

The Infection of Human Dendritic Cells with Recombinant Avipox Vectors Expressing a Costimulatory Molecule Transgene (*CD80*) to Enhance the Activation of Antigen-specific Cytolytic T Cells

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ABSTRACT

Human dendritic cells (DCs) express MHC class I and II molecules and several T-cell costimulatory molecules that contribute to their efficiency as antigen-presenting cells (APCs). Whereas most human DC populations uniformly express some costimulatory molecules such as B7-2 (CD86), previous studies have shown a wide variation in the expression of B7-1 (CD80) among different human DC preparations. In the studies reported here, we demonstrate that replication-defective avipox vectors expressing B7-1 can be used to rapidly and efficiently infect human DCs and can enhance the efficacy of human DCs to activate specific human T-cell populations. This has been demonstrated both in systems using peptide as a source of signal 1 and in systems using recombinant avipox vector to deliver signal 1. The antigen used in these studies was the tumor-associated human carcinoembryonic antigen (CEA). An immunodominant 9-mer CTL epitope for CEA (designated CAP-1) has been previously characterized (K. Y. Tsang *et al.*, *J. Natl. Cancer Inst. (Bethesda)*, 87: 982–990, 1995). The source of signal 1 used in these studies was (a) the CAP-1 peptide; (b) recombinant avipox-CEA; or (c) the dual transgene recombinant avipox-CEA/B7-1. These studies demonstrate that CEA-specific T cells are more efficiently activated using as APCs peptide-pulsed DCs infected with avipox-B7-1, as compared with peptide-pulsed DCs infected with wild-type vector, or with uninfected peptide-pulsed DCs. Greater activation of CEA-specific T cells was also obtained using as APCs DCs that were infected with avipox-CEA/B7-1 as compared with the use of DCs infected with avipox-CEA. A CEA tetramer was also used to isolate high- and low-tetramer-binding CEA-specific T-cell populations. Although both high- and low-tetramer-binding T cells had the ability to lyse CEA peptide-pulsed targets, only the high-tetramer-binding T cells had the ability to lyse colon carcinoma cells expressing CEA, which suggests the existence of tetramer-binding populations with different T-cell receptor (TCR) affinities. The demonstrated safety of recombinant avipox vectors in humans and the previously demonstrated ability to administer them multiple times without host immune response limitations indicate that these vectors expressing B7-1 have a potential use in enhancing the efficacy of human DC immunotherapy protocols using either peptide or recombinant vector to deliver signal 1.

INTRODUCTION

DCs² are believed to be the most potent of APCs. Peptide-pulsed human DCs have been used to activate human T cells *in vitro*, and clinical trials involving peptide-pulsed DCs have demonstrated the ability to generate T-cell responses in patients to both viral antigens

(1) and tumor-associated antigens (2). Various methodologies have been used to generate DCs and to enhance their ability to activate T cells; the most common methodology has involved the incubation of DC progenitors from peripheral blood with cytokines such as GM-CSF and IL-4. Further maturation of DCs has involved the use of cytokines and other factors. The phenotype of DCs has been shown to vary among different individuals. Whereas most human DCs that are generated express class I and class II MHCs and the T-cell costimulatory molecule B7-2 (CD86), many human DC preparations will vary in their expression of B7-1 (CD80); the percentage of B7-1-positive DCs, generated with GM-CSF and IL-4, has been reported to range from 2 to 60%, depending on the donor (3–12).

Induction of a T-cell response to an antigen usually requires two signals (13, 14). Signal 1 is delivered to the TCR via the peptide-MHC complex of the APC. Signal 2 involves the interaction of a costimulatory molecule of the APC with its ligand on the APC. Data are emerging that the level of signal 1 required for effective T-cell activation is influenced by the level of expression of signal 2 (15). 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 TCR (16). Recent studies have shown (17, 18) that T cells actually acquire the B7-1 costimulatory molecule from APCs on activation. The level of acquisition of B7-1 by T cells was shown to be related to both the level of expression of B7-1 on the APCs (including DCs) and the level of peptide-MHC complex on the APCs (17). This acquisition of B7-1 by T cells was shown to have both immunostimulatory and immunoregulatory properties, depending on levels of signals 1 and 2 and on whether the cell was a naive memory T cell. Thus, although human DCs express B7-1 (albeit in different levels for different donors), the augmentation level of B7-1 on DCs could have profound influences on T-cell activation. Previous studies with murine DCs have also shown that, although murine DCs express B7-1 on 100% of cells, infection with an avipox vector expressing B7-1 can enhance their ability to activate T cells. This was shown to be the case using either moderately mature or mature murine DCs (19).

The vast majority of human DC studies reported to date have used pulsing with 8–11 amino acid peptides as a source of antigen (signal 1) to generate CD8⁺ CTLs. This method conceivably will optimize the amount of peptide-MHC complex on the DCs for interaction with the TCR. On the other hand, several groups have used vectors that express an entire antigen transgene to deliver signal 1 to DCs. This methodology has the potential advantage of having the DCs present numerous class I epitopes (perhaps at lower levels than peptide pulsing) as well as class II helper epitopes. Studies have demonstrated that plasmid DNA in liposomes (2, 20), adenovirus (20, 21), vaccinia virus (22–24), and retroviruses (25–27) could be used to infect or transfect human DCs to present a variety of antigens.

In the studies reported here, we have demonstrated that one can use recombinant avipox vectors to rapidly and efficiently infect human DCs and to express transgenes on the cell surface. We first demon-

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² The abbreviations used are: DC, dendritic cell; APC, antigen-presenting cell; GM-CSF, granulocyte macrophage colony-stimulating factor; rhGM-CSF, recombinant human GM-CSF; IL, interleukin; TCR, T-cell receptor; rhIL, recombinant human IL; CEA, carcinoembryonic antigen; PBMC, peripheral blood mononuclear cell; RG, rabies glycoprotein; IVS, *in vitro* stimulation; DPBS, Dulbecco's phosphate buffered saline; PE, R-phycoerythrin; FACS, fluorescence-activated cell sorting/sorter; MFI, mean fluorescent intensity.

strated that peptide-pulsed DCs, infected with avipox-B7-1 recombinant, are more efficient in activating T cells than the use of uninfected peptide-pulsed DCs. The peptide used in these studies was the 9-mer CAP1-6D agonist epitope of the tumor-associated antigen CEA. Previous reports (28, 29) have demonstrated that the single amino change in the CAP1-6D epitope renders it more efficient in activating human T cells than the native CAP-1 epitope (30). We then compared the activation of human CAP-1-specific T cells by DCs infected with an avipox-CEA recombinant (expressing the entire CEA transgene) to that of DCs infected with an avipox recombinant simultaneously expressing both the CEA and B7-1 transgenes. T-cell responses were analyzed in terms of lysis of both peptide-pulsed targets and tumor cell targets expressing CEA. A tetramer, composed of the CEA-peptide-MHC (HLA-A2) molecule, was then used to isolate CEA-specific T cells. These T cells were separated into high- and low-tetramer-binding populations. Although little difference was observed in the two populations in terms of lysis of peptide-pulsed targets, a marked difference was observed in the lysis of tumor cell targets expressing CEA, with high-tetramer-binding T cells lysing tumor targets as compared with low-tetramer-binding T cells not demonstrating tumor cell lysis.

