Analyses of Recombinant Vaccinia and Fowlpox Vaccine Vectors Expressing Transgenes for Two Human Tumor Antigens and Three Human Costimulatory Molecules

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ABSTRACT

Purpose: The poor immunogenicity of tumor antigens and the antigenic heterogeneity of tumors call for vaccine strategies to enhance T-cell responses to multiple antigens. Two antigens expressed noncoordinately on most human carcinomas are carcinoembryonic antigen (CEA) and MUC-1. We report here the construction and characterization of two viral vector vaccines to address these issues.

Experimental Design: The two viral vectors analyzed are the replication-competent recombinant vaccinia virus (rV-) and the avipox vector, fowlpox (rF-), which is replication incompetent in mammalian cells. Each vector encodes the transgenes for three human costimulatory molecules (B7-1, ICAM-1, and LFA-3, designated TRICOM) and the CEA and MUC-1 transgenes (which also contain agonist epitopes). The vectors are designated rV-CEA/MUC/TRICOM and rF-CEA/MUC/TRICOM.

Results: Each of the vectors is shown to be capable of faithfully expressing all five transgenes in human dendritic cells (DC). DCs infected with either vector are shown to activate both CEA- and MUC-1–specific T-cell lines to the same level as DCs infected with CEA-TRICOM or MUC-1-TRICOM vectors. Thus, no evidence of antigenic competition between CEA and MUC-1 was observed. Human DCs infected with rV-CEA/MUC/TRICOM or rF-CEA/MUC/TRICOM are also shown to be capable of generating both MUC-1- and CEA-specific T-cell lines; these T-cell lines are in turn shown to be capable of lysing targets pulsed with MUC-1 or CEA peptides as well as human tumor cells endogenously expressing MUC-1 and/or CEA.

Conclusion: These studies provide the rationale for the clinical evaluation of these multigene vectors in patients with a range of carcinomas expressing MUC-1 and/or CEA.

INTRODUCTION

Various strategies are now being used toward the development of vaccines for the therapy and/or prevention of a range of human cancers. Because tumor-associated antigens are by definition poorly immunogenic or nonimmunogenic in the host, strategies are being developed to enhance the presentation of these antigens to the immune system and to enhance the activation of T cells directed toward those antigens. Evidence is now being accumulated in both preclinical and clinical studies that the use of two different vaccines in a diversified prime and boost regimen is superior to the continued use of one vaccine (1–5). Previous studies have shown that primary vaccination with a recombinant vaccinia virus (rV-) followed by multiple boosting with a replication defective recombinant avipox (fowlpox, rF-) vector is superior to the continued use of either vector alone (1, 4). Another strategy that has proven efficacious is the use of T-cell costimulation to enhance the immunogenicity of weak tumor antigens. Induction of a T-cell response to an antigen usually requires two signals. Signal 1 is delivered to the T-cell receptor via the peptide-MHC complex of antigen-presenting cells (APC). Signal 2 involves the interaction of costimulatory molecules (such as B7-1, ICAM-1, and LFA-3, designated TRICOM) of the APC with its ligand on the T cell. It has been shown that the level of signal 1 required for effective T-cell activation is influenced by the level of expression of signal 2. Conversely, the level of signal 2 required for effective T-cell activation is influenced by both the amount of peptide-MHC complex on the APC and, possibly, the avidity of the interaction of the peptide-MHC complex with the T-cell receptor. T-cell activation can also be enhanced by either the use of antibodies such as anti–CTLA-4 to block negative costimulatory signals or the insertion of T-cell costimulatory molecules into viral vectors (1, 6–14).

Poxvirus vectors are excellent vehicles for the insertion of multiple transgenes, each on its own promoter because they can accept large amounts of DNA, and numerous poxvirus promoters have now been identified (15). Preclinical studies have shown that the use of poxvirus recombinant vectors encoding the transgenes for an antigen and a triad of T-cell costimulatory molecules (TRICOM) results in far greater activation of antigen-specific CD4 and CD8 T cells and antitumor activity, as compared with the use of identical vectors encoding the transgene for antigen alone or antigen plus one or two costimulatory molecules (1, 8, 9, 11, 14).
Because tumors are heterogeneous, both genotypically and phenotypically, antigenic drift may occur as a consequence of vaccine therapy directed against a single tumor antigen. Two antigens overexpressed on a wide range of human carcinomas and other tumor types are carcinoembryonic antigen (CEA; refs. 16, 17) and MUC-1 (18–22). Several clinical trials involving poxvirus-CEA–based vaccines have shown the ability of patients with advanced CEA-expressing carcinomas to generate CEA-specific T-cell responses (4, 12, 23–26). These phase I/II clinical studies have used rV-CEA, avipox-CEA, avipox-CEA-B7, and rV- and rF-CEA-TRICOM. Clinical trials have also been carried out in patients with advanced breast cancer with the use of rV-MUC-1- and rV-MUC-1-IL-2–based vaccines (27, 28). The above phase I/II studies using CEA and MUC-1 vaccines have shown the generation of antigen-specific T-cell responses, decreases in serum markers, and evidence of tumor regression and increased survival after vaccination. These preliminary studies, however, will require larger randomized trials to confirm these early findings.

Numerous CEA- and MUC-1–specific T-cell epitopes have now been identified and characterized (29–40). Among these is the HLA-A2 CAP-1 epitope. Recent studies have shown that a T-cell receptor agonist epitope of CAP-1 (designated CAP1-6D) is capable of activating T cells to greater levels than CAP-1, and these T cells are capable of lysing tumor cells expressing native CEA to greater levels than T cells activated with CAP-1 (41, 42). A clinical trial using CAP1-6D–pulsed dendritic cells (DC) also showed objective clinical responses in patients with advanced CEA-expressing tumors (43). Recent studies (44) have also identified an agonist epitope of the P-93 HLA-A2 MUC-1 epitope, which has been designated P-93L. This agonist epitope has shown higher avidity for HLA-A2 than the native epitope and has shown the ability to activate MUC-1–specific T-cell lines from normal volunteers and patients with pancreatic cancer; these T-cell lines can in turn lyse human tumor cells expressing native MUC-1 to a greater level than T-cell lines generated using the native epitope.

