Carrier Cell-based Delivery of an Oncolytic Virus Circumvents Antiviral Immunity

Anthony T Power[1], Jiahu Wang[1], Theresa J Falls[1], Jennifer M Paterson[1], Kelley A Parato[1], Brian D Lichty[2], David F Stojdl[3], Peter AJ Forsyth[4], Harry Atkins[1] and John C Bell[1]

[1]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa Health Research Institute, Centre for Cancer Therapeutics, Ottawa, Ontario, Canada; [2]Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, 5023 Michael DeGroote Centre for Learning and Discovery McMaster University, Ottawa, Ontario, Canada; [3]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Apoptosis Research Center, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada; [4]Department of Medicine, University of Calgary, Tom Baker Cancer Centre, Calgary, Alberta, Canada

Oncolytic viruses capable of tumor-selective replication and cytolysis have shown early promise as cancer therapeutics. However, the host immune system remains a significant obstacle to effective systemic administration of virus in a clinical setting. Here, we demonstrate the severe negative impact of the adaptive immune response on the systemic delivery of oncolytic vesicular stomatitis virus (VSV) in an immune-competent murine tumor model, an effect mediated primarily by the neutralization of injected virions by circulating antibodies. We show that this obstacle can be overcome by administering virus within carrier cells that conceal viral antigen during delivery. Infected cells were delivered to tumor beds and released virus to infect malignant cells while sparing normal tissues. Repeated administration of VSV in carrier cells to animals bearing metastatic tumors greatly improved therapeutic efficacy when compared with naked virion injection. Whole-body molecular imaging revealed that carrier cells derived from solid tumors accumulate primarily in the lungs following intravenous injection, whereas leukemic carriers disseminate extensively throughout the body. Furthermore, xenogeneic cells were equally effective at delivering virus as syngeneic cells. These findings emphasize the importance of establishing cell-based delivery platforms in order to maximize the efficacy of oncolytic therapeutics.

Received 10 August 2006; accepted 2 October 2006.
doi:10.1038/sj.mt.6300039

INTRODUCTION

A variety of different replication-competent viruses have been selected or engineered to preferentially infect and kill cancer cells and are currently under clinical or preclinical study.[1] These therapeutics offer a high level of antitumor potency and, if administered systemically, could be particularly effective against disseminated disease. However, delivering an oncolytic virus to tumor cells through the circulatory system is a unique challenge. Virus particles injected into the bloodstream are vulnerable to inactivation by complement proteins,[2,3] uptake by the reticuloendothelial system,[4,5] and neutralization by circulating antibodies.[2,6–9] Of these, host antibodies are likely to be the most restrictive barrier to therapy, as they mediate a long-lasting state of immunity to repeated infection.

Vesicular stomatitis virus (VSV) is an effective oncolytic therapeutic when administered intravenously (IV) in a variety of murine cancer models;[10–13] however, the impact of a pre-existing or evolving immunity has yet to be tested. As robust adaptive immunity is elicited upon administration to mice,[14] this system offers the valuable opportunity to study oncolytic virotherapy in the context of a fully functional host immune system.

Here we demonstrate the negative impact of humoral immunity on systemic delivery of VSV, underscoring the need for alternative approaches to oncolytic virus administration. Infected cells have shown promise as delivery vehicles for viral therapeutics,[15–22] and we now demonstrate that cellular carriers can shield oncolytic virus from neutralizing antibodies during delivery, providing a simple and effective means to enhance therapy in the face of sterilizing antiviral immunity. Furthermore, we show that a wide variety of established, non-autologous cell lines, particularly those of hematological origin, could be a platform for the construction of specialized biotherapeutic delivery vehicles to maximize the efficacy of oncolytic viruses when applied in the clinic.

RESULTS

Circulating antibodies impair systemic therapy with VSV

To examine the impact of a pre-existing immune response, we compared virus delivery in naïve animals to those immunized with an IV dose of 5×10^8 plaque-forming units (PFU) of VSV 6 weeks before treatment. As we have reported previously,[10]

Correspondence: John C Bell, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa Health Research Institute Centre for Cancer Therapeutics, 503 Smyth Road, Ottawa, Ontario, Canada K1H 8L6. E-mail: jbell@ohri.ca

extensive tumor-specific replication, as evidenced by virus-associated fluorescence, is seen in CT26 tumors 24 h following IV administration of VSV-green fluorescent protein (GFP) to naïve mice (**Figure 1a**, upper panel). In contrast, no expression of virus-associated GFP was observed in tumors from pre-immunized mice (**Figure 1a**, lower panel). Interestingly, the inhibition of delivery correlated with the onset of humoral immunity. Neutralizing titers were detectable in serum as early as 4 days into therapy and reached long-lasting plateau levels by 21–28 days (**Figure 1b**), suggesting that circulating antibodies were responsible for impairing VSV delivery. To further investigate this possibility, we examined whether passive immunization with anti-VSV immune serum before treatment was sufficient to ablate infection of subcutaneous tumors. Quantitative analysis of tumor homogenates revealed nearly 10^{10} PFU/g in tumors from naïve mice, whereas no virions were detected in tumors from mice pre-immunized with either live virus or anti-VSV immune serum (**Figure 1c**). In contrast, adoptive transfer of purified immune T cells had no effect on tumor infection (**Figure 1d**). These results indicate that circulating antibodies rather than cellular responses elicited during oncolytic therapy are sufficient to ablate delivery of repeat virus doses, consistent with earlier observations that in the mouse, immunity to VSV is largely a humoral response.[14]

Systemic delivery of VSV using syngeneic carrier cells

We next tested whether an established syngeneic cell line could be used as a *trojan horse* vehicle to transiently sequester viral antigen during systemic delivery. As shown in **Figure 2a**, CT26 murine colon carcinoma cells are readily infected with VSV and begin to accumulate viral protein within 6 h. We therefore harvested cells after 3 h of infection, before the onset of antigen expression, for administration to mice with established lung metastases. Microscopic examination of lungs 24 h after IV injection of 10^6 VSV-infected cells revealed widespread expression of virally encoded GFP within tumor nodules, but not in surrounding normal lung tissue (**Figure 2b**). Nearly all visible tumor nodules were GFP positive, indicative of highly efficient carrier cell delivery to the lungs. Similarly, dual-color fluorescence experiments revealed significant accumulation of carboxyfluorescein succinimidyl ester (CFSE)-labeled carrier cells (**Figure 2c**, green fluorescent dye) in the lungs within 30 min of injection, which released VSV-red fluorescent protein (RFP) to infect nearby metastases within 12 h (**Figure 2c**, red). Notably, CFSE-labeled cells were found distributed throughout normal

