Virotherapy with a Type 2 Herpes Simplex Virus–Derived Oncolytic Virus Induces Potent Antitumor Immunity against Neuroblastoma

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Abstract

Purpose: We recently constructed an oncolytic virus from type 2 herpes simplex virus (HSV-2) that selectively targets and kills tumor cells with an activated Ras signaling pathway. Designated FusOn-H2, this virus has shown several discrete killing mechanisms. Here, we evaluated the antitumor immune responses after FusOn-H2–mediated virotherapy in a syngeneic murine neuroblastoma model.

Experimental Design: We directly injected FusOn-H2 into established tumors and then measured its antitumor effect and the accompanying tumor-specific immune responses. Several oncolytic HSVs constructed from HSV-1 were included in the same experiments for comparisons.

Results: Our data show that tumor destruction by FusOn-H2 *in vivo* induces potent antitumor immune responses in this syngeneic neuroblastoma model. The elicited cellular immunity not only eradicated neuroblastoma cells *in vitro* but also inhibited the growth of tumors at sites distant from the virus injection site. Moreover, adoptive transfer of splenocytes from mice receiving virotherapy to naïve mice resulted in a measurable antitumor effect.

Conclusion: We conclude that the ability of FusOn-H2 to induce tumor-specific cellular immunity expands the oncolytic repertoire of this virus and increases the likelihood that its use in patients would produce significant therapeutic benefits.

Oncolytic viruses are genetically modified to replicate in tumor cells but not in normal cells (1). Unlike more conventional forms of gene-based cancer therapy, these conditionally replicating viruses kill tumor cells directly through selective replication/cytolysis and consequent spread to surrounding tumor tissues (2). These properties represent a major advantage over the inherent inefficiency of therapeutic gene delivery and the resultant diminution of tumor cell killing activity. Moreover, tumor destruction *in situ* by an oncolytic virus should release large quantities of tumor antigens in their native forms and configurations, thus affording an attractive means to produce a whole tumor vaccine. Antitumor immune responses generated in this manner would be more likely to recognize legitimate tumor cell targets than would responses elicited by other types of vaccines prepared *in vitro* from extensively modified tumor antigens.

Tumor antigens can be presented to T cells either directly by tumor cells or indirectly via cross-presentation by professional antigen-presenting cells (3), such as dendritic cells. The latter route likely applies to antigens released by oncolytic virus–induced destruction of tumor cells. Several mechanisms facilitate the access of antigenic materials from malignant cells to the exogenous pathway of antigen-presenting cells for class I and class II presentation. Exosomes released from tumor cells provide one example. These small membrane vesicles contain abundant native tumor-associated antigens and are rich in molecules that promote antigen presentation (e.g., MHC class I and II molecules, costimulatory molecules, and heat shock proteins). After being released from tumor cells, exosomes are quickly internalized and efficiently processed by dendritic cells (4). It has been reported that syncytia formation induced by viral fusogenic glycoproteins can induce the release of large quantities of vesicles reminiscent of exosomes (syncytiosomes), which can potentiate the antitumor immune response and promote cross-presentation of tumor antigens (5–8). We have also shown that tumor destruction by a fusogenic oncolytic herpes simplex virus (HSV) induces potent antitumor immune responses to an otherwise nonimmunogenic murine mammary tumor (9). Another mechanism that can facilitate tumor antigen uptake by dendritic cells is the release of apoptotic bodies after tumor cell destruction by unconventional forms of apoptosis (10–12).

We recently constructed an oncolytic virus from type 2 HSV (HSV-2) that selectively targets tumor cells with an activated Ras signaling pathway (13). This conditionally replicating virus, designated FusOn-H2, kills tumor cells by several distinct mechanisms, such as a direct cytolytic effect from virus replication, as well as syncytia formation and apoptosis (13). The study reported here has investigated the possibility that, due to these distinct killing mechanisms, FusOn-H2 might also induce antitumor immune responses after its intratumoral injection in a syngeneic neuroblastoma model. The results show a robust tumor-specific immune response following local

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injection of FusOn-H2 that effectively inhibits the growth of distant tumor nodules. Thus, the antitumor properties of this novel oncolytic virus seem to include the induction of a potent systemic immune response against native tumor antigens released from virus-infected malignant cells.

Materials and Methods

Cell lines and viruses. African green monkey kidney (Vero) cells and the Neuro-2A murine neuroblastoma and Sa-I murine sarcoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). All of the cells were cultured in DMEM containing 10% fetal bovine serum.

FusOn-H2 was derived from the wild-type HSV-2 strain 186. Its construction as well as that of Baco-1 and Synco-2D, two HSV-1-derived oncolytic viruses, are described elsewhere (13–15). Virus stocks were prepared by infecting Vero cells with 0.01 plaque-forming units (pfu)/cell. Viruses were harvested 2 days later and purified as previously described (15). The purified viruses were titrated, divided into aliquots, and stored at −80°C until use.

For *in vitro* phenotypic characterization, cells were infected with the viruses at 0.1 pfu/cell and incubated for 24 h before photomicrographs were taken.