We report here the development and analysis of recombinant vaccinia and fowlpox vectors, each containing transgenes encoding CEA, MUC-1, and three human T-cell costimulatory molecules. Each of these transgenes is driven by its own viral promoter. Both the CEA and MUC-1 transgenes encode agonist epitopes (designated 6D and P-93L, respectively) capable of activating human T cells to greater levels than their native counterparts. The rV-CEA(6D)-MUC-1(P-93L)-B7-1-ICAM-1-LFA-3 vector has been designated rV-CEA/MUC/TRICOM and the rF-CEA(6D)-MUC-1(P-93L)-B7-1-ICAM-1-LFA-3 vector has been designated rF-CEA/MUC/TRICOM. Each of these vectors is shown to be capable of faithfully expressing each of the five transgenes in human DCs. Human DCs infected with either vector are shown to activate T-cell lines generated by MUC-1- and CEA-specific peptides. No evidence of antigenic competition between CEA and MUC-1 was observed. Moreover, human DCs infected with either recombinant vector are shown to be capable of generating both MUC-1- and CEA-specific T-cell lines. These T-cell lines are, in turn, shown to be capable of lysing targets pulsed with MUC-1 or CEA peptides, as well as human tumor cells expressing MUC-1 and/or CEA.

MATERIALS AND METHODS

Cell Cultures. The human breast adenocarcinoma cell line MCF-7 (ref. 45; HLA-A2 positive, CEA negative, and MUC-1 positive), the colorectal carcinoma cell line SW1463 (HLA-A2 positive, CEA positive, and MUC-1 positive), and the melanoma cell line SK-Mel-24 (HLA-A2 positive, CEA negative, and MUC-1 negative) were purchased from American Type Culture Collection (Manassas, VA). The cultures were free of mycoplasma and were maintained in complete medium [RPMI 1640 (Invitrogen Life Technologies Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Life Technologies)]. The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or B antigens (46). C1R-A2 cells are C1R cells that express a transfected genomics clone of HLA-A2.1 (47). These cells were obtained from Dr. William E. Biddison (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). The 174CEM-T2 cell line (T2) transport deletion mutant (48) was provided by Dr. Peter Cresswell (Yale University School of Medicine, New Haven, CT). C1R-A2 cells and T2 cells were mycoplasma free and were maintained in RPMI 1640 complete medium and Iscove’s modified Dulbecco’s complete medium (Invitrogen Life Technologies), respectively. The V8T cell line is a CD8+ CTL line directed against the CAP-1 epitope of CEA (23, 49). The T-1191-P93L cell line is a CD8+ MUC-1–specific CTL line generated from peripheral blood mononuclear cells (PBMC) from a healthy donor that was in vitro stimulated using a MUC-1 peptide (44). Both V8T and T-1191-P93L cell lines were cultured as described previously (23).

Peptides. The following HLA-A2 binding peptides were used in this study: (a) the CEA agonist peptide CAP1-6D (YLSGADLNL), designated CEA peptide (41), (b) the MUC-1 agonist peptide P-93L (ALWGQDVTSV), designated MUC-1 peptide (44), (c) the prostate-specific antigen (PSA) peptide PSA-3 (VISNDVCAQV; 50). All peptides were greater than 96% pure and manufactured by American Peptide Company, Inc. (Sunnyvale, CA).

Culture of Dendritic Cells from Peripheral Blood Mononuclear Cells. HLA-A2 normal donor PBMCs were obtained from heparinized blood. PBMCs were separated using lymphocyte separation medium gradient (Organon Teknika, Durham, NC), as described previously (51). DCs were prepared using a modification of the procedure described by Sallusto and Lanzavecchia (52). PBMCs (1.5 × 10⁶) were resuspended in AIM-V medium containing 2 mmol/L glutamine, 50 μg/mL streptomycin, and 10 μg/mL gentamycin (Invitrogen Life Technologies) and allowed to adhere to a T-150 flask (Corning Costar Corp., Cambridge, MA). After 2 hours at 37°C, the nonadherent cells were removed with a gentle rinse. The adherent cells were cultured for 6 to 7 days in AIM-V medium containing 100 ng/mL of recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) and 20 ng/mL of recombinant human interleukin 4 (rhIL-4). The culture medium was replenished every 3 days.

Recombinant Virus and Infection of Dendritic Cells with rV-CEA/MUC/TRICOM and rF-CEA/MUC/TRICOM. Both rV-CEA/MUC/TRICOM and rF-CEA/MUC/TRICOM encode the human CEA gene containing the 6D
modification (41), the human MUC-1 gene containing the 93L modification (44), and the genes for the human costimulatory molecules B7-1, ICAM-1, and LFA-3 (Fig. 1). Recombinant vectors were generated by homologous recombination as described previously (1). DCs ($10^6$) were incubated in 1 mL of Opti-MEM medium (Invitrogen Life Technologies) at 37°C with rF-CEA/MUC/TRICOM, rF-CEA/MUC/TRICOM, control avipox virus vector (FP-WT), or control vaccinia vector (V-WT). Titration experiments showed that infection of DCs for 2 hours with $4 \times 10^7$ plaque-forming units (pfu) per milliliter of rF-CEA/MUC/TRICOM, equal to a multiplicity of infection (MOI) of 40 pfu/cell was able to consistently induce transgene expression in $\sim 60\%$ of the infected DCs. Similar titration experiments showed that infection of DCs for 1 hour with $0.5 \times 10^7$ pfu/mL of rV-CEA/MUC/TRICOM, equal to an MOI of 5 pfu/cell, was able to consistently induce transgene expression in $\sim 35\%$ of the infected DCs. DCs from different donors were used for the infections with rF-CEA/MUC/TRICOM and rV-CEA/MUC/TRICOM, with the efficiency of infection ranging from $50\%$ to $65\%$ for rF-CEA/MUC/TRICOM and $30\%$ to $59\%$ for rV-CEA/MUC/TRICOM. The infected DCs were suspended in 10 mL of fresh, warm RPMI 1640 complete medium containing 100 ng/mL of rhGM-CSF and 20 ng/mL of rhIL-4, cultured for 24 hours, and subsequently used as APC.

**Flow Cytometric Analysis.** Dual-color flow cytometric analysis was done on T-cell lines by using the following antibody combinations: anti-CD56-FITC/anti-CD8-PE, anti-CD8-FITC/anti–CD54-PE, anti–MHC class I-FITC/anti–MHC class II-PE, and anti–IgG1-FITC/anti–IgG2a-PE (isotype controls). Other antibodies were purchased from BD Biosciences. The anti-CEA monoclonal antibody COL-1 (53) and anti–MUC-1 antibodies (DF3 and DF3-P; 54, 55) were also used. MOPC-104E (IgM, Cappel/Organon Teknika Corp., West Chester, PA) was used as negative control. After staining, cells were washed thrice and subsequently incubated with a 1:100 dilution of FITC-labeled goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Analysis was conducted as described above. Results were expressed in percentage of positive cells and mean fluorescence intensity. The mean fluorescence intensity value was collected in log scale and was used to express the levels of fluorescence determined by measuring the average for all the cells in the fluorescence dot plot.

