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Improving Vaccine Potency Through Intercellular Spreading and Enhanced MHC Class I Presentation of Antigen

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The potency of naked DNA vaccines is limited by their inability to amplify and spread in vivo. VP22, a HSV-1 protein, has demonstrated the remarkable property of intercellular transport and may thus provide a unique approach for enhancing vaccine potency. Therefore, we created a novel fusion of VP22 with a model Ag, human papillomavirus type 16 E7, in a DNA vaccine that generated enhanced spreading and MHC class I presentation of Ag. These properties led to a dramatic increase in the number of E7-specific CD8 T cell precursors in vaccinated mice (around 50-fold) and converted a less effective DNA vaccine into one with significant potency against E7-expressing tumors. In comparison, nonspreading VP22 1–267 mutants failed to enhance vaccine potency. Our data indicated that the potency of DNA vaccines may be dramatically improved through intercellular spreading and enhanced MHC class I presentation of Ag. The Journal of Immunology, 2001, 166: 5733–5740.

Materials and Methods

Plasmid DNA and vaccinia construction

We used pcDNA3 as an expression vector instead of a previously described pCMV-Neo-Bam vector (7, 8) that generates a stronger vector-related antigen. Our data indicated that the property of intercellular spreading generated by VP22 was important for enhancing vaccine potency. These data indicated that the strategy of linking VP22 to a model Ag can dramatically improve the potency of naked DNA vaccines.

Department of Pathology, Obstetrics and Gynecology, Molecular Microbiology and Immunology, and Oncology, Johns Hopkins Medical Institutions, Baltimore, MD 21205; and Department of Obstetrics and Gynecology, National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan

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W.-F.C. and C.-F.H. contributed equally to this paper.

Address correspondence and reprint requests to Dr. T.-C. Wu, Department of Pathology, Johns Hopkins University School of Medicine, Ross Research Building, Room 659, 720 Rutland Avenue, Baltimore, MD 21205. E-mail address: wutc@jhmi.edu

Abbreviations used in this paper: HPV, human papillomavirus; GFP, green fluorescent protein; LDH, lactate dehydrogenase; DC, dendritic cell; TAT, HIV TAT protein; MTS, membrane-translocating sequence; AH, Antennapedia homeodomain; HSV, herpes simplex virus; LAMP, lysosome-associated membrane protein.
vector. To generate pSC11-VP22, VP22 was isolated from pcDNA3-VP22 by NotI/Pmel and cloned into NotI/Smal sites of pSC11 vector. For the generation of pcDNA3-TAT/E7, we synthesized complementary oligomers encoding MRKKRRQRRR (11, 12) 5′-ctgctgctgcctccctcgcttgctgcctgcacccctg-3′ and 5′-agctggctgcctgcctgcacccctg-3′. The oligomers were annealed and cloned into the XbaI/EcoRI sites of pcDNA3-E7/E(B). For the generation of pcDNA3-E7/MTS, we synthesized complementary oligomers encoding AAVLPVLVAAAP (13): 5′-gcattgcctgctgcctgcacccctgc-3′. The oligomers were annealed and cloned into the BswHI/HindIII sites of pcDNA3-E7/E(B). For the generation of pcDNA3-AH/E7, we synthesized complementary oligomers encoding MRQIKIWQFNRRMKWKK (14): 5′-ctagattgcctgctgcctgcacccctg-3′. The oligomers were annealed and cloned into the XbaI/EcoRI sites of pcDNA3-E7/E(B). The accuracy of all the DNA constructs was confirmed by sequencing.

**DNA vaccination**

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination was performed with a helium-driven gene gun (Bio-Rad, Hercules, CA) according to a previously described protocol (9). DNA-coated gold particles (1 μg DNA/bullet) were delivered to the shaved abdominal region of C57BL/6 mice with a helium-driven gene gun (Bio-Rad) with a discharge pressure of 400 p.s.i.

