Modified Vaccinia Virus Ankara Recombinants Are as Potent as Vaccinia Recombinants in Diversified Prime and Boost Vaccine Regimens to Elicit Therapeutic Antitumor Responses

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ABSTRACT

Cancer vaccine regimens use various strategies to enhance immune responses to specific tumor-associated antigens (TAAs), including the increasing use of recombinant poxviruses (vaccinia [rV] and fowlpox [rF]) for delivery of the TAA to the immune system. However, the use of replication competent vectors with the potential of adverse reactions has made attenuation a priority for next-generation vaccine strategies. Modified vaccinia Ankara (MVA) is a replication defective form of vaccinia virus. Here, we investigated the use of MVA encoding a tumor antigen gene, carcinoembryonic antigen (CEA), in addition to multiple costimulatory molecules (B7-1, intercellular adhesion molecule-1, and lymphocyte function-associated antigen-3 designated TRICOM). Vaccination of mice with MVA-CEA/TRICOM induced potent CD4+ and CD8+ T-cell responses specific for CEA. MVA-CEA/TRICOM could be administered twice in vaccinia naïve mice and only a single time in vaccinia-immune mice before being inhibited by antivector-immune responses. The use of MVA-CEA/TRICOM in a diversified prime and boost vaccine regimen with rF-CEA/TRICOM, however, induced significantly greater levels of both CD4+ and CD8+ T-cell responses specific for CEA than that seen with rV-CEA/TRICOM prime and rF-CEA/TRICOM boost. In a self-antigen tumor model, the diversified MVA-CEA/TRICOM/rF-CEA/TRICOM vaccination regimen resulted in a significant therapeutic antitumor response as measured by increased survival, when compared with the diversified prime and boost regimen, rV-CEA/TRICOM/rF-CEA/TRICOM. The studies reported here demonstrate that MVA, when used as a prime in a diversified vaccination, is clearly comparable with the regimen using the recombinant vaccinia in both the induction of cellular immune responses specific for the “self”- TAA transgene and in antitumor activity.

INTRODUCTION

Experimental and clinical cancer vaccine regimens use various strategies to enhance immune responses to specific TAAs. Among these strategies is the use of the recombinant poxviruses for more efficient delivery of a TAA to the immune system (1–4). Among the poxvirus vectors are rV, which is replication competent, and rF, which is replication defective in mammalian cells. MVA was derived from vaccinia virus strain Ankara by over 570 serial passages in chicken embryo fibroblast cells. The resulting MVA strain lost the capacity to productively infect mammalian cells with six major deletions comprised by three successive rounds of plaque purification in chicken embryo dermal primary cultures and amplified further in these cells to produce research stocks. The abbreviations used are: TAA, tumor-associated antigen; rV, recombinant vaccinia; rF, recombinant avipox (fowlpox); MVA, modified vaccinia Ankara; TRICOM, TRRad of COstimulatory Molecules; ICAM-1, intercellular adhesion molecule-1; LFA-3, lymphocyte function-associated antigen-3; CEA, carcinoembryonic antigen; GM-CSF, granulocyte macrophage colony-stimulating factor; MVA-WT, nonrecombinant wild-type MVA virus; FP-WT, nonrecombinant wild-type fowlpox virus; V-WT, nonrecombinant vaccinia virus (Wyeth strain); pfu, plaque-forming unit(s); IL, interleukin.

Because of its high degree of attenuation, MVA has appeared as an attractive alternative to standard vaccinia strains for the development of viral vectors to be used in vaccination or immunotherapy. Currently, recombinant MVA-based vaccines have been examined for a variety of diseases. Progress has been made toward the clinical assessment of MVA-based vectors predominantly in the treatment of HIV and malaria (for review, see Refs. 7 and 8), either alone or in combination with DNA vaccines. Here, we sought to examine the potential of MVA as a vector for cancer vaccines. In previous experimental studies, we and others have demonstrated: (a) the advantages of vaccinia or fowlpox recombinants for the delivery of the TAA to the immune system (9–12); (b) recombinant vaccinia vectors can be used only once in vaccinia-immune hosts because of host-limiting immune responses directed against the vector (1, 13–16); (c) recombinant fowlpox vectors can be used multiple times without inhibition of transgene expression, as demonstrated in preclinical studies (1, 13, 17) and clinical studies (15, 18); (d) the advantages of diversified vaccine prime and boost regimens (3, 15, 19); and (e) the use of the T-cell costimulation via inserting costimulatory molecules into vectors (20–22) enhances T-cell responses to the TAA. The use of rV or rF vectors containing TRICOM (B7–1, ICAM-1, LFA-3) has been shown to activate T cells to greater levels than the use of any one or two of these costimulatory molecules in recombinant vectors (21). In this study, the model tumor antigen used was CEA, which is overexpressed on a high percentage of human adenocarcinomas, particularly those of the colon, pancreas, breast, lung, rectum, and stomach. Here, we used recombinant MVA to simultaneously deliver CEA with genes encoding the three costimulatory molecules (TRICOM) to the immune system for the enhancement of both CD4+ and CD8+ T-cell responses. The studies reported here demonstrate that although the MVA recombinant is not as efficient as the vaccinia recombinants in the induction of a primary antigen-specific T-cell response, when used as a prime in a diversified vaccination protocol (with recombinant avipox vector as a booster vaccination), the recombinant MVA prime is clearly comparable with the regimen using the rV in both the induction of CD4+ and CD8+ T-cell responses specific for the “self”- TAA transgene and in antitumor activity. These vaccine-mediated antitumor immune responses, coupled with the safety profile of MVA, thus demonstrate that MVA could be an effective and safe alternative to vaccinia virus for the immunization of patients with therapeutic cancer vaccines.

