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Synergy of Vaccine Strategies to Amplify Antigen-specific Immune Responses and Antitumor Effects

Douglas W. Grosenbach, Jacqueline C. Barrientos, Jeffrey Schlom,[1] and James W. Hodge

Laboratory of Tumor Immunology and Biology, National Cancer Institute [D. W. G., J. S., J. W. H.] and Howard Hughes Medical Institute, Research Scholars at the NIH [J. C. B.], NIH, Bethesda, Maryland 20892-1750

ABSTRACT

Several different vaccine strategies have been evaluated and combined in an attempt to amplify T-cell responses toward induction of antitumor immunity. The model tumor antigen used was carcinoembryonic antigen (CEA). While initial T-cell activation studies were conducted in conventional mice, combined vaccine strategy studies and antitumor studies were conducted in transgenic mice in which CEA is expressed in normal gastrointestinal tissue and CEA protein is found in sera. The studies reported here demonstrate: (*a*) A recombinant avipox (fowlpox, rF) vector expressing the signal 1 (CEA) and the B7-1 costimulatory molecule transgenes (designated rF-CEA/B7-1) is more potent in inducing CEA-specific T-cell responses than rF-CEA; one administration of recombinant fowlpox vector expressing CEA and three different costimulatory molecule transgenes (*B7-1, ICAM-1, LFA-3*, designated rF-CEA/TRICOM) was more potent in inducing CEA-specific T-cell responses than four vaccinations with rF-CEA or two vaccinations with rF-CEA/B7-1. Moreover, up to four vaccinations with rF-CEA/TRICOM induced greater CEA-specific T-cell responses with each vaccination. (*b*) A diversified prime and boost strategy using a prime with a recombinant vaccinia vector expressing CEA and the triad of costimulatory molecules (designated rV-CEA/TRICOM) and a boost with rF-CEA/TRICOM was more potent in inducing CEA-specific T-cell responses than the repeated use of rF-CEA/TRICOM alone. (*c*) The addition of granulocyte macrophage colony-stimulating factor (GM-CSF) to the rF-CEA or rF-CEA/TRICOM vaccinations via the simultaneous administration of a rF-GM-CSF vector enhanced CEA-specific T-cell responses. These strategies (TRICOM/diversified prime and boost/GM-CSF) were combined to treat CEA-expressing carcinoma liver metastases in CEA-transgenic mice; vaccination was initiated 14 days posttumor transplant. Antitumor effects in terms of survival and $CD8^+$ and $CD4^+$ responses specific for CEA were also observed in this CEA-transgenic mouse model. These studies demonstrate that the use of cytokines and diversified prime and boost regimens can be combined with the use of recombinant vectors expressing signal 1 and multiple costimulatory molecules to further amplify T-cell responses toward more effective vaccine strategies.

INTRODUCTION

The general hypothesis involving the induction of immune responses to TAAs[2] is that the antigens are extremely weak immunogens or functionally nonimmunogenic in the tumor-bearing host (1–5). Antitumor effects in many experimental vaccine studies have been correlated with T-cell responses to TAAs. However, this does not rule out the possibility that other immune mechanisms involving antibodies or other effector cells are also involved in antitumor effects. Nonetheless, the vast majority of experimental vaccine studies have demonstrated the role of both $CD8^+$ and $CD4^+$ T cells in antitumor responses (6–8).

Most experimental and some clinical vaccine studies to date have used various strategies to enhance T-cell responses to specific TAAs. Among these are: (*a*) the use of vectors for more efficient delivery of the TAA to the APC (9–12) and for more efficient processing with MHC molecules (13, 14); (*b*) the use of T-cell costimulation either by antibody-mediated blockade mechanisms (15, 16) or via inserting costimulatory molecules into vectors (17–19); (c) the use of cytokines to enhance either APC function or T-cell function (20–25); and (*d*) the use of diversified prime and boost vaccine strategies (26–32). Although several studies have used two or more of the above vaccine strategies, few studies, if any, have analyzed multiple strategies to determine maximum ability to activate T cells.

In previous experimental studies, we and others have demonstrated the following: (*a*) The use of recombinant orthopox vectors such as vaccinia or avipox [fowlpox and/or canarypox (ALVAC)] to enhance T-cell responses to TAAs and to induce antitumor immunity as compared to the use of the TAA protein in adjuvant (5, 27). (*b*) Diversified vaccine prime and boost regimens were advantageous in enhancing T-cell responses. A rV vector cannot be used multiple times because of the induction of host immunity to the virus (33–36). However, it has been shown that a primary vaccination with a rV vector followed by booster vaccinations with recombinant avipox viruses leads to optimal induction of T-cell responses (32). In these studies and others, it has been shown that the use of two different recombinant vectors, in prime and boost strategies, can be more potent in inducing T-cell responses than the continued use of one single vector. (*c*) The insertion of genes for a costimulatory molecule in vectors also containing the gene for a TAA enhances T-cell responses to the TAA. Moreover, the use of rV or rF vectors containing a triad of costimulatory molecules (B7-1, ICAM-1, LFA-3; designated TRICOM) 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 (18). (*d*) The use of cytokines such as GM-CSF has been shown to enhance the infiltration of APC, including dendritic cells, to regional nodes and consequently enhance T-cell responses when given for 4 consecutive days following vaccine (5, 37). More recently, it has been shown that a single administration of rF-GM-CSF given with vaccine is as potent as four daily administrations of recombinant GM-CSF in enhancing T-cell responses (38). In addition, it has been previously shown that when IL-2 has been used in low doses several days following vaccination, antigen-specific T-cell responses can be further enhanced (25).

Although the vast majority of experimental vaccine studies in mouse models have used conventional mice, and the target antigen is not "self," most human TAAs identified to date have been shown to be "self-antigens," with some expression on normal adult tissues. CEA is a TAA, which is overexpressed in most carcinomas, including gastrointestinal carcinomas, and is also expressed at lower levels in normal colonic mucosa (39). The most common site of metastases of CEA-positive malignancies in patients is the liver; CEA protein is also shed into the serum of many patients with metastatic CEA-positive malignancies (39). There are several clinical studies that have now

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[1] To whom requests for reprints should be addressed, at Laboratory of Tumor Immunology and Biology, National Cancer Institute, NIH, 10 Center Drive, Room 8B09, Bethesda, MD 20892. Phone: (301) 496-4343; Fax: (301) 496-2756; E-mail address: js141c@nih.gov.

[2] The abbreviations used are: TAA, tumor-associated antigen; APC, antigen-presenting cell; TRICOM, triad of costimulatory molecules; rV, recombinant vaccinia; rF, recombinant fowlpox; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-2, interleukin 2; CEA, carcinoembryonic antigen; FP-WT, fowlpox wild type; V-WT, vaccinia WT; pfu, plaque-forming unit; ConA, concanavalin A.

demonstrated that one can elicit CEA-specific T-cell responses in patients vaccinated with CEA-based vaccines (4, 40–46). Studies have also demonstrated that the CEA-specific T cells generated are capable of lysing CEA-expressing carcinoma cells in a MHC-restricted manner (4, 41–43, 47–49). Although indications of prolonged survival and some antitumor responses have been reported (42, 43), the main issue may well be the potency of the T-cell responses that have been generated in metastatic cancer patients to achieve therapeutic responses.

Many of the experimental studies described to date have used conventional mice to demonstrate antitumor immunity. A more appropriate model would be the use of mice in which the TAA is a self-antigen, such as CEA, with expression on some normal adult tissues. CEA-transgenic mice express CEA in normal adult gastrointestinal tissues in a manner similar to that expressed in humans and also express relatively high levels of CEA protein in serum, *i.e.*, at levels seen in many patients with advanced colon carcinoma (50). Moreover, CEA-transgenic mice have previously been shown to be tolerant to the induction of CEA-specific T-cell or antibody responses following vaccination with CEA protein in adjuvant (5). One study has shown that the administration of rV-CEA can reduce the growth rate of CEA-expressing tumors transplanted s.c. (25). Like many animal models published to date (17, 20, 51–55), the antitumor effect of the vaccine was seen only when vaccine therapy was initiated within 7 days posttumor transplant; waiting longer periods of time to initiate therapy was not successful.

In the studies described here, we have used these multiple vaccine strategies in an effort to obtain high levels of T-cell responses to a target antigen, CEA. Because of the limited availability of CEA-transgenic mice to conduct all of the studies reported here, initial studies were carried out in conventional mice to define optimal parameters of vaccine strategies. CEA-transgenic mice were then used to verify the induction of immune responses specific for CEA and in tumor therapy studies in which the TAA is a self-antigen. Using the multiple vaccine strategies described above, antitumor studies were carried out in the studies described here in CEA-transgenic mice bearing 14-day experimental CEA-expressing liver metastases.

MATERIALS AND METHODS

Recombinant Poxviruses. rF viruses were constructed by the insertion of foreign sequences into the *Bam*HI J region of the genome of the POXVAC-TC (Schering-Plough, Kenilworth, NJ) strain of fowlpox virus as described elsewhere (56). The rF virus designated rF-CEA contains the human *CEA* gene under the control of the 40k promoter (57). The recombinant fowlpox virus designated rF-CEA/B7-1 contains the human *CEA* gene under the control of the 40k promoter and the murine *B7-1* gene under control of the synthetic early/late promoter (58). The rF virus containing the human *CEA* gene and the murine *B7-1*, *ICAM-1*, and *LFA-3* genes (designated rV-CEA/TRICOM) has been described previously (18). The rV virus containing the human *CEA* gene and the murine *B7-1*, *ICAM-1*, and *LFA-3* genes (designated rV-CEA/TRICOM) has been described elsewhere (18). The rF virus containing the gene for murine GM-CSF under control of the 40k promoter has been described previously (59). Nonrecombinant wild-type fowlpox virus was designated FP-WT, while nonrecombinant vaccinia virus (Wyeth strain) was designated V-WT. Drs. Panicali, Mazzara, and Gritz of Therion Biologics Corp. (Cambridge, MA) kindly provided all orthopox viruses.