**Immune Blot Analysis.** Uninfected DCs, DCs infected with 40 MOI of rF-CEA/MUC/TRICOM, rF-CEA(6D)-TRICOM, or rF-MUC-1-TRICOM vectors, and DCs infected with 5 MOI of the rV-CEA/MUC/TRICOM vector were lysed by using the M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Protein concentration of the lysates was determined by using a MicroBCA Protein Extraction Assay Kit (Pierce), and 20 μg fractions of protein per sample were blotted onto a polyvinylidene difluoride membrane using a Bio-Dot Microfiltration apparatus (BioRad Laboratories, Hercules, CA), following the manufacturer’s instructions. After blotting, the membranes were blocked for 1 hour at room temperature with PBS containing 5% bovine serum albumin (Biosource International, Camarillo, CA). Membranes were then washed thrice with PBS containing 0.25% Tween 20 and incubated for 2 hours at room temperature with a solution at 1 μg/mL of COL-1, DF-3, or DF3-P antibodies. Membranes were then washed thrice as above and incubated with a 1:3,000 dilution of an anti-mouse IgG conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories) for 1 hour at room temperature. For immunodetection of the CEA and MUC-1 proteins, the SuperSignal West Pico Chemiluminescent Substrate was used (Pierce).

**Generation of T-Cell Lines.** Modification of the protocol described by Tsang et al. (23) was used to generate CEA- and or MUC-1–specific CTL. To generate T-cell lines T-rV and T-rF, autologous DCs infected with rV-CEA/MUC/TRICOM or rF-CEA/MUC/TRICOM, respectively, were used as APC. Autologous nonadherent cells were added to APC at an effector:APC ratio of 10:1; cultures were incubated for 3 days at 37°C, in a humidified atmosphere containing 5% CO2. The cultures were then supplemented with recombinant human IL-2 at a

<table>
<thead>
<tr>
<th>Virus</th>
<th>Designation</th>
<th>Promoters/Transgenes</th>
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<tr>
<td>rF-CEA(6D)/B7-1/ICAM-1/LFA-3</td>
<td>p40 CEA(6D) p30 LFA-3 B7-1</td>
<td></td>
</tr>
<tr>
<td>rF-CEA(6D)/B7-1/ICAM-1/LFA-3</td>
<td>p40 CEA(6D) p30 LFA-3 ICAM-1 B7-1</td>
<td></td>
</tr>
<tr>
<td>rF-MUC-1/B7-1/ICAM-1/LFA-3</td>
<td>p40 MUC-1 p30 LFA-3 ICAM-1 B7-1</td>
<td></td>
</tr>
<tr>
<td>rF-CEA(6D)/MUC-1(93L)/B7-1/ICAM-1/LFA-3</td>
<td>p40 CEA(6D) p30 LFA-3 ICAM-1 B7-1</td>
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<tr>
<td>rF-CEA(6D)/MUC-1(93L)/B7-1/ICAM-1/LFA-3</td>
<td>p40 CEA(6D) p30 LFA-3 ICAM-1 B7-1</td>
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Fig. 1 Schematic representation of the viral constructs.
concentration of 20 units/mL for 7 days; the IL-2 containing medium was replenished every 3 days. The 3-day incubation with peptide and 7-day IL-2 supplement constituted one in vitro stimulation (IVS) cycle. T-rV and T-rF were restimulated with rV-CEA/MUC/TRICOM- or rF-CEA/MUC/TRICOM–infected autologous DCs, respectively, as described above, on day 11 to begin the next IVS cycle. rV-CEA/MUC/TRICOM- and rF-CEA/MUC/TRICOM–infected autologous DCs were used as APC for three IVS cycles. For the generation of T-rF(CEA) and T-rF(MUC) cell lines, T cells were stimulated with autologous DCs infected with rF-CEA/MUC/TRICOM for one IVS, and then restimulated with uninfected autologous DCs pulsed with CAP1-6D or P-93L peptide, respectively, for two more IVS. After the third IVS cycle, irradiated (23,000 rad) autologous EBV-transformed B cells were used as APC. The EBV-transformed B cells were pulsed with 25 μg/mL of peptide and used for restimulation at an effector-APC ratio of 1:3. Cultures were then incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO₂. After removal of the peptide-containing medium, the cultures were supplemented with recombinant human IL-2 at a concentration of 20 units/mL for 7 days. T-cell lines from patients 55, 49, and 41 were generated by stimulation of PBMCs with autologous DCs infected with rF-CEA/MUC/TRICOM, using the same stimulation protocol described above. Patient 55 initially underwent a Whipple procedure for localized pancreatic cancer followed by adjuvant radiation therapy to the pancreatic bed. The patient had local recurrence and received chemotherapy with 5-fluorouracil/Leucovorin followed by an experimental vaccine study using both vaccinia-CEA and ALVAC-CEA before enrolling on this clinical trial. Patient 41 was diagnosed with colorectal carcinoma with liver metastasis. Before enrolling on study, this patient progressed on three different chemotherapy regimens, including 5-fluorouracil/leucovorin/CPT-11, 5-fluorouracil/leucovorin/oxaliplatin, and Xeloda. Patient 49 had colorectal carcinoma with liver metastasis. Before enrolling on study, the patient progressed on three different chemotherapy regimens, including 5-fluorouracil/leucovorin/CPT-11, 5-fluorouracil/leucovorin/oxaliplatin, and Xeloda. Patient 49 had colorectal cancer with both liver and lung metastasis. The patient progressed after four cycles of chemotherapy with CPT-11/5-fluorouracil/leucovorin before enrolling on the study.

**Cytotoxic Assay.** Target cells (C1R-A2 or tumor cells) were labeled with 50 μCi of indium-111–labeled oxyquinoline (Medi-Physics Inc., Arlington, IL) for 15 minutes at room temperature. Target cells (0.3 × 10⁷) in 100 μL of RPMI 1640 complete medium were added to each of 96 wells in flat-bottomed assay plates (Corning Costar Corp.). Labeled C1R-A2 target cells were incubated with peptides at the concentration indicated for 60 minutes at 37°C in 5% CO₂ before adding effector cells. No peptide was used when carcinoma cell lines were used as targets. Effector cells were suspended in 100 μL of RPMI 1640 complete medium supplemented with 10% pooled human AB serum and added to the target cells. The plates were then incubated at 37°C in 5% CO₂ for 6 or 16 hours. Supernatant was harvested for gamma counting with the use of harvester frames (Skatron Inc., Sterling, VA). Determinations were carried out in triplicate and SDs were calculated. Specific lysis was calculated with the use of the following formula (all values in counts per minute):

\[
\%\text{ lysis} = \frac{\text{Observed release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100
\]

Spontaneous release was determined from wells to which 100 μL of RPMI 1640 complete medium was added. Total releasable radioactivity was obtained after treatment of targets with 2.5% Triton X-100.