**Fluorescent microscopic examination**

293 D3,Kb cells were used for an in vitro assay of GFP expression. 293 D3,Kb cells were provided kindly by Dr. J. C. Yang (National Cancer Institute, National Institutes of Health, Bethesda MD) (15). A total of 20 μg of E7/GFP, VP22/E7/GFP, or VP22-267/E7/GFP DNA was transfected into 5 × 10^6 293 D3,Kb cells with lipofectamine 2000 (Life Technologies, Rockville, MD). Cells were collected 48 h after transfection and fixed with 100% methanol for 10 min at room temperature. The glass slides were mounted with anti-fading medium, Mowiol 4-88 (Calbiochem, La Jolla, CA) and covered with coverslips. Samples were examined on a fluorescent microscope.

**Immunohistochemical staining**

Vaccinated mice were sacrificed 3 days after the pcDNA3-VP22/GFP or pcDNA3-GFP DNA vaccination. Skin was biopsied, fixed, embedded in paraffin, and cut into 5-μm sections. After deparaffinization and hydration, the slides were incubated with rabbit anti-GFP polyclonal antibody (1:200 dilution; Custom Antibodies, La Jolla, CA) and covered with coverslips. Samples were examined on a fluorescent microscope.

**Intracellular cytokine staining**

Cell surface marker staining of CD8 or CD4 and intracellular cytokine staining for IFN-γ and IL-4 as well as FACScan analysis were performed under conditions described previously (9). Before FACScan, splenocytes from naïve or vaccinated groups of mice were incubated for 20 h with either 1 μg/ml of E7 peptide (aa 49–57; Ref. 16) containing an MHC class I epitope for detecting E7-specific CD8+ T cell precursors or 10 μg/ml of E7 peptide (aa 30–67; Ref. 17) containing an MHC class II epitope for detecting E7-specific CD4+ T cell precursors.

**CTL assays**

Splenocytes from vaccinated mice were harvested 2 wk after vaccination and cultured with E7 peptide (aa 49–57) containing an MHC class I epitope (16) at a concentration of 1 μg/ml in a total volume of 2 ml of RPMI 1640, supplemented with 10% (v/v) FBS, 50 U/ml penicillin and streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, and 2 mM non-essential amino acids in a 24-well tissue culture plate for 6 days. Spleno- cytoids were isolated as effector cells, and TC-1 tumor cells (15) were used as target cells. CTL assays were performed in 96-well round-bottom plates as described by Corr et al. (19). Cytolysis was determined by quantitative measurements of lactate dehydrogenase (LDH) (19). CTL assays were performed with effector cells and targets cells (1 × 10^5 per well) mixed together at various ratios (1:1, 5:1, 15:1, and 45:1) in a final volume of 200 μl. After a 5-h incubation at 37°C, 50 μl of the cultured medium was collected to assess the amount of LDH in the cultured medium according to the manufacturer’s protocol of the CytoTox assay kits (Promega, Madison, WI). The percentage of specific lysis was calculated from the fol- lowing equation: 100 × [(A - B)/(C - D)], where A is the reading of experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, and D is the target spontaneous background signal value.

**CTL assays with DNA-transfected or vaccinia-infected 293**

D3,Kb cells as target cells

A total of 20 μg of pcDNA3 (no insert), VP22, E7, or VP22/E7 DNA were transfected into 5 × 10^6 293 D3,Kb cells with lipofectamine 2000 (Life Technologies). Transfected 293 D3,Kb cells were used as target cells, and an E7-specific CD8+ T cell line (20) served as effector cells. Untransfected 293 D3,Kb cells were used as a negative control. Cells were collected 16 h after transfection. Cytolysis was determined by quantitative measurements of LDH as described above. CTL assays were performed with effector cells and targets cells (1 × 10^5 per well) mixed together at various ratios (1:1, 5:1, 15:1, and 45:1) in a final volume of 200 μl. After a 5-h incubation at 37°C, 50 μl of the cultured medium were collected to assess the amount of LDH in the cultured medium. The percentage of specific lysis was calculated as described above.