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. Maintenance of experimental mice continued under the same conditions. C57BL/6 mice transgenic for human CEA mice were 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-transgenic mouse has been previously described (50). Murine colon adenocarcinoma cells expressing human CEA (MC-38-CEA-2) have been described elsewhere (60). Prior to transplantation to mice, the cells were trypsinized and washed twice in HBSS before final suspension in the same.

Vaccine Dose and Route Studies. Six- to 8-week-old female C57BL/6 mice were vaccinated i.v. or s.c. with (*a*) 100 μl of HBSS; (*b*) either 10^8 or 10^9 pfu rF-CEA; or (*c*) either 10^8 or 10^9 pfu rF-CEA/TRICOM. All viruses used here and in subsequent experiments were diluted in HBSS such that the entire dose was delivered in 100 μl. Fourteen and 28 days later, mice were boosted using identical reagents. Fourteen days following the final vaccination, CEA-specific T-cell responses were analyzed as previously described (61).

Titration of Serum Antibodies. Anti-CEA and anti-fowlpox antibody (IgG) were quantified in the serum of each animal by ELISA. Microtiter plates were coated with either purified CEA (100 ng/well), FP-WT (1×10^7 pfu/well), or ovalbumin (100 ng/well). The plates were blocked with 5% BSA in PBS, dried, and stored at −20°C until used. The plates were incubated with serum serially diluted from 1:50 to 1:6250, as well as COL-1 or rabbit antifowlpox antiserum as controls, for 24 h at 4°C. Plates were washed several times with PBS containing 1% BSA and incubated at 37°C for 45 min with horseradish peroxidase-conjugated goat antimouse IgG-specific antiserum (1:4000) and antibody was detected by a horseradish peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. The absorbance of each well was read at 490 nm using a Bio-Tek EL310 microplate ELISA reader (Winooski, VT). Antibody titers were based on an absorbance of 1.5.

Prime and Boost Studies. C57BL/6 mice were vaccinated s.c. with 10^8 pfu of either rV-CEA/TRICOM or rF-CEA/TRICOM. Fourteen days later, mice were boosted with 10^8 pfu of rF-CEA/TRICOM. In a parallel experiment, mice were vaccinated with 10^8 pfu V-WT 14 days prior to beginning the above described vaccination strategies. Fourteen days following the final vaccination, CEA-specific T-cell responses were analyzed as described above.

rF-GM-CSF Studies. C57BL/6 mice were vaccinated s.c. with 10^8 pfu of either rF-CEA or rF-CEA/TRICOM alone or admixed with 10^7 pfu of rF-GM-CSF. Fourteen days later, CEA-specific T-cell responses were analyzed as described above.

Tumor Therapy Studies. CEA-transgenic mice were transplanted with MC38-CEA-2 cells as experimental hepatic metastases. The procedure for transplantation of hepatic metastases has been described (62). This dose of tumor cells (50,000) is lethal to >80% of mice within 12 weeks. Fourteen days following tumor transplant, mice were vaccinated as described in the legends to Figs. 6 and 7. The therapy studies were conducted twice with similar results, and the data shown in Fig. 7 are a composite of the results of both experiments.

In an additional study, CEA-transgenic mice were vaccinated in an identical manner but did not receive a tumor transplant. Fourteen days following the final vaccination, spleens were harvested and analyzed for CEA-specific lymphoproliferation as described above. To evaluate $CD8^+$ T-cell responses, spleens from three animals per group were removed, dispersed into single-cell suspensions, pooled, and coincubated with 10 μg/ml of the H-2^b-restricted peptide CAP-M8 ($CEA_{526-533}$, EIQNTTYL) (5, 63) for 6 days. Bulk lymphocytes were recovered by centrifugation through a Ficoll-Hypaque gradient and restimulated with fresh irradiated naive splenocytes and 10 μg/ml of either $CEA_{526-533}$ or VSVN (vesicular stomatitis virus N_{52-59}, RGYVYQGL) (64) peptide for 24 h. Supernatant fluids were collected and analyzed for murine IFN-γ by capture ELISA as described previously (65).

Statistical Analysis of the Data. Where indicated, the results of tests of significance are reported as *P* and are derived from Student's *t* test using a two-tailed distribution. Evaluation of survival patterns in mice bearing hepatic tumors was performed by the Kaplan-Meier method and ranked according to the Mantel-Cox log rank test using a Statview 4.1 (Abacus Concepts, Berkeley, CA) software package. For graphical representation of data, *y*-axis error bars indicate the SD of the data for each point on the graph. The *y*-axis error bars in Fig. 3 are reported as SE because the data are averaged from three individual mice analyzed in triplicate.

RESULTS

Initial studies involved the influence of the route of administration of rF vectors. The vector used was rF-CEA/TRICOM; control vector

FP-WT was used in all studies. Two weeks after vaccination via the s.c. or i.v. route, T cells were analyzed for reactivity to CEA and negative control protein ovalbumin using the T-cell lymphoproliferative assay described in "Materials and Methods." As seen in Fig. 1*A*, the administration of rF-CEA/TRICOM was clearly more effective in inducing CEA-specific proliferative T-cell responses when given s.c. than i.v. ($P = 0.026$). No immune responses were seen using control vector FP-WT, and no immune responses were observed with any of the vectors to ovalbumin control protein; these studies were conducted at 10^8 pfu vector per vaccination. Studies were also conducted using 10^9 pfu vector per vaccination (Fig. 1*B*). Here again a distinct and significant difference was seen between s.c. and i.v. vaccination, with s.c. vaccination being far superior ($P = 0.002$). As an additional control, splenocytes from mice vaccinated with rF-CEA/TRICOM or FP-WT were also analyzed for responses to ConA. As seen in Fig. 1*C*, there was a reduction in responses to ConA in mice receiving fowlpox vectors via the i.v. route. Although the reason for this effect of i.v. administered fowlpox is not known at this time, it was clear that subsequent studies should be carried out via the s.c. vaccination route.

Studies were then undertaken to determine the relative potency of different avipox (fowlpox) recombinant vectors. The vectors used were rF-CEA, rF-CEA/B7-1, or rF-CEA/TRICOM. FP-WT was used as a control virus. In previous studies, it was shown that one vaccination with a recombinant vaccinia virus expressing TRICOM was superior to one vaccination with rV-CEA or rV-CEA/B7-1 (18). However, it was not known whether this phenomenon would also hold for fowlpox vectors and for multiple vaccinations with these vectors, *i.e.*, would four vaccinations of rF-CEA be as potent or more potent than one vaccination with rF-CEA/TRICOM? To answer these questions, five groups of mice were vaccinated with one of the following: HBSS buffer (four vaccinations), FP-WT (four vaccinations), rF-CEA (four vaccinations), rF-CEA/B7-1 (one, two, three, or four vaccinations), or rF-CEA/TRICOM (one, two, three, or four vaccinations). Two weeks following vaccination, splenocytes of vaccinated mice were removed and analyzed for CEA-specific T-cell responses and for anti-CEA antibody responses. As seen in Fig. 2, three vaccinations with rF-CEA/B7-1 were clearly more potent ($P = 0.01$) than four vaccinations with rF-CEA in the induction of CEA-specific T-cell responses. Moreover, three vaccinations with rF-CEA/TRICOM were also more potent than three vaccinations with rF-CEA/B7-1 ($P = 0.03$) or four vaccinations with rF-CEA ($P = 0.004$) in the induction of CEA-specific T-cell responses. These studies also demonstrated that with consecutive vaccinations of rF-CEA/TRICOM CEA-specific T-cell responses continued to increase. There was no evidence of limited use of the fowlpox recombinant vector because of host antivector immune responses. This will be discussed in more detail in "Discussion." Anti-CEA IgG responses were also measured in these experiments. As seen in Fig. 3, anti-CEA IgG responses also increased with subsequent vaccinations of either rF-CEA/B7-1 or

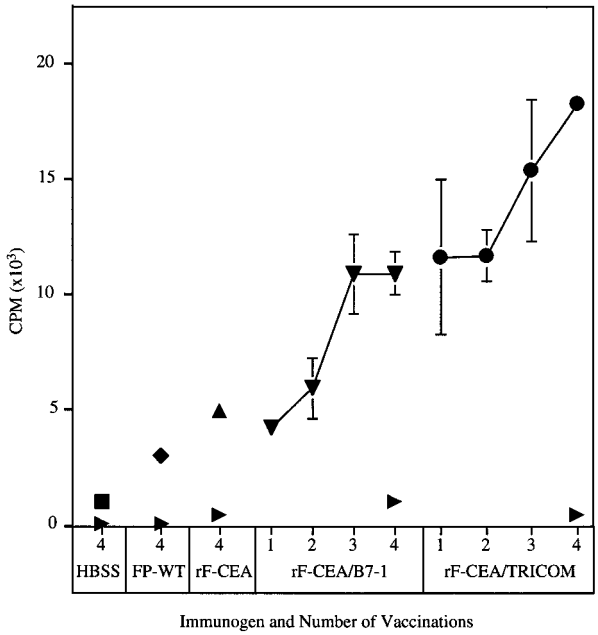

Fig. 2. Lymphoproliferation of splenic T cells in response to CEA protein following multiple boosts with rF-CEA vectors. Mice were vaccinated s.c. one, two, three, or four times at 2-week intervals as indicated. All vaccinations were at 10^8 pfu as follows: FP-WT (♦), rF-CEA (▲), rF-CEA/B7-1 (▼), rF-CEA/TRICOM (●), or HBSS buffer (■). Two weeks after the last vaccination, purified splenic T cells were tested for reactivity to CEA protein in an *in vitro* lymphoproliferation assay using naive splenocytes as APC and CEA protein as antigen. Proliferation in response to CEA was measured by [^{3}H]thymidine incorporation. Proliferation in response to the negative control protein ovalbumin is indicated by ▶. *Error bars*, SDs based on the mean of triplicate wells on 96-well culture plates. *Error bars* are obscured by the symbols for response to ovalbumin protein (▶), and response to CEA protein in mice receiving HBSS (■), FP-WT (♦), or four vaccinations of rF-CEA (▲).