Animal studies. Four- to 6-week-old immunocompetent A/J mice and Hsd athymic (nu/nu) mice were obtained from Harlan (Indianapolis, IN). All experimental procedures involving animals were approved by the Baylor College of Medicine Animal Care and Use Committee. On day 0, 5×10^5 cells were injected into the right flank as previously described (14). Approximately 7 days after tumor cell inoculation, when the local tumor became palpable in all mice, the animals received an intratumoral injection of either PBS or 2×10^6 pfu of each oncolytic HSV in a volume of 50 μL (n = 8 per group). Injections were done slowly at two to three different sites across the tumor to prevent leakage. The resultant local tumor growth was monitored weekly 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. For tumor rechallenge, 5×10^5 tumor cells were injected into the left flank on the same day that mice received the injection of therapeutic virus. The growth of the secondary tumors was measured exactly as described for the primary tumors.

Measurement of CTL activity. For assays of CTL activity *in vitro*, mice were sacrificed 14 days after intratumoral injection of virus. Splenocytes were isolated from mice in each treatment group. Effector cells were obtained by coculturing splenocytes (3×10^6) with irradiated (4,000 rad) Neuro-2A cells (1×10^6), supplemented with recombinant human interleukin-2 (20 units/mL). After 5 days of restimulation, we measured the ability of the effector cells to lyse target cells using the 4-h chromium release assay as described earlier (16). Briefly, the labeled tumor cell suspension was incubated for 45 min at 37°C before it was added to 96-well, U-bottomed plates. Purified splenocytes were added as effector cells to the 96-well plate at various effector-to-target 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 medium served as a negative control. Supernatants (100 μL) were harvested and ^{51}Cr release was measured with a scintillation counter. Spontaneous release was measured in wells containing target cells alone. Triton X-100 was used to lyse the target cells maximally. The percentage of specific lysis was calculated by the following formula: percent of specific lysis = [(experimental release cpm − spontaneous release cpm) / (maximum release cpm − spontaneous release cpm)] × 100.

ELISPOT assay and measurement of cytokine secretion. For ELISPOT assays, 96-well filter plates for high-throughput separations (Millipore, Bedford, MA) were precoated with anti-IFN-γ monoclonal antibody (BD Bioscience, San Diego, CA) 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 Neuro-2A or Sa-I tumor cells. After incubation at 37°C for 18 to 24 h followed by washing, biotinylated anti-IFN-γ antibody (BD Bioscience) 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 further incubation for 1 h, 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. For measurement of cytokine secretion, splenocytes were prepared as described above. Duplicate samples of effector cells (5×10^4 per well) were cocultured with either Neuro-2A tumor cells or Sa-I tumor cells at effector-to-target ratios of 2:1 in 96-well U-bottomed plates. After 24 h, the supernatants were harvested and analyzed for mouse IFN-γ, tumor necrosis factor-α, and interleukin-2 with the BD Mouse Th1/Th2 Cytokine Cytometric Bead Array Kit according to the manufacturer's instructions (BD Bioscience).

Adoptive transfer of CTLs. The procedure of treating tumor-bearing mice with adoptively transferred CTLs was essentially the same as previously described (17). Initially, Neuro-2A tumor cells were resuspended in HBSS and injected s.c. (5×10^5 in 100 μL) into the flanks of naïve, immunocompetent female A/J mice. Three days later, splenocytes were harvested from immunocompetent A/J mice of which the s.c. tumors were treated twice with either FusOn-H2 or Baco-1 or PBS. The harvested splenocytes were washed and resuspended in HBSS, and then injected i.v. into the tail veins of the tumor-bearing mice (1×10^7 in 100 μL per mouse). The tumor size was measured twice a week after animals received the adoptive immunotherapy and the tumor volume was determined with the formula described above.

Statistical analysis. Quantitative results are reported as means and SDs. The statistical analysis was done by one-way ANOVA using Statview 5.0 software (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered statistically significant.

Results

Phenotypic characterization of FusOn-H2 in murine neuroblastoma cells. Both the HSV-1-based Synco-2D and the HSV-2-based FusOn-H2 viruses have been shown to induce syncytia formation in tumor cells of different tissue origins, including breast, ovary, prostate, and pancreas (13, 15, 18, 19), but whether this effect extends to neuroblastoma cells remains uncertain. We therefore infected Neuro-2A cells with either of these two viruses at 0.1 pfu/cell. Uninfected cells or cells infected with a nonfusogenic HSV-1–based oncolytic virus (Baco-1) served as controls. Syncytia were clearly visible in both Synco-2D– and FusOn-H2–infected cells, but not in those cells infected with Baco-1 (Fig. 1). Hence, both Synco-2D and FusOn-H2 retain their fusogenic phenotype in this line of neuroblastoma cells.

***FusOn-H2 has potent oncolytic activity against established neuroblastoma cells* in vivo.** To test the overall activity of FusOn-H2 against primary neuroblastoma, we injected 5×10^5 freshly harvested Neuro-2A cells into the right flank of 6-week-old syngeneic A/J mice. At 7 days after tumor cell implantation, when the tumors had reached an approximate diameter of 5 mm, we injected them directly with 1×10^7 pfu of FusOn-H2, Synco-2D, or Baco-1 (PBS-treated tumors served as controls). Tumor sizes were measured twice a week and tumor volumes were calculated as described in Materials and Methods. Fourteen days after virus administration, the mice were euthanized and their spleens were harvested for immunologic assays.

