Preclinical Evaluation of “Whole” Cell Vaccines for Prophylaxis and Therapy Using a Disabled Infectious Single Cycle-Herpes Simplex Virus Vector to Transduce Cytokine Genes


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ABSTRACT

The development of genetically modified “whole” tumor cell vaccines for cancer therapy relies on the efficient transduction and expression of genes by vectors. In the present study, we have used a disabled infectious single cycle-herpes simplex virus 2 (DISC-HSV-2) vector constructed to express cytokine or marker genes upon infection. DISC-HSV-2 is able to infect a wide range of tumor cells and efficiently express the β-galactosidase reporter gene, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-2 genes. Gene expression occurred rapidly after infection of tumor cells, and the level of production of the gene product (β-galactosidase, GM-CSF, or IL-2) was shown to be both time- and dose-dependent. Vaccination with irradiated DISC-mGM-CSF or DISC-hIL-2-infected murine tumor cells resulted in greatly enhanced immunity to tumor challenge with live parental tumor cells compared with control vaccines. When used therapeutically to treat existing tumors, vaccination with irradiated DISC-mGM-CSF-infected tumor cells significantly reduced the incidence and growth rates of tumors when administered locally adjacent to the tumor site, providing up to 90% protection. The prophylactic and therapeutic efficacy of DISC-mGM-CSF-infected cells was shown initially using a murine renal cell carcinoma model (RENCA), and the results were confirmed in two additional murine tumor models: the M3 melanoma and 320R sarcoma. Therapy with DISC-infected RENCA “whole” cell vaccines failed to reduce the incidence or growth of tumor in congenitally T-cell deficient (nu/nu) mice or mice depleted of CD4+ and/or CD8+ T-lymphocytes, confirming that both T-helper and T-cytotoxic effector arms of the immune response are required to promote tumor rejection. These preclinical results suggest that this “novel” DISC-HSV vector may prove to be efficacious in developing genetically modified whole-cell vaccines for clinical use.

INTRODUCTION

Cancer vaccination strategies have focused on the use of autologous and allogeneic tumor cells genetically modified to express a range of different immunomodulatory genes which include cytokines, costimulatory molecules, and tumor antigens. Studies using animal models have shown that inoculation/immunization with tumor cells engineered to express IL-2, IFN-γ, IL-4, tumor necrosis factor α, GM-CSF, IL-7, or IL-6 enhances antitumor immunity (1–6). In most tumor models, this results not only in the rejection of the genetically modified tumor cells but also the induction of systemic immunity capable of mediating the rejection of a subsequent challenge with parental, unmodified tumor cells.

A major drawback and limitation in using autologous cellular vaccines to treat cancer patients is the need to establish in vitro tumor cell lines prepared from biopsy tumor tissue for the transduction of immuno-modulatory genes. Difficulties associated with establishing cell lines from human tumor biopsy material and the relative inefficiency of many of the transfection methodologies have led to renewed efforts to establish alternative strategies for the efficient delivery of genes into freshly prepared/isolated tumor cells. Vectors that efficiently deliver genes into tumor cells either in vivo or ex vivo are required, and several viral and nonviral vector systems have been investigated for their suitability in this regard (7). Viral vectors represent the most efficient means of transducing genes into tumor cells, and many replication-competent and replication-defective viruses have been used to deliver genes of interest to in vitro and in vivo targets. HSVs have been used recently for cancer therapy and gene transduction studies. Intratumoral injection of replication-competent attenuated mutants of HSV-1 were shown to be effective in killing malignant gliomas (8, 9), and Toda et al. (10) have reported recently that immunization with a defective HSV-1 vector encoding the IL-12 gene in combination with a HSV helper virus can induce local and systemic antitumor immunity to the CT26 murine colon carcinoma. Similarly, systemic therapy using a recombinant adenovirus encoding both subunits of IL-12 inhibited the formation of 3-day hepatic metastasis of murine tumors (11).