**Detection of Cytokines.** Supernatants of T cells exposed for 24 hours to peptide-pulsed autologous EBV-transformed B cells in IL-2–free medium, at various peptide concentrations, were screened for secretion of IFN-γ using an ELISA kit (Biosource International). The results were expressed in picograms per milliliter.

**Statistical Analysis.** Statistical analysis of differences between means was done using a two-tailed paired t test (StatView Statistical Software, Abacus Concepts, Berkeley, CA).

**RESULTS**

Studies were first undertaken to determine if infection of human DCs with rV-CEA/MUC/TRICOM would result in the expression of each of the five transgenes. Initial studies used an MOI of 5 and 10 for rV-CEA/MUC/TRICOM; both MOIs gave comparable results and thus an MOI of 5 was used in subsequent experiments. As seen in Fig. 2, uninfected human DCs do not express CEA (as detected by monoclonal antibody COL-1); expression of CD80, CD54, and CD58 and MHC class I and II by DCs is similar to that previously reported in several studies (13, 14). Infection with V-WT had little, if any, effect on any of these eight surface markers (Fig. 2). Infection with rV-CEA/MUC/TRICOM, however, is shown to substantially increase the level of expression of CEA, MUC-1, CD80, CD54, and CD58, and did not affect the level of expression of MHC class I and II. Consistent with results previously published, infection of DCs from the same donor with rV-CEA(6D)-TRICOM enhanced the level of CEA, CD80, CD54, and CD58 to levels similar to those seen with rV-CEA/MUC/TRICOM, but did not alter the expression of MUC-1 or MHC class I or II. In addition, infection of DCs with rV-MUC-1 admixed with rV-TRICOM (rV-MUC-1-TRICOM construct was not available) showed levels of enhanced expression of MUC-1 and the three costimulatory molecules similar to those seen with rV-CEA/MUC/TRICOM, but had no effect on the level of expression of MHC class I and II, and on the lack of expression of CEA (data not shown). Low levels of MUC-1 were detected in the uninfected and control vector–infected DCs. This is in agreement with that reported by Wykes et al. (56), which showed that MUC-1 is expressed in human DCs and monocyte-derived DCs when cultured in vitro.

Parallel studies were undertaken to determine if infection of human DCs with rF-CEA/MUC/TRICOM would result in the expression of each of the five transgenes. Initial studies used an MOI of 20 and 40 for rF-CEA/MUC/TRICOM; an MOI of 40 showed greater expression of transgenes and was thus used in subsequent experiments. As seen in Fig. 3, infection with FP-WT had little, if any, effect on any of the eight surface markers analyzed. Infection with rF-CEA/MUC/TRICOM, however, was shown to substantially increase the level of expression of CEA, MUC-1, CD80, CD54, and CD58, but did not affect the level of expression of MHC class I and II. Also consistent with results previously published, infection of DCs from the same donor with rF-CEA(6D)-TRICOM enhanced the level of CEA, CD80, CD54, and CD58 to similar levels as seen with rF-CEA/MUC/TRICOM, but did not alter
the expression of MUC-1 or MHC class I or II. In addition, infection of DCs with rF-MUC-1-TRICOM showed similar levels of enhanced expression of MUC-1 and the three costimulatory molecules as seen with rF-CEA/MUC/TRICOM, but had no effect on the level of expression of MHC class I and II and the lack of expression of CEA (data not shown).

The expression of CEA and MUC-1 on DCs infected with rF-CEA/MUC/TRICOM, rF-CEA(6D)-TRICOM, rF-MUC-1-TRICOM vector, or uninfected DCs was analyzed by immune blot analysis. As shown in Fig. 4, CEA was detected in DCs infected with rF-CEA(6D)-TRICOM, rF-CEA/MUC/TRICOM, and rV-CEA/MUC/TRICOM, but not in uninfected DCs or rF-MUC-1-TRICOM–infected DCs. As described above, DCs express a low level of MUC-1, but a great increase in MUC-1 expression was clearly observed in DCs infected with rF-MUC-1-TRICOM, rV-CEA/MUC/TRICOM, and rF-CEA/MUC/TRICOM, but not in DCs infected with rF-CEA(6D)-TRICOM (Fig. 4).

We have previously shown the ability of DCs pulsed with the CEA agonist peptide CAP-1(6D) and the MUC-1 agonist peptide P-93L, to activate human T cells. Studies were undertaken to determine if infection of human DCs with the rF-CEA/MUC/TRICOM vector could stimulate IFN-γ production by CEA- and MUC-1–specific T cells. These results were also compared with the ability of human DCs infected with rF-CEA(6D)-TRICOM or rF-MUC-1-TRICOM to activate these T cells. As seen in Table 1, uninfected DCs or DCs infected with FP-WT did not result in any IFN-γ production by the CEA-specific or the MUC-1–specific T-cell line. DCs pulsed with the CEA peptide induced IFN-γ production only by the CEA-specific T-cell line, whereas DCs pulsed with the MUC-1 peptide induced IFN-γ production only by the MUC-1–specific T-cell line. Similarly, DCs infected with rF-CEA(6D)-TRICOM induced IFN-γ production only by the CEA-specific T-cell line, whereas DCs infected with the rF-MUC-1-TRICOM induced IFN-γ production only by the MUC-1–specific T-cell line. Infection of DCs with rF-CEA/MUC/ TRICOM, however, induced IFN-γ production in both the CEA-specific and the MUC-1–specific T-cell lines, and at comparable levels to those seen when using the vectors containing only the single tumor-antigen transgene. These studies thus show the lack of antigenic competition between CEA and MUC-1 in the rF-CEA/MUC/TRICOM vector in the ability to activate T cells. Studies were then undertaken to determine if infection of human DCs with the rF-CEA/MUC/TRICOM vector could stimulate IFN-γ production by the CEA- and MUC-1–specific T cells. These results were also compared with the ability of human DCs infected with rV-CEA(6D)-TRICOM or rV-MUC-1 plus rV-TRICOM to activate these T cells. As seen in Table 2, uninfected DCs or DCs infected with V-WT did not result in any IFN-γ production by the CEA-specific or the MUC-1–specific T-cell line. DCs pulsed with the CEA peptide induced IFN-γ production only by the CEA-specific T-cell line, whereas DCs pulsed with the MUC-1 peptide induced IFN-γ production only by the MUC-1–specific T-cell line. Infection of DCs with rV-CEA(MUC/TRICOM, however, induced IFN-γ production in both the CEA- and MUC-1–specific T-cell lines.
and at comparable levels to those seen when using the vectors containing only the single tumor-antigen transgene. These studies thus show the lack of antigenic competition between CEA and MUC-1 in the ability to activate T cells with the use of the rF-CEA/MUC/TRICOM vector.