Among the three oncolytic HSVs, FusOn-H2 seemed to be the most effective at inhibiting the primary tumor growth

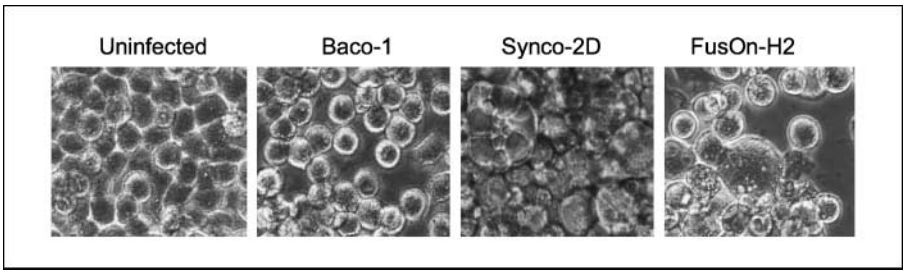

Fig. 1. Phenotypic characterization of oncolytic HSVs in Neuro-2A cells. Neuro-2A cell monolayers were infected with the indicated viruses at 0.1 pfu/cell. Micrographs were taken at 24 h after infection (original magnification, ×200).

(Fig. 2). On day 10 after virus injection, the tumors treated with FusOn-H2 were significantly smaller than those treated with either of the other two viruses (FusOn-H2 versus Synco-2D, $P < 0.05$; FusOn-H2 versus Baco-1, $P < 0.001$). The second most effective virus is Synco-2D. Although not as effective as the two fusogenic oncolytic HSVs, Baco-1 showed significant antitumor effect by comparison with the PBS control ($P < 0.05$ beginning on day 7). These data show that the recently constructed FusOn-H2 oncolytic virus has potent activity against primary neuroblastoma, in agreement with findings reported in the literature (20–22).

Tumor destruction by FusOn-H2 induces tumor-specific CTL responses. We previously showed that destruction of a weakly immunogenic mammary tumor by the doubly fusogenic Synco-2D induces a potent tumor-specific cellular immune response (9). Because FusOn-H2 also induces syncytia formation in tumor cells, it seemed reasonable to predict that the destruction of neuroblastoma cells *in vivo* by this virus would elicit effective antitumor immunity as well. We thus quantified the tumor-specific activities of CTLs collected from the spleens of mice used in the proceeding experiment. Our results show that both Synco-2D and FusOn-H2, but not Baco-1, induced measurable CTL activity that could readily lyse Neuro-2A tumor cells (Fig. 3A). At the highest effector-to-target ratio, the CTL activity induced by FusOn-H2 was significantly higher than the activity induced by Synco-2D ($P < 0.05$), indicating that FusOn-H2 is probably even more effective than Synco-2D at inducing antitumor immunity when used *in vivo*.

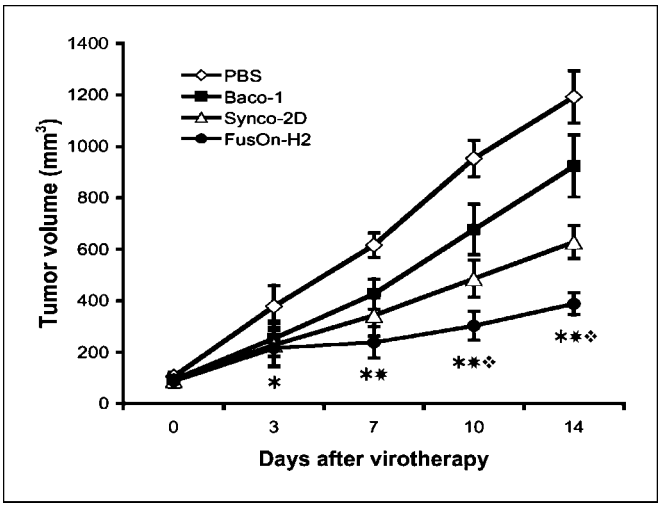

Fig. 2. Therapeutic effect of oncolytic HSVs against established neuroblastomas. Tumors were established on the right flank of immunocompetent A/J mice by s.c. implantation of 5×10^5 cells. Seven days later, viruses were injected into the tumor at a dose of 1×10^7 pfu. Tumor size was measured twice a week, and tumor volumes were determined as described in Materials and Methods. ⁎, $P < 0.05$, FusOn-H2 versus PBS; ⁑, $P < 0.05$, FusOn-H2 versus aco-1; ⁘, $P < 0.05$, FusOn-H2 versus Synco-2D.