We have reported previously the development of a genetically inactivated HSV-2 vector that is restricted to a single cycle of replication, DISC, for use as a vaccine against genital herpes infection (12–14). We have used this virus to deliver cytokine genes to tumor cells, and there are several reasons why this vector is potentially useful for cancer immunotherapy: (a) DISC-HSV-2 is unable to spread from cell to cell; replication of the virus is genetically restricted by deletion of the gH gene, which is essential for the production of infectious progeny; and (b) HSV-2 has a broad host cell range, making the DISC variant an appropriate vehicle for the delivery of genes to a variety of tumors. In addition, DISC-HSV infects nondividing as well as dividing cells and has been shown to rapidly and efficiently infect primary human leukemia and neuroblastoma cells (15), human carcinoma cells (16), and cultured murine tumor cells (17). In the present study, the DISC-HSV-2 vector has been used to deliver genes encoding murine GM-CSF, human IL-2, or the lacZ reporter genes to murine tumor cells and the efficacy of DISC-HSV-2-infected “whole” tumor cell vaccines for prophylactic immunization prior to tumor challenge and for the therapy evaluated in three murine tumor models. The results show that DISC-HSV-2 is an efficient vector for cytokine gene delivery into tumor cells, and that the expression of mGM-CSF or hIL-2 enhances the immunogenicity of whole-cell vaccines. In this study, the therapeutic response was

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shown to depend on the functionality of CD4+ and CD8+ lymphocytes.

MATERIALS AND METHODS

Tumors. RENCA-3 is a BALB/c renal carcinoma cell line of spontaneous origin and was generously provided by Dr. Robert Wiltrout (National Cancer Institute, Bethesda, MD). The immunogenicity of RENCA has been determined as being low to moderate. RENCA-3 cells were maintained by serial in vitro passage in RPMI 1640 supplemented with 10% FCS, sodium pyruvate, and NEAA. The M3 cell line is a DBA/2 melanoma cell line that was obtained from American Type Culture Collection. M3 cells were grown and maintained in Hams F-12 media supplemented with 15% FCS. The 302R is C57Bl/6 mouse sarcoma cell line, derived through repeated in vivo passage in mice, and was kindly supplied by Dr. B. Fox (Portland, OR). 302R cells were grown and maintained in DMEM media supplemented with 10% FCS.

Animals. Female DBA/2, C57Bl/6, BALB/c, and BALB/c N-c/Nea-1 mice were purchased from Harlan (UK) Ltd. and were maintained in accordance with the Home Office Codes of Practice for housing and care of animals.

Infection of Tumor cells with DISC-HSV lacZ Virus. Tumor cells were either cultured on glass slides precoated with fibronectin to increase the attachment of the cells or in 24-well plates and infected with DISC-HSV lacZ virus at a MOI of 1.25–10 pfu/cell. At various times postinfection, the cells were either fixed in acetone and stained for the presence of HSV-2 antigen using a polyclonal anti-HSV-2 antibody (Dako) or fixed in glutaraldehyde and stained for β-gal with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining (Promega).

Cytokine Assays. Expression of cytokines after infection of tumor cells with DISC-mGM-CSF and DISC-hIL-2 was determined by ELISA (R&D Systems, United Kingdom). Tumor cells were cultured in 24-well plates at a concentration of 1 × 10^5 cells/well overnight. The medium was removed, and cells were infected with 1.25–10 pfu/cell of each virus in a total volume of 100–200 μl for 1 h at 37°C. The medium was then removed and replaced by 1 ml of serum-free medium, and the plates were reincubated at 37°C for various times, up to 48 h. Supernatants were collected and stored at −20°C and assayed for mGM-CSF or hIL-2.

Apoptosis and HSP Expression. To determine whether DISC-HSV infection of tumor cells induced cell death by apoptosis, 5 × 10^5 cells were cultured in T25 flasks and infected with DISC-lacZ virus at 10 pfu/cell for 24 h. Floating and adherent (trypsinized) cells were pooled together for analysis. An ABO-BrdUrd kit from Pharmingen (San Diego, CA) was used according to the manufacturer’s instructions. Briefly, the cells were prefixed in 1% paraformaldehyde and then stored in 70% ethanol for 24 h. The cells were then washed and incubated with terminal deoxynucleotidyl transferase and bromo-dUTP, followed by FITC-anti-BrdUrd and propidium iodide. DNA breaks are indicative of apoptosis.

