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Advances in Brief

Intravesical Gene Therapy: *In Vivo* Gene Transfer Using Recombinant Vaccinia Virus Vectors[1]

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Abstract

Intratumoral gene transfer may be a significant tool in active immunotherapy. The ability to insert functional genes into a tumor *in vitro* and *in vivo* using recombinant vaccinia vectors was examined in the murine bladder tumor model. Vaccinia recombinants expressing the influenza hemagglutinin or nucleoprotein antigens infected/transfected murine (MB-49 and MBT-2) and human (T24) bladder tumor cell lines *in vitro*. Systemic vaccinia immunity was induced with as few as 10 plaque-forming units of recombinant vaccinia instilled intravesically, and the encoded protein was expressed *in vivo* in tumor and urothelium. However, preimmunity to vaccinia did not inhibit intravesical tumor transfection. Thus, recombinant vaccinia virus is effective in introducing foreign antigens locally into tumor *in vivo*, supporting its use in clinical immunotherapy.

Introduction

Efficient intratumoral foreign gene delivery/transfer would be a significant tool for stimulating host immune responses by potentially altering tumor immunogenicity. *In vitro* transduced cytokine and viral genes expressed by tumors have resulted in the elimination of transfected tumors and enhanced T-cell-mediated immunity to nontransduced tumor (1). *In vivo* gene delivery would obviate the need for *in vitro* manipulations of tumor cells and enhance the clinical applicability of this therapeutic approach. Viral expression vectors could be used to introduce a specific gene locally into the tumor site and thus modify the tumor into a more immunogenic form (2). One such method for foreign gene transfer *in vivo* is the direct administration of recombinant vaccinia virus into tumor tissues. Vaccinia virus, a double-stranded DNA poxvirus, has been well characterized since its successful use as a live vaccine to prevent smallpox. As a versatile eukaryotic expression vector, vaccinia virus can be genetically constructed to contain large fragments of foreign DNA (up to M_r 25,000) which have no effect on viral replication (2). Immunization with recombinant vaccinia can induce protective responses to the foreign gene(s) expressed (3). Previous studies have shown that percutaneous immunization with viral expression vectors containing foreign-encoded antigens can be used to enhance antitumor responses *in vivo* as measured by reduced tumorigenicity and the induction of protective host immune responses (4, 5). The purpose of this study was to determine if vaccinia virus recombinants could be administered intravesically to efficiently transfect bladder tumor cells. In an intravesically growing murine bladder tumor model, we have demonstrated *in vivo* vaccinia infectability as measured by the induction of systemic immunity to vaccinia virus antigens following intravesical instillation. Moreover, we show that vaccinia recombinants infect/transfect bladder tumor cell lines *in vitro* and intravesically established bladder tumor *in vivo*. Finally, we demonstrate that prior immunity to vaccinia, expected in preimmune mice, does not inhibit *in vivo* tumor transfection by recombinant vaccinia.

Materials and Methods

Mice. C57Bl/6 females, 4–6 weeks old, were purchased from The Jackson Laboratory, Bar Harbor, ME, and maintained at our facility for at least 2 weeks prior to use.

Tumor. TCC[3] cell lines MB-49 of C57Bl origin (obtained from Dr. Ratliff, Washington University, St. Louis, MO; Ref. 6), MBT-2 of C3H origin (obtained from Dr. W. Heston, Sloan-Kettering Memorial Hospital, New York, NY), and the human T24 bladder carcinoma (American Type Culture Collection, Rockville, MD) were carried *in vitro* in our laboratory.

Virus. Recombinant vaccinia viruses H1-VAC and NP-VAC expressing the hemagglutinin (H1) and nucleoprotein (NP) genes derived from influenza virus A/PR8/34 (7) were the kind gift of Dr. B. Moss (NIADID, Bethesda, MD). Expression of both influenza polypeptides is under the control of the early/late 7.5-kilobase promoter. Viral stocks quantitated in pfu were maintained in buffered salt solution/bovine serum albumin at −70°C until use.