The studies thus form the basis for the use of replication-defective avipox vectors to deliver B7-1 to peptide-pulsed DCs to enhance activation of T cells, or the use of recombinant avipox vectors to infect human DCs as a source of expression of both signal 1 and signal 2 to amplify the activation of antigen-specific T cells.

MATERIALS AND METHODS

Cell Cultures

Colorectal carcinoma cell lines SW1463 (HLA-A1, 2) and LS174T (HLA-A2) were purchased from American Type Culture Collection (Manassas, VA). The cultures were free of *Mycoplasma* and were maintained in complete medium [DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Inc.)]. The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or B antigens (31). C1R-A2 cells are C1R cells that express a transfected genomic clone of HLA-A2.1 (32). These cells were obtained from Dr. William E. Biddison (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). C1R-A2 culture was *Mycoplasma*-free and was maintained in RPMI 1640 complete medium (Life Technologies, Inc.). The V8T T-cell line is a CD8⁺ CTL line directed against the CEA CAP-1 epitope (33), and was established from a patient with metastatic colon carcinoma who was enrolled in a Phase I trial using an rV-CEA vaccine (33). V8T cells were cultured in RPMI 1640 complete medium containing 10% human AB serum and IL-2 (provided by the National Cancer Institute, Surgery Branch; 20 units/ml). V8T cells were restimulated with CAP-1 peptide (25 μ g/ml)-pulsed APCs on day 16 after prior stimulation at an effector:APC ratio of 1:3. Irradiated (23,000 rads) autologous EBV-transformed B cells were used as APCs.

Culture of DCs from PBMCs

PBMCs were obtained from heparinized blood from a patient (patient 15) with metastatic pelvic carcinoma who was enrolled in a Phase I trial using a combination of rV-CEA and avipox-CEA vaccines (34). All of the experiments involving patient materials were conducted according to NIH guidelines, and written informed consent was obtained from all individuals. PBMCs were separated using lymphocyte separation medium gradient (Organon Teknika, Durham, NC), as described previously (35). DCs were prepared using a modification of the procedure described by Sallusto and Lanzavecchia (36). PBMCs (1.5×10^8) were resuspended in AIM-V medium containing 2 mM glutamine, 50 μ g/ml streptomycin, 10 μ g/ml gentamicin (Life Technologies, Inc.) and allowed to adhere to a T-150 flask (Corning Costar Corp., Cambridge, MA). After 2 h at 37°C, the nonadherent cells were removed with a gentle rinse. The adherent cells were cultured for 6–7 days in AIM-V medium

containing 50 ng/ml of rhGM-CSF and 0.5 ng/ml of rhIL-4. The culture medium was replenished every 3 days.

Infection of DCs with Wild-Type and Recombinant Avipox Viruses

A 2109-bp DNA fragment encoding the entire open reading frame of CEA was obtained as described by Kaufman *et al.* (37). The following recombinant avipox (ALVAC) viruses were manufactured by Virogenetics Corp. (Troy, NY) using previously described methods (38–40): avipox-CEA, avipox-CEA/B7-1, avipox B7-1. Recombinant avipox (ALVAC)-RG was kindly supplied by Dr. James Tartaglia (Aventis Pasteur, Toronto, Canada). DCs (1×10^6) were incubated in 1 ml of Opti-MEM (Life Technologies, Inc.) at 37°C with avipox-B7-1, avipox-CEA, avipox-CEA/B7-1, and avipox-RG. Titration experiments indicated that 3×10^7 plaque-forming units/ml, equal to a multiplicity of infection of 30:1 for 2 h, were able to consistently induce expression of CEA in ~75% of the infected DCs. The infected DCs were suspended in 10 ml of fresh, warm RPMI 1640 complete medium containing 50 ng/ml of rhGM-CSF and 0.5 ng/ml rhIL-4 cultured for 24 h, and then subsequently used as stimulators.

Peptides

The CEA peptide CAP-1 (Ref. 30; amino acid position 571–579; YLSGANLNL), the CAP-1 agonist peptide CAP1-6D (Ref. 28; YLSGADLNL), and the Flu matrix protein peptide 58–66 (GILGFVFTL) were greater than 96% pure; they were manufactured by Multiple Peptide System (San Diego, CA).

Generation of T-Cell Lines

Modification of the protocol described by Tsang *et al.* (30) was used to generate CEA-specific CTLs. Uninfected DCs and DCs infected with avipox-B7-1, avipox-CEA, or avipox-CEA/B7-1 were used as APCs. CAP-1 peptide was added to the uninfected or avipox-B7-1-infected DCs at a final concentration of 25 μ g/ml. Autologous nonadherent cells were then added to APCs at an APC:effector ratio of 1:10. 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 then supplemented with rhIL-2 at a 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 IVS cycle. Primary cultures were restimulated with CAP-1 peptide (25 μ g/ml) on day 11 to begin the next IVS cycle. Irradiated (23,000 rads) autologous EBV-transformed B cells were used as APCs. A similar procedure was used for CTL generation when DCs that were infected with avipox-CEA or avipox-CEA/B7-1 were used as APCs, with the exception that no CAP-1 peptide was in the stimulation.

Construction of Peptide MHC Tetramers

Peptide-MHC complexes were synthesized as described by Altman *et al.* (41). The β_2 M clone was obtained from Dr. D. N. Garboczi (Harvard University, Cambridge, MA; Ref. 42) and the HLA-A2 construct was obtained from Immunotech (Beckman-Coulter, Marseille, France). The soluble HLA-A2 molecules, containing the 15-amino-acid substrate peptide for BirA-dependent biotinylation to the COOH-terminus of the HLA-A2 heavy chain and β_2 M, were grown separately in *Escherichia coli* and isolated as inclusion bodies. HLA-A2 and β_2 M were solubilized and renatured in the presence of CAP-1 or Flu-M1 58–66 peptide. The complex was purified by fast protein liquid chromatography on Superdex 200 (Pharmacia, Piscataway, NJ). Purified peptide-MHC complex was biotinylated using the BirA enzyme (Avidity, Denver, Co.). Tetramers were produced by mixing the biotinylated peptide-MHC complex with phycoerythrin-labeled UltraAvidin (Leinco Technologies, Inc., St. Louis, MO) at a molar ratio of 4:1.