Prophylactic Immunization and Therapy with DISC-HSV-infected Cells. Tumor cells were infected with 5–10 pfu/cell with either DISC-mGM-CSF, DISC-hIL-2, or DISC-lacZ viruses for 1 h. The virus inoculum was added for 1 h (experiments were performed in triplicate). Residual virus was removed, and fresh serum-free medium was added, and the cells were then irradiated (15,000 rads) using a Gamma cell cesium-137 source; uninfected tumor cells were prepared in a similar manner and used as control. Cells (1 × 10^5) cells infected with DISC-mGM-CSF or DISC-hIL-2 viruses (before and after irradiation) were cultured in 24-well plates to assess cytokine production (as detailed above). To assess the effect of prophylactic vaccination using RENCA cells, animals were immunized s.c. twice on the right flank at 2-week intervals with irradiated, DISC-infected RENCA cells in a volume of 200 μl (see individual experiments for details). Unless otherwise stated, animals were challenged s.c. 7 days after the second inoculation, with 5 × 10^5 (10 times TD50) parental RENCA cells on the opposite flank.

To assess the efficacy of DISC-HSV infected whole-cell vaccines in therapy, mice received injections in the right flank with 5 × 10^5 tumor cells and were vaccinated with 1 × 10^5 DISC-HSV infected-irradiated RENCA cells at the same site or contralaterally on day 0 or 3. Mice then received two additional immunizations at 3-day intervals. Similar protocols using the M3 melanoma and 302R sarcoma were used to confirm the findings obtained with the RENCA model.
Construction of the DISC-HSV Viruses. Construction of the basic vector DISC-HSV-2 (DISC-HSV) by plasmid recombination was previously described (17). A similar process was used to construct dH2B (DISC-mGM-CSF), which required a two-stage recombination strategy. For the first stage, sodium iodide-purified, wild-type DNA and plasmid DNA (pIMMB56) were transfected into gh expressing complementing CR1 cells. The plasmid pIMMB56 contains the lacZ gene under the control of the SV40 promoter; the expression cassette is flanked by HSV sequences to enable recombination into viral genome and is similar in construction to pIMMB47+ (17). The resulting virus is designated dH2D. For the second stage, sodium iodide-purified dH2B viral DNA and plasmid DNA pIMR3 were transfected into CR1 cells as described above. Plasmid pIMR3 was constructed by ligation of the mGM-CSF gene from plasmid pJL3.2 (received as a gift; Ref. 18) into the shuttle vector pIMBB46. Plasmid pIMMB46 had been adapted previously to contain the CMV promoter and bovine growth hormone poly(A) addition signal from the plasmid PPRC-CMV (R&D Systems). The resulting virus was passaged three times on BHK gH

Depletion of CD4⁺ and CD8⁺ Cells. The effect of the in vivo depletion of CD4⁺ or CD8⁺ T-cells or both CD4⁺ and CD8⁺ T-cells on the therapeutic efficacy of the DISC vaccine was investigated. Groups of 10 mice were given three i.p. injections of 1 mg of anti-CD4 (YTS191.1.2), anti-CD8 (YTS169.4.2.1), control isotype antibody (YTH24), or a combination of anti-CD4 and anti-CD8 antibodies over a period of 1 week (19). Ten days after the last injection, three representative animals from each group were tail bled to determine the efficiency of depletion by flow cytometric analysis of the blood cells using anti-CD4 and anti-CD8 antibodies (Serotec Ltd. Oxford, United Kingdom). Mice received injections of RENCA cells (5 × 10⁵ cells/mouse) 7 days after the last antibody injection. Three vaccinations, with irradiated DISC-infected RENCA cells, were given 3 days apart at an adjacent body site, commencing on day 3 after the injection of tumor cells.