We then asked if repeated administration of FusOn-H2 could boost the antitumor CTL response. Mice with established neuroblastoma received one or two intratumoral injections (1 week apart) of either FusOn-H2 or Baco-1, and tumor-specific CTL activity was measured 2 weeks after the last dose of virus. The results show that two doses of FusOn-H2 significantly enhanced antitumor immunity. The CTL killing activity against Neuro-2A cells in mice given a repeated injection of virus was nearly twice as potent as that in mice given a single injection (cf. Fig. 3A and B). The specificity of Neuro-2A lysis was confirmed by the demonstration that the same effector cells were unable to lyse the syngeneic sarcoma line Sa-I (Fig. 3C). We also compared the frequency of tumor-specific CTLs in FusOn-H2–treated versus Baco-1–treated mice. Overall, the results correlate with the CTL killing activities; the number of spot-forming cells per 10^5 splenocytes from FusOn-H2–treated mice was approximately thrice greater than the number from mice treated with Baco-1 ($P < 0.05$; Fig. 3D).

We also measured the levels of cytokines (IFN-γ, tumor necrosis factor-α, and interleukin-2) secreted by type 1 T helper (Th1) cells in the supernatant of cultured splenocytes after *in vitro* stimulation with the target Neuro-2A tumor cells or the control Sa-I sarcoma cells. The Th1 cells from mice treated with FusOn-H2 secreted from three to five times more of these cytokines than did the Th1 cells from Baco-1–treated mice (Fig. 4). There was a slight increase of IFN-γ and tumor necrosis factor-α release from the splenocytes that were stimulated with Sa-I cells. However, this increase was not statistically significant by comparison with results for the same two cytokines from the splenocytes of PBS-treated mice. These data are consistent with the results of CTL cytolysis testing and ELISPOT assays.

Virus-induced antitumor immunity against secondary neuroblastoma implant. A convenient way to assess the efficacy of virotherapy-induced antitumor immune responses is to measure their effect on distant metastases not injected with the oncolytic virus. Although Neuro-2A cells have been reported to metastasize to different organs after being implanted s.c. (23), we were unable to detect any notable metastases 4 weeks after tumor implantation in this study (data now shown). We therefore implanted Neuro-2A cells in the opposite flank (left) of A/J mice as "superficial metastases" to facilitate analysis of virus-induced tumor-specific cellular immunity. We initially implanted 5×10^5 Neuro-2A cell in the right flank of immunocompetent A/J mice. Seven days later, the same amount of Neuro-2A cells was implanted in the left flank on the same day