MATERIALS AND METHODS

Recombinant Poxviruses. MVA was generated from a stock (kindly provided by Dr. A. Mayr, Ludwig-Maximilians University, Munich, Germany) by three successive rounds of plaque purification in chicken embryo dermal primary cultures and amplified further in these cells to produce research stocks. MVA-B7–1/ICAM-1/LFA-3 (designated MVA-TRICOM) contains the murine B7–1 gene under control of the synthetic early/late (SE/L) promoter (23), the murine LFA-3 gene under control of the 30k (M2L) promoter (24), and the murine ICAM-1 gene under control of the vaccinia I3 promoter (25). MVA-CEA/B7–1/ICAM-1/LFA-3 (designated MVA-CEA/TRICOM) is the recom-
vaccinia TRICOM and fowlpox TRICOM, respectively) have been described (22). The rV and fV viruses containing the human CEA gene and the murine B7−1, ICAM-1, and LFA-3 genes (designated rV-CEA/TRICOM and rF-CEA/TRICOM, respectively) have been described (22). The rF virus containing the gene for murine GM-CSF under control of the 40k promoter has been described (17). Drs. D. Panicali, G. Mazzara, and L. Gritz of Therion Biologics Corporation (Cambridge, MA) kindly provided all orthopox viruses as part of an ongoing Collaborative Research and Development Agreement between the National Cancer Institute/NIH and Therion Biologics Corporation.

Animals/Cells. Female C57BL/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). Mice were housed and maintained under pathogen-free conditions in microisolator cages until used for experiments at 6–8 weeks of age. C57BL/6 mice transgenic for human CEA (designated CEA-Tg) were originally obtained from a breeding pair provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg, Freiburg, Germany). The generation and characterization of the CEA-Tg mouse has been described previously (27). These studies used murine colon adenocarcinoma cells (MC-38) or MC-38 cells expressing human CEA (MC-38-CEA; Ref. 28). Before transplantation to mice, the cells were trypsinized, dispersed through a 70-μM cell strainer (Falcon; Becton Dickinson, Franklin Lakes, NJ), and washed twice in HBSS before final suspension in HBSS.

Characterization of Recombinant Viruses. Confluent MC38 cells were infected with MVA constructs (MVA-WT, MVA-TRICOM, MVA-CEA/TRICOM), vaccinia constructs (V-WT, rV-TRICOM, rV-CEA/TRICOM), or fowlpox constructs (FP-WT, rF-TRICOM, rF-CEA/TRICOM) at 10 multiplicities of infection (pfu/cell) overnight. After infection, cells were harvested and immunostained with FITC-conjugated monoclonal antibodies specific for CEA (COL-1; Ref. 29), murine B7−1, ICAM-1, or CD48 (LFA-3). All antibodies, with the exception of COL-1, were purchased from BD PharMingen, Inc. (San Diego, CA). Cell fluorescence was analyzed and compared with the appropriate isotype-matched controls (BD PharMingen) with a FACScan cytometer (Becton Dickinson, Mountain View, CA) using Cellquest software. The levels of CEA protein in infected cells were quantified by ELISA (AMDL, Tustin, CA) according to the manufacturer’s instructions. The detection limit for CEA protein was 1 ng/ml.

Efficacy of Multiple Vaccinations. C57BL/6 mice (three per group) were vaccinated s.c. once with 1 × 10^8 pfu of either MVA-CEA/TRICOM or rV-CEA/TRICOM or control vectors MVA-TRICOM or rF-TRICOM admixed with recombinant murine GM-CSF (20 μg; ProProTech, Rocky Hill, NJ) and human IL-2 (16,000 IU, i.p.; Hoffmann-La Roche, Nutley, NJ). GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, IL-2 (16,000 IU) was administered twice a day for 4 days. This dose of IL-2 is designated low dose (33). Fourteen days after the primary vaccination, mice were boosted with 1 × 10^8 pfu rF-CEA/TRICOM admixed with 1 × 10^7 pfu of rF-GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 14-day intervals. Three weeks after the last vaccination, immune responses were analyzed. Purified splenic T cells were tested for reactivity to CEA protein in an in vitro lymphoproliferation assay as described above, and CD8 T-cell responses specific for CEA peptide were measured as described above.