Flow Cytometry

Single-Color Flow Cytometric Analysis. The method for single-color flow cytometric analysis has been described previously (43). Briefly, cells were washed three times with cold Ca²⁺ and Mg²⁺-free DPBS and then stained for 1 h with monoclonal antibody against HLA-A2 (A2,69; One Lambda, Inc., Canoga Park, CA), using 10 μ g of the $1 \times$ working dilution/ 10^6

cells. Mineral oil plasmacytoma-104E (Cappel/Organon Teknika Corp., West Chester, PA) was used as an isotype control. The cells were then washed three times and incubated with a 1:100 dilution of FITC-labeled goat antimouse immunoglobulin (IgG; Kirkegaard and Perry Laboratories, Gaithersburg, MD). The anti-CEA monoclonal antibody COL-1 (44) was used in 100 μ l of culture supernatant. The cells were then washed three times with DPBS and incubated for an additional hour in the presence of a 1:100 dilution (volume of 100 μ l of PBS containing 1% BSA) of FITC-conjugated goat antimouse immunoglobulin (Kirkegaard and Perry Laboratories). The cells were again washed three times with DPBS and resuspended in DPBS at a concentration of 1×10^6 cells/ml. The cells were immediately analyzed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 nW at 488 nm. Data were gathered from 10,000 live cells and were stored and used to generate results.

Dual-Color Flow Cytometric Analysis. The procedure for dual-color flow cytometric analysis was similar to that for single-color analysis with the following exceptions. The antibodies used were anti-CD86 FITC/anti-CD80 PE, anti-CD58 FITC/anti-CD54 PE, anti-MHC class I FITC/anti-MHC class II PE, and anti-IgG1 FITC/anti-IgG2a PE (isotype controls; Becton Dickinson, San Diego, CA, and Serotec, Oxford, United Kingdom). Staining was done simultaneously for 1 h, after which cells were washed three times, resuspended as above, and immediately analyzed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 nW at 488 nm with the use of the CellQuest program. DCs were gated for analysis.

Staining and Sorting of T Cells. CEA-tetramer-PE (CAP-1-MHC tetramer-PE) was used for the flow cytometric analysis and sorting of T cells. A procedure similar to that described above was used for tetramer staining. CEA-tetramer-PE was used at a concentration of $0.33 \mu\text{g}/2 \times 10^5$ cells. Cells were stained with CEA-tetramer-PE for 1 h at 4°C and then stained with anti-CD8 FITC for an additional hour. Cells were washed and analyzed on a Vantage Cell sorter (Becton Dickinson) or a FACScan (Becton Dickinson) using CellQuest software (Becton Dickinson). Sorter cells were cultured and expanded as described previously (30). Cells stained with UltraAvidin-PE and Flu-tetramer-PE were used as negative controls.

Cytotoxic Assay

Target cells were labeled with 50 μ Ci of ^{111}In -labeled oxyquinoline (Medi-Physics Inc., Arlington, IL) for 15 min at room temperature. Target cells (0.3×10^4) in 100 μ l of RPMI 1640 complete medium were added to each of 96 wells in flat-bottomed assay plates (Corning Costar Corp.). The labeled target cells were incubated with peptides for 60 min at 37°C in 5% CO_2 before effector cells were added. No peptide was used when carcinoma cell lines were used as targets. Effector cells were suspended in 100 μ l 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% CO_2 for 4 or 16 h. 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 cpm):

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

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

HLA Typing

The HLA phenotyping was performed by the Blood Bank of the NIH, using a standard antibody-dependent microcytotoxicity assay and a defined panel of anti-HLA antisera. The class I phenotype of the V8T cell line was: HLA-A2: B18 (W6), 44 (12, W4), and for patient 15, it was HLA-A2, 28: B13 (BW4), B51 (BW4); CW6.

Detection of Cytokine

Supernatant of T cells, stimulated for 24 h with DCs infected with avipox-CEA or avipox-CEA/B7-1 or to peptide-pulsed uninfected DCs and avipox-

B7-1-infected DCs in IL-2-free medium at various responder:stimulator ratios, were screened for secretion of IFN- γ using an ELISA kit (R&D Systems, Minneapolis, MN). The results are expressed in pg/ml.

Statistical Analysis

Statistical analysis of differences between means was done using a two-tailed *t* test.

RESULTS

Studies were first conducted to determine whether infection of human DCs with various recombinant avipox vectors would result in expression of the respective transgene on the DC surface. Human DCs were differentiated from adherent PBMCs by culturing with GM-CSF and IL-4 as described in "Materials and Methods." The yield of DCs was ~2–4% of the starting PBMC population. DCs were infected with avipox vectors as described in "Materials and Methods"; surface expression of six different markers was determined by FACS in terms of both the percentage of positive cells and the MFI. As seen in Fig. 1, >97% of DCs express CD86, CD 54, and class I and class II MHC; 20% of cells express CD80 (B7-1); and no expression of CEA was observed. Infection of DCs with a control avipox recombinant expressing the RG (avipox-RG) did not appreciably alter the expression of any of these markers.

Infection of DCs with avipox-B7-1 enhanced the expression of B7-1 in terms of both the percentage of positive cells and the MFI (Fig. 1); the MFI of class II was lower in the vector-infected DCs. None of the other markers were appreciably changed. Infection of DCs with the avipox-CEA recombinant resulted in enhanced expression of CEAs without affecting other markers. Infection of DCs with avipox-CEA/B7-1 dual transgene recombinant resulted in enhanced expression of both CEA and B7-1 (Fig. 1).

Studies were then carried out to determine the efficiency of avipox-B7-1-infected DCs to enhance antigen-specific T-cell responses as compared with control, uninfected DCs. For this purpose, DCs were pulsed with the CEA agonist peptide CAPI-6D. Previous studies (29) have shown that pulsing target cells with the CAPI-6D agonist peptide renders them more susceptible to CAP-1-specific T-cell lysis than pulsing with the CAP-1 peptide. As an effector cell, the allogeneic CAP-1-specific T-cell line V8T was used. All of the experiments were also conducted in the absence of peptide to rule out anti-allo-specific responses. Infection of DCs with avipox-RG was used to determine whether any effects observed were attributable to vector alone. T-cell responses were measured by the production of IFN- γ . As seen in Fig. 2, peptide-pulsed DCs, infected with avipox-B7-1, were more efficient in activating CEA-specific T cells than were peptide-pulsed uninfected DCs, or peptide-pulsed DCs infected with avipox-RG, at all six peptide concentrations. No IFN- γ production was observed in the absence of peptide.