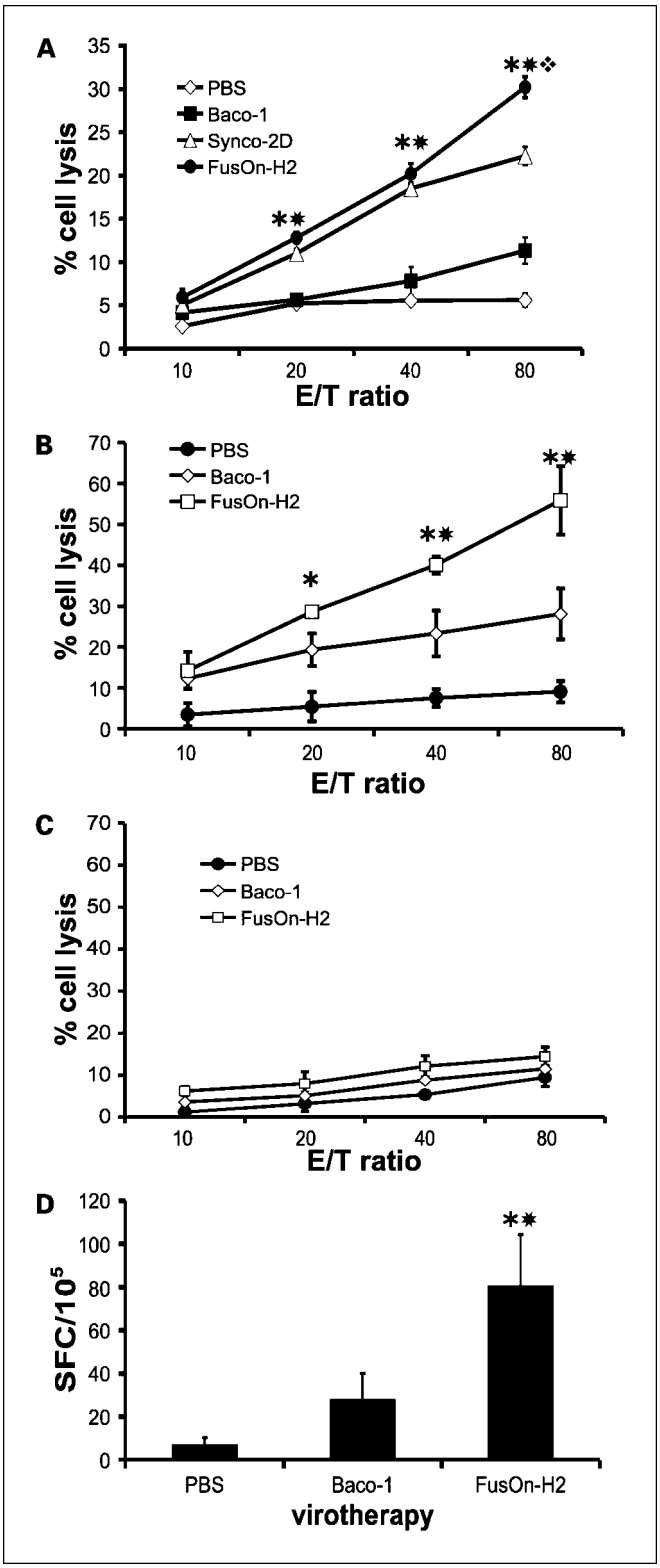

Fig. 3. Tumor-specific CTL activity after virotherapy. Neuroblastoma was established as described in the legend to Fig. 2. Mice then received either one (*A*) or two (*B*) injections of virus. Mice were sacrificed 7 d after the last virus injection and the cytotoxic activity of effector cells prepared from spleens was measured against either Neuro-2A cells (*A* and *B*) or syngeneic Sa-I sarcoma cells (*C*). Effector cells from the same preparation used in (*C*) were also studied with the ELISPOT assay (see Materials and Methods) to determine the frequency of tumor-specific CTLs (*D*). ∗, $P < 0.05$, FusOn-H2 versus PBS; ✱, $P < 0.05$, FusOn-H2 versus Baco-1; ❖, $P < 0.05$, FusOn-H2 versus Synco-2D.

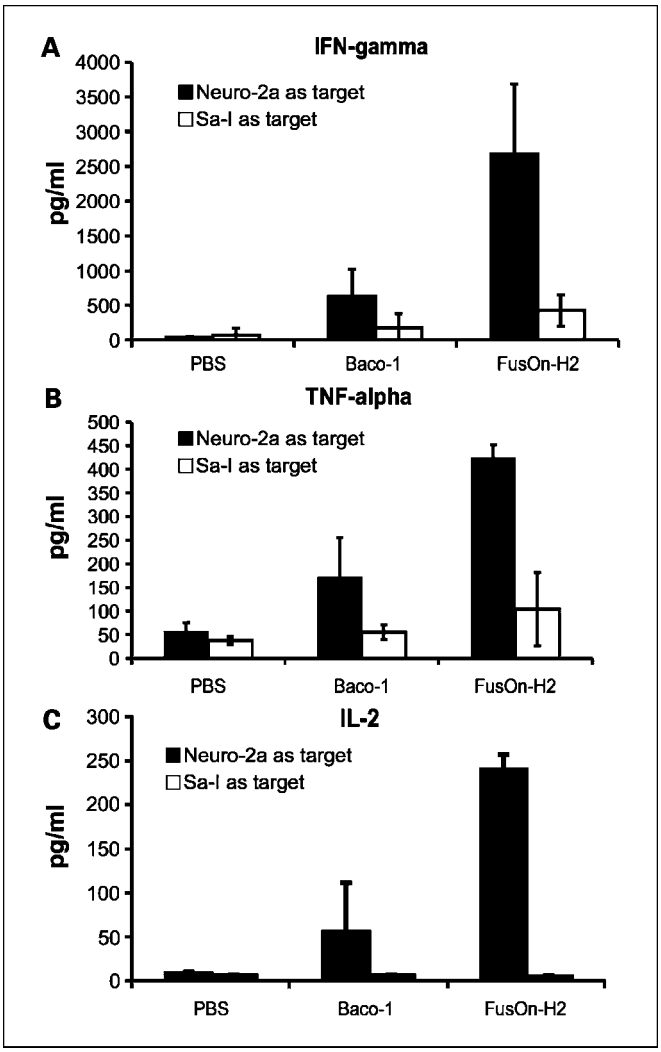

Fig. 4. Comparison of Th1 cytokine secretion by CTLs from mice treated with different oncolytic HSVs. The CTLs were from the same pool used in Fig. 3B. Supernatants were collected from wells containing 5×10^4 splenocytes that had been stimulated with either irradiated Neuro-2A or Sa-I cells. The concentration of cytokines was measured with the BD Mouse Th1/Th2 Cytokine Cytometric Bead Array Kit according to the manufacturer's instructions.

that mice received intratumoral injection of 2×10^6 oncolytic virus in the right flank. The tumor growth was monitored for 2 more weeks, after which the secondary tumors were explanted and weighted. Consistent with the data presented in Fig. 2, both Synco-2D and FusOn-H2 produced an efficient oncolytic effect on the primary tumors (data not shown). Direct injection of these two viruses into the primary tumor also produced a strikingly inhibitory effect on the secondary tumor implant (Fig. 5). Again, the most impressive result was seen in mice treated with FusOn-H2, in which the tumors showed only marginal growth after implantation. In contrast, Baco-1 injection produced only slight inhibition of tumor growth. These results, together with those in Fig. 3A, suggest that FusOn-H2 may be even more effective than Synco-2D at inducing host antitumor immune responses after *in vivo* administration, and that the elicited tumor-specific immunity may extend to tumor cells distant from the original injection site.