Antibodies, Reagents, and Staining. Supernatants from hybridoma cell lines specific for the influenza A hemagglutinin (H28-E23; Ref. 8) and nucleoprotein antigens (HB65; Ref. 9) were used to stain cells and tissues. The virus-infected bladder tumor cells and bladder urothelium sections were fixed with cold acetone and blocked with 0.1% fetal calf serum. HA and NP were detected with: (*a*) biotinylated primary mouse antibody plus avidin-HRP and 3′3′ diaminobenzidine substrate (Sigma, St. Louis, MO); or (*b*) avidin-biotin-complex method plus alkaline phosphatase with Fast red substrate (Vector Laboratories, Inc., Burlingame, CA). Tissue sections were counterstained with hematoxylin. In addition, H&E-stained sections were prepared.

Viral Infection and Transfection. For *in vitro* analysis, 2×10^6 cells of each cell line to be tested were plated into a 24-well flat-bottomed plate (Fisher, Pittsburgh, PA). Plates were incubated overnight, washed with PBS, and infected with H1-VAC or NP-VAC (10 pfu/cell) in buffered salt solution/bovine serum albumin by incubating at 37°C in 9% CO_2 for 1 h with rocking every 15 min. Virus was aspirated, media was added, and the plate was incubated for another 4 h. The cells were fixed with 1:1 acetone:methanol for 1 min and washed with PBS before immunohistochemical staining. Uninfected and recombinant virus-infected L929 fibroblasts, which are known to be susceptible to vaccinia virus infection, were used as a negative and positive control, respectively. For *in vivo* analysis, the experimental mice were intravesically instilled with recombinant virus as described for BCG treatment (10). Mice were anesthetized, catheterized via the urethra, then cauterized with a cautery wire (Birtcher Hyfricator, El Monte, CA) by applying a single 1-s pulse at 1 watt. After removal of the cautery wire, the bladders were instilled with: (*a*) 10^4 MB49 cells to establish intravesical growth of tumor; or (*b*) the indicated pfu concentration of vaccinia recombinants in PBS. At the indicated time points, mice were sacrificed, and bladders were removed and frozen in OCT media (Fisher) in liquid nitrogen. Bladder samples were stored at −70°C

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[3] The abbreviations used are: TCC, transitional cell carcinoma; pfu, plaque-forming unit(s); VAC, vaccinia virus; HA, hemagglutinin antigen; NP, nucleoprotein; HRP, horseradish peroxidase; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline; BCG, *Bacillus Calmette-Guérin*; CTL, cytotoxic T-lymphocyte.

until sectioned. For preimmunization studies, mice were immunized by injecting 10^7 pfu of wild-type vaccinia (WR strain) i.p. After 1 week, 10^4 MB49 bladder tumor cells were instilled into the bladder and allowed to grow for 2 weeks before being infected with 2×10^6 pfu of NP-VAC intravesically.

CTL Analysis. CTL responses to intravesical infection by vaccinia recombinants was determined by a 4-h ^{51}Cr assay (11). Spleens of virus-infected mice were isolated at 2 weeks postintravesical instillation, restimulated *in vitro* with live virus infected syngeneic spleen stimulators (3:1), and cultured for 7 days at 37°C in 5% CO_2. The responder cells were assayed for cytoxicity on ^{51}Cr-labeled vaccinia virus-infected MB49 tumor targets at effector:target ratios indicated. Percentage of specific lysis was calculated as:

$$\% \text{ specific lysis} = \frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Results

Intravesical Vaccinia Infects/Transfects Normal Urothelium and Induces Systemic Immunity. To determine if intravesical infection resulted in the generation of a systemic immune response to virus as defined by the generation of vaccinia-specific CTLs, spleens of intravesically infected C57BL/6 mice were tested for antigen-specific killing of vaccinia virus-infected MB-49 bladder tumor target cells in 4-h chromium release assays. No virus-induced target lysis was seen in the 4-h assay, and virus-specific CTL did not lyse uninfected targets (data not shown). As shown in Fig. 1, concentrations as low as 10 pfu intravesically were sufficient to induce a systemic antivaccinia CTL response. When the dose of intravesical vaccinia was titrated, concentrations of greater than 10^5 pfu per mouse were lethal to nonimmunized mice, which died within 5–6 days postinstillation. In contrast, mice receiving a single intravesical concentration of less than 10^5 pfu appeared normal and survived greater than 2 weeks postinstillation. Mice made preimmune with an i.p. injection of wild-type WR vaccinia virus (10^7 pfu) demonstrated no morbidity at intravesical concentrations as high as 2×10^6 pfu of vaccinia recombinants per mouse (data not shown).