Studies were also conducted to determine whether an avipox-CEA/B7-1 dual transgene recombinant vector could deliver both signal 1 and a costimulatory signal to the CEA-specific V8T cell line. Avipox-CEA infection of DCs led to enhanced activation of the CEA-specific T-cell line, whereas infection of DCs with the avipox-CEA/B7-1 recombinant led to even further production of IFN- γ (Table 1). The experiments shown in Fig. 1 demonstrated that infection of DCs with avipox-B7-1 had no effect on this cell line in the absence of exogenous peptides.

Studies were then conducted to determine whether the avipox-B7-1 vector (with peptide), avipox-CEA, and the avipox-CEA/B7-1 vector could be used to establish CEA-specific T-cell lines from PBMCs of a vaccinated patient; patient 15, with advanced colorectal cancer, had previously received recombinant CEA vaccine therapy. The specific-

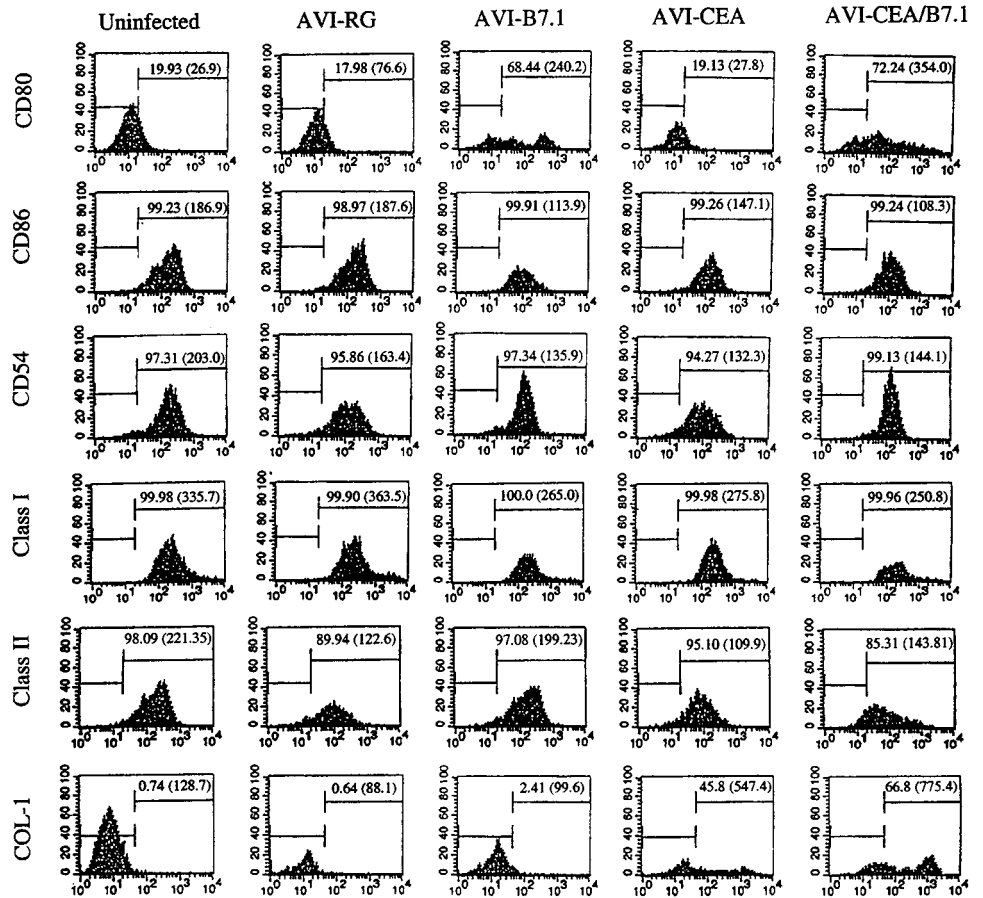


Fig. 1. Flow cytometric analysis of surface marker expression on human DCs before and after infection with avipox RG, avipox-B7-1, avipox-CEA, and avipox-CEA/B7-1. DCs (1×10^6) were incubated in 1 ml of Optim-MEM at 37°C with the avipox vector at a multiplicity of infection of 30:1 for 2 h. The infected DCs were suspended in 10 ml of fresh, warm RPMI 1640 complete medium containing 100 ng/ml of rhGM-CSF and 20 ng/ml rhIL-4, and cultured for 24 h. DCs were generated in GM-CSF and IL-4 for 7 days prior to infection with recombinant vectors. Numbers in each histogram, the percentage of positive cells; numbers in parentheses, the MFI.

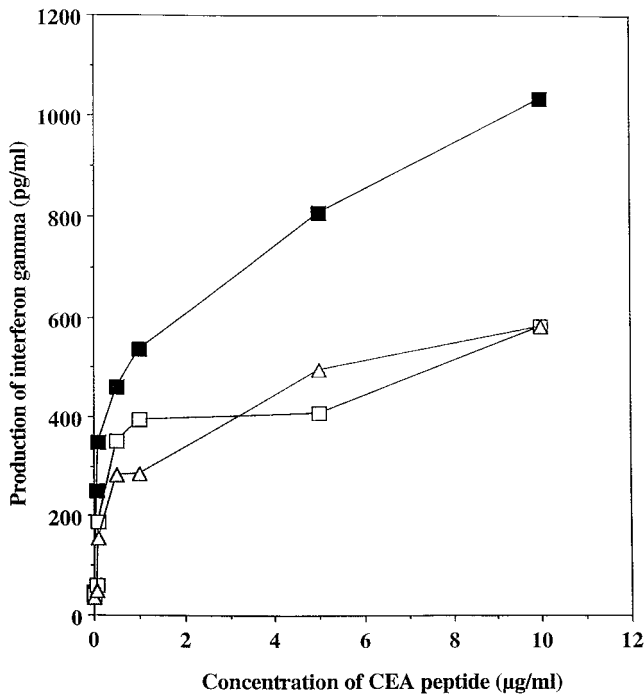


Fig. 2. Production of IFN- γ by CEA-specific T cells on stimulation by CEA peptide-pulsed DCs, uninfected, or infected with avipox-B7-1. Effector cells were the CEA-specific T-cell line V8T. Stimulator cells were DCs pulsed with no peptide or different concentrations of the CEA peptide CAPI-6D. DCs were uninfected (Δ), infected with avipox-RG control recombinant vector (\square), or infected with avipox-B7-1 (\blacksquare). This experiment was performed three times with similar results.