In a second CTL assay, vaccinia-infected 293 D3,Kb cells served as target cells. Briefly, 1 × 10^7 PFU of wild-type, VP22, E7, or VP22/E7 vaccinia was used to infect 1 × 10^6 293 D3,Kb cells. Cells were collected 16 h after infection. Cytolysis was determined by quantitative measurements of LDH as described above. CTL assays were performed with effector cells and targets cells (1 × 10^5 per well) mixed together at various ratios (1:1, 5:1, 15:1, and 45:1) in a final volume of 200 μl. After a 5-h incubation at 37°C, 50 μl of the cultured medium were collected to assess the amount of LDH in the cultured medium. The percentage of specific lysis was calculated as described above.

**In vivo tumor protection experiment**

For the tumor protection experiment, mice (five per group) were vaccinated via gene gun with 2 μg of pcDNA3 (no insert), VP22, E7, VP22-267/E7, or VP22/E7 DNA. One week later, mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were s.c. challenged with 5 × 10^5 cells/mouse TC-1 tumor cells in the right leg and then monitored twice a week.

**In vivo tumor treatment experiment**

Tumor cells and DNA vaccines were prepared as described earlier. Mice (five per group) were i.v. challenged with 1 × 10^5 cells/mouse TC-1 tumor cells via tail vein on day 0. Three days after challenge with TC-1 tumor cells, mice were administered 2 μg of pcDNA3 (no insert), VP22, E7, or VP22/E7 DNA via gene gun. One week later, these mice were boosted with the same regimen as the first vaccination. Mice were sacrificed and their lungs were explanted on day 21 after tumor challenge. The pulmonary nodules in each mouse were evaluated and counted by experimenters blinded to sample identity. Compared with the percentage of tumor-free mice in the s.c. tumor model, the mean number of tumor nodules in the pulmonary tumor model is a more sensitive indicator of the antitumor effect generated by each vaccine. The pulmonary tumor model may allow us to evaluate subtle differences in the antitumor effects that might not be appreciable by using our s.c. tumor model.

**In vivo Ab depletion experiment**

In vivo Ab depletions have been described previously (18). Briefly, mice were vaccinated with 2 μg VP22/E7 DNA via gene gun, boosted 1 wk later, and then challenged with 1 × 10^5 cells/mouse TC-1 tumor cells. Depletions were started 1 wk before tumor challenge. mAb GK1.5 was used for CD4 depletion, mAb 2.43 was used for CD8 depletion, and mAb PK136 was used for NK1.1 depletion. Depletion was terminated on day 63 after tumor challenge.

**Results**

We initially generated several DNA constructs (E7, VP22, VP22/E7, VP22-267/E7, E7/GFP, VP22/GFP, VP22/E7/GFP, and VP22-267/E7/GFP) with a mammalian cell expression vector (pcDNA3). VP22-267 is a mutant that lacks 34 C-terminal residues of VP22 and has been shown to be unable to spread intercellularly (4). To demonstrate whether VP22/E7 protein generated enhanced intercellular spreading of E7 in 293 D3,Kb cells, we used GFP as a marker protein and examined green fluorescence. We performed fluorescent microscopic examination of 293 D3,Kb cells transfected with E7/GFP, VP22/E7/GFP DNA, or VP22-267/E7/GFP. Downloaded from http://www.jimmunol.org/ by guest on April 13, 2017
GFP 48 h after transfection to investigate the distribution of GFP protein. We observed significant spread of GFP protein in cells transfected with VP22/E7/GFP DNA but not in cells transfected with E7/GFP or VP22(1–267)/E7/GFP DNA (Fig. 1A).