An alternative explanation for the apparent growth inhibition of secondary tumor in oncolytic virus–treated

mice is that the viruses injected into the opposite flank leaked into the bloodstream and were subsequently transported to the sites of secondary tumor implantation. We therefore repeated the above experiment in nude mice, which lack thymus and thus cannot generate mature T lymphocytes. As such, they are unable to mount cell-mediated immune responses and also have an accompanying defect in antibody formation (due to the lack of required $CD4^+$ helper T cells). Our assumption was that virus leakage and subsequent transport to the sites of secondary tumor would be much more likely in nude mice compared with immunocompetent mice due to the lack of antiviral immunity in the former model. Intratumoral injection of FusOn-H2 in nude mice produced essentially the same local effect as in immunocompetent mice (cf. Fig. 6A with Fig. 2), but failed to inhibit the growth of tumors growing in the opposite flank (Fig. 6B). These data support our suggestion that the tumor-specific CTL activity elicited by FusOn-H2–mediated oncolysis directly inhibited the growth of secondary tumor implant, as seen in Fig. 5.

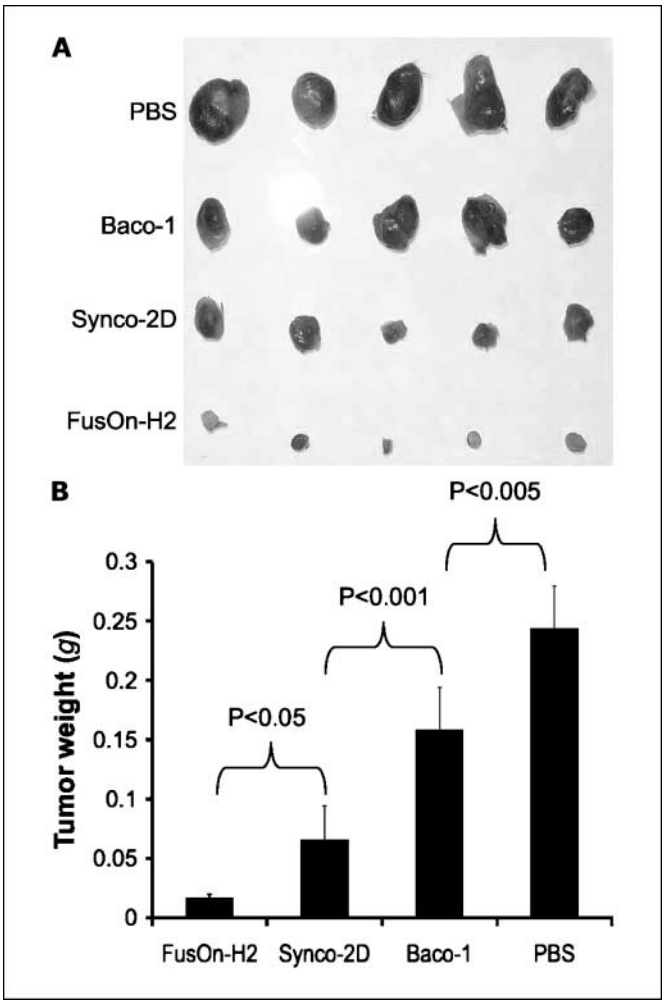

Fig. 5. Growth of tumors implanted in the opposite flank of mice receiving virotherapy. Neuroblastoma was established in the right flank of A/J mice by s.c. injection of 5×10^5 Neuro-2A cells. Seven days later, the tumor nodules were injected with oncolytic virus, and new tumors (5×10^5 Neuro-2A cells) were established in the left flank. At 2 wk postimplantation, the resultant secondary tumor nodules were explanted and their gross appearance (*A*) and weight (*B*) were recorded. Statistical comparison of tumor weights is shown on the graph.

Antitumor effect of adoptively transferred splenocytes from mice receiving virotherapy. Any firm conclusion about the capacity of FusOn-H2–induced antitumor immunity to affect distant metastases requires direct demonstration of that ability. We thus adoptively transferred the splenocytes harvested from mice treated with two injections of oncolytic viruses into naïve mice that had been implanted with tumor cells 3 days earlier. Splenocytes from PBS-treated mice served as a negative control. Tumor sizes were measured once a week and tumor volumes were calculated as described in Materials and Methods. Overall, tumor growth in this experiment was slower than shown in Fig. 3. This discrepancy likely reflects the variable passage conditions of the tumor cells used in the individual experiments, although we cannot rule out a nonspecific inhibitory effect from the adoptively transferred splenocytes. Nonetheless, by comparison with the effects of splenocytes from PBS controls or Baco-1–treated mice, the adoptively transferred splenocytes from FusOn-H2–treated mice produced significantly greater inhibition of tumor growth (Fig. 7; $P < 0.05$ and $P < 0.01$, respectively, at days 21 and 27 after tumor implantation by comparison with PBS; $P < 0.05$, at day 27 by comparison with the Baco-1 group). Adoptive transfer of splenocytes from mice treated with Baco-1 produced inhibitory effect by comparison with the PBS control ($P < 0.05$, at days 21 and 27). Part of the moderate antitumor effect of splenocytes from mice treated with Baco-1 may reflect the enhanced tumor-specific CTL activity in mice receiving two injections of the virus (Fig. 3B). These results correlate well with the data presented in Fig. 5 and argue strongly for a tumor-specific immune response to oncolysis induced by virotherapy.