RESULTS

Reporter and Cytokine Gene Expression in RENCA Cells. DISC-HSV-2 viruses have been constructed to express the genes for LacZ, mGM-CSF, and hIL-2, respectively, after infection. In vitro studies were performed to confirm the ability of these viruses to infect murine tumor cells and to express the gene of interest. Expression of the LacZ gene was observed in RENCA cells coexpressing HSV viral glycoproteins, as shown by dual staining for β-gal and HSV protein expression. RENCA cells infected with DISC-LacZ (0.5–10 pfu) demonstrated an increase in immunostaining for the virus using anti-HSV fluorescent-labeled antibody only in cells expressing the β-gal gene (Fig. 1A). The infectability of RENCA cells using a MOI ranging from 0.3 to 10 pfu/cell was determined. A MOI of 1.25 pfu/cell resulted in 50% of the cells staining for β-gal protein 24 h after infection, whereas a MOI of 2.5 pfu/cell or greater resulted in β-gal protein expression in virtually all of the cells (Fig. 1B) 3 h after infection (Fig. 1C). Similar results were obtained for 302R and M3 cells (results not shown).

RENCA cells infected with DISC-mGM-CSF released up to 400 pg of m-GM-CSF/ml/10⁵ cells in 24 h (Fig. 2A), which was the maximum amount detected in this time course. The mGM-CSF release from RENCA cells increased proportionally with virus MOI (0.3 to 10 pfu/cell). Similar results were obtained for RENCA cells infected with DISC-hIL-2 virus, with the maximum release occurring 48 h after infection (Fig. 2B). For RENCA cells infected with DISC-hIL-2 virus at a MOI of 10 pfu/cell, 548 × pg/ml/10⁵ cells of hIL-2 was released into the supernatant at 24 h. In comparison, the mouse sarcoma 302R cells released 440 pg/ml/10⁵ cells 24 h after infection with 10 pfu/cell of DISC-hIL-2 virus (B) for 1 h, washed twice in serum-free medium, and incubated for up to 72 h. Supernatant from individual wells was collected at each indicated time points and stored at −20°C for cytokine analysis. Bars, SD.

![Fig. 2. Time course for the production of mGM-CSF and hIL-2 by cells infected with DISC-mGM-CSF and DISC-hIL-2 viruses. RENCA cells were cultured in 24-well plates overnight and infected with 10 pfu/cell of DISC-mGM-CSF virus (A) and DISC-hIL-2 virus (B) for 1 h, washed twice in serum-free medium, and incubated for up to 72 h. Supernatant from individual wells was collected at each indicated time points and stored at −20°C for cytokine analysis. Bars, SD.](Image)

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increase in the amount of mGM-CSF release of ~65% and M3 cells a decrease of ~40% (results not shown). A significant ($P \leq 0.01$) increase in hIL-2 release occurred after irradiation of DISC-IL-2-infected RENCA cells (Table 1; mean of three experiments).

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>+ Irradiation (pg/ml)</th>
<th>− Irradiation (pg/ml)</th>
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<tr>
<td>A. mGM-CSF</td>
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</tr>
<tr>
<td>1</td>
<td>244</td>
<td>304</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>4</td>
<td>290</td>
<td>214</td>
</tr>
<tr>
<td>Average ± SD</td>
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<td>251.5 ± 37.8</td>
</tr>
<tr>
<td>B. hIL-2</td>
<td>present in supernatant (pg/ml)</td>
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</tr>
<tr>
<td>1</td>
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<td>548</td>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
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<tr>
<td>Average ± SD</td>
<td>1076 ± 81.7</td>
<td>522.6 ± 37.1</td>
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* RENCA cells were infected with 10 pfu/cell of DISC-HSV-mGM-CSF virus or DISC-HSV-IL-2 virus for 1 h, and the cells were washed and exposed to irradiation. Cells ($1 \times 10^6$) in 1 ml of serum-free medium were cultured in 24-well plates, and supernatants were collected after 24 h.

* Irradiation (15,000 rads) of RENCA cells was performed 1 h after virus infection.

* Cytokine release was measured by ELISA; assays were performed in duplicate (values are mean ± SD).

* Significant at $P < 0.01$.

Treatment of RENCA cells with the protein synthesis inhibitor cycloheximide resulted in a ~95% inhibition of the reporter (LacZ) and cytokine gene expression (results not shown), confirming that virus replication is essential for gene expression.