We also administered VP22/GFP or GFP intradermally into C57BL/6 mice via gene gun. To demonstrate whether the linkage of VP22 to protein led to enhanced intercellular spreading of the linked protein in vaccinated mice, we used GFP as a marker protein because we had difficulty detecting E7 in paraffin-embedded tissue sections with available E7-specific Abs. pcDNA3, pcDNA3-VP22/GFP, or pcDNA3-GFP was administered intradermally into C57BL/6 mice via gene gun. The skin at the vaccinated sites was biopsied 3 days after vaccination and processed for the detection of GFP by immunocytochemical staining. As shown in Fig. 1B, a widespread distribution of positive brown-staining was observed in the epidermis and skin appendices from mice vaccinated with pcDNA3-VP22/GFP, with some positive staining of nuclei in the epidermis. In contrast, only a few positive cells were observed in the epidermis of pcDNA3-GFP-vaccinated mice. These results suggested that the linkage of VP22 to protein led to enhanced intercellular spreading of the linked protein.

The observed increase in intercellular spreading of the marker protein within the epidermis raises the possibility of generating an increased number of APCs that present the linked protein because the epidermis is rich with Langerhans’ cells, the professional APC precursors. To further investigate whether such increased spreading can lead to enhanced Ag-specific T cell activities, we linked VP22 to a model Ag, HPV-16 E7, which is associated with a majority of cervical cancers. E7 is important in the induction and maintenance of cellular transformation and is coexpressed in most HPV-containing cervical cancers and their precursor lesions; therefore, it represents an ideal target for vaccine development (21).

The importance of CD8+ CTLs for the control of viral infections and neoplasms has been demonstrated in several preclinical models (9, 22). To determine whether vaccination of mice with the pcDNA3-VP22/E7 DNA vaccine can enhance the number of E7-specific CD8+ T cell precursors, we performed intracellular cytokine staining on splenocytes from vaccinated mice. As shown in Fig. 2A, mice vaccinated with VP22/E7 DNA exhibited around a 50-fold increase in E7-specific IFN-γ+ T cell precursors (576/3 × 105 splenocytes) compared with mice vaccinated with wild-type E7 DNA (12/3 × 105 splenocytes). These results indicated that the addition of VP22 to E7 significantly enhanced E7-specific CD8+ T cell-mediated immune responses and that fusion of E7 to VP22 was essential for this observed enhancement because VP22 mixed with E7 (VP22 + E7 DNA, 14/3 × 105 splenocytes) did not generate a significant increase in the number of CD8+ T cell precursors. Furthermore, the linkage of irrelevant proteins (such as GFP) to E7 did not generate enhancement of E7-specific CD8+ T cell activity (data not shown).

To explore whether the property of intercellular spreading of VP22 is important for the observed significant increase in E7-specific CD8+ T cell precursors, we tested the VP22(1–267) truncated mutant for its ability to enhance the number of E7-specific CD8+ T cell precursors in vaccinated mice. As shown in Fig. 2A, linkage of the mutant VP22(1–267) to E7 failed to significantly enhance the number of E7-specific CD8+ T cell precursors in vaccinated mice (17/3 × 105 splenocytes). These results suggested that the property of intercellular spreading generated by full-length VP22 was important for increasing the number of E7-specific CD8+ T cell precursors.

Although addition of VP22 to E7 led to enhanced E7-specific CD8+ T cell activities, we did not detect a significant difference in the number of E7-specific IFN-γ-secreting CD4+ T cells (Fig. 2B) or IL-4-secreting CD4+ T cells (data not shown) among the various vaccination groups. By direct ELISA, we also detected no significant difference in E7-specific Ab responses in the sera of mice vaccinated with various DNA vaccines (data not shown).