Discussion

Immunotherapy directed to known tumor antigens or whole tumor cells can potentially eradicate locally invasive or metastatic tumors that are difficult to manage with conventional agents and thus represents a highly promising strategy of cancer treatment. Cancer vaccines derived from whole tumor cells have a major advantage over those based on identified tumor antigens, in that a single vaccine preparation would, in principle, include the entire antigenic repertoire of tumor cells (24, 25). Virotherapy-mediated oncolysis affords a simple and efficient means to generate whole tumor vaccines, as it releases tumor antigens in their native forms and configurations without any requirements for *in vitro* manipulations.

We previously showed that tumor destruction by a doubly fusogenic oncolytic virus constructed from HSV-1 (Synco-2D) induces robust antitumor immunity to otherwise weakly immunogenic murine mammary tumor cells (9). It was suggested that syncytia formation induced by the fusogenic virus might function as an adjuvant to enhance the presentation of tumor antigens (9). In the current study, we show that Synco-2D, but not the nonfusogenic HSV-1–based oncolytic virus Baco-1, effectively induces tumor-specific immune responses in a syngeneic murine neuroblastoma model, confirming the ability of syncytia formation to enhance tumor antigen presentation. A third oncolytic virus, FusOn-H2 constructed from HSV-2, also has the ability to induce syncytia formation, as well as apoptosis, in tumor cells (13, 19) and was

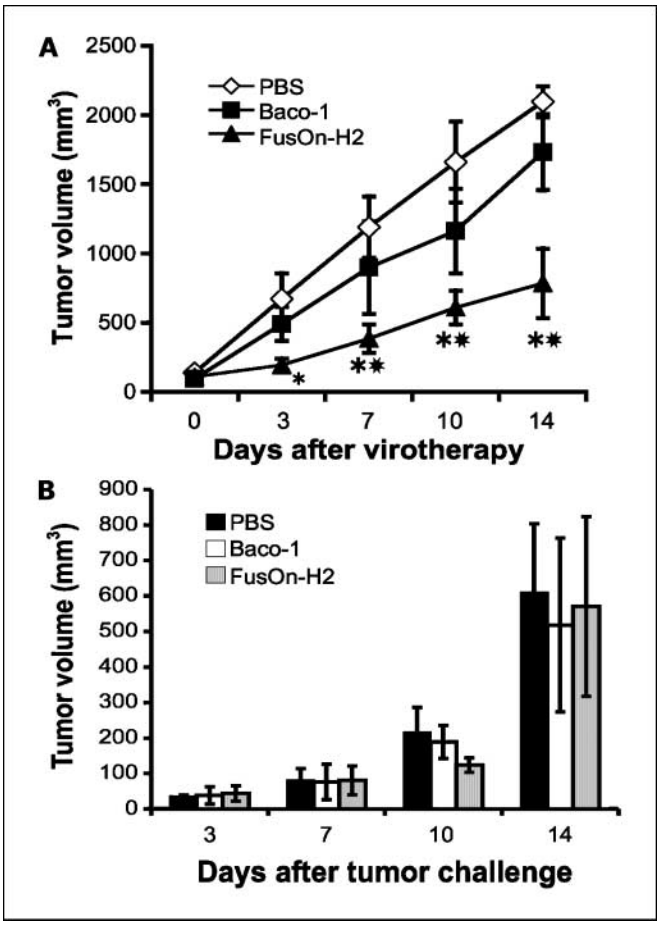

Fig. 6. Therapeutic effect of oncolytic HSVs against neuroblastoma in nude mice. Neuroblastoma was initially established on the right flank of Hsd nude mice. Subsequent virotherapy and Neuro-2A tumor cell rechallenge were done as described in the legend to Fig. 5. *A,* growth curve for primary tumors implanted on the right flank and subsequently injected with oncolytic HSVs. *B,* growth profiles for tumors that were implanted in the opposite flank and not treated with virus. ∗, $P < 0.05$, FusOn-H2 versus PBS; ⁕, $P < 0.05$, FusOn-H2 versus Baco-1. Tumor growth rates among the three groups in (*B*) did not differ significantly at any time points.

shown to elicit effective antitumor immunity when directly injected into established neuroblastomas in mice. Moreover, direct compassion of the antitumor activities of Synco-2D and FusOn-H2 indicated that the latter virus is more effective than the former. We attribute this difference in efficacy to the additional ability of FusOn-H2 to induce apoptosis, a property that likely also contributed to the stimulation of antitumor immune responses, because such induction in tumor cells has been reported to increase tumor antigen cross-priming and cross-presentation (10). It has also been reported that vaccination with an HSV-2 mutant deleted for the pyruvate kinase domain mainly induces a Th1 HSV-specific immune response (26). Interestingly, our data show that tumor destruction mediated by FusOn-H2 predominately elicits tumor-specific cytokine secretion from Th1 cells, indicating that this unique property of the mutant HSV-2 may constitute one of the underlying mechanisms by which it induces cell-mediated antitumor immunity.