### Apoptosis and Heat Shock Protein Expression in RENCA Cells Infected with DISC-HSV Virus

Cell death by apoptosis was investigated in RENCA cells infected with the DISC-lacZ virus. RENCA cells infected with DISC-lacZ at a MOI of 10 pfu/cell for 24 h induced apoptotic cell death in 7.8% of all cells compared with 2.1% of noninfected control cells (Fig. 3). Analysis of the DNA profile of cells showed that infected cells contained more DNA than uninfected cells, indicative of a block in the S or G2-M phases of the cell cycle, together with an increase in sub-G1 cells, which is suggestive of an increase in necrotic cell death (Fig. 3). Furthermore, infecting RENCA cells with DISC-lacZ did not induce the expression of the HSP protein HSP70, as shown by quantitative mRNA expression (results not shown), inferring that virus infection does not induce the early onset of stress response proteins that has been reported previously to influence the immunogenicity of tumor cells (20).

### Prophylactic Immunization Using Irradiated Tumor Cells Infected with DISC-HSV

Having demonstrated that RENCA cells release mGM-CSF and hIL-2 after infection with DISC-mGM-CSF and DISC-hIL-2 viruses, respectively, experiments were performed to compare the immunogenicity of DISC-infected whole-cell vaccines with standard vaccines prepared from irradiated, uninfected RENCA cells. Cultured RENCA cells were infected with DISC-HSV-2-containing either the lacZ, mGM-CSF, or hIL-2 gene for 1 h, irradiated, and injected.

**Fig. 3.** Necrosis versus apoptosis in RENCA cells infected with DISC-lacZ virus. RENCA cells ($5 \times 10^5$) were seeded into T25 tissue culture flasks and infected with 10 pfu/cell of DISC-lacZ for 24 h. Floating and adherent (trypsinized) cells were pooled and analyzed using an APO-BrdUrd kit to determine the apoptosis-indicative DNA breaks. The DNA profile (a and b) and DNA breaks (c and d) for noninfected (a and c) and infected (b and d) tumor cells are shown.
protective immunity was increased after immunization with 1 representative of several experiments are shown in Fig. 4. The degree of were injected with 1 irradiated (15,000 rads). Groups of up to 10 mice control (nonimmunized) mice (Fig. 4 with mice immunized with irradiated (noninfected) RENCA cells and, cells were injected s.c. on the right flank on two occasions 2 weeks apart; control mice were untreated. One week after the second immunization, the mice were challenged s.c with 5 × 10^6 (10 × TD<sub>50</sub>) parental RENCA cells on the opposite flank, and tumor growth and incidence were monitored on a regular basis. Animals immunized with 10<sup>6</sup> cells and challenged with 5 × 10<sup>5</sup> RENCA cells (B), immunized with 10<sup>5</sup> cells and challenged with 5 × 10<sup>4</sup> RENCA cells (C), or immunized with 10<sup>3</sup> cells and challenged with 2 × 10<sup>3</sup> RENCA cells (D) are shown.

into groups of up to 10 mice. Two immunizations were performed at 2-week intervals, and the animals were challenged s.c. with 5 × 10<sup>4</sup> live RENCA cells (10 × TD<sub>50</sub>) 7 days after the second immunization. Results representative of several experiments are shown in Fig. 4. The degree of protective immunity was increased after immunization with 1 × 10<sup>4</sup> DISC-infected cells expressing mGM-CSF compared with nonimmunized control mice or mice receiving irradiated RENCA cells or irradiated RENCA cells infected with DISC-lacZ (Fig. 4A). In addition, mice immunized with 1 × 10<sup>5</sup> DISC-mGM-CSF-infected RENCA cells showed an increased level of protection to tumor challenge compared with mice immunized with irradiated (noninfected) RENCA cells and control (nonimmunized) mice (Fig. 4B). It was observed that immunization with 1 × 10<sup>3</sup> irradiated RENCA cells infected with DISC-lacZ could also increase resistance to tumor challenge compared with nonimmunized control mice, although the degree of protective immunity was consistently lower than that observed for mice immunized with DISC-mGM-CSF-infected cells.