ity of the T-cell lines established from patient 15 was analyzed by the ability of the T-cell lines to lyse C1R-A2 targets with or without pulsing with the CEA peptide CAPI-6D. Four T-cell lines could be established as designated in Table 2: (a) the T-15-1 line was established using CEA peptide-pulsed DCs; (b) the T-15-2 line was established using CEA peptide-pulsed DCs that had been infected with avipox-B7-1; (c) the T-15-3 line was established using DCs infected with avipox-CEA; and (d) the T-15-4 line was established using DCs infected with avipox-CEA/B7-1. All four T-cell lines were then assayed for lytic potential at IVS-3, as described in "Materials and Methods." As seen in Table 2, the T-15-2 cell line was more lytic for C1R-A2 cells pulsed with the CEA peptide than was the T-15-1 cell line. The only difference in the establishment of these two cell lines was that peptide-pulsed DCs were infected with avipox-B7-1 to establish the T-15-2 line. Analogous results were seen when signal 1 (CEA) was delivered via the avipox vector. The T-15-4 cell line demonstrated more lysis of peptide-pulsed targets than did the T-15-3 line. No difference in lysis (both negative) was seen using nonpeptide-

Table 1 Specific release of IFN- γ by CEA-specific cells stimulated with human DCs infected with avipox-B7-1 or avipox-CEA/B7-1

CEA-specific V8T cells were stimulated with irradiated allogeneic DCs that were either uninfected or were infected with avipox-CEA or avipox-CEA/B7-1. Effector:APC ratio was 10:1. Twenty-four-h culture supernatants were collected and screened for the secretion of IFN- γ . Infections were performed at 30 multiplicity of infection for 2 h. Infected cells were then incubated in complete medium at 37°C overnight and then used as APCs.

APCs:DCs	Production of IFN- γ (pg/ml)
Uninfected	<15
Infected with avipox-CEA	250
Infected with avipox-CEA/B7-1	545

Table 2 CTL activity of four CEA-specific T-cell lines generated with and without vector-driven T-cell costimulation

Four different CEA-specific T-cell lines were established using autologous PBMCs from a patient (patient 15) who had previously received CEA-based vaccine therapy (see "Materials and Methods"). Line T-15-1 was established using as APCs, DCs pulsed with CEA-peptide CAP-1. Line T-15-2 was established in the same manner as T-15-1, except that DCs were infected with avipox-B7-1. Line T-15-3 was established using DCs infected with avipox-CEA to deliver signal 1. Line T-15-4 was established using DCs infected with avipox-CEA/B7-1 to deliver signal 1 and signal 2. T-cell lines were assayed for lytic potential at *in vitro* stimulation cycle 3 (see "Materials and Methods"). A 4-h ¹¹¹In release assay was performed. Labeled C1R-A2 cells were used as target. CAPI-6D peptide was used at a concentration of 25 μg/ml. The results are expressed as percentage specific lysis at E:T ratios of 25:1 and 12.5:1. The number in parentheses is the SD.

APC used to establish T-cell line	T-cell line designation	Target cell pulsed with CAPI-6D	E:T = 12.5:1		E:T = 25:1	
			% specific lysis (SD)		% specific lysis (SD)	
Uninfected DCs pulsed with CEA peptide	T-15-1	+ ^b	11.5 (0.49)	18.5 (4.32) ^c		
	T-15-1	-	4.5 (0.88)	9.0 (2.85)		
Peptide-pulsed DCs infected with avipox-B7-1	T-15-2	+	33.9 (1.73) ^c	52.8 (0.26) ^c		
	T-15-2	-	4.2 (0.25)	14.3 (1.77)		
DCs infected with avipox-CEA	T-15-3	+	19.4 (0.61) ^c	36.4 (3.64) ^c		
	T-15-3	-	5.1 (0.87)	8.4 (0.34)		
DCs infected with avipox-CEA/B7-1	T-15-4	+	33.8 (0.81) ^c	62.4 (0.24) ^c		
	T-15-4	-	3.4 (0.94)	5.7 (1.76)		

^a Statistically significant differences ($P < 0.01$, two-tailed *t* test) were observed between groups using target cells pulsed with peptide: the *P* for T-15-1 versus T-15-2 at 25:1 and 12.5:1 were 0.0038 and 0.0042, respectively; for T-15-1 versus T-15-3 at 25:1 and 12.5:1 were 0.0465 and 0.0460, respectively; for T-15-1 versus T-15-4 at 25:1 and 12.5:1 were 0.025 and 0.0014, respectively; for T-15-3 versus T-15-4 at 25:1 and 12.5:1 were 0.0096 and 0.001, respectively.

^b +, yes; -, no.

^c Statistically significantly different ($P < 0.01$, two-tailed *t* test) from values obtained without peptide.

Table 3 Ability of CEA-specific T-cell lines to lyse CEA-expressing tumor cells

T cells were assayed at IVS-4. An 18-h ¹¹¹In release assay was performed. Labeled colorectal carcinoma cell lines SW1463 (HLA-A1, 2) and LS174T (with a very low expression of HLA-A2) were used as targets. The results are expressed as percentage of specific lysis at E:T ratios of 50:1 and 25:1. The number in parentheses is the SD.

T-cell line	SW1463		LS174T	
	50:1	25:1	50:1	25:1
T-15-1 ^a	12.2 (4.5) ^b	6.3 (1.9)	1.3 (0.2)	1.8 (0.3)
T-15-2 ^c	18.7 (3.4) ^b	14.6 (1.2) ^b	1.4 (0.8)	0.9 (0.2)
T-15-3 ^d	19.4 (1.9) ^b	12.4 (0.8) ^b	1.7 (0.3)	1.5 (0.6)
T-15-4 ^e	26.8 (2.3) ^b	18.3 (0.6) ^b	2.1 (0.2)	1.3 (0.4)

^a T-cell line established by stimulating with uninfected DCs pulsed with CAP-1 peptide.

^b Statistically significantly different ($P < 0.01$, two-tailed *t* test) from values obtained from lysis of LS174T cells.

^c T-cell line established using DCs infected with avipox-B7-1 and pulsed with CAP-1 peptide.

^d T-cell line established using DCs infected with avipox-CEA.

^e T-cell line established using DCs infected with avipox-CEA/B7-1-infected DCs.

pulsed target cells (Table 2). The only difference between the APCs used to establish these two T-cell lines was that DCs were infected with avipox-CEA/B7-1 to establish T-15-4, whereas DCs were infected with avipox-CEA to establish T-15-3. It should be pointed out that the T-15-3 and T-15-4 T-cell lines are most likely polyclonal in nature because the entire CEA transgene was expressed. The read-out for these assays, however, was lysis of cells pulsed with the CAPI-6D CEA peptide. Thus, only part of the CEA-specific response was most likely being seen in these assays. The peptide-specific read-out was used here to compare the use of pulsing with a single peptide to deliver signal 1, versus the infection of a DC with a vector to deliver signal 1. Five different donors were studied in this investigation. Results from two donors are shown in Tables 1–4. Similar results

were obtained using DCs from three other donors (Table 5).