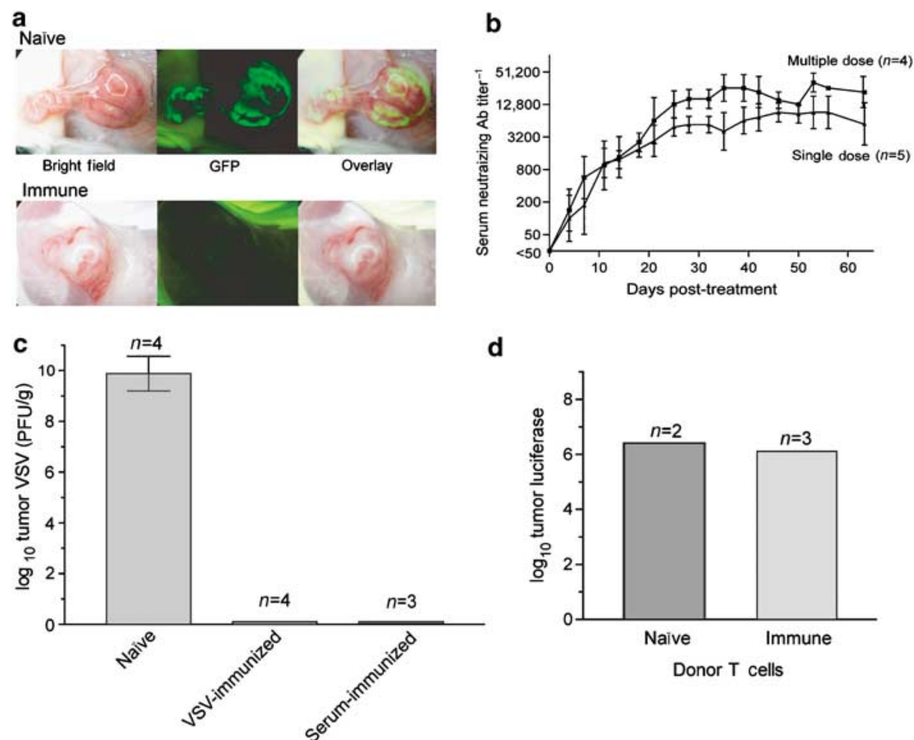

Figure 1 Humoral immune response impairs systemic tumor therapy with oncolytic VSV. (**a**) Composite bright-field/fluorescent microscopy images of subcutaneous tumors from either naïve (upper panels) or pre-immunized (lower panels) mice, 24 h following IV administration of 5×10^8 PFU VSV-GFP. (**b**) Kinetics of neutralizing antibody (NAb) response to either single (▲) or multiple dose (■) therapy with oncolytic VSV. The multiple dose group received three IV doses of 5×10^8 PFU per week over a total period of 7 weeks. Geometric mean NAb titers for each group ± SD are shown. (**c**) Quantitative analysis of VSV titers in subcutaneous tumors removed from naïve, VSV-immunized, or passively immunized mice 24 h following IV therapy as described above. Bars represent mean $\log_{10}$ titers ± SD. (**d**) Quantitation of tumor luciferase activity in mice receiving a transfer of T lymphocytes from either naïve (dark bar) or VSV-immune (light bar) donors 24 h before IV therapy with VSV luciferase. Tumors were homogenized and assayed for luciferase activity 24 h post-treatment. Bars represent mean activity of tumor lysates from mice receiving naïve T cells ($n = 2$) or immune T cells ($n = 3$).

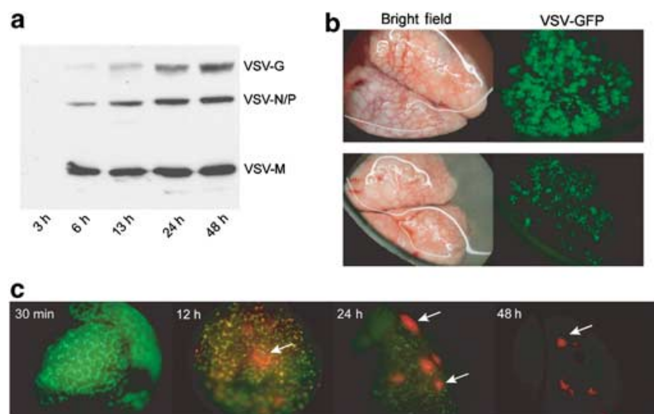

Figure 2 Delivery of oncolytic VSV to lung tumors in syngeneic carrier cells. (**a**) Western blot analysis showing time course of VSV protein synthesis in CT26 colon carcinoma carrier cells infected *in vitro.* (**b**) Balb/c mice with established CT26 lung tumors were treated IV with 10^6 cells infected with VSV-GFP. Mice were killed and lungs were imaged under a dissecting microscope at 24 h post-treatment. Bright-field images (left) show distribution of tumor nodules, whereas green fluorescent images (right) show localization of viral replication. (**c**) Two-color fluorescent imaging of delivery to lung metastases. The cellular fluorochrome CFSE was used to label CT26 cells before infection with VSV-RFP and subsequent systemic infusion as above. At indicated time points, mice were killed and lungs were examined under a fluorescent dissecting microscope. Images shown are composites from CFSE (green, cellular label) and RFP (red, virus replication) channels. Lungs were examined under bright field to identify tumor nodules (arrows).

lung tissue, whereas the replication of virus was confined exclusively to tumor nodules (**Figure 2c**, white arrows).