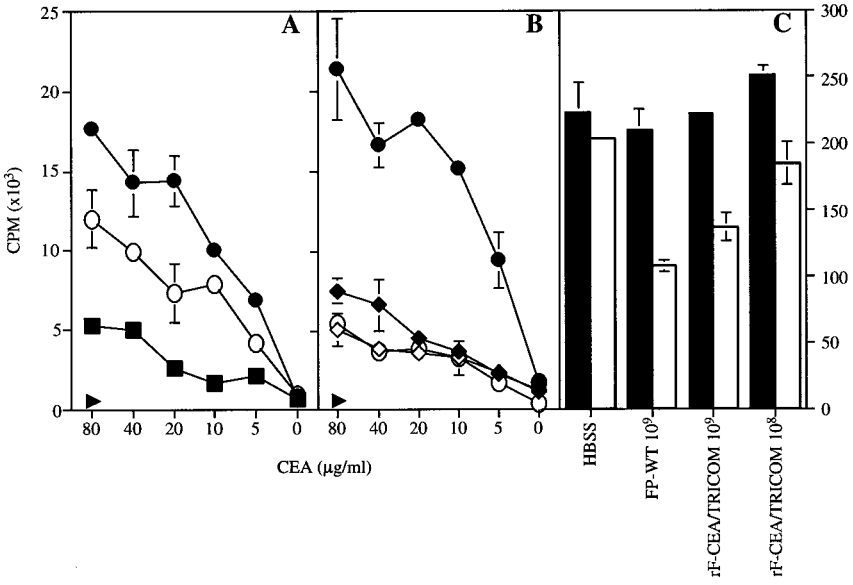

Fig. 1. Lymphoproliferation of splenic T cells in response to CEA protein following vaccination with rF-CEA/TRICOM administered i.v. or s.c. at two doses. Mice were vaccinated with 10^8 (*A*) or 10^9 (*B*) rF-CEA/TRICOM by the i.v. route (○) or s.c. route (●) three times at 2-week intervals. Two weeks after the last injection, purified splenic T cells were tested in an *in vitro* lymphoproliferation assay using naive splenocytes as APC and CEA protein or ovalbumin protein as antigen. Proliferation in response to CEA antigen was measured by [^{3}H]thymidine incorporation and proliferation was graphed as a function of cpm *versus* protein concentration. Proliferation in response to three i.v. injections of HBSS buffer (*A*, ■) or 10^9 FP-WT control vector (*B*, i.v. route, ◇; s.c. route, ♦) is also shown. Proliferation of all groups in response to the negative control protein ovalbumin (80 μg/ml) is indicated in *A* and *B* by ▶. Proliferation in response to the T-cell mitogen ConA is shown in *C*. Equal numbers of T cells from mice vaccinated as described were cultured with 2.5 μg/ml ConA and naive splenocytes as APC. Proliferation of T cells from mice vaccinated by the s.c. route is shown using ■, and proliferation of T cells from mice vaccinated by the i.v. route is shown using □. *Error bars* (*A–C*), SDs based on the mean of triplicate wells on 96-well culture plates.

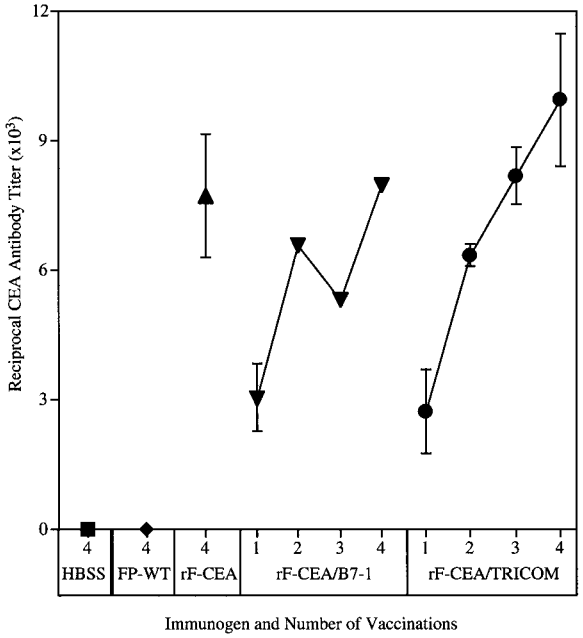

Fig. 3. Induction of CEA-specific humoral immunity following vaccination with fowlpox CEA vectors. Mice were vaccinated s.c. one, two, three, or four times at 2-week intervals as indicated. All vaccinations were at 10^8 pfu as follows: FP-WT (♦), rF-CEA (▲), rF-CEA/B7-1 (▼), rF-CEA/TRICOM (●), or HBSS buffer (■). Two weeks after the last vaccination, serum samples were analyzed for CEA-specific IgG by ELISA as outlined in "Materials and Methods." These data are represented as reciprocal antibody titers corresponding to absorbance of 1.5 at an absorbance of 490 nm as determined by ELISA. *Error bars*, SE of data generated from three individually analyzed mice.

rF-CEA/TRICOM. These studies further demonstrated the potential for multiple vaccinations of rF vectors. Note that the use of costimulatory molecules (B7-1 or TRICOM) did not significantly increase anti-CEA IgG responses as they did CEA-specific T-cell responses.

Previous studies have shown that diversified prime and boost vaccine strategies are more effective in enhancing T-cell responses as compared to the continued use of one immunogen (32). It has been previously shown that rV viruses can be used only one or two times because of the generation of host antivaccinia immune responses (33, 34). On the other hand, the question was raised as to the necessity for the use of diversified vaccination strategies when vectors contain multiple costimulatory molecules. To address this, mice were vaccinated at biweekly intervals with either two vaccinations of rF-CEA/TRICOM or priming with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM. Two weeks following the last vaccination, splenocytes of vaccinated animals were analyzed for CEA-specific T-cell responses. As seen in Fig. 4*A*, using rV-CEA/TRICOM as a prime vaccination followed by a boost with rF-CEA/TRICOM was significantly more potent in inducing CEA-specific T-cell responses than priming and boosting with rF-CEA/TRICOM ($P = 0.048$). This study was also carried out in mice that had been vaccinated 2 weeks earlier with wild-type vaccinia virus and shown to contain high levels of antivaccinia antibody (32). This scenario mimicked that commonly observed in the clinical situation in which most adults exhibit preexisting antivaccinia immunity as a result of prior smallpox vaccination. As seen in Fig. 4*B*, the diversified prime and boost strategy of priming with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM remained significantly more potent in inducing T-cell responses than the priming and boosting with rF-CEA/TRICOM ($P = 0.0415$). Thus, even when using vectors containing multiple costimulatory molecules, diversified prime and boost strategies appeared to be advantageous.

It has previously been shown that recombinant GM-CSF enhances the infiltration of dendritic cells to regional nodes, and when recombinant GM-CSF is given with a vaccine, antigen-specific T-cell responses to the vaccine can be enhanced (37). Recent studies have demonstrated that the use of a rF vector containing the transgene for GM-CSF (designated rF-GM-CSF) also enhances the level of APC and dendritic cells in regional nodes and has been shown to enhance antigen-specific T-cell responses when given with a recombinant vaccine (59). Moreover, it has been shown that a single administration of rF-GM-CSF is as potent as four daily administrations of recombinant GM-CSF in enhancing both the number of and duration of dendritic cells in regional nodes. Experiments were conducted to determine whether rF-GM-CSF was required when using vectors containing three costimulatory molecules. Mice were vaccinated with rF-CEA, rF-CEA/TRICOM, or FP-WT, all with or without rF-GM-CSF being admixed in the vaccine. Two weeks later, splenocytes of vaccinated mice were removed and analyzed for CEA-specific T-cell responses using a lymphoproliferation assay. As seen in Fig. 5, rF-GM-CSF had no effect on the induction of CEA-specific T-cell responses when administered with FP-WT. rF-GM-CSF showed a marked and significant enhancement of CEA-specific T-cell responses when used with rF-CEA ($P = 0.015$). This level of CEA-specific T-cell responses was similar to that achieved using the rF-CEA/TRICOM vector alone. When rF-CEA/TRICOM was used in combination with rF-GM-CSF, however, a marked difference was seen as compared to the use of rF-CEA/TRICOM alone in the induction of CEA-specific T-cell responses. As seen in Fig. 5 (*inset*, *A*), no differences in responses to ConA for any of the groups with or without rF-GM-CSF were observed. Moreover, no responses were seen in any of the groups to the negative control antigen ovalbumin.