Four T-cell lines were tested for their ability to lyse CEA-expressing carcinoma cells in an 18-h assay. No killing was observed in a 4-h assay. As seen in Table 3, all four were capable of lysis at the SW1463 colon carcinoma cell line, which expresses CEA and HLA-A2. They were not capable, however, of lysing the LS174T colon carcinoma line that expresses CEA, but little, if any, HLA-A2 (<2.0% HLA-A2-positive cell with MFI of 14). Cold target inhibition assays were also performed using the T-15-2 and T-15-4 cell lines. As seen in Table 4, the lysis of CEA peptide-pulsed ¹¹¹In-labeled C1R-A2 cells by either of the T-cell lines was inhibited by the addition of unlabeled CEA-expressing SW1463 cells. In separate experiments, the addition of unlabeled C1R-A2 cells pulsed with CEA peptide CAPI-6D also decreased the lysis of SW1463 cells by the two T-cell lines.

To further characterize the ability of an avipox vector to deliver both signal 1 and signal 2 to activate T cells, T-cell populations were isolated and characterized with the use of a peptide-specific tetramer. A tetramer for the MHC-CAP-1 complex was constructed as described in "Materials and Methods"; a Flu-MHC tetramer was similarly constructed as a control. The CEA-specific V8T line and a Flu T-cell line were used to evaluate the tetramers. Flow cytometry was

Table 4 Cold target inhibition assay: the ability of CEA-expressing SW1463 cells to inhibit the lysis of CEA peptide CAPI-6D-pulsed C1R-A2 cells by T-cell lines

An 18-h ¹¹¹In release assay was performed. CAPI-6D peptide was used at a concentration of 25 μg/ml. The results are expressed as percentage of specific lysis at an E:T ratio of 25:1. The number in parentheses is the SD. Unlabeled CEA-expressing SW1463 colon carcinoma cells and labeled C1R-A2 cells were used at a ratio of 10:1. See "Materials and Methods" for details.

T-cell line	Target cells	
	C1R-A2 pulsed with CAPI-6D	SW1463 + C1R-A2 pulsed with CAPI-6D (10:1)
T-15-2 ^a	76.7 (4.32) ^b	11.8 (0.44)
T-15-4 ^c	67.9 (1.97) ^b	5.7 (1.03)

^a T-cell line established by stimulating with DCs infected with avipox-B7-1 and pulsed with CAP-1 peptide.

^b Statistically significantly different ($P < 0.01$, two-tailed *t* test) from value obtained without the addition of SW1463 cells.

^c T-cell line established by stimulating with DCs infected with avipox-B7-1 and pulsed with avipox-CEA/B7-1-infected DCs.

Table 5 Specific release of IFN-γ by a CEA-specific T-cell line (V8T) stimulated with human DCs from different donors infected with avipox vectors

CEA-specific T-cell line V8T was stimulated with irradiated allogeneic DCs from three normal donors that were either uninfected or infected with control vector (avipox-RG), avipox-B7-1, avipox-CEA, or avipox-CEA/B7-1. Effector:APC ratio was 10:1. Twenty-four-h culture supernatants were collected and screened for the secretion of IFN-γ. Infections were performed at 30 MOI for 2 h. Infected cells were then incubated in completed medium at 37°C overnight and then used as APCs. CAPI-6D peptide was used at a concentration of 10 μg/ml.

DCs infected with	Donor of DCs	Production of IFN-γ (pg/ml)	
		No peptide	Plus peptide
Uninfected	1	<26	312
	2	<26	545
	3	<26	456
Control vector	1	<26	401
	2	<26	515
	3	NT ^a	NT
Avipox-B7-1	1	<26	777
	2	28	1271
	3	NT	NT
Avipox-CEA	1	259	NT
	2	305	NT
	3	250	NT
Avipox-CEA/B7-1	1	486	NT
	2	696	NT
	3	555	NT

^a NT, not tested.

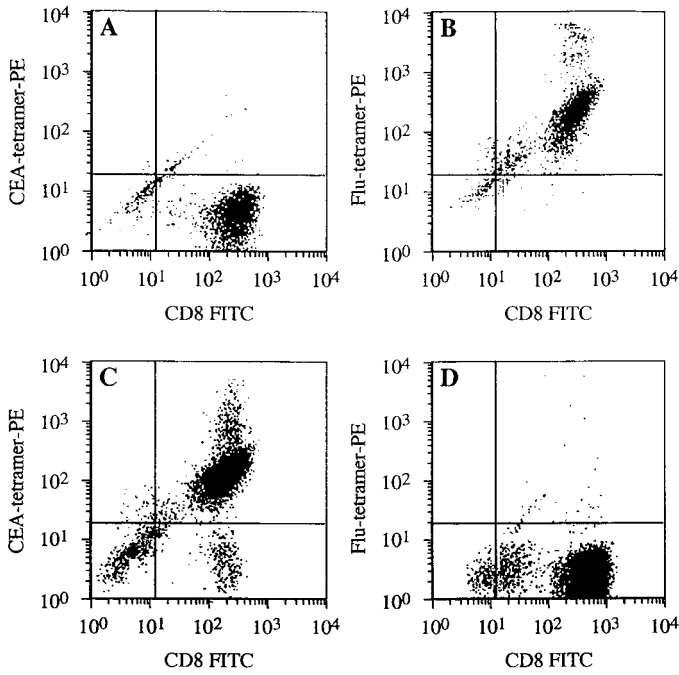


Fig. 3. Binding of CEA tetramer and Flu tetramer to T-cell lines. The CEA tetramer contained the CAP-1 peptide, and the Flu tetramer contained the 58–66 Flu peptide. T cells were the CEA-specific V8T and the Flu-specific CD8⁺ T-cell lines. A, the CEA tetramer binding to the Flu-specific T cells. B, the Flu tetramer binding to the Flu-specific T cells. C, the CEA tetramer binding to the CEA-specific T cells. D, the Flu tetramer binding to the CEA-specific T cells.

used to analyze the ability of the tetramers to bind the TCR of these two CD8⁺ T cell lines. As seen in Fig. 3, the Flu tetramer did not bind the V8T line (Fig. 3D), whereas it bound 91% of the Flu T cells (Fig. 3B). The CEA CAP-1 tetramer, on the other hand, bound 95% of the CEA-specific V8T cells (Fig. 3C) and only minimally to Flu T cells (Fig. 3A).

The CEA tetramer that was then used characterized the T cells activated by the four methods outlined in Table 2. T cells at IVS-2 were used in these studies. As seen in Fig. 4A, Panel A, the CAP-1 tetramer bound to only 0.93% of the T-15-1 cells activated with CAP-1-pulsed DCs but bound 24.62% of the T-15-2 cells activated with CAP-1 pulsed DCs infected with avipox-B7-1 (Fig. 4A, Panel B). Similarly, the CEA tetramer bound only 0.55% of T cells activated with DCs infected with avipox-CEA (Fig. 4A, Panel C) but bound 15.89% of CD8⁺ cells activated with DCs infected with avipox-CEA/B7-1 (Fig. 4A, Panel D).