To confirm infection of the urothelium, C57BL/6 female mice were given a single intravesical instillation with vaccinia recombinant H1-VAC or NP-VAC (10^4 pfu). At the indicated time points postinstillation, the mice were sacrificed, and their bladders were recovered for sectioning and staining. Histological analysis by H&E staining demonstrated that urothelial cells lining the bladder lumen were virus-infected as indicated by characteristic morphological changes including cell enlargement and nuclear and cytoplasmic vacuolization, as well as atypical chromatin pattern (Fig. 2).

Vaccinia Recombinants Transfect TCC *in Vitro* to Express Encoded Antigens. To determine if vaccinia recombinants could infect and transfect TCC cells, the murine MBT-2 and MB-49 TCC cells were infected *in vitro* with H1-VAC (see "Materials and Methods"). When compared to uninfected tumor cells, immunohistochemical staining with specific antibodies showed positive expression for encoded HA or NP antigens, indicated by the cytoplasmic staining of virus-infected TCC cells (Fig. 3). In addition, the human bladder tumor cell line T24 was similarly infected *in vitro* (data not shown). Thus, both murine and human bladder tumor cells can be infected/transfected by vaccinia recombinants demonstrating susceptibility to the virus and supporting their use in *in vivo* studies.

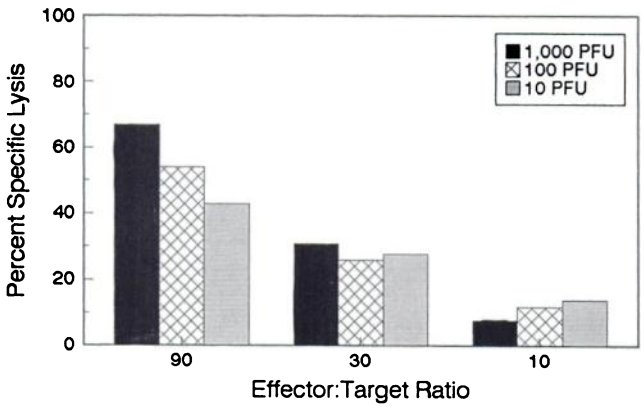

Fig. 1. Intravesical instillation of vaccinia virus results in systemic immunity. Mice received intravesical instillation of VAC at the indicated doses. Two weeks later, mice spleen cells were removed and tested for their ability to lyse VAC-infected MB-49 cells as described in "Materials and Methods."