Because CD8+ CTL activity has been implicated as important for generating an antitumor effect, we used CTL assays to study E7-specific CTL killing generated in mice vaccinated with various DNA vaccines. We used splenocytes from mice vaccinated with various DNA vaccines as effector cells and E7-expressing TC-1 tumor cells as target cells. We observed the highest CTL activity

**FIGURE 1.** Characterization of in vitro and in vivo distribution of GFP protein that is linked to VP22. A, Fluorescent microscopic examination to demonstrate the in vitro spread of chimeric VP22/E7/GFP protein. 293 D9,Kb cells were transfected with E7/GFP, VP22/E7/GFP or VP22(1–267)/E7/GFP DNA. Significant intercellular spread of GFP was observed in cells transfected with VP22/E7/GFP DNA but not in cells transfected with E7/GFP or VP22(1–267)/E7/GFP DNA. B, Immunohistochemical staining to demonstrate the in vivo spread of chimeric VP22/GFP protein. Mice were vaccinated with 2 μg/mouse of pcDNA3-GFP (left panel) or pcDNA3-VP22/GFP DNA (right panel). Skin from vaccinated mice was stained with rabbit anti-GFP polyclonal Ab. Positive cells are stained brown. Significant spread of GFP was noted in skin obtained from mice vaccinated with pcDNA3-VP22/GFP (right panel).
FIGURE 2. Flow cytometry analysis of IFN-γ-secreting E7-specific CD8⁺ and CD4⁺ T cell precursors in mice vaccinated with various recombinant DNA vaccines. Mice were vaccinated via gene gun with 2 μg of E7, VP22, VP22 mixed with E7 (VP22 + E7), VP22₁–₂₆₇/E7, VP22/E7 DNA, or pcDNA3 (no insert). One week later, mice were boosted with the same regimen as the first vaccination. A, Determination of E7-specific CD8⁺ T cells. Splenocytes from vaccinated mice were cultured in vitro with 1 μg/ml of E7 peptide (aa 49–57) overnight and analyzed for CD8 and intracellular IFN-γ by flow cytometry analysis. Vaccination of mice with VP22/E7 DNA generated the highest frequency of IFN-γ secreting E7-specific CD8⁺ double-positive T cells compared with the other groups. The data from intracellular cytokine staining shown here are from one representative experiment of two performed. B, Determination of E7-specific CD4⁺ T cells. The number of IFN-γ-secreting CD4⁺ T cells was analyzed by flow cytometry. No significant difference in the number of E7-specific IFN-γ-secreting CD4⁺ cells was observed in mice immunized with various recombinant DNA vaccines.

in mice vaccinated with VP22/E7 compared with mice vaccinated with pcDNA3 (no insert), VP22, or E7 DNA (Fig. 3). Our data indicated that CTL activity correlated with the frequency of E7-specific CD8⁺ T cell precursors generated by each DNA vaccine.

One potential explanation for the observed enhancement of E7-specific CD8⁺ T cell activity in mice vaccinated with VP22/E7 DNA may be the enhanced MHC class I presentation of E7 in cells transfected with this chimeric vaccine. Thus, we conducted CTL assays to determine whether cells transfected with pcDNA3 (no insert), VP22, E7, or VP22/E7 DNA presented E7 directly to a Db-restricted E7-specific CD8⁺ T cell line. We chose 293 Dkb cells (15) as target cells because they have been shown to have a stable high transfection efficiency (up to 80%) and express the murine MHC class I molecule, Dkb. As shown in Fig. 4A, 293 Dkb cells transfected with VP22/E7 DNA displayed significantly higher percentages of specific lysis at the 9:1 (22.8 ± 1.8% vs 6.2 ± 1.5%; p < 0.001) and 27:1 (48.1 ± 2.4% vs 13.0 ± 1.6%; p < 0.001) E:T ratios compared with cells transfected with wild-type E7 DNA. Because intercellular spreading of VP22/E7 may affect the results of this assay by generating more E7-positive target cells, we performed a CTL assay with vaccinia-infected target cells to alleviate this concern. Vaccinia have been shown to infect nearly 100% of target cells. We used 293 Dkb cells infected with wild-type E7, VP22, or VP22/E7 vaccinia as target cells. Our results demonstrated that cells infected with VP22/E7 vaccinia generated a higher percentage of lysis compared with cells infected with other vaccinia (Fig. 4B). Taken together, our data from Fig. 4, A and B suggested that cells expressing VP22/E7 may present E7 Ag through the MHC class I pathway more efficiently than cells expressing wild-type E7.