In vivo imaging of cell-based delivery

In order to non-invasively visualize both the distribution of the cellular vehicle and the viral payload delivered *in vivo*, we created a cell line that expresses *Renilla reniformis* luciferase from an integrated cellular gene, $CT26^{RLUC}$, and a viral strain (VSV^{FLUC}) that expresses *firefly* luciferase from a viral promoter. This allowed us to independently follow the biodistribution of the carrier cell and virus-associated luciferase enzymes by administering the appropriate substrate to treated mice and imaging bioluminescence with an *in vivo* imaging system. Using the *in vivo* imaging system, we observed that syngeneic carrier cells accumulate rapidly in the lungs within the first hour following IV infusion, where they remained until releasing virus and undergoing lysis by 24 h (**Figure 3a**, upper panel). The lung-associated localization was not tumor specific, as the same pattern was observed in the lungs of tumor-free mice (**Figure 3b** upper panel). In contrast, virus-associated *FLUC* activity continued to increase and persisted well after the elimination of carrier cells in tumor-bearing but not tumor-free lungs (**Figure 3a** and **b** lower panels), confirming the release of virus and tumor-specific replication as detected by fluorescence imaging in the preceding experiments.

Xenogeneic carrier cells mediate systemic delivery of VSV in immune-competent animals

Past experience with cell-based therapies has led to the belief that autologous cells are the preferred source for viral carriers,[23] as unlike histoincompatible allogeneic and xenogeneic cells they are not subject to immune rejection. To test this hypothesis experimentally, we used whole-body imaging to examine the capacity of labeled human $A549^{RLUC}$ carcinoma cells to deliver VSV^{FLUC} to tumors in immune-competent mice. Human carcinoma cells, like their mouse counterparts, accumulated in the lung within the first hour following IV administration and were eliminated by 24 h (**Figure 3c**, upper panels). Surprisingly, the xenogeneic cells were equally capable of releasing VSV to infect lung tumors (**Figure 3c**, lower panels). Thus, cell-mediated delivery of VSV can be achieved using immunologically incompatible cells of allogeneic or xenogeneic background.

Leukemic carrier cells deliver VSV to disseminated tumor sites

Although murine and human carcinoma cell lines efficiently delivered and released VSV to tumor cells *in vivo*, their apparent accumulation within lung microcapillaries prompted us to investigate whether a cell line derived from a hematological malignancy might more readily distribute throughout the circulatory system upon IV administration. In sharp contrast to carcinoma cells, a murine leukemia cell line ($L1210^{RLUC}$) showed a much more disseminated pattern of delivery following IV administration, with *RLUC*-tagged cells detectable not only in the lungs but also throughout the abdominal cavity and lymphoid organs (**Figure 4a** and **b**, left panels). Infected $L1210^{RLUC}$ cells retained the ability to deliver VSV^{FLUC} to lung tumors (**Figure 4b**, right) and were also able to bypass the lung and deliver virus to subcutaneous tumors located on the hind flank of mice (**Figure 4c**). As observed with carcinoma carriers, replication of VSV^{FLUC} was confined to carrier cells following injection into tumor-free mice, with no detectable replication persisting in normal tissues. Therefore, leukemic carrier cells appear to be less restricted in their passage through the circulatory system and can disseminate virus to diverse anatomical locations.

Immune evasion by cell-based delivery of VSV

Given the effective systemic delivery achievable using cellular carriers, we asked whether this approach could protect a therapeutic dose of VSV from the detrimental effects of antiviral antibodies. Administration of naked VSV^{FLUC} particles to mice did not lead to detectable infection of lung tumors when anti-VSV antibodies were present (**Figure 5a**, lower panel). A transient, low level of replication was detected only in the spleen at 7 h. In contrast, carrier cells could bypass circulating antibody to reach the lungs and transfer virus to pre-existing tumor cells, with robust viral infection persisting for up to 6 days post-injection (**Figure 5a**, upper panel). In a separate experiment, quantitative titers of virus were undetectable in lung tumors 24 h following administration of naked virions to mice with pre-existing humoral immunity, whereas tumors treated with infected cells contained on the order of 10^9 virus particles, despite the presence of circulating antibodies (**Figure 5b**). Consistent with the ablation of viral delivery, mice with pre-existing immunity to VSV show no therapeutic response to multiple systemic doses of naked virions, succumbing to