The above studies were conducted in conventional C57BL/6 mice and not CEA-transgenic mice because of the lack of sufficient numbers of CEA-transgenic mice. Subsequent studies used the knowledge gained from the experiments described above toward antitumor therapy studies in a CEA-transgenic mouse model. CEA-transgenic mice have previously been shown to be tolerant to CEA when CEA protein in adjuvant was used as an immunogen (5). Therefore, our initial

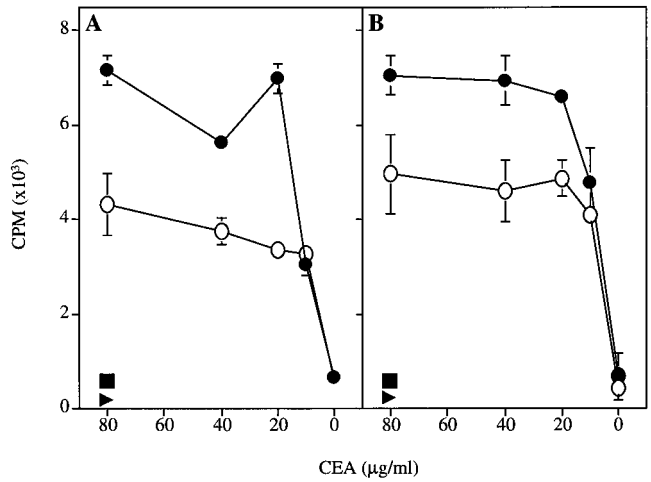

Fig. 4. Lymphoproliferation of splenic T cells in response to CEA protein following heterologous prime and boost with recombinant CEA vectors. Naive mice (*A*) or mice that had been prevaccinated with wild-type vaccinia virus 14 days prior (*B*) were used. Mice received either two vaccinations (prime and boost) with rF-CEA/TRICOM (○) or a prime vaccination with rV-CEA/TRCOM and a boost with rF-CEA/TRICOM (●). All vaccinations were at 10^8 pfu/mouse s.c. with a 2-week interval between prime and boost. Two weeks following the boost, purified splenic T cells were tested for reactivity to CEA protein in an *in vitro* lymphoproliferation assay using naive splenocytes as APC and whole CEA protein as antigen. Proliferation in response to CEA was measured by [^{3}H]thymidine incorporation. Proliferation in response to CEA protein (80 μg/ml) following vaccination with HBSS buffer is indicated in *A* and *B* by ■. Proliferation in response to the negative control protein ovalbumin (80 μg/ml) is indicated in *A* and *B* by ▶. *Error bars*, SDs based on the mean of triplicate wells on 96-well culture plates.

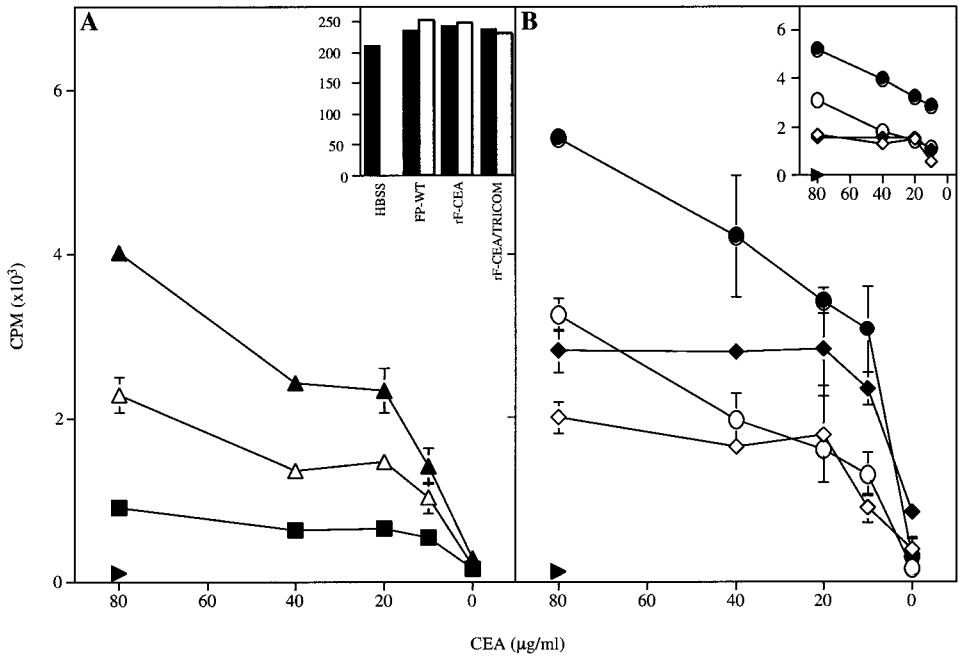

Fig. 5. Lymphoproliferation in response to CEA vaccines admixed with rF-GM-CSF. *A*, two groups of mice were vaccinated with rF-CEA. In one group, the rF-CEA vector was admixed with rF-GM-CSF (▲). The other group received only rF-CEA (△). Control mice were injected with only HBSS buffer (■). Proliferation in response to the control protein ovalbumin is shown by ►. *Inset* in *A*, lymphoproliferation of T cells from vaccinated mice in response to the T-cell mitogen ConA. Equal numbers of T cells from mice vaccinated as described were cultured with 2.5 μg/ml ConA and naive splenocytes as APC. Proliferation of T cells from mice vaccinated without rF-GM-CSF is shown using ■, and proliferation of T cells from mice vaccinated with the admixture of vector and rF-GM-CSF is shown using □. *B*, two groups of mice were vaccinated with rF-CEA/TRICOM. In one group, the rF-CEA/TRICOM vector was admixed with rF-GM-CSF (●). The other group received only rF-CEA/TRICOM (○). Control mice received injections of FP-WT. In one control group, the FP-WT vector was admixed with rF-GM-CSF (♦). The other control group received only FP-WT vector (◇). Proliferation in response to the control protein ovalbumin is shown by ►. The data in the *inset* of *B* is identical to the graph in *B* with the exception that it is graphed as a function of Δcpm (experimental cpm − cpm in response to media only) *versus* protein concentration. rF-CEA vectors and FP-WT vectors were used at 10^8 pfu/mouse and rF-GM-CSF was used at 10^7 pfu/mouse. Two weeks postvaccination, purified splenic T cells were tested for reactivity to either CEA protein (80 to 10 μg/ml) or ovalbumin (80 μg/ml) in an *in vitro* lymphoproliferation assay using naive splenocytes as APC. Proliferation was measured by [^{3}H]thymidine incorporation. *Error bars*, SDs based on the mean of triplicate wells on 96-well culture plates.

studies were carried out to determine whether the vaccine regimen determined to be optimal in conventional mice resulted in the generation of enhanced CEA-specific T-cell responses in CEA-transgenic mice. Three groups of mice were used in these studies. Group 1 received a primary vaccination of rV-CEA/TRICOM followed by three weekly booster vaccinations with rF-CEA/TRICOM. This group also received recombinant GM-CSF protein with the rV-CEA/TRICOM vaccination and rF-GM-CSF with rF-CEA/TRICOM; group 1 also received low-dose systemic IL-2. Group 2 received treatment identical to group 1, except that the mice received control vector V-WT as the primary vaccination and control vector FP-WT for the subsequent three booster vaccinations. Group 2 also received GM-CSF and IL-2 in a manner identical to group 1. Group 3 received no treatment. The reason for the use of recombinant GM-CSF protein with vaccinia vectors in groups 1 and 2 will be discussed in "Discussion." Fourteen days following the last vaccination, splenocytes were analyzed for CEA-specific immune responses. $CD8^+$ responses were measured using the CEA 8-mer peptide $CEA_{526-533}$ and IFN-γ production was measured. A control peptide (VSV-N) was also used. As seen in Fig. 6*A*, $CD8^+$ CEA-specific responses could be observed in the CEA-transgenic mice receiving the recombinant CEA/TRICOM vaccines (group 1) which were significantly greater than those observed for the vector treatment group (group 2; $P = 0.001$) or the untreated group (group 3; $P = 0.001$). $CD4^+$ CEA-specific responses were also observed in the group receiving the CEA/TRICOM vaccines and were significantly greater than those in the group receiving control wild-type vector vaccines with cytokines ($P = 0.0013$; Fig. 6*B*). No significant responses were observed to the control ovalbumin protein.

Concomitant to this study, a tumor therapy study was initiated to determine the therapeutic efficacy of a CEA-based vaccine in CEA-transgenic mice. In these studies, MC-38 murine colon carcinoma cells, which had been transduced with the *CEA* gene using a retroviral vector, were inoculated intrasplenically into CEA-transgenic mice. After dissemination of the cells into the liver through the portal vein, mice were then splenectomized; previous studies have shown that large hepatic metastases develop in similar models at 4–5 weeks posttumor transplant (66, 67). Mice also developed large experimental liver metastases in the model used here that were lethal in >80% of mice by 12 weeks posttransplant if left untreated. Three groups were used in these tumor therapy studies. Mice in all groups received tumor at day 0. Fourteen days after tumor transplant, vaccination was initiated in groups 1 and 2. Group 1 received a primary vaccination of rV-CEA/TRICOM followed by three weekly booster vaccinations with rF-CEA/TRICOM. This group also received recombinant GM-CSF protein with the rV-CEA/TRICOM vaccination and rF-GM-CSF with rF-CEA/TRICOM; group 1 also received low-dose systemic IL-2. Group 2 received treatment identical to group 1, except that the mice received V-WT as the primary vaccination and FP-WT for the subsequent three booster vaccinations. Group 2 also received GM-CSF and IL-2 in a manner identical to group 1. Group 3 received no treatment following tumor transplant.