Studies were then conducted to determine whether the degree of CEA tetramer binding to T cells correlated with the ability of that T-cell population to lyse targets. The T-15-2 T-cell line, which showed the highest level of tetramer binding of the four T-cell lines, was chosen for further characterization at IVS-4. The T-15-2 cells showed a broad range of tetramer-positive CD8⁺ cells, which could be sorted into high-tetramer-binding (T-15-2H) and low-tetramer-binding (T-15-2L) populations (see Fig. 4B). Each of these two populations was then restimulated with CEA-pulsed autologous EBV-transformed B cells. The two T-cell populations were then analyzed for their ability to lyse C1R-A2 cells with or without CEA peptide pulsing. As seen in Fig. 5A, the T-15-2H cells lysed the CEA peptide-pulsed targets at a greater efficiency than did the T-15-2L cells. Little if any lysis was observed using either cell line *versus* using targets without peptide. The difference in lysis between the high- and low-tetramer-binding lines was further magnified, however, when a CEA-positive, HLA-A2-positive colon carcinoma cell line was used as a

target. As seen in Fig. 5B, the low-tetramer-binding cells were not capable of lysis of the SW1463 (CEA⁺, HLA-A2⁺) carcinoma cells or the LS174T (CEA⁺, HLA-A2) carcinoma cells, whereas the high-tetramer-binding cells were capable of lysing the SW1463 carcinoma cells and not the LS174T cells. This was seen at two different ratios of E:T cells.

DISCUSSION

The studies reported here demonstrate that recombinant avipox vectors can efficiently deliver and express transgenes for signal 1, signal 2, or both, to human DCs; this in turn was shown to lead to enhanced activation of human T cells. Two models were used in these studies. In the autologous model, T-cell lines and DCs were derived from the same patient with advanced carcinoma who had previously

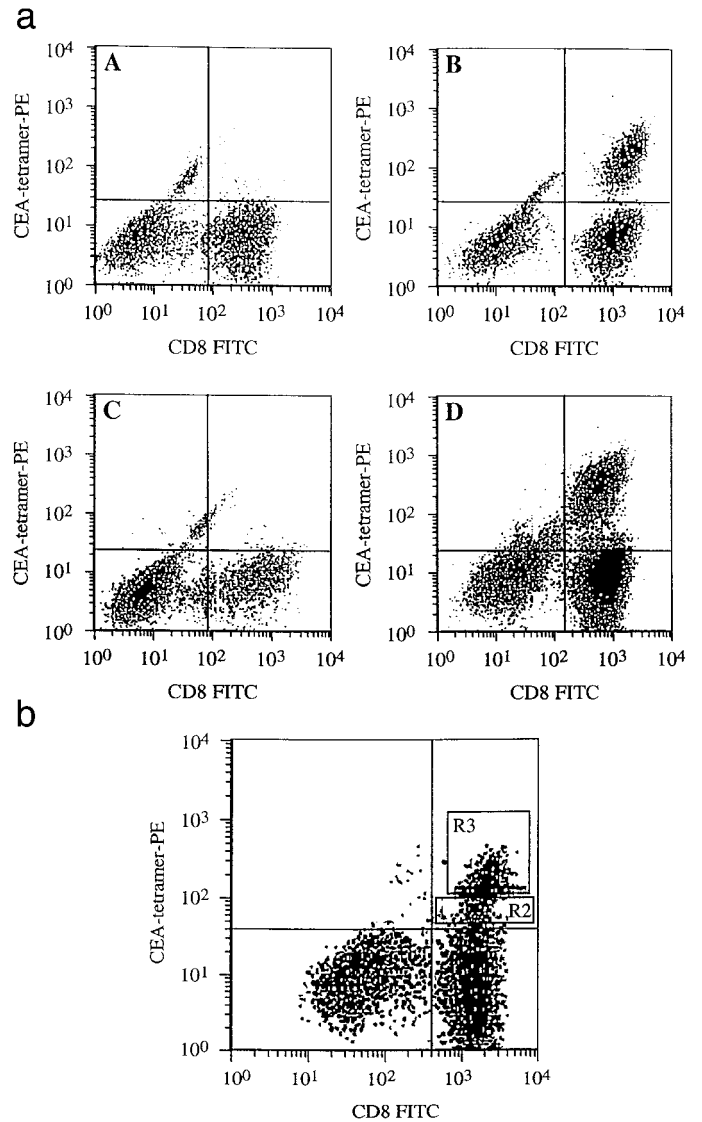


Fig. 4. a, binding of CEA tetramer to different CEA-specific T-cell lines. Binding of the CEA tetramer to T cells established by the following APCs: CEA peptide CAP-1-pulsed DCs (Panel A); CAP-1-pulsed DCs infected with avipox-B7-1 (Panel B); DCs infected with avipox-CEA (Panel C); DCs infected with avipox-CEA/B7-1 (Panel D). b (bottom panel), separation of CEA-specific T cells into high- and low-CEA-tetramer binders. The T-cell line used was T-15-2, which was generated by incubation with CEA peptide CAP-1-pulsed DCs that had been infected with avipox-B7-1. The T cells were stained with FITC-conjugated anti-CD8 antibody and the PE-labeled CAP-1-MHC-tetramer and sorted for CD8⁺ and CAP-1-MHC high-tetramer-binding (R3, designated T-15-2H) and low-tetramer-binding (R2, designated T-15-2L) cells.