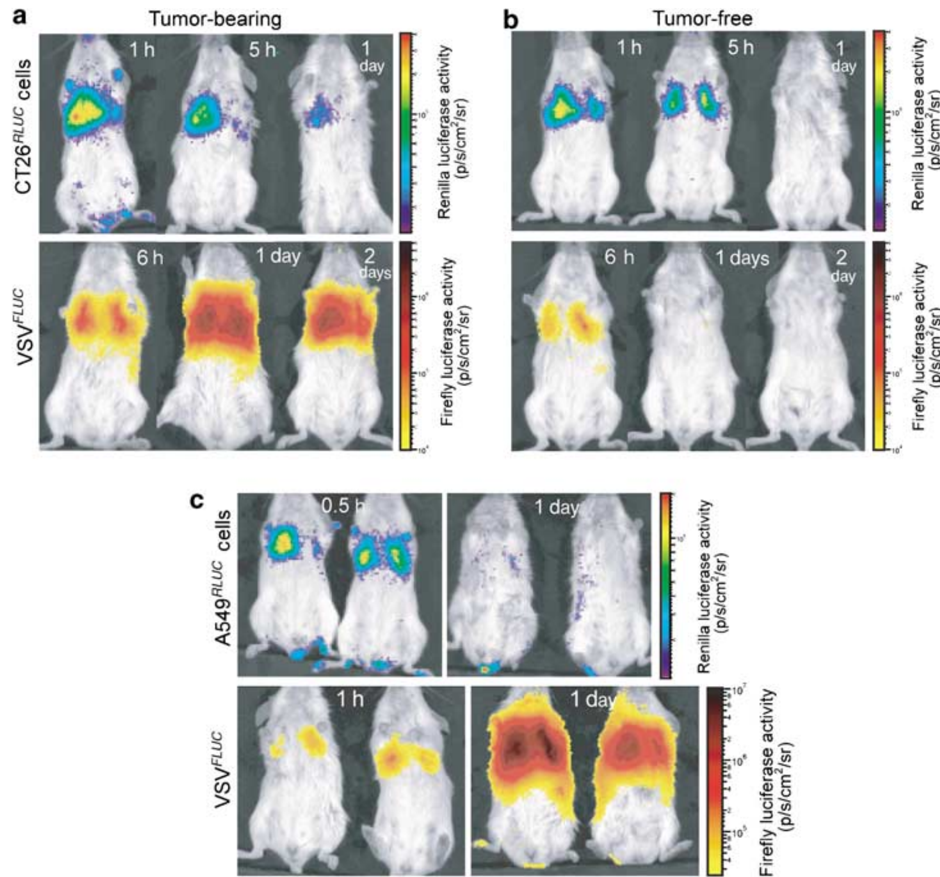

Figure 3 Dual-enzyme *in vivo* luminescence imaging of VSV delivery by autologous and xenogeneic carcinoma cells. (**a**) Lung tumor-bearing mice were injected IV with 10^6 autolgous CT26^{RLUC} carcinoma cells infected with VSV^{FLUC}. (**b**) Tumor-free mice were injected IV with 10^6 autolgous CT26^{RLUC} carcinoma cells infected with VSV^{FLUC}. (**c**) Lung tumor-bearing mice were injected IV with 10^6 xenogeneic A549^{RLUC} human carcinoma cells infected with VSV^{FLUC}. At time points indicated, mice were treated with the appropriate substrate to independently image cell-associated (*RLUC*, rainbow color scale) and virus-associated (*FLUC*, yellow-red color scale) enzyme expression.

extensive tumor burden within the same time period (15 days) as saline-treated controls (**Figure 5c**). However, administration of VSV-infected carrier cells could induce significant tumor regression (**Figure 5c**, right). On autopsy, we observed clearance of nearly all tumor nodules from the lungs and only one small residual nodule that had presumably escaped infection (white arrow). These data indicate that carrier cells can enable systemic VSV delivery and tumor oncolysis despite the presence of virus-neutralizing humoral immunity.

Enhanced efficacy of systemic therapy with repeated administration of VSV-infected cells

We next examined whether the cell-based delivery approach could enhance the therapeutic efficacy of a clinically relevant dosing regimen in an animal tumor model. As VSV is not a common pathogen in humans, patients are likely to be immunological naïve at the outset of therapy, but repeat virus doses will be exposed to an evolving adaptive response. We therefore chose to compare the antitumor efficacy of multiple VSV doses administered as naked virions or within infected carrier cells to initially naïve, but immune-competent mice with pre-established lung tumors. In this highly aggressive model of disease, saline-treated controls rapidly succumb to disease within 14 days owing to the invasion of normal lung tissue by hundreds of metastatic tumor nodules (**Figure 6b, —○—, c** upper-left). We have previously shown that delivery of a single dose of VSV directly to the lungs via intranasal instillation induces lasting disease regression in this model.[10] However, repeated IV infusion of VSV particles has only a modest effect on tumor progression (**Figure 6b —□—,** and **c** upper-right), presumably as delivery to all tumor nodules is not achieved before continued dosing is rendered ineffective by the onset of humoral immunity (**Figure 1**). In contrast, 12 administrations of VSV-infected carrier cells led to lasting cures in the majority of mice and significantly increased survival in all others (**Figure 6b —∇—, c** lower-left). These results suggest that hiding oncolytic virus within carrier cells during delivery can greatly increase the cumulative therapeutic benefit of systemic dosing regimens in the face of evolving antiviral immunity.

DISCUSSION

The adaptive immune system, evolved over millions of years to defend animals from invading pathogens, is a serious impediment to the prospect of using viruses as systemic therapeutics. We have shown that circulating antibodies elicited against an oncolytic strain of VSV in immune-competent animals are

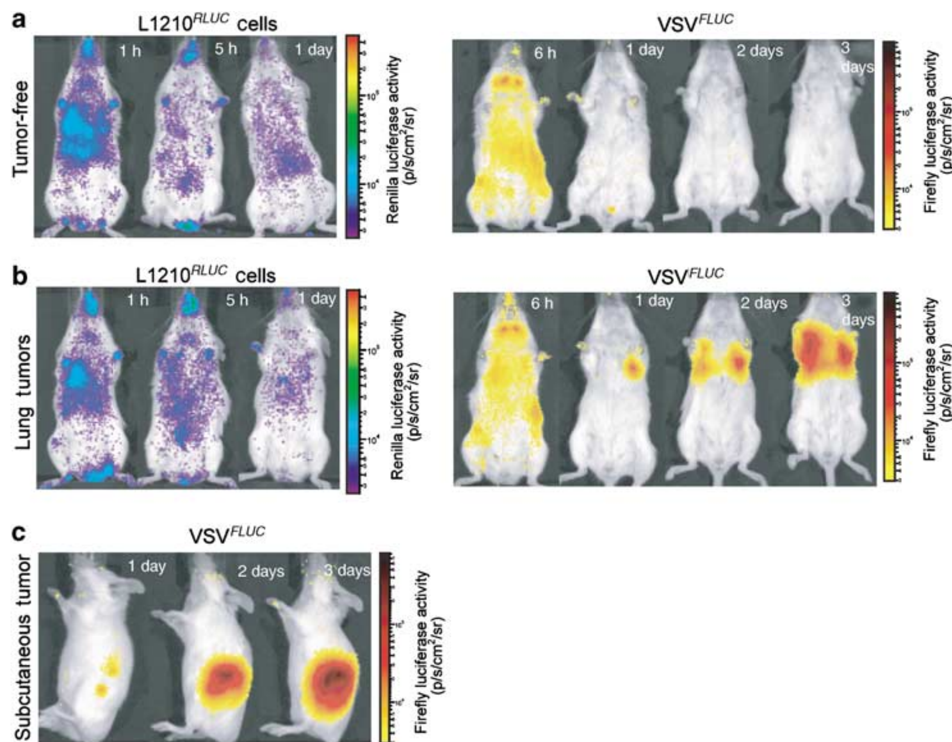

Figure 4 Dual-enzyme *in vivo* luminescence imaging of VSV delivery by murine leukemia cells. (**a**) Tumor-free mice were injected IV with 10^6 murine L1210^{RLUC} cells infected with VSV^{FLUC}. (**b**) Lung tumor-bearing mice were injected IV with 10^6 murine L1210^{RLUC} cells infected with VSV^{FLUC}. (**c**) Subcutaneous tumor-bearing mice were injected IV with 10^6 murine L1210^{RLUC} cells infected with VSV^{FLUC}. At time points indicated, mice were treated with the appropriate substrate to image cell-associated (*RLUC*, rainbow color scale) or virus-associated (*FLUC*, yellow-red color scale) enzyme expression.