To determine whether the observed enhancement in E7-specific CD8⁺ T cell-mediated immunity translated to a significant E7-specific antitumor effect, we performed an in vivo tumor protection experiment with a previously characterized E7-expressing tumor model, TC-1 (18). As shown in Fig. 5A, 100% of mice receiving the VP22/E7 DNA vaccine remained tumor-free 63 days after TC-1 challenge. In contrast, all of the unvaccinated mice and mice receiving pcDNA3 (no insert), VP22, or wild-type E7 developed tumors within 14 days after tumor challenge. We also observed that fusion of E7 to VP22 was required for antitumor immunity, because VP22 mixed with E7 (VP22 + E7 DNA) did not generate enhancement of tumor protection. Furthermore, the linkage of the mutant VP22₁–₂₆₇ to E7 failed to generate a significant antitumor effect in vaccinated mice (Fig. 5A). These data suggested that the property of intercellular spreading generated by linkage of full-length VP22 to E7 was important for the observed enhancement of E7-specific tumor protection.

We then investigated the therapeutic potential of the chimeric VP22/E7 DNA construct in treating TC-1 tumor metastases in the lungs. As shown in Fig. 5B, mice vaccinated with VP22/E7 DNA exhibited the lowest mean number of pulmonary nodules (0.75 ± 0.95) compared with mice vaccinated with wild-type E7 DNA (32.6 ± 2.5) or VP22 DNA (13 ± 2.58; one-way ANOVA, p < 0.001). Interestingly, vaccination with VP22 DNA generated a
weak nonspecific antitumor effect in the lung metastasis model. The results from the tumor protection and treatment experiments indicated that linkage of VP22 to E7 dramatically enhanced anti-tumor effects against the growth of TC-1 tumors.

To determine the subset of lymphocytes that are important for the antitumor effect generated by VP22/E7 DNA vaccine, we performed in vivo Ab depletion experiments. As shown in Fig. 5C, all naive mice and VP22/E7 DNA-vaccinated mice depleted of CD8 T cells grew tumors within 14 days after tumor challenge. In contrast, all of the nondepleted mice and all of the mice depleted of CD4 T cells remained tumor-free 63 days after tumor challenge, and 40% of NK1.1-depleted mice grew tumors 6 wk after tumor injections. These results suggested that CD8 T cells but not CD4 T cells are essential for the antitumor immunity generated by the VP22/E7 DNA vaccine. NK1.1 cells may also contribute to the antitumor effect generated by the chimeric VP22/E7 DNA vaccine.

The success of the VP22/E7 DNA vaccine warrants the consideration of strategies that use proteins with trafficking properties. For example, HIV TAT protein (TAT) has been shown to have transcellular transport properties (11, 12). In addition, a peptide containing nine amino acids of TAT (RKKRRQRRR) conjugated with OVA was able to generate higher CTL activity in mice that were vaccinated with TAT-OVA compared with mice vaccinated with OVA (23). Another molecule with similar properties is the membrane-translocating sequence (MTS), which also has been shown to have transmembrane permeability. The amino acid residues accounting for this property have been narrowed down to 12 amino acids (AAVLLPVLLAAP; Ref. 13). Finally, the third helix of the Antennapedia homeodomain (AH) of the Drosophila also has been shown to have membrane translocation properties, and 16 amino acid residues have been shown to account for this effect (RQIKIWFQNRRMKWKK; Ref. 14). These studies suggest that chimeric proteins with these molecules also may result in an effect similar to that observed with VP22/E7.