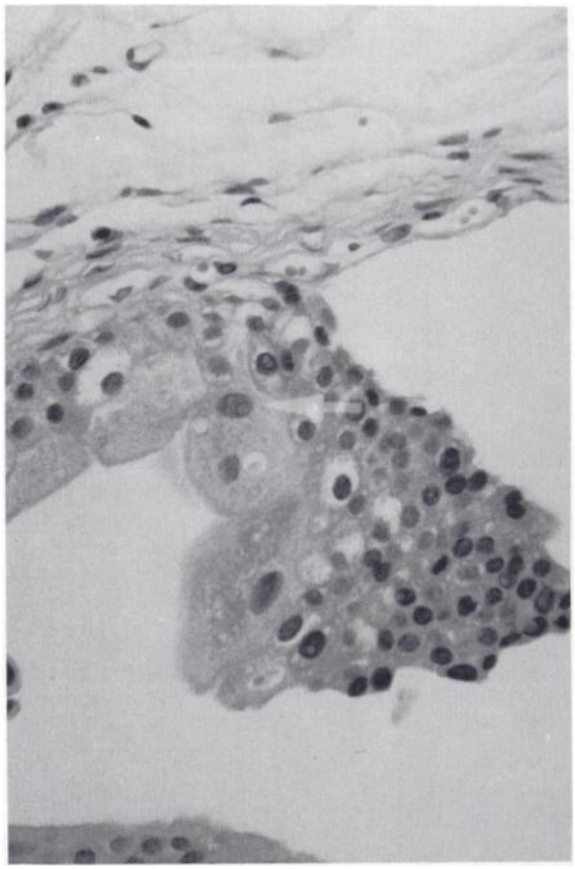

Fig. 2. Infection of normal urothelium by intravesically administered H1-VAC. Sections of infected urothelium stained by H&E demonstrate characteristic morphological changes (*arrow*). × 200.

Vaccinia Recombinant NP-VAC-infected Intravesically Established Bladder Tumor in Vaccinia Immune Mice. To determine whether vaccinia recombinants could also infect established tumor *in vivo*, a requirement for any clinical use of the virus, NP-VAC (10^4 pfu) was instilled intravesically into mice that had been implanted with syngeneic MB-49 tumor. Mice, preimmunized i.p. with wild-type WR vaccinia (10^7 pfu), were implanted intravesically with MB-49 tumor cells. Two weeks following tumor development, a single intravesical instillation of NP-VAC (2×10^6 pfu, shown not to have systemic toxicity in preimmune mice) was given. At 8 and 22 h postinstillation, bladders were removed, sectioned, and stained. As shown in Fig. 4, *in vivo* expression of encoded NP was demonstrated at 22 h after instillation. Similar results were seen at the 8-h time point (data not shown). Thus, significant infection/transfection of established tumor is possible following intravesical administration, and systemic immunity to vaccinia does not inhibit tumor transfection of intravesically instilled vaccinia recombinants.

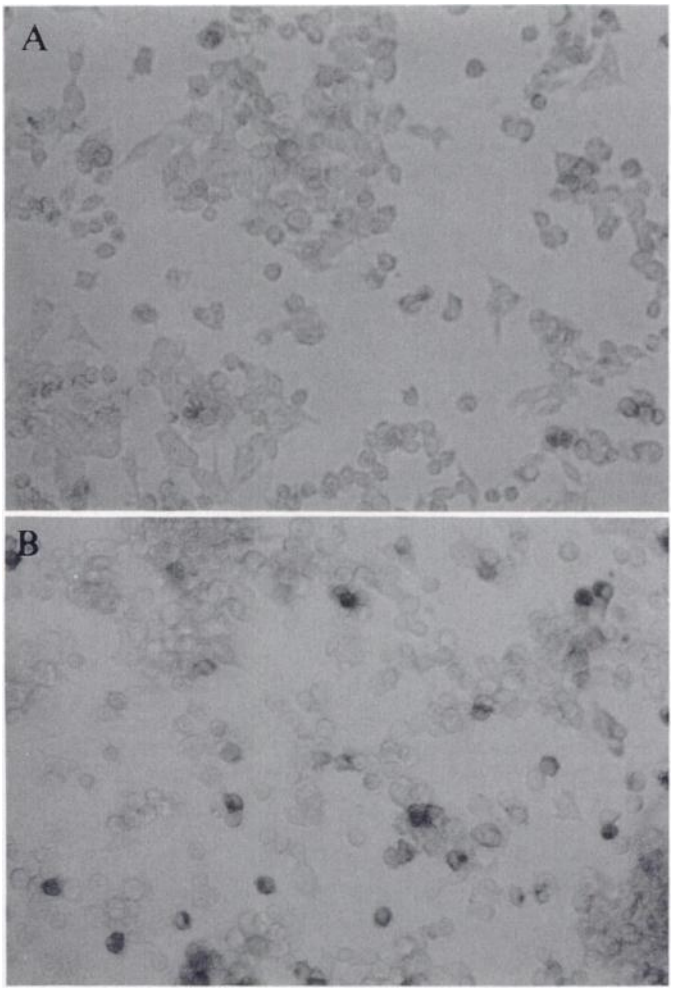

Fig. 3. H1-VAC infection of the MB-49 TCC *in vitro* results in expression of the HA antigen. Five-h H1-VAC-infected MB-49 cells were stained with avidin-HRP alone (*A*) or biotinylated anti-HA (H28-E23) plus avidin-HRP (*B*). × 100.

