treated with FusOn-H2 alone versus FusOn-H2 + cyclophosphamide at each of the E:T ratios ($P < 0.05$).

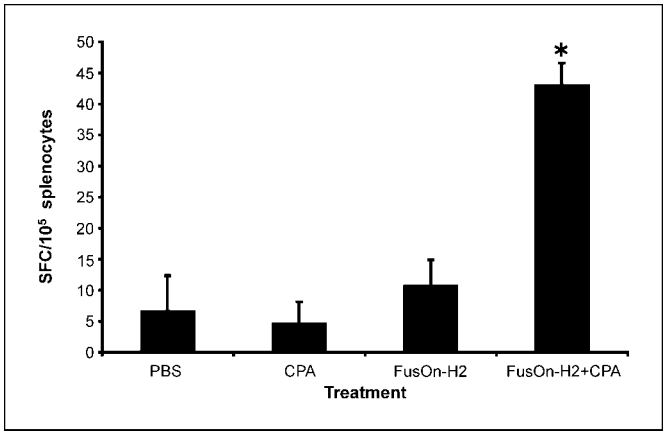

Figure 6. Comparison of CTL frequencies after treatment with FusOn-H2 virotherapy alone or in combination with cyclophosphamide. The effector cells were from the same preparation used in Fig. 5. ELISPOT assay was done as described in Materials and Methods to determine the frequency of tumor-specific CTLs. *, $P < 0.005$, FusOn-H2 + cyclophosphamide versus FusOn-H2 alone. *SFC,* spot-forming cells.

The results reported here, using the LLC tumor model, indicate that coadministration of cyclophosphamide with FusOn-H2 can significantly potentiate the antitumor effect of this HSV-2–based oncolytic virus. Indeed, when given alone to tumor-bearing mice, neither cyclophosphamide nor FusOn-H2 produced notable oncolytic effects, but their coadministration significantly enhanced and prolonged the replication of FusOn-H2 in tumor tissues, leading to an antitumor effect that clearly exceeded the additive results of monotherapy with these agents. This outcome agrees with previous studies in which oncolytic viruses were combined with commonly used cancer drugs (22–24); however, the use of cyclophosphamide for this purpose may provide additional benefit by virtue of the ability of the drug to inhibit host innate immunity, which would be predicted to further enhance the therapeutic potential of a potent oncolytic virus, such as FusOn-H2.

In earlier studies, we showed that FusOn-H2–mediated destruction of syngeneic murine tumors, such as mammary tumor (established from 4T1 tumor cells) and neuroblastoma (established from Neuro2A tumor cells), induces robust antitumor immune responses that contribute to the overall efficacy of this virotherapy (14, 15). In the current study, by contrast, tumor-specific immune responses were barely detectable when FusOn-H2 was used alone. We attribute this discrepancy to the fact that FusOn-H2 does not induce syncytia formation in this particular murine tumor cell line (data not shown). Another reason for this discrepancy is that LLC cells are less permissive than either 4T1 or Neuro2A cells to FusOn-H2 infection. Consequently, the release of tumor antigens by LLC cells was likely greatly decreased compared with results with the other two tumor models, although the same oncolytic virus was used. We would stress that coadministration of cyclophosphamide not only increased virus replication (thus tumor antigen release) but also prolonged the duration of virus replication, both of which likely contributed to the enhanced antitumor immune responses. Finally, it has been reported that cyclophosphamide can selectively deplete $CD4^+CD25^+$ regulatory T cells in cancer patients (25). Thus, this clinically approved antineoplastic drug may enhance tumor-specific immune responses when coadministered with FusOn-H2 by ablating one or more of the host's immunosuppressive defenses within the tumor microenvironment.

Because tumor cells in cancer patients are likely to be more primitive (and thus more resistant to oncolytic viruses) than tumor cells in xenograft models that have been maintained in the laboratory, our observation of a strong synergistic antitumor effect between cyclophosphamide and FusOn-H2 in the marginally sensitive LLC tumor model seems highly relevant to clinical settings. Indeed, given our experience with oncolytic viruses in preclinical tumor models, we suggest that coadministration of cyclophosphamide or another agent with similar properties may be necessary in future clinical trials to realize the optimal therapeutic benefits of oncolytic HSVs in cancer patients.

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