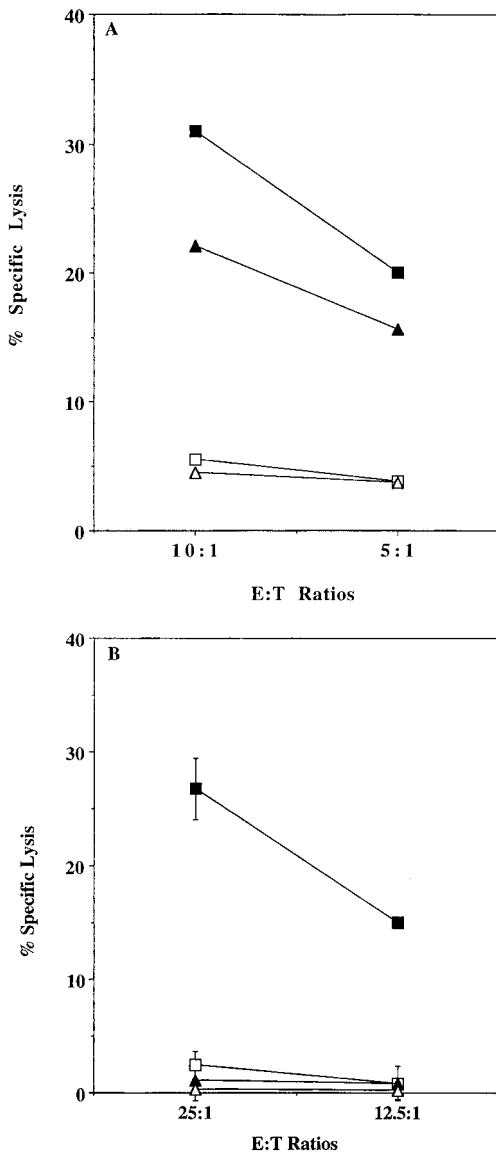


Fig. 5. A, cytotoxicity of high- and low-CEA-tetramer-binding T cells using peptide-pulsed targets. High-CEA-tetramer-binding CEA-specific T cells (T-15-2H) were analyzed for lysis of C1R-A2 cells pulsed with CEA peptide (■) or without peptide (□). Low-CEA-tetramer-binding (T-15-2L) T cells were analyzed for lysis of C1R-A2 cells pulsed with CEA peptide (▲) or without peptide (△). The E:T ratios were 10:1 and 5:1, and cells were pulsed with 25 μ g/ml CEA peptide CAP1-6D. B, cytotoxicity of high- and low-CEA-tetramer-binding T cells using tumor cells as targets. Target cells were colon carcinoma cell lines SW1463 (CEA⁺, A2⁺) and LS174T (CEA⁺, A2⁻). Cytotoxicity of high tetramer binding (T-15-2H) T cells for SW1463 (■) and LS174T (▲). Cytotoxicity of low-tetramer-binding (T-15-2L) T cells for SW1463 (□) and LS174T (△). CTL activity was determined in a 16-h ¹¹¹In release assay at E:T ratios of 25:1 and 12.5:1.

received CEA-based vaccine therapy. In the allogeneic model, a previously characterized (29, 33) CEA-specific T-cell line was used to evaluate T-cell responses using allogeneic DCs. Allospecific reactions were ruled out in all of these studies by the fact that no T-cell activation was observed in the absence of peptide or with the use of uninfected DCs or DCs infected with wild-type vector (FP-WT).

CEA remains an intriguing potential target for cancer therapy. CEA is overexpressed on the vast majority of colorectal, gastric, and pancreatic cancers, 50% of breast cancers, and 70% of non-small cell lung cancers. Although it is a self-antigen expressed in fetal tissue and to a lesser extent on normal colonic epithelium, clinical trials using several types of CEA-based vaccines have demonstrated the ability of these vaccines to generate both CEA-specific CD4⁺ and CD8⁺ T-cell

responses in patients with advanced cancers (30, 34, 45–47). A recent study (48), using DCs pulsed with the CEA TCR-enhancer agonist peptide, CAP1-6D (28), showed the generation of T-cell responses, decreases or stabilization in serum CEA, and antitumor activity in patients with advanced carcinoma. A recent study (49), using rV-CEA as a prime vaccination followed by multiple booster vaccinations with avipox CEA, showed a statistical correlation ($P = 0.04$) with the generation of CEA-specific T-cell responses and survival at 2-plus years in vaccinated patients with advanced carcinomas.

The experiments reported here on the use of the CEA peptide CAP-1 tetramer demonstrate the specificity of the CEA-specific T-cell lines. They also demonstrate that CAP-1-specific T cells, isolated by MHC-CAP-1 tetramers, can recognize the CAP-1 epitope on human colon carcinoma cells manifested by the lysis of those tumor cells. The finding that only the high-tetramer binders can lyse the colon carcinoma cell indicates that the CAP-1 epitope is present on tumor cells either at a low concentration or perhaps at a low avidity. The studies reported here, however, are analogous to recent findings of Yee *et al.* (50). In those studies, MART-1 tetramer could be used to separate different T-cell populations with different abilities to lyse melanoma cells expressing MART-1. Those findings and the findings reported here with the CEA tetramer suggest that a more detailed analysis of the T cells of cancer patients, before and after vaccination, may lead to valuable information concerning the potential success of a given vaccine strategy.

Avian orthopox viruses (fowlpox or canarypox, *e.g.*, ALVAC) are replication competent in avian cells but are replication defective in mammalian cells. The recombinant avipox vectors used here express their transgene(s) on early orthopox promoters; these transgenes are expressed for ~14–21 days, at which time the infected cells die. Although some avipox proteins are also expressed in virus-infected mammalian cells and host immune responses to these proteins can be detected, most avipox structural proteins are not synthesized in mammalian cells; no evidence of host-neutralizing antibodies to avipox recombinants has been observed in either preclinical or clinical studies after repeated recombinant avipox administration. In a recent clinical trial, avipox CEA recombinants have been administered five to nine times at monthly intervals, with resultant increases in CEA-specific precursors seen after each successive vaccination (34, 51). Murine studies have also demonstrated that avipox recombinants can be given multiple times without any evidence of host-virus neutralizing activity (52–54). Because of their ability to be given to humans multiple times and their demonstrated lack of toxicity in several human clinical trials, avipox recombinants appear to be attractive vectors for the delivery of transgenes to human DCs. It should also be pointed out that because of their large size, avipox vectors can accommodate and simultaneously express several different transgenes, each on different orthopox promoters. Indeed, it has recently been demonstrated that murine tumor cells and murine DCs can be infected with avipox recombinant vectors and vaccinia recombinant vectors each containing a triad of murine costimulatory molecules, and each of these molecules was expressed on the cell surface (19). Recent studies have also demonstrated that recombinant avipox vectors expressing four transgenes simultaneously (*CEA*, *B7-1*, *ICAM-1*, and *LFA-3*) can be used in experimental murine studies to enhance CEA-specific T-cell responses and antitumor activity (53).

It is unclear at this point whether the use of peptide-pulsed DCs or the use of vectors to deliver signal 1 to DCs will be most efficacious in human clinical trials. Although the use of peptide-pulsed DCs may display the maximum amount of an immunodominant peptide-MHC complex to the TCR, the use of a vector to deliver the entire transgene (perhaps including multiple CTL epitopes as well as helper epitopes) would appear to be more advantageous. Each of these expressed

epitopes, however, would most likely be expressed in the context of MHC at a lower concentration than peptide-pulsed DCs. Perhaps a combination of both of these methods would be most efficacious. The studies reported here demonstrate, however, the advantage of using (a) peptide-pulsed DCs infected with avipox-B7 as compared with the use of uninfected peptide-pulsed DCs in activating peptide-specific T-cell responses, and (b) DCs infected with avipox-CEA-B7 dual recombinant, as compared with the use of DCs infected with avipox-CEA, in the generation of CEA-specific T-cell responses.

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