As can be seen in Fig. 7, 56.3% (9 of 16) of the mice in group 1 receiving the recombinant CEA vaccines and cytokines remained alive and apparently healthy through the 25-week observation period. However, only 5.3% (1 of 19) of the mice that received control vectors along with cytokine treatment (group 2) survived past 16 weeks. Only 8.3% (1 of 12) of the mice in the group receiving no treatment (group 3) survived past 16 weeks. The *P* for survival between mice receiving no treatment and treatment with control

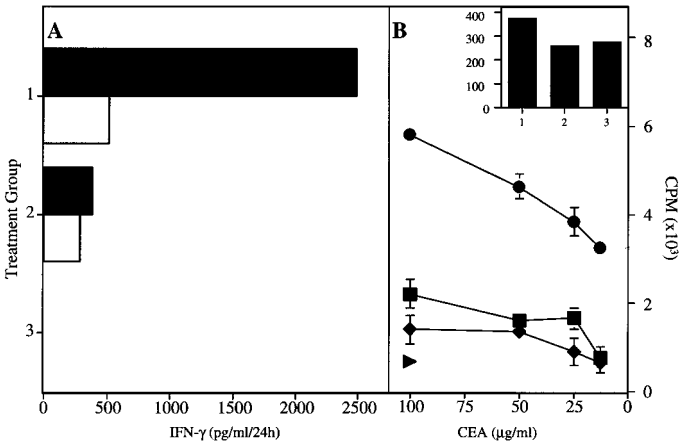

Fig. 6. Immune responses to CEA vaccines in CEA-transgenic mice. CEA-transgenic mice were divided into three vaccination groups: group 1 received a rV-CEA/TRICOM prime vaccination with recombinant GM-CSF followed by three weekly boosts with rF-CEA/TRICOM admixed with rF-GM-CSF. IL-2 was given 4 consecutive days beginning on the day of vaccination as described in "Materials and Methods." Group 2 received a V-WT prime vaccination with recombinant GM-CSF followed by three weekly boosts with FP-WT admixed with rF-GM-CSF. Low-dose IL-2 was given as in group 1. Group 3 received only HBSS buffer injections. *In vitro* assays were performed 2 weeks after the last vaccination. Each group contains splenic T cells pooled from three mice. *A*, IFN-γ production in response to the 8-mer $CEA_{526-533}$ peptide. Splenic T cells from vaccinated mice were bulk-cultured for 1 week with 10 μg/ml $CEA_{526-533}$ peptide. T cells were purified and restimulated an additional 24 h with $CEA_{526-533}$ (■) or VSV-N (□) peptides. Culture supernatants were assayed for IFN-γ production by ELISA. *B*, lymphoproliferation of splenic T cells in response to CEA protein. Group 1 is indicated by ●, group 2 by ◆, and group 3 by ■. Purified splenic T cells were tested for reactivity to CEA protein in an *in vitro* lymphoproliferative assay using naive splenocytes as APC and whole CEA protein as antigen. Proliferation in response to CEA was measured by [^{3}H]thymidine incorporation. Proliferation in response to the negative control protein ovalbumin (80 μg/ml) is indicated by ▶ in *B*, proliferation in response to the T-cell mitogen ConA is shown in the *inset*. Equal numbers of T cells from mice vaccinated as described were cultured with 2.5 μg/ml ConA and naive splenocytes as APC. Proliferation of T cells from all groups in response to ConA was measured by [^{3}H]thymidine incorporation and graphed as cpm. Recombinant CEA/TRICOM and control vectors were given s.c. and used at 10^8 pfu/mouse/injection. Twenty micrograms of recombinant GM-CSF were injected once a day for 4 consecutive days following the prime vaccination in groups 1 and 2. Recombinant IL-2 (16,000 IU) was given by the i.p. route every 12 h for 4 days following each vaccination in groups 1 and 2. rF-GM-CSF was used at 10^7 pfu/mouse/injection and was admixed with the fowlpox vectors in groups 1 and 2. *Error bars*, SDs based on the mean of triplicate wells on 96-well culture plates.

vectors plus cytokines was not significant ($P = 0.43$). The P for the survival of mice receiving CEA-TRICOM vaccines plus cytokines (group 1) *versus* the untreated group was statistically significant ($P = 0.015$). Furthermore, survival of mice receiving the CEA-TRICOM vectors plus cytokines was statistically significant as compared with survival of mice receiving the control vectors plus cytokines ($P = 0.026$).

DISCUSSION

Although numerous studies have demonstrated the advantage of one or two vaccine strategies in enhancing antigen-specific T-cell responses, few have sequentially evaluated multiple strategies. It should be pointed out that the initial analyses of these strategies were conducted in conventional mice and not in the more appropriate transgenic mice model where the TAA is clearly a self-antigen. The lack of sufficient numbers of CEA-transgenic mice to carry out all of the variables analyzed prohibited the use of transgenic mice for the initial studies. Previous studies, however, have shown that both conventional C57BL/6 and CEA-transgenic mice on the C57BL/6 background respond to a rV-CEA vaccine, but the T-cell response and antibody response to CEA is approximately five times more vigorous in the conventional C57BL/6 mice than in CEA-transgenic mice (5). The CEA-transgenic mouse, however, was used for both antitumor therapy studies and to demonstrate the induction of both $CD8^+$ and $CD4^+$ responses to CEA. The use of conventional mice to analyze these different strategies is also appropriate when one considers that these strategies can be used for vaccines for non-self-determinants such as point-mutated oncogenes and for vaccines for infectious disease agents.

The animal model used here for antitumor therapy employed a murine colon carcinoma cell expressing CEA to induce experimental liver metastases in which 14 days posttumor transplant vaccine therapy was initiated. The vast majority of tumor therapy models to date have initiated vaccine therapy 3–5 days posttumor transplant (14, 17, 31, 51, 68). To our knowledge, this is the longest time interval between tumor transplant and vaccine therapy for a tumor therapy model in which the vaccine is directed against a self-antigen. The fact that only half of the animals remained tumor free during the 23-week observation period, however, affirms the need for further studies to improve antitumor responses. These studies will involve the mechanisms of antitumor immunity as well as potential tumor escape mechanisms. More specifically, these studies will deal with histochemical and immunohistochemical analyses of the tumor site to identify both the type and quantity of effector cells and cell subsets present within the tumor infiltrate. Adoptive transfer of effector cell subsets and/or antibody-blocking experiments will best define the mechanisms of antitumor responses. The study of tumor escape mechanisms will include analysis of tumors that have escaped vaccine therapy for possible antigen loss variants, possible down-regulation of MHC molecules on tumor cells, and Fas-FasL interactions. Mice bearing tumors that have escaped vaccine therapy will also be analyzed for the presence of T cells displaying down-regulation of the ζ chain of the T-cell receptor. The studies reported here do demonstrate, however, that both $CD4^+$ and $CD8^+$ responses specific for CEA were generated in vaccinated CEA-transgenic mice.

One of the issues that also needs to be resolved in future studies is that of pathological autoimmunity as a result of the use of vectors containing a self-antigen and costimulatory molecules. In a previous study, it was shown that repeated vaccinations with rV-B7-1 did not induce any evidence of autoimmunity (69). In recent toxicology studies, no evidence of autoimmunity, as analyzed by histochemistry, altered blood chemistry, or antinuclear antibody, was observed when CEA-transgenic mice received four vaccinations of rV-CEA/TRICOM or rF-CEA/TRICOM vectors. It is unclear at this time, however, if the more potent vaccine strategies used in the studies reported here in tumor-bearing animals would lead to any evidence of pathological autoimmunity. Long-term studies using CEA-transgenic mice will be required to answer this question; at the 23-week observation period in the studies reported here (Fig. 7), however, such mice exhibited normal body weight gain and appeared otherwise healthy.

The studies reported here demonstrated that rF-CEA and rF-CEA/TRICOM induced more potent T-cell responses when given s.c. than when administered i.v. It should also be pointed out that the i.v. dosing also reduced the ability of splenic T cells to respond to ConA mitogen. The reason for this is unclear at this time. This was seen not only with the rF-CEA and rF-CEA/TRICOM vectors, but also with mice receiving FP-WT vector via the i.v. route. Although it has been reported that the i.v. administration of rF-β-galactosidase was effective in treating 3-day lung metastases expressing β-galactosidase in conventional mice (51, 68), no comparison of i.v. *versus* s.c. administration was reported in those studies and it should be noted that much lower doses (10^6 pfu) were used in those experiments. Additionally, in a separate study, the observation was made that the immune response to antigen administered by the i.v. route could not be augmented by the use of GM-CSF (70). It is apparent from the data presented here and in other studies that the local administration of

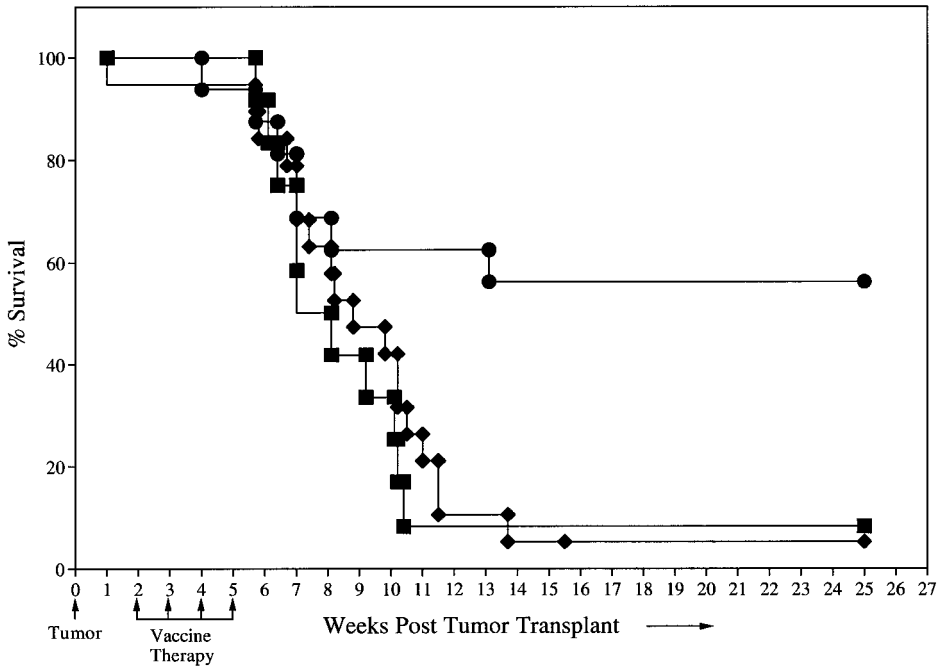

Fig. 7. Induction of antitumor responses by CEA-TRICOM vectors. CEA-transgenic mice bearing 14-day established hepatic metastases were divided into three treatment groups. Tumors were induced by intrasplenic injection of MC-38 colon carcinoma cells that were transduced with CEA. Group 1 (n = 16, ●) received 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. Group 2 (n = 19, ◆) received a V-WT prime vaccination with recombinant GM-CSF and low-dose IL-2 followed by three weekly boosts with FP-WT admixed with rF-GM-CSF and low-dose IL-2. Group 3 (n = 12, ■) received only HBSS buffer injections. Mice in each group were monitored weekly for survival. Recombinant CEA/TRICOM vectors were given s.c. and used at 10^8 pfu/mouse/injection. Twenty micrograms of recombinant GM-CSF were injected once a day for 4 consecutive days following the prime vaccination in groups 1 and 2. Recombinant IL-2 (16,000 IU) was given by the i.p. route every 12 h for 4 days following each vaccination in groups 1 and 2. rF-GM-CSF was used at 10^7 pfu/mouse/injection and was admixed with the fowlpox vectors in groups 1 and 2.