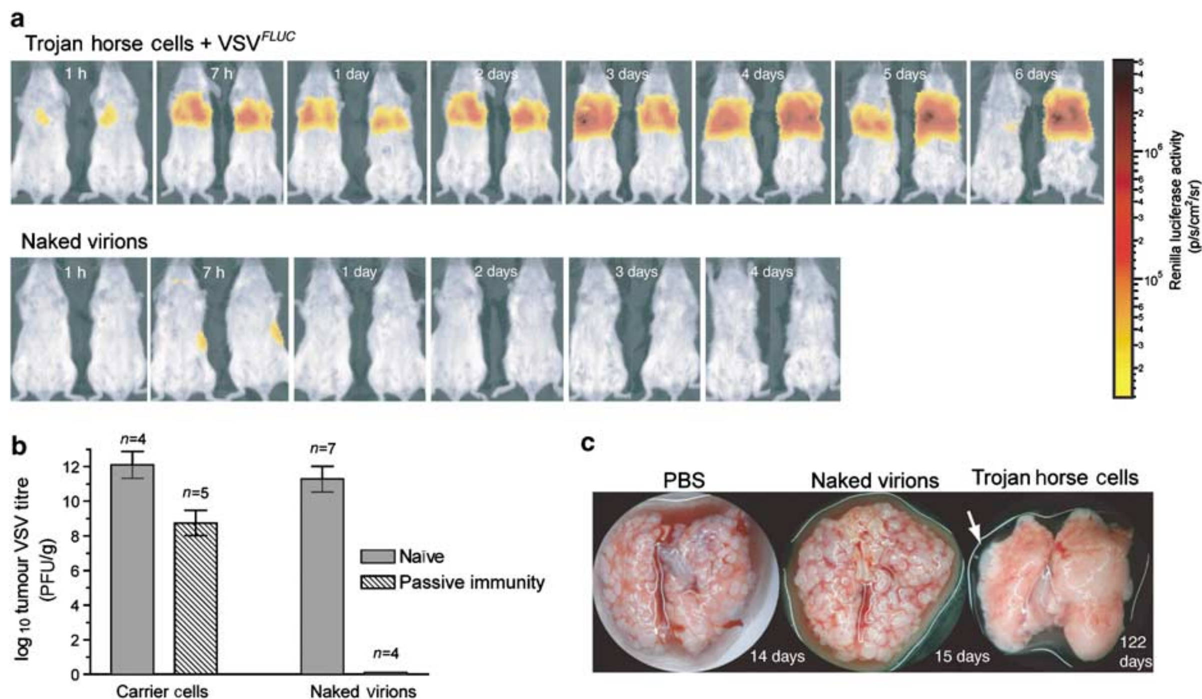

Figure 5 Cell-mediated systemic delivery and antitumor activity of VSV in the presence of circulating antibody. (**a**) Mice were were injected intraperitoneally with immune serum containing antibodies against VSV and treated with VSV in carrier cells (upper panels) or as naked virions (lower panels). Mice were injected with luciferin and imaged at the indicated time points. (**b**) Quantitation of VSV in subcutaneous tumors 24 h following IV administration of infected carrier cells or naked virions in the presence (shaded bars) or absence (hatched bars) of circulating antiviral antibodies. Mean $\log_{10}$ tumor titers $\pm$ SD are plotted. (**c**) Post-mortem images of lung metastases following IV repeated administration of saline (PBS), 5×10^8 naked VSV virions, or 10^6 VSV-infected carrier cells (three doses/week over 4 weeks) in mice with pre-existing antiviral immunity.

© The American Society of Gene Therapy

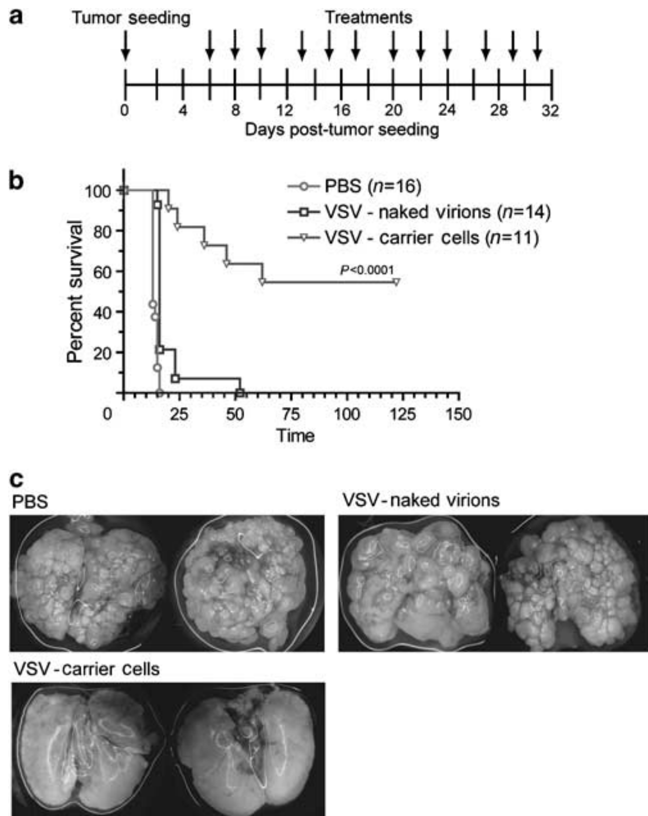

Figure 6 Enhancement of systemic therapy with VSV delivered in carrier cells. Tumors were seeded in balc/c mice at day 0 by IV injection of 3×10^5 CT26 cells. Beginning at day 6, mice were treated IV with saline (PBS), 5×10^8 PFU VSV as naked virions, or 10^6 VSV-infected syngeneic carrier cells. Animals were dosed 3 days/week for 4 weeks as shown in (**a**) or until reaching tumor burden end points as plotted in (**b**). Survival of mice receiving carrier cells was significantly different to that of PBS-treated controls by log-rank test with the indicated *P*-value. (**c**) Representative photographs of lung tumor burdens at experimental end points for each treatment group.