Cytokine Studies. CEA-Tg mice were vaccinated s.c. once with 1 × 10^8 pfu of either MVA-CEA/TRICOM or rV-CEA/TRICOM, or with MVA-CEA/TRICOM or rV-CEA/TRICOM admixed with recombinant murine GM-CSF (20 μg; ProProTech, Rocky Hill, NJ) and human IL-2 (16,000 IU, i.p.; Hoffmann-La Roche, Nutley, NJ). GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, IL-2 (16,000 IU) was administered twice a day for 4 days. This dose of IL-2 is designated low dose (33). Fourteen days after the primary vaccination, mice were boosted with 1 × 10^8 pfu rF-CEA/TRICOM admixed with 1 × 10^7 pfu of rF-GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 14-day intervals. Three weeks after the last vaccination, immune responses were analyzed. Purified splenic T cells were tested for reactivity to CEA protein in an in vitro lymphoproliferation assay as described above, and CD8 T-cell responses specific for CEA peptide were measured as described above.

Tumor Therapy Studies. CEA-Tg mice were transplanted with 50,000 MC38-CEA cells to form experimental peripancreatic metastases, as described (34). Briefly, the spleens of anesthetized mice were exteriorized by means of a small costal incision. Cells were directly injected in 100 μl of HBSS using 1-ml syringes with 26-gauge 5/8-inch needles. Spleenectomy was performed −2 min after tumor cell injection by cauterization using a high-temperature cautery (Roboz, Rockville, MD). The abdominal cavity was closed in one layer using 9-mm wound clips. This dose of tumor cells is lethal to >80% of mice within 8–10 weeks, with the primary tumor arising in the peripancreatic environment (34).

Fourteen days after tumor transplant, mice were vaccinated s.c. once with 1 × 10^8 pfu of either MVA-CEA/TRICOM or rV-CEA/TRICOM, or control vectors MVA-TRICOM or rV-TRICOM admixed with recombinant murine GM-CSF (20 μg) and human IL-2 (16,000 IU, i.p.). GM-CSF was administered at the injection site for the following 3 days. Concurrently, low-dose IL-2 was administered twice a day for 4 days. Seven days after the primary vaccination, mice were boosted with 1 × 10^8 pfu of the corresponding fowlpox vector: rF-CEA/TRICOM or rF-TRICOM, admixed with 1 × 10^7 pfu of rF-GM-CSF. Low-dose IL-2 was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 7-day intervals. Mice were monitored weekly for survival.

In a parallel experiment, CEA-Tg mice were vaccinated (day −6 before tumor transplant) with 1 × 10^8 PFU V-WT. Fourteen days after tumor transplantation, mice were vaccinated s.c. once with 1 × 10^8 pfu of either MVA-CEA/TRICOM or rV-CEA/TRICOM, or control vectors MVA-TRICOM or rV-TRICOM admixed with recombinant murine GM-CSF (20 μg) and human IL-2 (16,000 IU, i.p.). GM-CSF was administered at the injection site for the following 3 days. Concurrently, low-dose IL-2 was administered twice a day for 4 days. Seven days after the primary vaccination, mice were boosted with 1 × 10^8 pfu of the corresponding fowlpox vector: rF-CEA/TRICOM or rF-TRICOM, admixed with 1 × 10^7 pfu of rF-GM-CSF. This booster vaccination regimen was repeated two additional times at 7-day intervals. Mice were monitored weekly for survival.

Statistical Analysis of the Data. Where indicated, the results of tests of significance are reported as P values and are derived from Student’s t test using a two-tailed distribution. Ps were calculated at 95%. Evaluation of survival patterns in mice bearing peripancreatic tumors was performed by the Kaplan-Meier method and ranked according to the Mantel-Cox log-rank test using Stavview 4.1 (Abacus Concepts, Inc., Berkeley, CA) software package. For graphical representation of data, Y-axis error bars representing ±1 SE are shown. In some cases, the variation was such that the error bars were obscured by the plot symbol.

RESULTS

Recombinant MVA: Expression of Transgenes and Comparison with rV and rF. To confirm that the recombinant modified vaccinia Ankara (MVA-CEA/TRICOM) virus expressed the appropriate transgenes and to compare these expression levels with those of
comparable vaccinia and fowlpox recombinants, the murine adenocarcinoma cell line MC38 was infected with the various recombinants, and cell-surface expression of the transgene(s) was demonstrated by flow cytometry (Fig. 1). Mock infected (uninfected) cells and cells infected with control virus wild-type MVA (MVA-WT) failed to express CEA, or any of the three costimulatory molecules. In contrast, cells infected with MVA-CEA/TRICOM became positive for the expression of CEA, B7–1, ICAM-1, and LFA-3 (Fig. 1). MC38 cells infected with MVA-TRICOM, although remaining negative for the expression of CEA, became positive for B7–1, ICAM-1, and LFA-3 at expression levels similar to those measured when cells were infected with MVA-CEA/TRICOM (data not shown). To determine whether the rV and rF viruses expressed their recombinant proteins to levels similar to that of MVA, MC38 cells were infected with the vaccinia and fowlpox constructs in a similar manner (Fig. 1). Again, cells infected with control viruses V-WT or FP-WT failed to express CEA or any costimulatory molecule. However, cells infected with rV-CEA/TRICOM or rF-CEA/TRICOM expressed CEA and all three costimulatory molecules. Moreover, the expression levels of the recombinant proteins, as determined by mean fluorescent intensity, were similar between the recombinant MVA, vaccinia, and fowlpox vectors. As an additional comparison of transgene expression, MC38 cells were infected with the recombinant MVA, vaccinia or fowlpox vectors, and the quantity of CEA protein produced by each recombinant virus was measured by ELISA. MVA-CEA/TRICOM-infected cells expressed 1360 ng/mg total protein, and rV-CEA/TRICOM-infected cells expressed 1309 ng/mg total protein; rF-CEA/TRICOM-infected cells expressed 154 ng/mg total protein.