The level of protective immunity was decreased after immunization with whole-cell vaccines (1 × 10<sup>6</sup> cells/inoculum) and challenge with 20 × TD<sub>50</sub> or 40 × TD<sub>50</sub> RENCA cells (Fig. 4, C and D, respectively). Vaccination with DISC-hIL-2-infected RENCA cells gave results that were similar to those obtained using DISC-mGM-CSF-infected RENCA cells (results not shown). These experiments were repeated in a second tumor model. Prophylactic immunization with irradiated DISC- mGM-CSF- or DISC-hIL-2-infected 302R cells enhanced immunity to tumor challenge and confirmed the results obtained using the RENCA model (results not shown).

**Tumor Therapy Using DISC-HSV-infected Tumor Cells.** The ability of DISC-infected tumor cells to influence the growth of established tumors was investigated in three tumor models: RENCA, 302R, and M3. Groups of 10 mice were injected s.c. on the right flank with 5 × 10<sup>4</sup> (10 × TD<sub>50</sub>) viable RENCA cells prior to vaccination with irradiated DISC-infected or irradiated noninfected RENCA cells. Three immunizations (each containing 1 × 10<sup>6</sup> irradiated tumor cells) were given s.c. on the same or contralateral flank on days 3, 6, and 9, and the tumor incidence and tumor size were recorded during a 9-week observation period. By day 3 after inoculation of live tumor cells, defined tumor foci were detected by H&E histological analysis (results not shown), and for most of the experiment performed, this was the start date for initiation of therapy. The results (Fig. 5A) demonstrate a slight but insignificant delay in the onset of tumors in mice receiving the irradiated (noninfected) RENCA cell vaccine compared with control mice; this difference was not apparent when the average tumor sizes of the groups were compared (results not shown). Mice receiving DISC-mGM-CSF-infected RENCA cells showed a significant delay in the onset of tumors, and a high proportion of mice remained tumor free up to 9 weeks after challenge. In addition, vaccination with DISC-hIL-2-infected cells significantly inhibited tumor growth; 60% of mice remained free of tumor throughout the observation period (Fig. 5A). These results were reproducible and occurred when immunization was initiated on day 0 or day 3 after tumor cell inoculation. Up to 80% of mice immunized with either DISC-mGM-CSF or DISC-hIL-2 vaccines remained free of tumor throughout the study period (data from several experiments, not shown).

Similar results were obtained with the M3 melanoma (Fig. 5B) model. Vaccination, beginning on day 0 after live tumor cell implantation, with DISC-mGM-CSF or DISC–hIL-2-infected M3 cells inhibited tumor growth in 60 and 20% of vaccinated animals, respectively (Fig. 5C), and caused a delay in tumor growth in the remaining mice (results not shown). Inhibition of established M3 tumor growth also occurred in 40% of mice treated with DISC-lacZ M3-infected cells.

One important feature of this immunotherapy model was the development of local immunity in vaccinated mice. Vaccination at a site...
adjacent to the tumor implantation site with the DISC-mGM-CSF RENCA cell vaccine was effective in delaying the onset and growth of tumors; however, immunization on the contralateral flank was less effective (Fig. 5C). These results demonstrate that an increased therapeutic benefit can be derived by local administration of tumor cells infected with DISC-HSV-2 engineered to express either mGM-CSF or hIL-2 and were confirmed using the 203R tumor model (results not given).