Studies were then undertaken to determine if human T cells could be established from PBMCs using autologous DCs infected with rF-CEA/MUC/TRICOM and/or rV-CEA/MUC/TRICOM as APC. After three IVS, as described in MATERIALS AND METHODS, resultant T cells were analyzed for their ability to be activated by DCs pulsed with peptides or infected with vector. As can be seen in Table 3, neither of the T-cell lines established using rV-CEA/MUC/TRICOM- or rF-CEA/MUC/TRICOM–infected DCs as APC could be activated to produce IFN-γ, when stimulated by uninfected DCs, or DCs infected with FP-WT. These results are consistent with previous observations in the murine system that there is no cross-reactivity in terms of T-cell epitopes between vaccinia virus and fowlpox (57). On the other hand, the T-cell lines generated with rV-CEA/MUC/TRICOM- or rF-CEA/MUC/TRICOM–infected APC were both activated to produce IFN-γ when using as APC DCs pulsed with either the CEA or the MUC-1 peptides (Table 3). These results indicate that DCs infected with either vector will generate T cells from PBMCs that are directed against both the CEA and MUC-1 antigens. As expected, T cells generated by DCs infected with either rV-CEA/ MUC/TRICOM or rF-CEA/MUC/TRICOM produced IFN-γ when exposed to DCs infected with rF-CEA/MUC/TRICOM.

In additional experiments, T cells were generated initially with the use of rF-CEA/MUC/TRICOM–infected DCs as APC, and were then passaged with CEA peptide–pulsed APC for two IVS. As can be seen in Table 3, these T cells lost their ability to be activated by DCs pulsed with the MUC-1 peptide but retained their ability to be activated to produce IFN-γ by DCs pulsed with the CEA peptide. Conversely, when T cells generated initially using rF-CEA/MUC/TRICOM–infected DCs were then passaged in the presence of DCs pulsed with MUC-1 peptide, they lost their ability to be activated by DCs pulsed with CEA peptide but retained their ability to be activated by MUC-1 peptide.

Fig. 3 Flow cytometric analysis of surface marker expression on human DCs uninfected, infected with control vector (FP- WT), or infected with rF-CEA/ MUC/TRICOM. DCs (1 × 10^6) were incubated in 1 mL of Opti- MEM medium at 37°C with rF-CEA/MUC/TRICOM or control vector (FP-WT) for 2 hours at an MOI of 40:1. The infected DCs were suspended in 10 mL of fresh, warm complete medium containing 100 ng/mL of rhGM-CSF and 20 ng/mL of rhIL-4 and then cultured for 24 hours. Numbers in each histogram indicate the percentage of positive cells and the mean fluorescence intensity (in parentheses).

Multigene Vaccine Constructs
Uninfected were used at a concentration of 25 MUC/TRICOM, rF-CEA(6D)-TRICOM, or rF-MUC-1/TRICOM. Peptides were used at a concentration of 25 μg/mL. The effector-APC ratio was 10:1. Twenty-four-hour culture supernatants were collected and screened for the production of IFN-γ.

**Fig. 4** Immunoblotting analysis of human DCs uninfected or infected with rF-CEA-TRICOM, rF-MUC-1-TRICOM, rF-CEA/MUC/TRICOM, and rV-CEA/MUC/TRICOM. Monoclonal antibodies CO1-1 and DF-3 were used for the detection of CEA and MUC-1, respectively.

HLA-A2+ target cell lines were evaluated: the MCF-7 human breast carcinoma line, which is positive for MUC-1 and negative for CEA; the human colon carcinoma cell line SW1463, which is positive for both CEA and MUC-1; and the SK-Mel-24 human melanoma line, which is negative for both MUC-1 and CEA expression. We were unable to identify an HLA-A2+ cell line that was negative for MUC-1 and positive for CEA. As seen in Table 4, the T-cell lines generated using DCs infected with rV-CEA/MUC/TRICOM or rF-CEA/MUC/TRICOM both were able to lyse the breast and colon carcinoma lines, but were unable to lyse the melanoma line. On the other hand, the T-cell line generated using DCs infected with rF-CEA/MUC/TRICOM and then restimulated with DCs pulsed with the CEA peptide for two IVS was able to lyse the CEA positive/MUC-1 positive colon carcinoma line, but was unable to lyse the CEA negative/MUC-1 positive breast cancer line and the CEA negative/MUC-1 negative melanoma line. The T-cell line generated using DCs infected with rF-CEA/MUC/TRICOM and then restimulated with DCs pulsed with the MUC-1 peptide for two IVS was able to lyse both the colon and breast cell lines but not the melanoma line. Collectively, these data show that both recombinant vaccinia and avipox vectors can be constructed to each faithfully express five human transgenes, and that no antigenic competition is observed in the ability of these vectors to activate human T cells directed against two human tumor-associated antigens.

The T-rV, T-rF, T-rF(CEA), and T-rF(MUC) T-cell lines were generated from an apparently healthy individual. All four cell lines were shown to be >97% CD8 positive, <2% CD56 positive, >75% CD45RA positive, and >81% CD27 positive. Studies were then conducted to determine whether specific T-cell lines could be derived from a patient with pancreatic cancer (patient 55). A T-cell line was generated using rF-CEA/MUC/TRICOM–infected DCs as APC, and was designated T-55. As determined by flow cytometric analysis, the T-55 cell line was 99.9% CD8 positive, <2% CD56 positive, 73.6% CD45RA positive, and 87% CD27 positive. As seen in Table 5, this T-cell line was shown to produce IFN-γ when stimulated with rF-CEA/MUC/TRICOM and DCs pulsed with either the CEA peptide or the MUC-1 peptide but not the PSA-3 peptide. Studies were then conducted to determine whether this T-cell line could lyse CEA and/or MUC-1 positive and HLA-A2 positive cancer cell lines. The melanoma cell line SK-Mel-24 (MUC-1 negative, CEA negative, and HLA-A2 positive) was used as a negative control. As seen in Table 6, the T-55 cell line lysed MCF-7 and SW1463 cells at various E:T ratios but showed no lysis of the melanoma cell line. Two additional T-cell lines were generated from colon carcinoma patients. These T-cell lines were designated T-41 and T-49. The T-41 cell line was 98.8% CD8 positive, <1% CD56 positive, 33.6% CD45RA positive, and 96.8% CD27 positive. The T-49 cell line was 98.9% CD8 positive, <2% CD56 positive, >75% CD45RA positive, and >81% CD27 positive.