Therefore, we have chimerically linked these molecules to E7 in a DNA format and vaccinated mice with each construct to examine the E7-specific immune responses generated by these vaccines in comparison to VP22/E7 DNA. We performed intracellular cytokine staining for E7-specific CD8 T cell precursors with splenocytes from vaccinated mice. As shown in Fig. 6, the linkage of the DNA sequence encoding the characterized membrane translocation region of each of these proteins to E7 did not generate as dramatic an enhancement of E7-specific CD8 T cell immune responses as we had observed with VP22 linked to E7. These results indicated that VP22/E7 DNA may have an unique property that is not shared with the other constructs that we tested.
Discussion

In this study, we demonstrated that VP22 is capable of enhancing intercellular spreading of the linked protein. We demonstrated that mice vaccinated with VP22/E7 DNA generated a greater number of E7-specific CD8$^+$ T cell precursors than mice vaccinated with E7 DNA or truncated VP22_{1-267}/E7 DNA which lacks the property of...
intercellular spreading (4). We also found that vaccination with VP22/E7 DNA generated a stronger antitumor effect than E7 or VP22_1–267/E7 DNA and that this antitumor effect was CD8 dependent. Finally, our study revealed that DNA vaccines encoding E7 linked to molecules derived from proteins with trafficking properties (HIV TAT, MTS, or AH) did not generate a CD8+ T cell-mediated immune response as potent as that generated by VP22/E7 DNA.

In this study, we observed a dramatic increase in the number of E7-specific CD8+ T cell precursors in mice vaccinated with VP22/E7 (Fig. 2). One potential explanation for this enhancement is that intradermal administration of VP22/E7 DNA can introduce DNA directly into professional APCs of the skin (24), allowing transfected APCs to directly present E7 through the MHC class I pathway. Another important reason for this observed enhancement is that the linkage VP22 to E7 may facilitate the spreading of Ag from VP22/E7 DNA-transfected cells to surrounding APCs, increasing the number of APCs that present E7 through MHC class I pathway. Finally, our data indicated that the linkage of VP22 to E7 is capable of presenting the Ag more efficiently in transfected cells. All of these factors may have played a role in enhancing the number of E7-specific CD8+ T cell precursors observed in vaccinated mice.

Another potential explanation for the observed enhancement of E7-specific CD8+ T cell immune responses in vivo may be the so-called “cross-priming effect”, whereby release of chimeric VP22/E7 protein may lead to uptake and processing by dendritic cells (DCs) for presentation via the MHC-I restricted pathway (25). Previously, we observed that DCs pulsed with apoptotic cells expressing VP22/E7 (26) or cell lysates containing VP22/E7 fusion protein (C.-F.H., unpublished observation) were capable of presenting E7 Ag through the MHC class I pathway more efficiently than wild-type E7 counterparts. However, other studies have suggested that direct priming of CD8+ T cells by gene-transfected DCs may be a more important mechanism in gene gun-mediated DNA immunization than the cross-priming mechanism (27, 28). Thus, the extent of cross-priming in contributing to CD8+ T cell-mediated immune responses remains unclear and requires additional experiments to characterize its role in the observed enhancement of Ag-specific CD8+ T cell activity.

The property of intercellular spreading may be important for the Ag-specific immune response and antitumor effect generated by VP22/E7 DNA. In this study, we tested a construct lacking the property of intercellular spreading, VP22_1–267/E7 DNA, and found that this vaccine was unable to generate significant enhancement of E7-specific CD8+ T cell activity or protection against TC-1 tumors. However, it is not clear whether the property of intercellular spreading alone is sufficient to account for the enhancement of the E7-specific CD8+ T cell immune response. Our examination of certain molecules with trafficking properties revealed that E7 linked to molecules derived from TAT, MTS, or AH did not generate E7-specific CD8+ T cell immune responses comparable to that generated by VP22/E7 DNA (Fig. 5). It is likely that the trafficking properties of these molecules are not the same as those of VP22. It would be interesting to further characterize the trafficking properties of these molecules and correlate their ability to enhance immune responses.