recombinant GM-CSF protein (24, 37, 59) or the rF vector rF-GM-CSF (38) enhances immune responses to s.c. injected antigen. Whereas the i.v. administration of vaccines may represent a viable alternative to the s.c. route, the use of locally acting cytokines such as GM-CSF cannot be used in those vaccine protocols to enhance the immune response. To our knowledge, the studies reported here are the first comparison of the use of fowlpox vectors by these routes and should discourage the administration of high doses of this vector by the i.v. route.

A previous study has demonstrated that one dose of rV-CEA/TRICOM was superior to one dose of rV-CEA/B7-1 or rV-CEA in inducing T-cell responses (32). In the experiments reported here (Fig. 2), it was shown that one vaccination of rF-CEA/TRICOM elicits greater T-cell responses than even four vaccinations with either rF-CEA or one or two vaccinations with rF-CEA/B7-1. Moreover, with repeated administration of rF-CEA/TRICOM, CEA-specific T-cell responses as well as CEA-specific antibody responses continued to increase (Figs. 2 and 3). This is most likely attributable to the fact that avipox viruses, which are replication competent in avian species, are defective for replication in mammalian cells. The CEA and TRICOM transgenes inserted into the avipox vector were done so under the transcriptional control of early pox virus promoters, while most structural viral proteins are under the control of late viral promoters (71, 72). Indeed, in the studies conducted here, antifowlpox virus antibody was induced after each rF vector vaccination (data not shown), but as depicted in the data in Figs. 2 and 3, there is little if any evidence of virus-neutralizing activity. These results support previous findings that avipox recombinant vectors can be given repeatedly to increase antigen-specific T cell and antibody responses. This was observed in both a murine model as reported here and recently in clinical studies (42).

Previous studies have shown that rV viruses can be given no more than one or two times in mice or humans that are vaccinia immune. Several groups have now demonstrated the validity of diversified vaccination protocols where two different immunogens are superior to the repeated use of one immunogen (26, 29, 32). It was unclear, however, whether this diversified vaccination scheme would be necessary with vectors containing multiple costimulatory molecules that are capable of enhancing T-cell responses to far greater levels than vectors devoid of such molecules. Fig. 4 demonstrates that a prime with rV-CEA/TRICOM followed by a boost with rF-CEA/TRICOM was superior to the repeated use of rF-CEA/TRICOM alone. This was demonstrated in both vaccinia naive and vaccinia immune mice. In recent clinical trials (42), patients receiving a primary vaccination with rV-CEA and up to eight avipox-CEA booster vaccinations showed increasing precursor frequencies of CEA-specific T cells with each vaccination. These data were obtained using an enzyme-linked immunospot assay in which the cells of patients were only incubated with CEA peptide overnight, to rule out the possibility of artifacts attributable to *in vitro* stimulation cycles.

Another concept examined in the studies reported here is the use of the cytokine GM-CSF as a biological adjuvant. Recombinant GM-CSF has been used in numerous vaccine protocols in both experimental and clinical studies to demonstrate enhanced T-cell responses to antigens. The vast majority of these studies have administered recombinant GM-CSF on the day of vaccine and for 3 or 4 consecutive days following vaccination. Recent studies have shown that a single administration of rF-GM-CSF enhances APCs and dendritic cells in regional nodes to levels greater than those achieved with four daily administrations of recombinant GM-CSF (38). Studies were conducted here to determine whether rF-GM-CSF could induce greater T-cell responses in combination with rF-CEA/TRICOM. The results demonstrate (Fig. 5) an additive effect in the use of a rF-TRICOM vector, and that of a rF-GM-CSF vector, in inducing systemic antigen-specific T-cell responses. Recombinant GM-CSF was used in the primary vaccination with rV-CEA/TRICOM in the antitumor studies reported here to alleviate the concern that the replication competent rV-CEA/TRICOM would lyse cells infected with rF-GM-CSF and thus limit the paracrine effect of the expressed GM-CSF over the 2 or 3 weeks that fowlpox vectors have been shown to express their transgene. Additional studies will be required, and are in progress, to determine the optimal use of the rF-GM-CSF vector with a range of different types of immunogens, *i.e.*, peptides, proteins, and various vectors.

It should be noted, though, that if rF-GM-CSF is used in conjunction with poxvirus vaccines, it may be more accurate to graph the

lymphoproliferative response as a function of Δcpm (experimental cpm − cpm of the media control) rather than cpm. As seen in Fig. 5*B*, the FP-WT vector, when admixed with rF-GM-CSF, appears to induce a CEA-specific T-cell proliferative response that is equal to or greater than rF-CEA/TRICOM used alone. Even in the absence of antigen, T cells from FP-WT-vaccinated mice proliferate to some degree if rF-GM-CSF is included in the vaccine. When the background proliferation is subtracted and the data graphed as Δcpm (*inset*, *B*), the response to antigen (CEA) in the proliferation assay is specific for those mice receiving rF-CEA/TRICOM. This phenomenon should thus be considered if *in vitro* lymphoproliferation is used as a readout for vaccine efficacy where rF-GM-CSF is used.

Low-dose IL-2 was also used in these studies with CEA-transgenic mice because it has been previously shown in several vaccine studies (14, 22, 23, 25) to enhance T-cell responses. It should be pointed out, however, that the induction of antitumor immunity as depicted in Fig. 7 and the CEA-specific $CD4^+$ and $CD8^+$ responses depicted in Fig. 6 were dependent on inclusion of rF-CEA/TRICOM in the vaccine regimen. No antitumor effect or CEA-specific T-cell responses were observed in mice receiving only control vectors, rF-GM-CSF, and low-dose IL-2. In fact, the survival rates and T-cell responses did not significantly differ from those of the control mice receiving only buffer.

Although the studies reported here demonstrate that the repeated use of rF-CEA/TRICOM is more potent in inducing CEA-specific T-cell responses than the repeated use of rF-CEA or rF-CEA/B7-1, they also demonstrate that vaccine strategies such as the use of diversified prime and boost regimens can also be used along with these vectors to further amplify T-cell responses. Although CEA was used as an antigen in these studies, there is no reason to believe that the strategies reported here cannot be used with other antigens associated with infectious diseases or malignancies. Moreover, it is hoped that the studies reported here will work toward a more rational design of vaccine-based clinical trial strategies.