**Table 1** Production of IFN-γ by CEA-specific and MUC-1–specific T-cell lines stimulated with rF-CEA/MUC/TRICOM

<table>
<thead>
<tr>
<th>Treatment of DCs</th>
<th>CEA-specific CTL</th>
<th>MUC-1–specific CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>&lt;15</td>
<td>&lt;15</td>
</tr>
<tr>
<td>FP-WT</td>
<td>&lt;15</td>
<td>&lt;15</td>
</tr>
<tr>
<td>Uninfected + CEA peptide</td>
<td>772</td>
<td>&lt;15</td>
</tr>
<tr>
<td>Uninfected + MUC-1 peptide</td>
<td>&lt;15</td>
<td>458</td>
</tr>
<tr>
<td>FP-WT + CEA peptide</td>
<td>689</td>
<td>&lt;15</td>
</tr>
<tr>
<td>FP-WT + MUC-1 peptide</td>
<td>689</td>
<td>&lt;15</td>
</tr>
<tr>
<td>rF-CEA(6D)-TRICOM</td>
<td>&lt;15</td>
<td>404</td>
</tr>
<tr>
<td>rF-MUC-1-TRICOM</td>
<td>&lt;15</td>
<td>298</td>
</tr>
<tr>
<td>rF-CEA/MUC/TRICOM</td>
<td>455</td>
<td>278</td>
</tr>
</tbody>
</table>

**NOTE.** CEA-specific T cells (V8T) and MUC-1–specific T cells (T-1191-P93L) were stimulated with autologous uninfected DCs alone or pulsed with either the CEA peptide (CAP1-6D) or the MUC-1 peptide (P-93L); DCs infected with the control vector FP-WT alone or pulsed with either the CEA or MUC-1 peptides; DCs infected with rF-CEA/MUC/TRICOM, rF-CEA(6D)-TRICOM, or rF-MUC-1/TRICOM. Peptides were used at a concentration of 25 μg/mL. The effector-APC ratio was 10:1. Twenty-four-hour culture supernatants were collected and screened for the production of IFN-γ.

**Table 2** Production of IFN-γ by CEA-specific and MUC-1–specific T-cell lines stimulated with rV-CEA/MUC/TRICOM

<table>
<thead>
<tr>
<th>Treatment of DCs</th>
<th>CEA-specific CTL</th>
<th>MUC-1–specific CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>&lt;15</td>
<td>&lt;15</td>
</tr>
<tr>
<td>V-WT</td>
<td>&lt;15</td>
<td>&lt;15</td>
</tr>
<tr>
<td>Uninfected + CEA peptide</td>
<td>820</td>
<td>&lt;15</td>
</tr>
<tr>
<td>Uninfected + MUC-1 peptide</td>
<td>&lt;15</td>
<td>550</td>
</tr>
<tr>
<td>V-WT + CEA peptide</td>
<td>720</td>
<td>&lt;15</td>
</tr>
<tr>
<td>V-WT + MUC-1 peptide</td>
<td>&lt;15</td>
<td>358</td>
</tr>
<tr>
<td>rV-CEA(6D)-TRICOM</td>
<td>384</td>
<td>&lt;15</td>
</tr>
<tr>
<td>rV-MUC-1 + rV-TRICOM</td>
<td>&lt;15</td>
<td>213</td>
</tr>
<tr>
<td>rV-CEA/MUC/TRICOM</td>
<td>285</td>
<td>256</td>
</tr>
</tbody>
</table>

**NOTE.** CEA-specific T cells (V8T) and MUC-1–specific T cells (T-1191-P93L) were stimulated with autologous uninfected DCs alone or pulsed with either the CEA peptide (CAP1-6D) or the MUC-1 peptide (P-93L); DCs infected with the control vector V-WT alone or pulsed with either the CEA or MUC-1 peptides; DCs infected with rV-CEA/MUC/TRICOM, rV-CEA(6D)-TRICOM, or rV-MUC-1 plus rV-TRICOM. Peptides were used at a concentration of 25 μg/mL. The effector-APC ratio was 10:1. Twenty-four-hour culture supernatants were collected and screened for the production of IFN-γ.
positive, <1% CD56 positive, 29.8% CD45RA positive, and 95.3% CD27 positive. As seen in Table 5, both T-41 and T-49 cell lines were shown to produce IFN-γ when stimulated with autologous DCs infected with rF-CEA/MUC/TRICOM and DCs pulsed with either the CEA peptide or the MUC-1 peptide, but not the PSA-3 peptide. As seen in Table 7, both T-41 and T-49 cell lines showed lysis of MCF-7 and SW1463 at various E:T ratios, but showed no lysis of the SK-Mel-24 cell line.

**DISCUSSION**

Two of the major concerns in the development and use of vaccines for cancer therapy are (a) the poor immunogenicity of tumor-associated antigens and (b) antigenic heterogeneity of tumors. The vectors described here were developed to address both these issues. These vectors are the first in which five complete transgenes are inserted into an avipox vector and, to our knowledge, any replication-incompetent vector. A parallel five-transgene construct recombinant vaccinia virus has also been developed. For both vectors, each transgene was driven by its own promoter. Previous studies in both preclinical models and clinical trials have shown that diversified prime and boost vaccine regimens using two different vaccines are superior to the continued use of one vaccine. It is for this reason that both the recombinant vaccinia and recombinant fowlpox vectors were developed. It should be pointed out that previous multigene constructs using vaccinia virus recombinants have been recorded. In one report, six Plasmodium genes were expressed using four poxvirus promoters. In another report, the murine B7-1, IL-12, and Lac-Z genes were expressed along with an E. coli GPT selection gene (58). In a third report, a murine polytope recombinant vaccinia virus coding for 10 contiguous minimal murine CTL epitopes (each 8-10 amino acids in length) was constructed as a polyprotein. In another study, a recombinant vaccinia polyepitope vaccine was constructed that contained 10 murine CTL epitopes (59).