**Vaccine Therapy in T-Cell-deficient Mice.** To determine that T-lymphocytes were required for effective immunotherapy using DISC whole tumor-cell vaccines, BALB/c nude mice (Nu/−/Nu−) received injections s.c. with 10×10^3 TD50 of RENCA cells on the right flank and vaccinated (starting on day 0) three times (3 days apart) on the same flank with irradiated noninfected RENCA cells or irradiated DISC-mGM-CSF-infected RENCA cells. The tumor incidence and growth rate were similar in control and vaccinated Nu/−/Nu− mice, indicating that T lymphocytes play a pivotal role in promoting tumor rejection (results not given). To establish the involvement of CD4+ and CD8+ T lymphocytes in immunotherapy, mice were depleted of the respective T-cell populations by the administration of Mabs raised to either CD4 or CD8 antigens. Seven days after antibody treatment, mice were injected with 5×10^4 RENCA cells and vaccine therapy (irradiated RENCA cells infected with DISC-mGM-CSF) given on days 3, 6, and 9. The results demonstrate that CD4, CD8, and CD4/CD8 “knock out” mice failed to respond to whole-cell vaccine therapy, whereas mice inoculated with isotype control serum or untreated mice were successfully treated by vaccination with DISC-mGM-CSF-infected RENCA cells (Fig. 6). The abrogation of therapeutic efficacy was proportional to the reduction in the subpopulations of T lymphocytes; the administration of CD8 Mab caused a 50% depletion of circulating CD8+ T cells and abrogated the effect of immunotherapy in 60% of mice. Administration of CD4 Mab reduced circulating CD4+ T cells by <95% and completely abrogated the effect of vaccine therapy. Collectively, these results demonstrate an absolute requirement for both CD4+ and CD8+ T lymphocytes for effective immunotherapy using DISC-mGM-CSF-infected whole-cell vaccination.

**DISCUSSION**

Murine tumor models have been used to evaluate whole tumor cell vaccines, genetically modified by gene transfection to produce cytokines, for their ability to promote anticancer immunity. These vaccines have been shown to elicit systemic immunity against tumor challenge and in some instances induce the regression of small tumors when given therapeutically (1–6). Thus, a number of viral and nonviral vector systems have been investigated as vehicles for gene delivery into tumor cells (21). In the present study, a DISC-HSV-2 was used as a vector for gene transfection of tumor cells in preclinical studies to assess its potential for human application.

We previously constructed gH-deleted HSV-2 to be used as a vaccine for the prevention of HSV-induced disease. This virus, which we term DISC, can only complete one replication cycle in normal cells and was shown to stimulate broad humoral and cell-mediated antiviral immune responses (12). DISC-HSV offers advantages as a vector system for gene transfer; they are safe because they are unable to spread from cell to cell.
within the patient, and they have a broad host cell range, making them suitable for the delivery of genes to a variety of tumors. Initial studies have shown that the DISC-HSV-2 will infect a wide range of murine and human tumor cells, including primary human leukemia and neuroblastoma cells (15, 16, 22). Here we show that DISC-HSV is able to infect murine carcinoma, sarcoma, and melanoma cells.

We have used three murine tumors, RENCA, 302R, and M3, as models to assess the ability of DISC-HSV-2 to deliver cytokine genes into tumor cells and undertaken preclinical evaluation of whole-cell vaccines expressing the cytokines mGM-CSF or hIL-2 to assess their ability to promote protective and therapeutic immunity. The RENCA tumor model was used extensively in this study, and the results were confirmed using 302R and M3 tumors. The relationship between the expression of the reporter β-gal gene and viral proteins after in vitro infection with the DISC-HSV-lacZ virus was established by dual staining for the expression HSV glycoprotein and the β-gal protein. Infection and reporter gene expression were time and dose dependent. A correlation between β-gal expression and the MOI was shown and confirmed that the virus was incapable of lateral spread. In a study by Lowstein et al. (23), recombinant HSV type 1 mutant tsk vectors containing β-gal were shown to infect neurocortical cells; β-gal expression was directly related with the MOI of the virus.

Infected RENCA cells with DISC-HSV-2 encoding mGM-CSF and recombinant hIL-2 genes resulted in the release of cytokines into the culture supernatant in a time-dependent manner. After infection with DISC-HSV-β-gal, an increase in necrotic cell death versus apoptosis occurred. RENCA cells undergoing death by necrosis may provide addition activation of the immune system in vivo by promoting tumor antigen processing and presentation by professional antigen-presenting cells, leading to an increase in T-cell activation (24, 25). In situ killing of tumor cells using suicide gene transfer to induce cell death through a nonapoptotic pathway is associated with enhanced immunogenicity and may in some cases require the induction of HSP expression (20), although in the present study RENCA cells infected with DISC-HSV failed to show elevated expression of HSP.