**Efficiency of Multiple Administrations of MVA-CEA/TRICOM.** In previous studies, it was demonstrated in naive mice that after two vaccinations with rV-CEA, additional vaccinations with rV-CEA were ineffective (3). In vaccinia-immune mice (3), and in human clinical studies (15), it was shown that rV-CEA boosting was ineffective after only one vaccination of rV-CEA. It was shown in mice that vaccination with rV virus vaccines induces antivector antibodies that potentially interfere with the efficiency of subsequent rV boosts. However, it was not known whether this phenomenon would also hold for modified vaccinia virus Ankara vectors. To address this, eight groups of mice were vaccinated with one of the following: (a) HBSS buffer (four vaccinations); (b) MVA-WT (four vaccinations); (c) MVA-CEA/TRICOM (one to four vaccinations); (d) V-WT (four vaccinations); (e) rV-CEA/TRICOM (one to four vaccinations); (f) FP-WT (four vaccinations); or (g) rF-CEA/TRICOM (one to four vaccinations). These vaccinations were performed via the s.c. route, because it was previously determined that there were no significant differences in the CD4\(^+\) or CD8\(^+\) CEA-specific responses induced by s.c. or i.m. vaccination (data not shown). When antibodies specific for vaccinia or fowlpox were analyzed (Fig. 2, A and B), it was noted that vaccination with V-WT (○), rV-CEA/TRICOM (●), MVA-WT (□), or MVA-CEA/TRICOM (■) all induced IgG specific for vaccinia (Fig. 2B) and not fowlpox. Vaccination with FP-WT or rF-CEA/TRICOM induced IgG specific for fowlpox (Fig. 2C) and not vaccinia. Thus, there was no cross-reactivity observed between vaccinia and fowlpox at the antibody level. As shown in Fig. 2C, one vaccination with rV-CEA/TRICOM (●) induced marked and significant CD4\(^+\) CEA-specific proliferation (P < 0.0001 versus V-WT; ○). Boosting of these mice once with rV-CEA/TRICOM further increased CEA-specific proliferation (P < 0.0001 versus rV-CEA/TRICOM prime). However, additional boosting of these mice with rV-CEA/TRICOM failed to enhance CEA-specific T-cell proliferation over that seen with rV-CEA/TRICOM administered twice. In contrast, one vaccination with rF-CEA/TRICOM (△) induced significant CD4\(^+\) CEA-specific proliferation (P < 0.0001 versus FP-WT; △), which was further potentiated with each subsequent boost with rF-CEA/TRICOM, despite rising levels of anti-fowlpox antibodies (Fig. 2B). One vaccination with MVA-CEA/TRICOM (■) induced significant CD4\(^+\) CEA-specific proliferation (P < 0.002 versus MVA-WT; □). Boosting of these mice with MVA-CEA/TRICOM further increased CEA-specific proliferation. Additional boosting of these mice with MVA-CEA/TRICOM failed to enhance CEA-specific T-cell proliferation over that seen with MVA-CEA/TRICOM administered twice. When mice were prevaccinated with V-WT and then vaccinated with MVA-CEA/TRICOM, significant CD4\(^+\) CEA-specific proliferation was noted. However, these mice could not be boosted further with MVA-CEA/TRICOM (data not shown). These data, taken together, indicate that like vaccinia, MVA could effectively be administered twice in a vaccinia naive setting and only a single time in a vaccinia-immune setting.

**Use of Recombinant MVA in a Diversified Prime and Boost Vaccination Regimen.** Previous studies have shown that diversified prime and boost vaccine strategies are more effective in enhancing T-cell responses compared with the continued use of one immunogen. It has been shown above (Fig. 2) that rV and MVA viruses can be used only one or two times because of the generation of host antivaccinia-immune responses and that rF viruses can be administered multiple times. Experiments were conducted to determine whether the contin-