sufficient to ablate delivery of naked virions to tumors. However, infected 'trojan horse' cells conceal VSV from the host immune system to bypass this obstacle. Many oncolytic viruses are potent antitumor agents when directly injected into tumors,[24–27] but vulnerability to host defenses complicates their administration as systemic therapeutics. Serum factors such as complement proteins and antibodies can bind to and inactivate virus,[2,6–9,28] whereas organ sinks such as the liver can remove it from the circulation altogether.[4,5] Excessive uptake of virus by the liver can also be responsible for toxic side effects following systemic administration.[29] The use of carrier cells to shield virions from systemic defenses thus offers a simple solution to the key problems associated with delivering VSV and other viral therapeutics to disseminated tumor sites.

We have shown that multiple doses of VSV administered systemically within carrier cells can induce complete disease regression in a lung model (**Figure 6b** and **c**), whereas a single dose cannot (data not shown). In contrast, even multiple doses of IV-injected naked VSV virions were largely ineffective in this model (**Figure 6b** and **c**). These findings indicate that stealth delivery contributes to the overall efficacy of multiple dosing with VSV-laden carrier cells administered in the face of an evolving antiviral immune response. It will be important in future studies to determine whether an immune response is mounted against the carrier cell vehicle itself, and whether this has any effect on virus delivery. On the other hand, the immunogenicity of carrier cells may enhance therapy, as the activation of antitumor immunity during virotherapy appears to contribute some degree to eliminating tumors and may help to protect from disease recurrence. Indeed, herpes simplex virus[30] and VSV (data not shown) appear to invoke cell-based antitumor responses in addition to killing malignant cells by direct infection and oncolysis. In a similar manner, tumor cells infected with Newcastle disease virus and injected into tumor-bearing animals have also been reported to activate antitumor immunity.[31] Thus, the destruction of tumors by oncolytic viruses, whether administered as naked virions or within infected cells, is likely achieved through multiple mechanisms. Significant effort is now being made in our lab to understand how these mechanisms contribute to the overall potency of oncolytic virus therapy with these different delivery approaches.

The possibility of seeding *de novo* tumor growth is an important safety consideration if transformed cells are to be used as systemic therapeutics. To ensure that cells do not persist to form tumors, Coukos *et al.*[16] have shown that herpes simplex virus-infected carriers can be lethally irradiated before administration. Similarly, we have observed that the production of VSV is unimpaired by irradiation of infected cells (data not shown), and are currently investigating whether they retain the ability to deliver virus *in vivo*. Alternatively, allogeneic or xenogeneic carrier cells such as those described here would be eliminated by the patient's own immune system following virus delivery, avoiding the need for any special manipulation of carrier cells before administration. Inducible suicide programs could also be engineered into carrier cell lines to ensure that they do not persist following injection into the body. Combining these types of safeguards should ensure the safety of transformed cells lines for clinical administration, a tremendous advance over the use of autologous tumor[15,16] or normal cells[17,18,20] as the former are renewable, rapidly propagated to large quantities, and generally more permissive to oncolytic virus infection.

Previous studies have clearly demonstrated efficient delivery of therapeutic viruses to lung tumors,[15,32] and we have shown here that certain cell types, particularly those derived from solid tumors, accumulate rapidly and exclusively within the lungs, the site of the first microcapillary bed encountered following IV injection. Although such a vehicle could be useful for delivering virus specifically to the tumor site in patients with primary or metastatic disease localized to the lung, it is clear that carrier cells must be capable of bypassing lung capillaries to reach metastases found at other anatomical locations. Blood cells normally traffic throughout the circulation and our studies suggest that they may be the ideal vehicle for delivering virus to disseminated sites. We found that a leukemia cell line was able to largely bypass the lungs and could deliver VSV to more distally located tumors. Similarly, previous studies have established that leukocyte-derived cytokine-induced killer (CIK) cells can deliver oncolytic vaccinia virus to hind flank tumors upon IV administration.[18]

The small size of leukocyte-derived carriers is likely a crucial parameter that facilitates their passage through blood vessels to achieve this widespread dissemination, although other factors such as structural rigidity,[33] chemotactic responses,[34] and adhesion molecule expression[35] are also known to influence blood cell circulation.

A live-cell carrier for therapeutic delivery is an attractive alternative to the various chemicals that can be used to encapsulate virions.[36–41] Infected-cell carriers have the unique ability to produce a burst of virus upon delivery to the tumor site, thereby amplifying the effective therapeutic dose by several orders of magnitude. In addition, the cell can be engineered to have limited or no innate antiviral response, thus increasing the output of therapeutic virus at the target site. Carrier cells could be made to express tumor antigens on their surface in the ideal context to stimulate immunity or perhaps surface molecules to facilitate retention in tumor beds. Our results suggest that an immortalized cell line of hematological origin could be an ideal foundation for constructing custom-built biotherapeutic vehicles, with the potential to significantly enhance the delivery, selectivity, and antitumor efficacy of systemic virotherapy.

MATERIALS AND METHODS

Viruses. The recombinant AV3 strain of VSV with an attenuating deletion of methionine 51 of the matrix protein and a transgene encoding enhanced GFP,[10] RFP, or enhanced GFP-luciferase (see below) were propagated in Vero cells. Virions were purified from cell culture supernatants by passage through a 0.2 μm Steritop filter (Millipore, Billerica, MA) and centrifugation at $30\,000 \times g$ before resuspension in phosphate-buffered saline (PBS) (Hyclone, Logan, UT) for all animal studies.