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REFERENCES

1. Restifo, N. P., Esquivel, F., Kawakami, Y., Yewdell, J. W., Mule, J. J., Rosenberg, S. A., and Bennink, J. R. Identification of human cancers deficient in antigen processing. J. Exp. Med., *177:* 265–272, 1993.
2. Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., Pardoll, D., and Levitsky, H. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. Proc. Natl. Acad. Sci. USA, *95:* 1178–1183, 1998.
3. Shrikant, P., and Mescher, M. F. Control of syngeneic tumor growth by activation of $CD8^+$ T cells: efficacy is limited by migration away from the site and induction of nonresponsiveness. J. Immunol., *162:* 2858–2866, 1999.
4. Zhu, M. Z., Marshall, J., Cole, D., Schlom, J., and Tsang, K. Y. Specific cytolytic T-cell responses to human CEA from patients immunized with recombinant avipox-CEA vaccine. Clin. Cancer Res., *6:* 24–33, 2000.
5. Kass, E., Schlom, J., Thompson, J., Guadagni, F., Graziano, P., and Greiner, J. W. Induction of protective host immunity to carcinoembryonic antigen (CEA), a self-antigen in CEA transgenic mice, by immunizing with a recombinant vaccinia-CEA virus. Cancer Res., *59:* 676–683, 1999.
6. Yang, S., Vervaert, C. E., Burch, Jr., J., Grichnik, J., Seigler, H. F., and Darrow, T. L. Murine dendritic cells transfected with human GP100 elicit both antigen-specific $CD8^+$ and $CD4^+$ T-cell responses and are more effective than DNA vaccines at generating anti-tumor immunity. Int. J. Cancer, *83:* 532–540, 1999.
7. Abrams, S. I., Hodge, J. W., McLaughlin, J. P., Steinberg, S. M., Kantor, J. A., and Schlom, J. Adoptive immunotherapy as an *in vivo* model to explore antitumor mechanisms induced by a recombinant anticancer vaccine. J. Immunother., *20:* 48–59, 1997.
8. Chen, L., McGowan, P., Ashe, S., Johnston, J., Li, Y., Hellstrom, I., and Hellstrom, K. E. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. J. Exp. Med., *179:* 523–532, 1994.
9. Liu, D. W., Tsao, Y. P., Hsieh, C. H., Hsieh, J. T., Kung, J. T., Chiang, C. L., Huang, S. J., and Chen, S. L. Induction of CD8 T cells by vaccination with recombinant adenovirus expressing human papillomavirus type 16 *E5* gene reduces tumor growth. J. Virol., *74:* 9083–9089, 2000.
10. Akagi, J., Hodge, J. W., McLaughlin, J. P., Gritz, L., Mazzara, G., Kufe, D., Schlom, J., and Kantor, J. A. Therapeutic antitumor response after immunization with an admixture of recombinant vaccinia viruses expressing a modified *MUC1* gene and the murine T-cell costimulatory molecule B7. J. Immunother., *20:* 38–47, 1997.
11. Hodge, J. W., Schlom, J., Donohue, S. J., Tomaszewski, J. E., Wheeler, C. W., Levine, B. S., Gritz, L., Panicali, D., and Kantor, J. A. A recombinant vaccinia virus expressing human prostate-specific antigen (PSA): safety and immunogenicity in a non-human primate. Int. J. Cancer, *63:* 231–237, 1995.
12. Hodge, J. W., McLaughlin, J. P., Abrams, S. I., Shupert, W. L., Schlom, J., and Kantor, J. A. Admixture of a recombinant vaccinia virus containing the gene for the costimulatory molecule B7 and a recombinant vaccinia virus containing a tumor-associated antigen gene results in enhanced specific T-cell responses and antitumor immunity. Cancer Res., *55:* 3598–3603, 1995.
13. Bronte, V., Carroll, M. W., Goletz, T. J., Wang, M., Overwijk, W. W., Marincola, F., Rosenberg, S. A., Moss, B., and Restifo, N. P. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. Proc. Natl. Acad. Sci. USA, *94:* 3183–3188, 1997.
14. Chen, P. W., Wang, M., Bronte, V., Zhai, Y., Rosenberg, S. A., and Restifo, N. P. Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. J. Immunol., *156:* 224–231, 1996.
15. Leach, D. R., Krummel, M. F., and Allison, J. P. Enhancement of antitumor immunity by CTLA-4 blockade. Science (Wash DC), *271:* 1734–1736, 1996.
16. Hurwitz, A. A., Yu, T. F., Leach, D. R., and Allison, J. P. CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 10067–10071, 1998.
17. Chamberlain, R. S., Carroll, M. W., Bronte, V., Hwu, P., Warren, S., Yang, J. C., Nishimura, M., Moss, B., Rosenberg, S. A., and Restifo, N. P. Costimulation enhances the active immunotherapy effect of recombinant anticancer vaccines. Cancer Res., *56:* 2832–2836, 1996.
18. Hodge, J. W., Sabzevari, H., Gomez Yafal, A., Gritz, L., Lorenz, M. G. O, and Schlom, J. A triad of costimulatory molecules synergize to amplify T cell activation. Cancer Res., *59:* 5800–5807, 1999.
19. Hodge, J. W., Rad, A. N., Grosenbach, D. W., Sabzevari, H., Yafal, A. G., Gritz, L., and Schlom, J. Enhanced activation of T cells by dendritic cells engineered to hyperexpress a triad of costimulatory molecules. J. Natl. Cancer Inst., *92:* 1228–1239, 2000.
20. Carroll, M. W., Overwijk, W. W., Surman, D. R., Tsung, K., Moss, B., and Restifo, N. P. Construction and characterization of a triple-recombinant vaccinia virus encoding B7-1, interleukin 12, and a model tumor antigen. J. Natl. Cancer Inst., *90:* 1881–1887, 1998.
21. Hara, I., Nagai, H., Miyake, H., Yamanaka, K., Hara, S., Micallef, M. J., Kurimoto, M., Gohji, K., Arakawa, S., Ichihashi, M., and Kamidono, S. Effectiveness of cancer vaccine therapy using cells transduced with the interleukin-12 gene combined with systemic interleukin-18 administration. Cancer Gene Ther., *7:* 83–90, 2000.
22. Overwijk, W. W., Theoret, M. R., and Restifo, N. P. The future of interleukin-2: enhancing therapeutic anticancer vaccines. Cancer J. Sci. Am., *6* (Suppl. 1)*:* S76–S80, 2000.
23. Lotze, M. T., Shurin, M., Esche, C., Tahara, H., Storkus, W., Kirkwood, J. M., Whiteside, T. L., Elder, E. M., Okada, H., and Robbins, P. Interleukin-2: developing additional cytokine gene therapies using fibroblasts or dendritic cells to enhance tumor immunity. Cancer J. Sci. Am., *6* (Suppl. 1)*:* S61–S66, 2000.
24. McLaughlin, J. P., Abrams, S., Kantor, J., Dobrzanski, M. J., Greenbaum, J., Schlom, J., and Greiner, J. W. Immunization with a syngeneic tumor infected with recombinant vaccinia virus expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) induces tumor regression and long-lasting systemic immunity. J. Immunother., *20:* 449–459, 1997.
25. McLaughlin, J. P., Schlom, J., Kantor, J. A., and Greiner, J. W. Improved immunotherapy of a recombinant carcinoembryonic antigen vaccinia vaccine when given in combination with interleukin-2. Cancer Res., *56:* 2361–2367, 1996.
26. Pancholi, P., Liu, Q., Tricoche, N., Zhang, P., Perkus, M. E., and Prince, A. M. DNA prime-canarypox boost with polycistronic hepatitis C virus (HCV) genes generates potent immune responses to HCV structural and nonstructural proteins. J. Infect. Dis., *182:* 18–27, 2000.
27. Ruitenberg, K. M., Walker, C., Love, D. N., Wellington, J. E., and Whalley, J. M. A prime-boost immunization strategy with DNA and recombinant baculovirus-expressed protein enhances protective immunogenicity of glycoprotein D of equine herpesvirus 1 in naive and infection-primed mice. Vaccine, *18:* 1367–1373, 2000.
28. Hanke, T., Samuel, R. V., Blanchard, T. J., Neumann, V. C., Allen, T. M., Boyson, J. E., Sharpe, S. A., Cook, N., Smith, G. L., Watkins, D. I., Cranage, M. P., and McMichael, A. J. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. J. Virol., *73:* 7524–7532, 1999.
29. Barnett, S. W., Klinger, J. M., Doe, B., Walker, C. M., Hansen, L., Duliege, A. M., and Sinangil, F. M. Prime-boost immunization strategies against HIV. AIDS Res. Hum. Retroviruses, *14* (Suppl. 3)*:* S299–S309, 1998.
30. Tartaglia, J., Excler, J. L., El Habib, R., Limbach, K., Meignier, B., Plotkin, S., and Klein, M. Canarypox virus-based vaccines: prime-boost strategies to induce cell-mediated and humoral immunity against HIV. AIDS Res. Hum. Retroviruses, *14 (*Suppl. 3)*:* S291–S298, 1998.