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**Fig. 1.** Tumor antigen and costimulatory molecule surface expression after infection with recombinant vectors. MC38 tumor cells were infected for 24 h at 10 multiplicities of infection with the indicated virus. After infection, cells were immunostained with tumor antigen (CEA) or costimulatory molecule-specific FITC-labeled monoclonal antibodies. *Shaded* histograms show fluorescence intensity of the specific monoclonal antibody, whereas *unshaded* histograms show the fluorescence intensity of the appropriate isotype control antibody. The numbers in each panel indicate the percentage of positive cells and mean fluorescent intensity (in parentheses).
used administration of rF-CEA/TRICOM could induce CEA-specific immune responses comparable with those induced by rV-CEA/TRICOM in a diversified prime and boost vaccination regimen. CEA-Tg mice were used in these experiments. Previous studies have shown that CEA is expressed as a self-antigen in gastrointestinal tissues in these mice and that vaccination with CEA protein in adjuvant does not induce a CEA-specific T-cell response. CEA-Tg mice were primed once with either rF-CEA/TRICOM or rV-CEA/TRICOM. Mice were then boosted three times with rF-CEA/TRICOM. Control mice were administered buffer (HBSS). The regimen using priming with rV-CEA/TRICOM (Fig. 3A, ●) significantly enhanced CEA-specific lymphoproliferation compared with the regimen involving priming with rF-CEA/TRICOM (Fig. 3A, □; \( P < 0.0021 \) at 50 \( \mu \)g/ml CEA). T cells from the buffer control group (△) failed to react with CEA protein. CD8\(^+\) T-cell responses were measured using a CEA 8-mer peptide, and IFN-\( \gamma \) production was measured. A control peptide (VSV-N) was also used. IFN-\( \gamma \) production by T cells stimulated with CEA peptide was significantly enhanced in rV-CEA/TRICOM-primed animals (VFFF) compared with rF-CEA/TRICOM-primed animals (FFFF; \( P < 0.0001 \); Fig. 3B).

Because the VFFF vaccine regimen induced superior CEA-specific immune responses, experiments were then conducted to determine whether MVA-CEA/TRICOM could effectively replace rV-CEA/TRICOM in the diversified prime and boost vaccination regimen. CEA-Tg mice were primed once with either MVA-CEA/TRICOM or rV-CEA/TRICOM. Mice were then boosted three times with rF-CEA/TRICOM. Control mice were primed with either MVA-TRICOM or rV-TRICOM and boosted with rF-TRICOM (i.e., vectors devoid of the CEA transgene). The regimen using priming with MVA-TRICOM (Fig. 4A, □) significantly enhanced CEA-specific lymphoproliferation compared with the regimen involving priming with rV-CEA/TRICOM (Fig. 4A, ●; \( P < 0.0001 \) at 50 \( \mu \)g/ml CEA). T cells from control (no CEA) groups (open symbols) failed to react with CEA protein. Next, CD8\(^+\) T-cell responses were examined. Again, IFN-\( \gamma \) production by T cells stimulated with CEA peptide was significantly enhanced in MVA-CEA/TRICOM-primed animals (MFFF) compared with rV-CEA/TRICOM-primed animals (VFFF; \( P < 0.001 \); Fig. 4B).

**Determination of Cytokine Requirements for Optimal Immunity.** The above results suggested that priming with MVA-CEA/TRICOM, followed by continued boosting with rF-CEA/TRICOM, was more effective in generating CEA-specific T-cell responses than rV-CEA/TRICOM priming, followed by rF-CEA/TRICOM boosting. Previous preclinical and clinical studies have shown that the addition of GM-CSF at the vaccine site can enhance the recruitment of antigen-presenting cells (including dendritic cells) to regional nodes, resulting in increases in both antigen-specific T-cell responses and antitumor immunity. Similarly, the addition of IL-2 has been shown to enhance immune responses to vaccination. To determine whether the addition of GM-CSF and IL-2 would further enhance antigen-specific immune responses using MVA-CEA/TRICOM vectors, CEA-Tg mice were vaccinated with MVA-CEA/TRICOM or rV-CEA/TRICOM as a prime and boosted three times weekly with rF-CEA/TRICOM. These vaccination regimens were performed with or without the addition of GM-CSF and low-dose IL-2, as described in “Materials and Methods.” As shown in Fig. 5A, the addition of GM-CSF and IL-2 to mice primed with either rV-CEA/TRICOM (●) or MVA-CEA/TRICOM (■) enhanced T-cell proliferation to a greater level (\( P = 0.021 \) and \( P < 0.001 \), respectively, at 50 \( \mu \)g/ml CEA) than mice vaccinated with rV-CEA/TRICOM (○) or MVA-CEA/TRICOM (□) without the addition of cytokine. Moreover, CD8\(^+\) T-cell responses as measured by IFN-\( \gamma \) production in response to an 8-mer peptide were significantly enhanced by the addition of cytokines to either MVA-CEA/TRICOM or rV-CEA/TRICOM vaccination (Fig. 5B; \( P < 0.001 \)).

**MVA-CEA/TRICOM Prime/rF-CEA/TRICOM Boost for Therapy of Established Experimental Metastases.** Tumor therapy studies were initiated to determine the therapeutic efficacy of a MVA-CEA/TRICOM/rF-CEA/TRICOM-based vaccination regimen versus the same regimen but using rV-CEA/TRICOM as a prime. In these studies, MC-38 murine colon carcinoma cells, which had been transduced with the CEA gene using a retroviral vector, were used as targets in CEA-Tg mice as described previously (1, 35). Peripancreatic metastases develop at 4–5 weeks after tumor transplant (36, 37) and are lethal in >80% of mice by 8–10 weeks after transplant if left untreated. Tumor therapy was initiated on day 14 after tumor trans-
Concanavalin A is shown in the splenic T cells in response to CEA protein. Proliferation in response to the T-cell mitogen FTRICOM (F). Control mice were administered buffer (HBSS). A, lymphoproliferation of or VSV-N (V) peptides.