Construction and rescue of recombinant VSV strains. Two additional recombinant VSV genomes were generated by insertion of either mRFP1[42] or EGFPLuc (Clontech Laboratories, Mountain View, CA) reporter genes and rescued as described previously.[10] Rescued viruses were propagated and purified as above.

Lentiviral transductions. A bicistronic lentivector was rescued by insertion of the *Renilla reniformis* luciferase gene (pGL3 R2.1, Promega, Madison, WI) into the *Pme*I restriction site downstream from the encephalomyocarditis virus internal ribosome entry site-driven GFP marker in pWPI and used to transduce target cell lines as described elsewhere.[43] Fluorescence-activated cell sorting based on GFP expression yielded a population consisting of $>90\%$ transduced cells.

Cell lines. Murine CT26 colon carcinoma, human A549 lung carcinoma, and L1210 murine leukemia cell lines (American Type Tissue Collection) were propagated in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal calf serum (Cansera, Etobicoke, Ontario, Canada).

Serum-neutralizing antibody assay. Two-fold serial dilutions of mouse serum were incubated with 300 PFU of VSV in a total volume of 60 μl for 90 min at 37°C and applied to Vero cell monolayers in a 96-well plate. Wells were examined for cytopathic effects 48 h post-inoculation. Neutralizing titer was taken as the highest dilution factor of serum that prevented the appearance of cytopathic effects.

Passive immunizations. Serum was collected from donor mice 6 weeks following IV immunization with 5×10^8 PFU of VSV. Recipients were injected intraperitoneally with 100 μl immune serum 24 h before therapeutic administration of VSV.

T-cell purification and adoptive transfer. Single-cell suspensions of splenocytes from donor mice were purified on a magnetic-activated cell sorter column using a Pan T-Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) to obtain an enriched population of untouched T cells. Each recipient was injected IV with a single spleen equivalent of purified T cells 24 h before treatment with VSV.

Mice and tumor models. Female, 8- to 10-week-old balb/c mice were obtained from Charles River Laboratories (Wilmington, MA), and injected IV with 3×10^5 CT26 cells to establish lung tumors, or injected subcutaneously with 10^6 CT26 cells to establish hind flank tumors. Animals bearing lung tumors were killed upon signs of severe respiratory distress. All experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Service.

In vivo *imaging.* Mice were injected with either native coelenterazine (NanoLight Technology, Pinetop, AZ) (40 μl IV at 1 mg/ml in 50% v/v methanol/PBS) to image *Renilla* luciferase activity, or *d*-luciferin (Molecular Imaging Products Company, Ann Arbor, MI) (200 μl intraperitoneally at 10 mg/ml in PBS) for *Firefly* luciferase imaging. Mice were anesthesized under 3% isofluorane (Baxter Corp., Deerfield, IL) and imaged with the *in vivo* imaging system 200 Series Imaging System (Xenogen Corporation, Hopkinton, MA). Data acquistion and analysis was performed using Living Image v2.5 software. For each experiment, images were captured under identical exposure, aperture and pixel binning settings, and bioluminescence is plotted on identical color scales.

Infection of carrier cells. Cells were infected at a multiplicity of infection of 10 at 37°C for 2.5 h, harvested (adherent lines were detached with 0.05% Trypsin-EDTA), washed to remove cell-free virions, and resuspended at a concentration of 10^7 cells/ml in PBS before IV tail-vein injection into mice at 3 h post-infection (10^6 cells/mouse in 100 μl). For fluorescent imaging experiments, cells were stained with 5 μm CFSE (Molecular Probes/Invitrogen, Carlsbad, CA) for 30 min before infection following the manufacturer's protocol.

Fluorescent imaging. Mice were killed and tumors were examined using a Leica MZFLIII dissecting microscope with a standard GFP filter set. Images were captured with a Nikon Coolpix 100 camera. Overlays were generated using Adobe Photoshop CS v8.0 software.

Western blot detection of VSV proteins. Cell lysates were collected in 4% sodium dodecyl sulfate sample buffer, run on a NuPAGE Bis-Tris 4–12% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with polyclonal anti-VSV serum from hyperimmune rabbits.

Quantitation of VSV infection in tumors. Tumors were excised from killed mice and homogenized in PBS. The extent of VSV infection was measured by either plaque assay on Vero cells or luciferase assay (Promega) read on a luminometer (EG&G Berthold, Oak Ridge, TN, Lumat LB9507).

ACKNOWLEDGMENTS

This work was supported by a Terry Fox Program Project Grant through the National Cancer Institute of Canada and the Ontario Cancer Research Network. ATP was supported by an Ontario Graduate

Scholarship in Science and Technology and the Ottawa Health Research Institute. JW was supported by a Lymphoma Research Foundation of Canada Research Fellowship.