On the basis of these in vitro results and because of the potential of these cytokines to activate effector T cells (26), DISC-mGM-CSF and DISC-hIL-2 vectors were chosen for in vivo studies. In animal models, mGM-CSF expression by tumor cells results in potent systemic antitumor immunity, which can potentiate the rejection of weakly immunogenic murine tumors (27). IL-2 is also a potent mediator of antitumor immunity and can promote CTL activation and T-cell differentiation, enhance the activation status of natural killer cells, and induce lymphokine-activated killer cell activity (28, 29). Interestingly, hIL-2 production and release by DISC-hIL-2-infected cells were significantly increased after irradiation, an effect observed previously by Simova et al. (30), where administration of irradiated IL-2-secreting plasmacytoma cells was shown to be more effective than nonirradiated cells in promoting tumor immunity. Here, we demonstrate that immunization with irradiated RENCA cells infected with DISC encoding either mGM-CSF or hIL-2 cytokine genes protects mice against challenge with parental tumor cells in a dose-related manner.

DISC-mGM-CSF vaccine therapy prevented tumor growth in a high percentage of mice. The response to therapy was T-lymphocyte dependent and required the participation of both CD4+ and CD8+ T lymphocytes. One important consideration in this therapy model is the relative contribution of HSV infection versus cytokine production. Partial protection was observed after immunization with tumor cells infected with the DISC β-gal virus (used as a control for cells expressing cytokine), indicating that protection against tumor challenge may, in part, be a consequence of viral infection of the tumor cells; immunization with the β-gal protein alone does not illicit a measurable antitumor immune response (10). We suggest that DISC-HSV infection can act as an additional stimulus to enhance the immunogenicity of the tumor cells. HSV infection of mice has been shown to lead to the up-regulation of IL-12 expression (31) and to have potent antitumor effects in animal models (32), most probably by promoting a Th1 response to tumor antigen(s). Preexisting immunity to HSV infection did not seem to affect the efficacy of this vaccine because no significant difference was shown when animals were preimmunized with HSV prior to tumor implantation and subsequent therapy (data not shown). Irradiated RENCA cell vaccination also induced a degree of protection against rechallenging with the parental tumor line. These data are consistent with previous observations demonstrating that RENCA cells are weakly to moderately immunogenic (33), and where irradiation itself may affect the immunogenicity of tumor cells through the up-regulation of H-2Kd class I MHC antigens (34). For the reasons outlined, there is a precedent for using DISC-HSV to deliver immune response genes to tumor cells, in the present study by ex vivo
infection of the tumor cells, but additionally by direct in vivo injection into the tumor using a murine colon carcinoma model, where 40% of tumors regressed completely (16); we have shown that both approaches are efficacious for therapy in animal models.

GM-CSF has been used to potentiates antitumor immunity by promoting the maturation and function of professional antigen-presenting cells (35) and by recruiting additional antigen-specific and nonspecific effector cells. One noticeable feature of the immune response after inoculation of whole-cell vaccines expressing GM-CSF is the prevalence of a delayed-type hypersensitivity response at the site of vaccination and at the site of tumor rejection. Infiltration of tumors by eosinophils in response to GM-CSF has been reported in preclinical and clinical studies (27, 36), and a similar response is also observed after immunization with IL-4 gene-transduced vaccines (37, 38). There is also evidence that patients treated with an autologous GM-CSF gene-transduced vaccine can undergo an objective clinical response (36), although it is unclear to what extent eosinophils and effector cells other than CD8+ and CD4+ lymphocytes actually contribute to tumor rejection. Eosinophil infiltration of small established RENCA tumors occurs within 24 h after vaccine therapy with irradiated DISC-mGM-CSF RENCA cells and may represent a response associated with the production of Th2 cytokines IL-4 and IL-5 (36). In conclusion, the results obtained in this study allow us to propose a clinical approach to cancer immunotherapy based on the use of a novel DISC-HSV vector for the efficient delivery of cytokine genes to tumor cells.

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REFERENCES


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