Our selection of a syngeneic neuroblastoma model for the present study was intended to provide a contrasting experimental system with the syngeneic mammary tumor model used earlier to show the antitumor properties of a fusogenic HSV (9). The similarity of outcomes in these two systems suggests that the diverse antitumor mechanisms of FusOn-H2 and Synco-2D are not restricted to tumors of a particular tissue origin but operate across tumor types. The ability of these oncolytic viruses to induce syncytia formation or induction of apoptosis (or both) seems to underlie their ability to induce antitumor immunity *in vivo*. Moreover, the inflammatory environment created by virotherapy provides strong proinflammatory signals that may promote dendritic cell maturation (27) and help to breach the tumor-related immunosuppressive "firewalls," thus facilitating the infiltration of tumor-specific immune cells to the tumor site. Indeed, all these factors operating in concert seem to be necessary for the efficient induction and the subsequent effect of antitumor immunity by either FusOn-H2 or Synco-2D.

Amplification/overexpression of MYCN, a member of the *MYC* proto-oncogene family, occurs in 25% to 30% of primary untreated neuroblastomas (28, 29). Amplification of this gene has been reported to be highly correlated with advanced-stage disease, rapid progression, and a poor prognosis (30). Thus, it would be desirable to assess the antitumor effect of any new therapeutic modality for neuroblastoma in the context of MYCN amplification status. However, because the murine neuroblastoma Neuro-2A cell line used in this study contains only single-copy MYCN (31), the effect of its amplification status on the results of FusOn-H2 – mediated virotherapy could not be fully evaluated. Nonetheless, as it has been reported that cooperation between Ras and MYC is required for cellular transformation and active Ras is needed to block MYCN degradation (32), it seems that cell cycle progression of neuroblastoma cells would, by necessity, depend on Ras-MYCN interaction (33). Thus, an oncolytic virus such as FusOn-H2 that targets tumor cells with an activated Ras signaling pathway

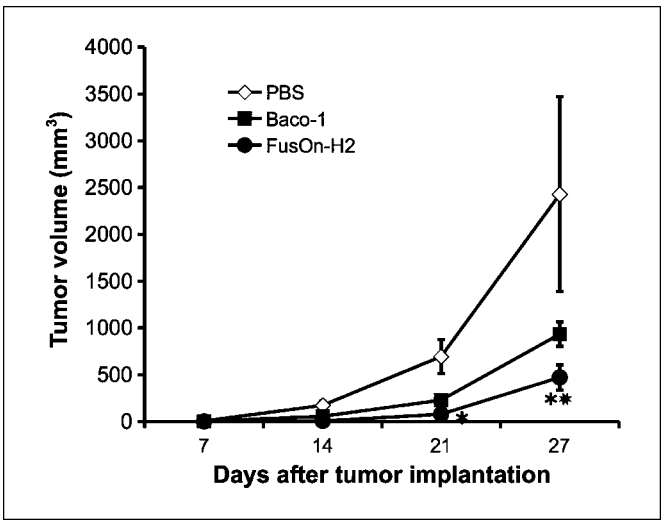

Fig. 7. Antitumor effect of adoptively transferred splenocytes. Splenocytes from the experiment described in Fig. 3B were used. Initially, 5×10^5 Neuro-2A cells were implanted in the right flank of A/J mice. Three days later, 1×10^7 splenocytes in 100 μL of HBSS were adoptively transferred to the mice ($n = 5$ mice per group) by tail vein injection. Tumor size was measured once a week and tumor volume was determined as described in Materials and Methods. ∗, $P < 0.05$, FusOn-H2 versus PBS; ⁕, $P < 0.05$, FusOn-H2 versus Baco-1.

would likely be effective at destroying murine neuroblastoma cells harboring MYCN amplification.

A repeated injection of FusOn-H2 enhanced the antitumor immunity induced by the virus almost 2-fold. Although we did not investigate whether more than two virus injections would boost antitumor immunity even higher, that definitely remains a possibility. Because virotherapy is likely to be used in frequent repeated injections in cancer patients, we would anticipate more robust antitumor immune responses in the setting than were achieved in our mouse neuroblastoma model. However, it is envisaged that the elicited antitumor immunity, regardless of its strength, is unlikely to significantly shrink vascularized bulky tumors, but may contribute to the overall antitumor effect by clearing residual tumor cells not eradicated by virotherapy. Thus, in the experiment reported in Fig. 7, we chose to adoptively transfer the splenocytes from mice treated with virotherapy to naïve mice that had been implanted with tumor only 3 days earlier, when the tumor was relatively small. This might partly explain why the limited transfer of splenocytes efficiently controlled tumor growth.

In conclusion, we have shown that a novel oncolytic virus derived from HSV-2 can induce a potent antitumor immune response in addition to its recognized killing mechanisms. Thus, initial treatment with this virus could be expected to reduce the primary tumor mass, whereas residual tumor cells and possibly metastatic tumor would be destroyed by immune responses to the native antigens released from lysed tumor. This combination of antitumor effects might be sufficient to eradicate bulky solid tumors and their metastases in cancer patients, a possibility we plan to test in the near future.

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