REFERENCES

1. Parato, KA, Senger, D, Forsyth, PA and Bell, JC (2005). Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* **5**: 965–976.
2. Ikeda, K *et al.* (1999). Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses. *Nat Med* **5**: 881–887.
3. Wakimoto, H *et al.* (2002). The complement response against an oncolytic virus is species-specific in its activation pathways. *Mol Ther* **5**: 275–282.
4. Worgall, S, Wolff, G, Falck-Pedersen, E and Crystal, RG (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. *Hum Gene Ther* **8**: 37–44.
5. Ye, X, Jerebtsova, M and Ray, PE (2000). Liver bypass significantly increases the transduction efficiency of recombinant adenoviral vectors in the lung, intestine, and kidney. *Hum Gene Ther* **11**: 621–627.
6. Hirasawa, K *et al.* (2003). Systemic reovirus therapy of metastatic cancer in immune-competent mice. *Cancer Res* **63**: 348–353.
7. Lang, SI, Giese, NA, Rommelaere, J, Dinsart, C and Cornelis, JJ (2006). Humoral immune responses against minute virus of mice vectors. *J Gene Med* **12**: 12–1222.
8. Chen, Y, Yu, DC, Charlton, D and Henderson, DR (2000). Pre-existent adenovirus antibody inhibits systemic toxicity and antitumor activity of CN706 in the nude mouse LNCaP xenograft model: implications and proposals for human therapy. *Hum Gene Ther* **11**: 1553–1567.
9. Tsai, V *et al.* (2004). Impact of human neutralizing antibodies on antitumor efficacy of an oncolytic adenovirus in a murine model. *Clin Cancer Res* **10**: 7199–7206.
10. Stojdl, DF *et al.* (2003). VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **4**: 263–275.
11. Ebert, O, Harbaran, S, Shinozaki, K and Woo, SL (2005). Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice. *Cancer Gene Ther* **12**: 350–358.
12. Ahmed, M, Cramer, SD and Lyles, DS (2004). Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses. *Virology* **330**: 34–49.
13. Obuchi, M, Fernandez, M and Barber, GN (2003). Development of recombinant vesicular stomatitis viruses that exploit defects in host defense to augment specific oncolytic activity. *J Virol* **77**: 8843–8856.
14. Gobet, R, Cerny, A, Ruedi, E, Hengartner, H and Zinkernagel, RM (1988). The role of antibodies in natural and acquired resistance of mice to vesicular stomatitis virus. *Exp Cell Biol* **56**: 175–180.
15. Garcia-Castro, J *et al.* (2005). Tumor cells as cellular vehicles to deliver gene therapies to metastatic tumors. *Cancer Gene Ther* **12**: 341–349.
16. Coukos, G *et al.* (1999). Use of carrier cells to deliver a replication-selective herpes simplex virus-1 mutant for the intraperitoneal therapy of epithelial ovarian cancer. *Clin Cancer Res* **5**: 1523–1537.
17. Komarova, S, Kawakami, Y, Stoff-Khalili, MA, Curiel, DT and Pereboeva, L (2006). Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther* **5**: 755–766.
18. Thorne, SH, Negrin, RS and Contag, CH (2006). Synergistic antitumor effects of immune cell-viral biotherapy. *Science* **311**: 1780–1784.
19. Raykov, Z, Balboni, G, Aprahamian, M and Rommelaere, J (2004). Carrier cell-mediated delivery of oncolytic parvoviruses for targeting metastases. *Int J Cancer* **109**: 742–749.
20. Jevremovic, D *et al.* (2004). Use of blood outgrowth endothelial cells as virus-producing vectors for gene delivery to tumors. *Am J Physiol Heart Circ Physiol* **287**: H494–H500.
21. Crittenden, M *et al.* (2003). Pharmacologically regulated production of targeted retrovirus from T cells for systemic antitumor gene therapy. *Cancer Res* **63**: 3173–3180.
22. Raykov, Z, Balboni, G, Aprahamian, M and Rommelaere, J (2004). Carrier cell-mediated delivery of oncolytic parvoviruses for targeting metastases. *Int J Cancer* **109**: 742–749.
23. Harrington, K *et al.* (2002). Cells as vehicles for cancer gene therapy: the missing link between targeted vectors and systemic delivery? *Hum Gene Ther* **13**: 1263–1280.
24. Mineta, T, Rabkin, SD, Yazaki, T, Hunter, WD and Martuza, RL (1995). Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat Med* **1**: 938–943.
25. Bischoff, JR *et al.* (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **274**: 373–376.
26. Coffey, MC, Strong, JE, Forsyth, PA and Lee, PW (1998). Reovirus therapy of tumors with activated Ras pathway. *Science* **282**: 1332–1334.
27. Grote, D *et al.* (2001). Live attenuated measles virus induces regression of human lymphoma xenografts in immunodeficient mice. *Blood* **97**: 3746–3754.
28. Wakimoto, H *et al.* (2002). The complement response against an oncolytic virus is species-specific in its activation pathways. *Mol Ther* **5**: 275–282.
29. Schiedner, G *et al.* (2003). A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells. *Hum Gene Ther* **14**: 1631–1641.
30. Hummel, JL, Safroneeva, E and Mossman, KL (2005). The role of ICP0-null HSV-1 and interferon signaling defects in the effective treatment of breast adenocarcinoma. *Mol Ther* **12**: 1101–1110.
31. Plaksin, D, Porgador, A, Vadai, E, Feldman, M, Schirrmacher, V and Eisenbach, L (1994). Effective anti-metastatic melanoma vaccination with tumor cells transfected with MHC genes and/or infected with Newcastle disease virus (NDV). *Int J Cancer* **59**: 796–801.
32. Cole, C *et al.* (2005). Tumor-targeted, systemic delivery of therapeutic viral vectors using hitchhiking on antigen-specific T cells. *Nat Med* **11**: 1073–1081.
33. Skalak, R and Branemark, PI (1969). Deformation of red blood cells in capillaries. *Science* **164**: 717–719.
34. Stein, JV and Nombela-Arrieta, C (2005). Chemokine control of lymphocyte trafficking: a general overview. *Immunology* **116**: 1–12.
35. Carlos, TM and Harlan, JM (1994). Leukocyte-endothelial adhesion molecules. *Blood* **84**: 2068–2101.
36. Fisher, KD *et al.* (2001). Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Therapy* **8**: 341–348.
37. O'Riordan, CR *et al.* (1999). PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum Gene Ther* **10**: 1349–1358.
38. Chillon, M, Lee, JH, Fasbender, A and Welsh, MJ (1998). Adenovirus complexed with polyethylene glycol and cationic lipid is shielded from neutralizing antibodies *in vitro*. *Gene Therapy* **5**: 995–1002.
39. Matthews, C, Jenkins, G, Hilfinger, J and Davidson, B (1999). Poly-L-lysine improves gene transfer with adenovirus formulated in PLGA microspheres. *Gene Therapy* **6**: 1558–1564.
40. Pearce, OM, Fisher, KD, Humphries, J, Seymour, LW, Smith, A and Davis, BG (2005). Glycoviruses: chemical glycosylation retargets adenoviral gene transfer. *Angew Chem Int Ed Engl* **44**: 1057–1061.
41. Green, NK *et al.* (2004). Extended plasma circulation time and decreased toxicity of polymer-coated adenovirus. *Gene Therapy* **11**: 1256–1263.
42. Campbell, RE *et al.* (2002). A monomeric red fluorescent protein. *Proc Natl Acad Sci USA* **99**: 7877–7882.
43. Naldini, L *et al.* (1996). *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**: 263–267.