31. Irvine, K. R., Chamberlain, R. S., Shulman, E. P., Surman, D. R., Rosenberg, S. A., and Restifo, N. P. Enhancing efficacy of recombinant anticancer vaccines with prime/boost regimens that use two different vectors. J. Natl. Cancer Inst., *89:* 1595–1601, 1997.
32. Hodge, J. W., McLaughlin, J. P., Kantor, J. A., and Schlom, J. Diversified prime and boost protocols using recombinant vaccinia virus and recombinant non-replicating avian pox virus to enhance T-cell immunity and antitumor responses. Vaccine, *15:* 759–768, 1997.
33. Kundig, T. M., Kalberer, C. P., Hengartner, H., and Zinkernagel, R. M. Vaccination with two different vaccinia recombinant viruses: long-term inhibition of secondary vaccination. Vaccine, *11:* 1154–1158, 1993.
34. Belyakov, I. M., Moss, B., Strober, W., and Berzofsky, J. A. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. Proc. Natl. Acad. Sci. USA, *96:* 4512–4517, 1999.
35. Rooney, J. F., Wohlenberg, C., Cremer, K. J., Moss, B., and Notkins, A. L. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: long-term protection and effect of revaccination. J. Virol., *62:* 1530–1534, 1988.
36. Cooney, E. L., Collier, A. C., Greenberg, P. D., Coombs, R. W., Zarling, J., Arditti, D. E., Hoffman, M. C., Hu, S. L., and Corey, L. Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. Lancet, *337:* 567–572, 1991.
37. Disis, M. L., Bernhard, H., Shiota, F. M., Hand, S. L., Gralow, J. R., Huseby, E. S., Gillis, S., and Cheever, M. A. Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. Blood, *88:* 202–210, 1996.
38. Kass, E., Panicali, D., Mazzara, G., Schlom, J., and Greiner, J. Granulocyte/macrophage-colony stimulating factor produced by recombinant avian poxviruses enriches the regional lymph nodes with antigen-presenting cells and acts as an immunoadjuvant. Cancer Res., *61:* 206–214, 2001.
39. Guadagni, F., Roselli, M., Cosimelli, M., Spila, A., Cavaliere, F., Arcuri, R., D'Alessandro, R., Fracasso, P. L., Casale, V., Vecchione, A., Casciani, C. U., Greiner, J. W., and Schlom, J. Quantitative analysis of CEA expression in colorectal adenocarcinoma and serum: lack of correlation. Int. J. Cancer, *72:* 949–954, 1997.
40. Foon, K. A., John, W. J., Chakraborty, M., Das, R., Teitelbaum, A., Garrison, J., Kashala, O., Chatterjee, S. K., and Bhattacharya-Chatterjee, M. Clinical and immune responses in resected colon cancer patients treated with anti-idiotype monoclonal antibody vaccine that mimics the carcinoembryonic antigen. J. Clin. Oncol., *17:* 2889–2885, 1999.
41. Marshall, J. L., Hawkins, M. J., Tsang, K. Y., Richmond, E., Pedicano, J. E., Zhu, M. Z., and Schlom, J. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. J. Clin. Oncol., *17:* 332–337, 1999.
42. Marshall, J., Hoyer, R., Toomey, M., Faraguna, K., Chang, P., Richmond, E., Pedicano, J., Gehan, E., Peck, R., Arlen, P., Tsang, K., and Schlom, J. Phase I study in cancer patients of a diversified prime and boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen responses. J. Clin. Oncol., *18:* 3964–3973, 2000.
43. Fong, L., Hou, Y., Benike, C., Yuen, A., Fisher, G., and Engleman, E. Immunization with CEA agonist epitope pulsed FLT-3L expanded dendritic cells for human immunotherapy. Proc. Am. Assoc. Cancer Res., *41:* 217, 2000.
44. Samanci, A., Yi, Q., Fagerberg, J., Strigard, K., Smith, G., Ruden, U., Wahren, B., and Mellstedt, H. Pharmacological administration of granulocyte/macrophage-colony-stimulating factor is of significant importance for the induction of a strong humoral and cellular response in patients immunized with recombinant carcinoembryonic antigen. Cancer Immunol. Immunother., *47:* 131–142, 1998.
45. Horig, H., Lee, D., Conkright, W., DiVito, J., Hasson, H., LaMare, M., Rivera, A., Park, D., Tine, J., Guito, K., Tsang, K., Schlom, J., and Kaufman, H. Phase I clinical trial of a recombinant canarypoxvirus (ALVAC) vaccine expressing human carcinoembryonic antigen (CEA) and the B7.1 co-stimulatory molecule. Cancer Immunol. Immunother., *49:* 504–514, 2000.
46. von Mehren, M., Arlen, P., Tsang, K. Y., Rogatko, A., Meropol, N., Cooper, H. S., Davey, M., McLaughlin, S., Schlom, J., and Weiner, L. M. Pilot study of a dual gene recombinant avipox vaccine containing both carcinoembryonic antigen (CEA) and B7.1 transgenes in patients with recurrent CEA-expressing adenocarcinomas. Clin. Cancer Res., *6:* 2219–2228, 2000.
47. Tsang, K. Y., Zaremba, S., Nieroda, C. A., Zhu, M. Z., Hamilton, J. M., and Schlom, J. Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J. Natl. Cancer Inst., *87:* 982–990, 1995.
48. Tsang, K. Y., Zhu, M., Nieroda, C. A., Correale, P., Zaremba, S., Hamilton, J. M., Cole, D., Lam, C., and Schlom, J. Phenotypic stability of a cytotoxic T-cell line directed against an immunodominant epitope of human carcinoembryonic antigen. Clin. Cancer Res., *3:* 2439–2449, 1997.
49. Alters, S. E., Gadea, J. R., Sorich, M., O'Donoghue, G., Talib, S., and Philip, R. Dendritic cells pulsed with CEA peptide induce CEA-specific CTL with restricted TCR repertoire. J. Immunother., *21:* 17–26, 1998.
50. Eades-Perner, A. M., van der Putten, H., Hirth, A., Thompson, J., Neumaier, M., von Kleist, S., and Zimmermann, W. Mice transgenic for the human carcinoembryonic antigen gene maintain its spatiotemporal expression pattern. Cancer Res., *54:* 4169–4176, 1994.
51. Kaufman, H. L., Rao, J. B., Irvine, K. R., Bronte, V., Rosenberg, S. A., Restifo, N. P., and Irivine, K. R. Interleukin-10 enhances the therapeutic effectiveness of a recombinant poxvirus-based vaccine in an experimental murine tumor model. J. Immunother., *22:* 489–496, 1999.
52. Carroll, M. W., Overwijk, W. W., Chamberlain, R. S., Rosenberg, S. A., Moss, B., and Restifo, N. P. Highly attenuated modified vaccinia virus Ankara (MVA) as an effective recombinant vector: a murine tumor model. Vaccine, *15:* 387–394, 1997.
53. Rao, J. B., Chamberlain, R. S., Bronte, V., Carroll, M. W., Irvine, K. R., Moss, B., Rosenberg, S. A., and Restifo, N. P. IL-12 is an effective adjuvant to recombinant vaccinia virus-based tumor vaccines: enhancement by simultaneous B7-1 expression. J. Immunol., *156:* 3357–3365, 1996.
54. Sivanandham, M., Shaw, P., Bernik, S. F., Paoletti, E., and Wallack, M. K. Colon cancer cell vaccine prepared with replication-deficient vaccinia viruses encoding B7.1 and interleukin-2 induce antitumor response in syngeneic mice. Cancer Immunol. Immunother., *46:* 261–267, 1998.
55. Qin, H., and Chatterjee, S. K. Cancer gene therapy using tumor cells infected with recombinant vaccinia virus expressing GM-CSF. Hum. Gene Ther., *7:* 1853–1860, 1996.
56. Jenkins, S., Gritz, L., Fedor, C. H., O'Neill, E. M., Cohen, L. K., and Panicali, D. L. Formation of lentivirus particles by mammalian cells infected with recombinant fowlpox virus. AIDS Res. Hum. Retroviruses, *7:* 991–998, 1991.
57. Gritz, L., Destree, A., Cormier, N., Day, E., Stallard, V., Caiazzo, T., Mazzara, G., and Panicali, D. Generation of hybrid genes and proteins by vaccinia virus-mediated recombination: application to human immunodeficiency virus type 1 env. J. Virol., *64:* 5948–5957, 1990.
58. Chakrabarti, S., Sisler, J. R., and Moss, B. Compact, synthetic, vaccinia virus early/late promoter for protein expression. BioTechniques, *23:* 1094–1097, 1997.
59. Kass, E., Parker, J., Schlom, J., and Greiner, J. W. Comparative studies of the effects of recombinant GM-CSF and GM-CSF administered via a poxvirus to enhance the concentration of antigen-presenting cells in regional lymph nodes. Cytokine, *12:* 960–971, 2000.
60. Robbins, P. F., Kantor, J. A., Salgaller, M., Hand, P. H., Fernsten, P. D., and Schlom, J. Transduction and expression of the human carcinoembryonic antigen gene in a murine colon carcinoma cell line. Cancer Res., *51:* 3657–3662, 1991.
61. Kantor, J., Irvine, K., Abrams, S., Kaufman, H., DiPietro, J., and Schlom, J. Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. J. Natl. Cancer Inst., *84:* 1084–1091, 1992.
62. Lafreniere, R., and Rosenberg, S. A. A novel approach to the generation and identification of experimental hepatic metastases in a murine model. J. Natl. Cancer Inst., *76:* 309–322, 1986.
63. Kalus, R. M., Kantor, J. A., Gritz, L., Gomez Yafal, A., Mazzara, G. P., Schlom, J., and Hodge, J. W. The use of combination vaccinia vaccines and dual-gene vaccinia vaccines to enhance antigen-specific T-cell immunity via T-cell costimulation. Vaccine, *17:* 893–903, 1999.
64. Esquivel, F., Yewdell, J., and Bennink, J. RMA/S cells present endogenously synthesized cytosolic proteins to class I-restricted cytotoxic T lymphocytes. J. Exp. Med., *175:* 163–168, 1992.
65. Abrams, S. I., Dobrzanski, M. J., Wells, D. T., Stanziale, S. F., Zaremba, S., Masuelli, L., Kantor, J. A., Schlom, J., and Masuelle, L. Peptide-specific activation of cytolytic $CD4^+$ T lymphocytes against tumor cells bearing mutated epitopes of K-*ras* p21. Eur. J. Immunol., *25:* 2588–2597, 1995.
66. Gnant, M. F., Puhlmann, M., Bartlett, D. L., and Alexander, H. R., Jr. Regional versus systemic delivery of recombinant vaccinia virus as suicide gene therapy for murine liver metastases. Ann. Surg., *230:* 352–360, 1999.
67. Gnant, M. F., Puhlmann, M., Alexander, H. R., Jr., and Bartlett, D. L. Systemic administration of a recombinant vaccinia virus expressing the cytosine deaminase gene and subsequent treatment with 5-fluorocytosine leads to tumor-specific gene expression and prolongation of survival in mice. Cancer Res., *59:* 3396–3403, 1999.
68. Wang, M., Bronte, V., Chen, P. W., Gritz, L., Panicali, D., Rosenberg, S. A., and Restifo, N. P. Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. J. Immunol., *154:* 4685–4692, 1995.
69. Freund, Y. R., Mirsalis, J. C., Fairchild, D. G., Brune, J., Hokama, L. A., Schindler-Horvat, J., Tomaszewski, J. E., Hodge, J. W., Schlom, J., Kantor, J. A., Tyson, C. A., and Donohue, S. J. Vaccination with a recombinant vaccinia vaccine containing the B7-1 co-stimulatory molecule causes no significant toxicity and enhances T cell-mediated cytotoxicity. Int. J. Cancer, *85:* 508–517, 2000.
70. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Sznol, M., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Seipp, C. A., Einhorn, J. H., Rogers-Freezer, L., and White, D. E. Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. J. Immunol., *163:* 1690–1695, 1999.
71. Drillien, R., Spehner, D., Bohbot, A., and Hanau, D. Vaccinia virus-related events and phenotypic changes after infection of dendritic cells derived from human monocytes. Virology, *268:* 471–481, 2000.
72. Ramsey-Ewing, A., and Moss, B. Restriction of vaccinia virus replication in CHO cells occurs at the stage of viral intermediate protein synthesis. Virology, *206:* 984–993, 1995.