The addition of GM-CSF and low-dose IL-2. As shown in Fig. 6B, IFN-γ production in response to CEA526–533 (I) or VSV-N (III) peptides.

Plant by vaccination with either MVA-CEA/TRICOM (M) or rV-CEA/TRICOM (V). All mice were boosted three times with rF-CEA/TRICOM (F). All prime and boost vaccinations were accompanied by the addition of GM-CSF and low-dose IL-2. As shown in Fig. 6A, 71% (five of seven) of the mice receiving the MFFF vaccination regimen with cytokines (I) remained alive and apparently healthy through the 16-week observation period. However, only 14% (one of seven) of the mice that received control vectors along with cytokine treatment (II) survived past 16 weeks (P = 0.02). These results are similar to those seen with therapy consisting of the VFFF vaccination regimen (Fig. 6B), in which 57% (four of seven) of the mice receiving rV-CEA/TRICOM/rF-CEA-TRICOM vaccine regimen with cytokines (III) survived. There was, thus, no statistical difference (P = 0.821) in survival in the MFFF-treated group versus the VFFF-treated group. Again, only 14% (one of seven) of the mice that received control vectors along with cytokine treatment (II) survived past 16 weeks (P = 0.03).

Tumor therapy was then examined in vaccinia-immune CEA-Tg mice (Fig. 6, C and D) in which mice were prevaccinated with V-WT 14 days before tumor transplant. This immunization regimen could be analogous to that of a patient vaccinated against smallpox as a child. Tumor therapy was again initiated on day 14 by vaccination with either MFFF or the VFFF regimens. All prime and boost vaccinations were accompanied by the addition of GM-CSF. As shown in Fig. 6C, 50% (5 of 10) of the mice receiving the MFFF vaccination regimen with GM-CSF (I) remained alive through the 16-week observation period. In contrast, 10% (1 of 10) of the mice that received control vectors along with GM-CSF treatment (II) survived. Vaccine therapy consisting of the VFFF vaccination regimen (Fig. 6D) resulted in 60% (6 of 10) of mice surviving, whereas no mice (0 of 10) that received control vectors along with GM-CSF (II) survived past 16 weeks. There was, thus, no statistical difference (P = 0.729) in survival in the MFFF-treated group versus the VFFF-treated group.

**DISCUSSION**

rV virus-based vaccines are being used for the therapy of infectious disease and cancer. The safety of vaccinia virus immunization has been well documented during the smallpox eradication program, in which over 1 billion people were vaccinated (38). Indeed, the success of that program led to the discontinued routine use of the smallpox vaccine in the 1970s. The occurrence of rare adverse reactions to vaccinia vaccination (in the form of the smallpox vaccine) and the emerging threat of bioterrorism have generated considerable new interest in vaccinia and the coincident safety concerns and contraindications for vaccination of the general population. Contraindications for vaccine include eczema or other skin conditions, pregnancy, altered immunocompetance, HIV, infants, children, and cancer therapy (38, 39). The adverse reaction rate for vaccinia immunization has been estimated at 1253/million in naïve populations and 108/million in immune populations (38, 40). In view of these safety issues, more attenuated strains of vaccinia virus have been examined, including MVA (41).

MVA is a highly attenuated, nonreplicating vaccinia virus that was used during the end stage of the smallpox eradication program in Germany and Turkey. Prevaccination of high-risk patients with MVA, followed by vaccination with a standard Lister/Elstree vaccinia strain, reportedly reduced side effects dramatically (42–44). The properties of the MVA strain that make it so attractive as a vector include its inability to produce infectious virus in mammalian cells, avirulence in a variety of animals even under immunosuppressive conditions, and little or no local or systemic reaction on inoculation of humans, including high-risk individuals (5, 43, 45, 46).

Here, we investigated the use of recombinant MVA expressing a TAA in combination with multiple T-cell costimulatory molecules as a model for a vaccine for cancer immunotherapy. The safety of MVA has been confirmed in several experimental systems. Hanke et al. (47), using MVA-HIV in murine studies, found no significant toxic effects, either
local or systemic. In a more rigorous model, Stittelaar et al. (48) demonstrated the safety of MVA administration in nonhuman primates that were deliberately immunosuppressed with total-body irradiation or antithymocyte globulin to closely model immune-suppressed humans. These immunosuppressed macaques, given high doses of MVA, remained normal with regard to clinical, hematological, and pathological parameters. In addition, there was no detectable replicating MVA virus or MVA genome by PCR (48). In humans, recombinant MVA was used to vaccinate 18 HIV-negative patients with MVA-HIV in Kenya (49); in another study, MVA-HIV(nef) was given to 14 HIV-infected patients with CD4⁺ T-cell counts < 50 cells/µl in order to model HIV infection in the gut (50). In both studies, MVA administration was determined to be safe and well tolerated (50). On the basis of these results, recombinant MVA vectors are being evaluated for the therapy of cancer, preclinically in a murine tumor model (51), and in patients with colorectal carcinoma (52) and prostate cancer (53).