Previous studies have shown that signal 1 (the antigen) and signal 2 (the costimulatory signal or signals) must be expressed on the same cell in order to achieve enhanced activation of T cells. Previous attempts in our laboratory with admixing avipox vectors have proven to be unsuccessful in enhancing activation of T cells. It should also be pointed out that previous attempts in admixing DNA vectors were also unsuccessful in enhancing activation of T cells. It is for this reason that the two tumor-antigen genes and the three costimulatory molecule genes must be on the same vector if one is to achieve successful enhanced activation of T cells. Previous studies (60) have shown that one can actually admix recombinant vaccinia viruses because they are replication competent. However, those same studies also showed that a delicate balance must be achieved in the ratios of the admixed vaccinia vectors. It is thus also advantageous to have all five transgenes expressed on the same recombinant vaccinia. Another reason for the development and use of such multigene constructs involves the conservation of energy and resources, which must be considered in translational efforts to develop useful clinical agents. For example, the development of an rF-CEA(6D)-TRICOM vector and an rF-MUC-1-TRICOM vector, as opposed to the rF-CEA/MUC/TRICOM vector, would result in twice the effort in terms of GMP production of agent, vector purification and vialing, and quality control issues such as

### Table 3 Production of IFN-γ by CEA- and MUC-1–specific T cells established by using rV-CEA/MUC/TRICOM or rF-CEA/MUC/TRICOM vectors

<table>
<thead>
<tr>
<th>T-cell line</th>
<th>Uninfected</th>
<th>FP-WT</th>
<th>rF-CEA/MUC/TRICOM</th>
<th>Uninfected + CEA peptide</th>
<th>Uninfected + MUC-1 peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-rV</td>
<td>&lt;15.6</td>
<td>&lt;15.6</td>
<td>&gt;1,000</td>
<td>976.3</td>
<td>514.0</td>
</tr>
<tr>
<td>T-rF</td>
<td>&lt;15.6</td>
<td>&lt;15.6</td>
<td>&gt;1,000</td>
<td>550.0</td>
<td>446.0</td>
</tr>
<tr>
<td>T-rF(CEA)</td>
<td>&lt;15.6</td>
<td>&lt;15.6</td>
<td>403.9</td>
<td>729.2</td>
<td>&lt;15.6</td>
</tr>
<tr>
<td>T-rF(MUC)</td>
<td>&lt;5.6</td>
<td>&lt;15.6</td>
<td>381.4</td>
<td>626.8</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Human T-cell lines T-rV, T-rF, T-rF(CEA), and T-rF(MUC) were generated as described in MATERIALS AND METHODS. These T-cell lines were stimulated with autologous uninfected DCs alone or pulsed with either the CEA or MUC-1 peptide, DCs infected with the control vector FP-WT, and DCs infected with rF-CEA/MUC/TRICOM. Peptides were used at a concentration of 25 μg/mL; the effector-APC ratio was 10:1. Twenty-four-hour culture supernatants were collected and screened for the secretion of IFN-γ.

### Table 4 Ability of T-cell lines established by using rV-CEA/MUC/TRICOM and rF-CEA/MUC/TRICOM as APC to lyse human tumor cells

<table>
<thead>
<tr>
<th>CIR-A2 cells pulsed with</th>
<th>No peptide</th>
<th>CEA peptide*</th>
<th>MUC-1 peptide</th>
<th>PSA peptide</th>
<th>MCF-7</th>
<th>SW1463</th>
<th>SK-Mel-24</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-rV</td>
<td>-2.1 (1.26)</td>
<td>57.9 (4.5)*</td>
<td>50.0 (1.3)*</td>
<td>0.6 (2.4)</td>
<td>34.4 (0.1)†</td>
<td>27.8 (0.5)†</td>
<td>1.6 (1.1)</td>
</tr>
<tr>
<td>T-rF</td>
<td>3.58 (3.9)</td>
<td>62.0 (4.6)*</td>
<td>59.7 (0.8)*</td>
<td>3.8 (0.2)</td>
<td>31.0 (2.4)†</td>
<td>25.6 (1.2)†</td>
<td>2.7 (2.0)</td>
</tr>
<tr>
<td>T-rF(CEA)</td>
<td>-4.3 (1.6)</td>
<td>62.8 (1.9)*</td>
<td>-1.9 (1.0)</td>
<td>2.7 (1.0)</td>
<td>4.2 (3.2)</td>
<td>32.3 (2.1)†</td>
<td>0.6 (3.5)</td>
</tr>
<tr>
<td>T-rF(MUC)</td>
<td>-1.6 (3.2)</td>
<td>-3.6 (5.5)</td>
<td>46.4 (3.3)*</td>
<td>1.4 (4.5)</td>
<td>38.2 (1.3)†</td>
<td>25.2 (1.3)†</td>
<td>0.1 (1.1)</td>
</tr>
</tbody>
</table>

NOTE. Results are expressed in % lysis (SD). The human T-cell lines T-rV, T-rF, T-rF(CEA), and T-rF(MUC) were established as described in MATERIALS AND METHODS. A 6-hour release assay was done on CIR-A2 cells and a 16-hour release assay was done on CIR-A2 cells and SW1463, and SK-Mel-24 cells. CEA peptide (CAP1-6D), MUC-1 peptide (P-93L), and PSA peptide (PSA-3) were used at a concentration of 25 μg/mL. MCF-7 (human breast carcinoma cell line: HLA-A2+, MUC-1 positive and CEA-negative), SW1463 (colon carcinoma cell line: HLA-A2+, MUC-1 positive and CEA positive), SK-Mel-24 (human melanoma cell line: HLA-A2+, MUC-1 negative, CEA negative). The effector-target ratio was 25:1.

*P < 0.01, two-tailed t test, when comparing lysis to CIR-A2 cells.
†P < 0.01, two-tailed t test, when comparing lysis to SK-Mel-24 cells.
sterility, potency, and stability. Although one may not consider these to be “scientific issues,” they are often the bottleneck or even the barrier between good scientific and intellectual efforts and clinical trials in patients with terminal diseases. The constructs reported here all have individual poxvirus promoters driving each transgene. Previous studies have shown that each of these promoters has different strengths, and thus the sequence in which they are placed in the genome is important to achieve faithful expression of the multiple transgenes. This has been achieved for the five genes expressed by the rV-CEA/MUC/TRICOM and rF-CEA/MUC/TRICOM vectors.

One of the concerns in the use of recombinant vectors encoding transgenes for multiple antigens is that of antigenic competition, specifically CEA and MUC-1 in this case. As shown in Tables 2 to 7, infection of DCs with the rV-CEA/MUC/TRICOM or rF-CEA/MUC/TRICOM vector resulted in the activation of T cells as efficiently as the use of DCs as APC that were infected with either CEA-TRICOM or MUC-1-TRICOM vectors. Moreover, T cells generated using DCs infected with rV-CEA/MUC/TRICOM or rF-CEA/MUC/TRICOM were able to lyse target cells expressing either CEA or MUC-1.