Discussion

This study describes a novel application of a viral expression vector for local delivery of foreign DNA in a syngeneic, murine bladder tumor model. Using vaccinia virus recombinants, we show that intravesical instillation with as few as 10 pfu induced systemic immunity. Vaccinia recombinants infect/transfect murine TCC cells *in vitro* and normal urothelium and established bladder tumor *in vivo*, as determined by expression of the encoded gene products. In addition, we show that vaccinia recombinant NP-VAC can infect intravesically established bladder tumor in vaccinia immune mice with detectable NP expression at 8–22 h postinstillation (see Fig. 4), supporting the utility of this approach in subjects immunized to vaccinia.

Localized therapy has been successful in human bladder carcinoma and melanoma with intralesional BCG (12, 13). We and others have reported significant infiltration with T-lymphocytes in human and murine superficial bladder tumor lesions treated with intravesical BCG (14). In addition to T-cell recruitment, we have shown induction of T-cell-derived lymphokines in the bladder and tumor sites with mRNA expression of γ-interferon and tumor necrosis factor present following intravesical BCG (15). Collectively, these data lend support to developing strategies involving approaches to localized therapy designed to engender tumor-specific immunity.

Introduction of foreign genes into tumor *in vitro* has been effective in some cases at enhancing subsequent *in vivo* tumor regression. Genetic modification of various tumors to express viral gene products (16) and cytokine genes (17) has been effective in enhancing tumor immunogenicity, inhibiting tumor growth, and, in a minority of studies, inducing host immunity. Of particular interest are studies using cytokine-modified tumor cells as vaccines. Administration of transduced tumor cells, including bladder tumors, expressing interleukin 2 (17), interleukin 4(1), γ-interferon (18), and granulocyte-macrophage colony-stimulating factor (19) has been demonstrated in different tumor models to induce local and, in limited cases, systemic immunity, including elimination of preexisiting tumor. Different host effector cells have been shown to mediate antitumor responses, depending on the cytokine expressed. These data support the use of cytokine gene transfer into tumor for immunotherapy. However, *in vitro* manipulations of tumor to express cytokine has its limitations, particularly in the clinical setting. Genetic modification of tumors for cellular vaccines is dependent upon and limited by the ability to resect and to grow each patient tumor *in vitro* and to reinject viable, modified tumor. In hopes of bypassing the need for *in vitro* tumor manipulation, we have examined ways in which similar genes could be introduced *in vivo*, avoiding these potential limitations.

In vivo delivery and expression of recombinant genes for gene therapy without great cost and technical difficulty can be achieved by using vaccinia recombinants. Unlike *in vitro* methods of gene transfer,