Here, it was observed that a single vaccination with MVA-CEA/TRICOM induced less potent T-cell responses than a single vaccination with rV-CEA/TRICOM (Fig. 2). These data are in contrast to that of Sutter et al. (54), who demonstrated that MVA was equal to, or more efficient than, the Western Reserve strain in protecting mice against the influenza virus. In those studies, the main effect of vaccination was an antibody response against the influenza products.

Preclinical studies (1, 13, 17) and now clinical studies (15, 18) have clearly demonstrated that rV vectors can be used only once because of host-limiting immune responses directed against the vector. In experimental settings, MVA vaccines have been administered multiple times; however, preexposure to MVA has been shown to decrease subsequent MVA vaccine immunogenicity in mice (55), and this effect has been confirmed in primates (56). To determine how many times MVA-CEA/TRICOM could be administered effectively, mice were vaccinated one to four times with MVA-CEA/TRICOM (Fig. 2). MVA-CEA/TRICOM could be administered effectively twice in vaccinia naïve mice and only a single time in vaccinias-immune mice before being inhibited by antivector-immune responses. Indeed, mice vaccinated with rV-CEA/TRICOM developed significant antivaccinia antibody titers (>1:6000) after two administrations (Fig. 2B), which corresponded with the cessation of further improvement of CEA-specific CD4⁺ T-cell proliferation (Fig. 2A). In contrast, repeated vaccination with rF-CEA/TRICOM, despite generating significant anti-fowlpox antibody titers, continued to increase the magnitude of T-cell responses to CEA. In this regard, recombinant MVA vectors behaved like rV vectors (1, 13, 17).

To circumvent this potential limitation, an effective strategy to potentiate vaccine-induced immune responses has been the use of alternative vectors for the boosting phase of vaccination. It has been demonstrated that rF vectors can be used multiple times without inhibition of transgene expression (1, 13, 15, 17, 18). The use of rV to prime the immune response, followed by subsequent boosting with rF, has been shown to be superior to the continued use of one vector (Fig. 3). These studies were conducted in CEA-Tg mice, to model the situation of breaking tolerance to a self-TAA. In these mice, human CEA is a self-antigen and is expressed in normal adult gastrointestinal

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Fig. 5. The effect of cytokine addition to the diversified prime and boost vaccine regimen in CEA-specific immune responses in CEA-Tg mice. CEA-Tg mice (three per group) received a MVA-CEA/TRICOM prime vaccination (M), followed by three weekly boosts with rF-CEA/TRICOM (F, □), or received a MVA-CEA/TRICOM prime vaccination with recombinant GM-CSF and low-dose IL-2, followed by three weekly boosts with rF-CEA/TRICOM admixed with rF-GM-CSF and low-dose IL-2 (■). For comparison, CEA-Tg mice (three per group) received a rV-CEA/TRICOM prime vaccination (V), followed by three weekly boosts with rF-CEA/TRICOM (F, ○), or received a rV-CEA/TRICOM prime vaccination with recombinant GM-CSF and low-dose IL-2, followed by three weekly boosts with rF-CEA/TRICOM admixed with rF-GM-CSF and low-dose IL-2 (●). A, lymphoproliferation of splenic T cells in response to CEA protein. Proliferation in response to the T-cell antigen concanavalin A is shown in the insets. B, IFN-γ production in response to CEA (526–533) or VSV-N (56) peptides.

Fig. 6. Therapy of 14-day established experimental metastases. CEA-Tg mice were transplanted with tumor intrasplenically on day 0 (solid arrow). A, Fourteen days later, mice received MVA-CEA/TRICOM prime vaccination (M) with recombinant GM-CSF and low-dose IL-2, followed by three weekly boosts with rF-CEA/TRICOM (F) admixed with rF-GM-CSF and low-dose IL-2 and rF-CEA/TRICOM (no CEA) prime vaccination with cytokines, followed by three weekly boosts with rF-CEA/TRICOM (no CEA) admixed with rF-GM-CSF and low-dose IL-2 (●). Control CEA-Tg mice received a MVA-TRICOM (no CEA) prime vaccination with cytokines, followed by three weekly boosts with rF-TRICOM (no CEA) admixed with rF-GM-CSF and low-dose IL-2 (■). B, Fourteen days later, mice received rV-CEA/TRICOM prime vaccination (V) with cytokines, followed by three weekly boosts with rF-CEA/TRICOM (F) admixed with rF-GM-CSF and low-dose IL-2 (○). Control CEA-Tg mice received a rV-TRICOM (no CEA) prime vaccination with cytokines, followed by three weekly boosts with rF-TRICOM (no CEA) admixed with rF-GM-CSF and low-dose IL-2 (□). C, CEA-Tg mice (10/group) were prevaccinated with high-dose V-WT (day −6). Six days later, the mice were transplanted with tumor intrasplenically on day 0 (solid arrow). Fourteen days later, mice received MVA-CEA/TRICOM prime vaccination (M) admixed with rF-GM-CSF, followed by three weekly boosts with rF-CEA/TRICOM (F) admixed with rF-GM-CSF (●). Control CEA-Tg mice received a MVA-TRICOM (no CEA) prime vaccination admixed with rF-GM-CSF, followed by three weekly boosts with rF-TRICOM (no CEA) admixed with rF-GM-CSF (□). D, rV-CEA/TRICOM prime vaccination (V) admixed with rF-GM-CSF, followed by three weekly boosts with rF-CEA/TRICOM (F) admixed with rF-GM-CSF (○). Control CEA-Tg mice received a rV-TRICOM (no CEA) prime vaccination admixed with rF-GM-CSF, followed by three weekly boosts with rF-TRICOM (no CEA) admixed with rF-GM-CSF (□). Mice in each group were monitored weekly for survival.
tissues, in a manner similar to its expression in humans (27). These mice have been shown to be tolerant to CEA, as defined by a lack of T-cell responses after vaccination with CEA protein in adjuvant (12), indicating that more efficient vaccination strategies are required than in conventional mice. This is in agreement with preclinical studies by Hodge et al. (3) and Aarts et al. (35), and now clinically (15). This diversified prime and boost regimen has also shown to be effective with MVA vectors, predominately in conjunction with DNA vaccines (47, 57–60). MVA has also been used in conjunction with fowlpox-based vaccines (51, 60). Because the diversified prime and boost regimen (vaccinia, followed by fowlpox) induced significantly greater CEA-specific CD4+ and CD8+ T-cell responses, subsequent experiments analyzed the effects of MVA-CEA/TRICOM, followed by boosting with rF-CEA/TRICOM.