Virtually all tumors express multiple tumor-associated antigens and most of them are heterogeneously expressed in tumor masses. This has been shown to be attributable to inherent antigenic heterogeneity, environmental factors in the tumor milieu such as spatial configuration, or antigenic drift due to therapeutic intervention. Thus, vaccines expressing multiple transgenes may well help to alleviate this obstacle of antigenic heterogeneity. CEA is expressed on most colorectal, pancreatic, and gastric tumors and in ~70% of non–small cell lung cancers and 50% of breast cancers, as well as other tumor types such as head and neck carcinoma and subsets of ovarian carcinoma (16, 17). MUC-1, on the other hand, is overexpressed on most colorectal, pancreatic, breast, and ovarian cancers as well as other carcinoma types (18–22). Thus, the multitargeting of these two antigens may prove advantageous for those cancer types expressing both antigens.

This report deals with analyses of both recombinant vaccinia and replication-defective recombinant avipox (fowlpox) vectors, because preclinical and clinical studies have shown the advantage of priming with vaccinia and boosting with avipox recombinants. Whereas previous data showed that the triad of costimulatory molecules in TRICOM enhances the level of antigen-specific T cells in experimental models, studies have also shown that TRICOM enhances the avidity of antigen-specific T cells. Recent studies have also shown that in vitro activation of human T cells directed against CEA using human DCs infected with TRICOM results in the generation of higher-avidity CEA-specific T cells, as compared with T cells generated with uninfected DCs. This was shown to be the case using either immature or CD40L mature DCs. The inclusion of HLA-A2 enhancer agonist epitopes for both CEA and MUC-1 into both the rV- and rF-CEA/MUC/TRICOM vectors should also enhance T-cell responses directed against those epitopes in HLA-A2 positive patients. It should be pointed out, however, that the entire CEA gene is also contained in these vectors, and numerous A2, A3, and A24 class I epitopes for CEA have now been identified along with

### Table 5 Establishment of T-cell lines from patients with cancer using rF-CEA/MUC/TRICOM–infected autologous DCs shows reactivity to both CEA and MUC-1 epitopes

<table>
<thead>
<tr>
<th>T-cell line</th>
<th>Uninfected</th>
<th>FP-WT</th>
<th>rF-CEA/MUC-1/TRICOM</th>
<th>Uninfected + CEA peptide</th>
<th>Uninfected + MUC-1 peptide</th>
<th>Uninfected + PSA peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-55</td>
<td>&lt;15.6</td>
<td>&lt;15.6</td>
<td>&gt;1,000</td>
<td>1,000</td>
<td>985.8</td>
<td>&lt;15.6</td>
</tr>
<tr>
<td>T-49</td>
<td>&lt;15.6</td>
<td>&lt;15.6</td>
<td>&gt;1,000</td>
<td>974.2</td>
<td>819.0</td>
<td>&lt;15.6</td>
</tr>
<tr>
<td>T-41</td>
<td>&lt;15.6</td>
<td>&lt;15.6</td>
<td>846.4</td>
<td>933.4</td>
<td>745.0</td>
<td>&lt;15.6</td>
</tr>
</tbody>
</table>

NOTE. T-55, T-49, and T-41 were established by stimulating T cells isolated from a patient with pancreatic cancer (patient 55) and patients with colon carcinoma (patients 49 and 41) with autologous DCs infected with rF-CEA/MUC/TRICOM (40 MOI) for three IVS. The effector-APC ratio was 10:1. Peptides were used at a concentration of 25 μg/mL. Twenty-four-hour culture supernatants were collected and screened for the secretion of IFN-γ. Results are expressed in picograms per milliliter of IFN-γ.

### Table 6 Ability of a T-cell line (T-55) established from a pancreatic patient using rF-CEA/MUC/TRICOM–infected DCs as APC to lyse human tumor cells

<table>
<thead>
<tr>
<th>E:T</th>
<th>No peptides</th>
<th>CEA peptide*</th>
<th>MUC-1 peptide</th>
<th>PSA peptide</th>
<th>MCF-7</th>
<th>SW1463</th>
<th>SK-Mel-24</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:1</td>
<td>0 (0.6)</td>
<td>52.4 (3.3)*</td>
<td>53.3 (0.4)*</td>
<td>0 (0.1)</td>
<td>23.8  (1.2)*</td>
<td>24.1  (1.2)*</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>25:1</td>
<td>0 (0.9)</td>
<td>30.3 (1.3)*</td>
<td>34.4 (0.8)*</td>
<td>0.6 (0.5)</td>
<td>20.4  (1.1)*</td>
<td>17.6  (0.3)*</td>
<td>0.4 (0.3)</td>
</tr>
<tr>
<td>12.5:1</td>
<td>0 (0.2)</td>
<td>16.4 (1.8)*</td>
<td>26.2 (5.0)*</td>
<td>0 (0.2)</td>
<td>13.8  (1.2)*</td>
<td>15.2  (0.4)*</td>
<td>0 (0.3)</td>
</tr>
</tbody>
</table>

NOTE. A 6-hour 111In release assay was done on C1R-A2 cells and a 16-hour 111In release assay was done on MCF-7, SW1463, and SK-Mel-24 cells. Results are expressed in % lysis (SD). CEA peptide (CAP1-6D), MUC-1 peptide (P-93L), and PSA peptide (PSA-3) were all used at a concentration of 25 μg/mL. MCF-7 (human breast carcinoma cell line: HLA-A2+, MUC-1 positive and CEA negative); SW1463 (colon carcinoma cell line: HLA-A2+, MUC-1 positive and CEA positive); SK-Mel-24 (human melanoma cell line: HLA-A2+, MUC-1 negative, CEA negative). T-55 was established by stimulating T cells isolated from a pancreatic patient (patient 55) with autologous DCs infected with rF-CEA/MUC/TRICOM (40 MOI) for three IVS.

*P < 0.01, two-tailed t test, when comparing lysis to C1R-A2 cells.

†P < 0.01, two-tailed t test, when comparing lysis to SK-Mel-24 cells.
Table 7  Ability of T-cell lines (T-49 and T-41) established from patients with colon carcinoma using rF-CEA/MUC/TRICOM–infected DCs as APC to lyse human tumor cells.

<table>
<thead>
<tr>
<th>T-cell lines</th>
<th>MCF-7</th>
<th>SW1463</th>
<th>SK-Mel-24</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-49</td>
<td>40:1</td>
<td>20.6</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>T-41</td>
<td>40:1</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>20.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

*p < 0.01, two-tailed t test, when comparing lysis to SK-Mel-24 cells.

several helper epitopes. It is believed that for weak tumor antigens such as CEA and MUC-1, both class I and class II epitopes should be present to maximize the immune response. A major portion of the MUC-1 gene is also present in the recombinant vectors described here, and likewise, numerous MUC-1 epitopes have previously been identified.

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