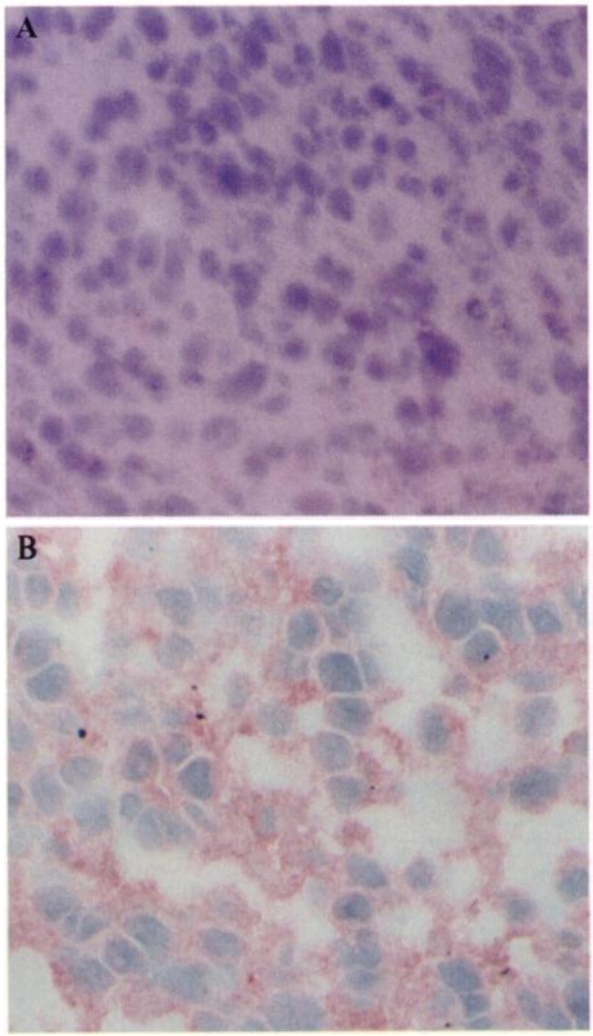

Fig. 4. Systemic immunity to vaccinia does not inhibit *in vivo* infection/transfection. Established MB-49 tumor in vaccinia immune mice was infected/transfected using NP-VAC following intravesical instillation. *A*, second antibody control; *B*, anti-NP (HB65) plus second antibody. × 100.

infection and transfection using recombinant vaccinia is a simple, rapid, and highly efficient procedure (Ref. 20 and our studies presented here). Important for CTL induction, vaccinia recombinants can efficiently deliver antigens to the class I presentation pathway and have been proposed as feasible vectors for expressing protective antigens for vaccine delivery (2). As an added advantage, the vaccinia genome can accept large fragments of foreign DNA (< M_r 25,000) and thus express (multiple) recombinant antigens (reviewed in Ref. 2). Based upon its ability to infect and induce expression of recombinant proteins in a wide range of host cells, viral vectors could stimulate the immune system by expressing immunogenic antigens or inducing cytokine production at the tumor site. In addition, tumor cell lysis by the cytopathic virus may lead to enhanced antigen processing and presentation by phagocytic antigen-presenting cells. In this fashion, local introduction of genes into tumor can serve to trigger host antitumor effectors by modulating the immunogenicity of the existing superficial bladder cancer. This direct delivery approach for gene transfer can be applied to other tumors accessible to intratumoral injection. We have also determined that human melanoma cells are able to be transfected by vaccinia recombinants *in vitro* (data not shown). There is also the potential for inserting into a tumor cell any immunogenic protein to enhance host immunity.

Potential problems in using vaccinia recombinants *in vivo* include the risk of systemic infection and/or the inhibition of vaccinia infection/transfection in immune hosts. This latter concern exists since: (*a*) multiple injections may be necessary for optimal treatment; and (*b*) preimmunity to vaccinia is common in the clinical population previously immunized for smallpox. In addressing this possibility, we demonstrated intravesical transfection of established tumors with vaccinia recombinants in virus-immune mice. In addition, our findings showed that higher, local doses of vaccinia recombinants could be administered intravesically, despite preimmunization with vaccinia. To our benefit, vaccinia immunity would facilitate resolution of systemic virus infection and allow transient, local expression of recombinant protein. While normal cells were also infected/transfected, approaches aimed at transfecting cytokine genes at the tumor site would not be negatively affected by such activity. Transfected normal urothelium would, in the latter case, add to the local production of the desired cytokine and thus contribute to the favorably modified immune milieu at the tumor site.

In summary, we have demonstrated *in vivo* infectability of vaccinia recombinants by showing that instillation of recombinant vaccinia into the bladder can induce virus-specific systemic immunity and infect/transfect urothelium and tumor. In support of this finding, we demonstrate virus susceptibility of murine bladder tumor by immunohistochemical staining for encoded recombinant gene products of virus infected/transfected bladder tumor cells lines *in vitro* and established bladder tumor *in vivo*. Preimmunity to vaccinia, expected in the clinical population, was found not to inhibit *in vivo* tumor transfection of established tumor by vaccinia recombinants. These data suggest that the use of vaccinia virus recombinants may be an effective and therapeutically important means of introducing genes into tumor following local administration.

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