The use of MVA-CEA/TRICOM in a diversified prime and boost vaccine regimen with rF-CEA/TRICOM induced significantly greater levels of both CD4+ and CD8+ T-cell responses specific for CEA than that seen with rV-CEA/TRICOM prime, rF-CEA/TRICOM boost (Fig. 4). One explanation of how MVA vaccination could make a host more responsive to fowlpox boosting is the reported expression of immune evasion or suppression molecules by different poxviral vectors. Vaccinia is known to express several immune evasion factors, many of which are soluble secreted homologues of cytokine receptors (61). The potential absence of these immune evasion molecules in the attenuated MVA could enhance the resulting immune response to the target antigen (48).

To potentially further enhance CEA-specific immune responses, the effect of the cytokines GM-CSF and IL-2, in addition to MVA-TRICOM vector, was investigated in CEA-Tg mice. IL-2 has been shown to promote the proliferation and differentiation of T and B cells and to enhance cytolytic activity of natural killer cells and lymphokine-activated killer cells (62). The infiltration of antigen-presenting cells to regional lymph nodes has been shown to be enhanced by locally administered GM-CSF (63, 64), which potentiates T-cell responses (17). In addition, it was shown previously that the coadministration of GM-CSF and IL-2 to effect of the cytokines GM-CSF and IL-2, in addition to CEA/TRICOM viral vectors, was investigated in CEA-Tg mice. IL-2 has been shown to be a positive finding in terms of the safety profile of MVA vectors, predominately in conjunction with DNA vaccines (63, 64), which potentiates T-cell responses (17). In addition, it was shown previously that the coadministration of GM-CSF and IL-2 to MVA vectors, predominately in conjunction with DNA vaccines (63, 64), which potentiates T-cell responses (17). In addition, it was shown previously that the coadministration of GM-CSF and IL-2 to T-cell responses specific for CEA than that seen with rV-CEA/TRICOM prime, rF-CEA/TRICOM boost (Fig. 4).

The studies reported here demonstrate that when MVA-CEA/TRICOM is used in a diversified prime and boost regimen with rF-CEA/TRICOM, there is no statistical difference in antitumor activity when compared with the regimen using rV-CEA/TRICOM as a prime and boosting with rF-CEA/TRICOM. This was shown to be the case using CEA-Tg mice that were either vaccinia naive or vaccinia immune (Fig. 6). This is a positive finding in terms of the safety profile of MVA compared with vaccinia, but it is surprising in light of the fact that the MFFV regimen was superior to the VFFV regimen in the induction of both CD4+ and CD8+ T-cell responses specific for CEA. Previous studies using depleting antibodies and the exact model used here have demonstrated that the antitumor effect using the VFFV regimen is primarily caused by the induction of CD4+ and CD8+ T-cell responses (65). Perhaps the fact that the VFFV and MFFV regimens are similar in antitumor effects is because of the fact that the VFFV regimen further enhances natural killer cell activity or other effector functions. Alternatively, the similarity in results could be because of the tumor model itself (i.e., perhaps a certain percentage of tumors (~40%) are unable to be cured by any regimen because of the overexpression of immunoregulatory substances such as transforming growth factor-β and/or IL-10).

The studies reported here demonstrate for the first time that the use of replication-defective MVA vectors containing as many as three costimulatory molecules induce potent and effective antigen-specific T-cell responses and can be used in diversified prime and boost vaccine regimens in conjunction with replication-defective rF-CEA/TRICOM for the induction of even greater T-cell responses and antitumor therapy. These MVA-CEA/TRICOM vaccine-mediated antitumor-immune responses, coupled with the extensive safety profile of MVA, thus demonstrate that MVA could be an effective and safe alternative to vaccinia virus for the immunization of patients with therapeutic cancer vaccines.

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References


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