Recently, two additional examples of proteins with purported intercellular spreading properties have emerged, bovine herpesvirus VP22 (BVP22) (29) and Marek’s disease virus VP22 (30, 31), both of which are VP22 homologues. Bovine herpesvirus VP22 shares ~22% amino acid identity to human herpesvirus VP22. A previous study found that BVP22 trafficking may be more proficient than that of human VP22 after endogenous synthesis (29). Marek’s disease virus VP22 shares ~17% amino acid identity to human herpesvirus VP22 (30) and may be capable of intercellular transport after exogenous application (31). Interestingly, mice vaccinated with DNA encoding Marek’s disease virus VP22 linked to E7 exhibited enhanced E7-specific CD8+ T cell activities compared with mice vaccinated with wild-type E7 DNA (C.-F.H., unpublished observation). It would be important to perform a head-to-head comparison of these molecules in the future to identify the fusion construct that is most potent at generating Ag-specific immunity and antitumor effects.

Recently, we also have tested the VP22 strategy in a naked Sindbis virus self-replicating RNA vector (26). We found that naked DNA and naked Sindbis virus RNA vector generated enhancement of E7-specific CD8+ T cell immune responses and an antitumor effect in vaccinated mice, although studies of the immune effector cells essential to the antitumor effect generated by these vectors revealed some interesting differences. Our studies demonstrated that CD8+ T cells were important for the antitumor effects generated by VP22/E7 vaccines with naked DNA or naked Sindbis virus RNA vector, whereas CD4+ T cells were not essential for the antitumor effect generated by either vaccine. Depletion of CD4+ T cells after treatment with naked VP22/E7 DNA or naked VP22/E7 Sindbis virus RNA did not lead to any significant loss in antitumor effect (26). In contrast, we found that NK cells were essential for the antitumor effect generated by the naked VP22/E7 Sindbis virus RNA vaccine but were not as important in the naked VP22/E7

FIGURE 6. Flow cytometry analysis of IFN-γ-secreting E7-specific CD8+ T cell precursors in mice vaccinated with various recombinant DNA vaccines. Mice were vaccinated via gene gun with 2 μg of pcDNA3, E7, TAT/E7, E7/MTS, AH/E7, or VP22/E7 DNA. One week later, mice were boosted with the same regimen as the first vaccination. Splenocytes from vaccinated mice were cultured in vitro with 1 μg/ml of E7 peptide (aa 49–57) overnight and analyzed for both CD8 and intracellular IFN-γ by flow cytometry analysis. Vaccination of mice with VP22/E7 DNA generated the greatest number of IFN-γ+ CD8+ double-positive T cells compared with the other vaccination groups. The data from intracellular cytokine staining shown here are from one representative experiment of two performed.
DNA vaccine. Thus, different types of vaccine vectors encoding the same gene may activate different subsets of effector cells that are important to the antitumor effect.

The intercellular spreading strategy can potentially be used in conjunction with other strategies to further enhance vaccine potency. We previously have used *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) linked to E7 to significantly enhance MHC class I presentation of E7 Ag to CD8+ T cells (9). Furthermore, we have developed an enveloped/lysosomal targeting strategy with E7 chimerically linked to a signal peptide (Sig) and the enveloped/lysosomal sorting signal (derived from lysosome associated membrane protein, LAMP-1) to significantly enhance MHC class II presentation of Ag to CD4+ T cells (10). Because these two approaches may act through a different mechanism than VP22/E7, the vaccine potency of HSP70 or Sig/E7/LAMP-1 vaccines may be further enhanced if the intercellular spreading strategy is also implemented in the vaccine design.

In summary, our findings illustrate the promise of enhancing vaccine potency by linkage of VP22 to Ag, allowing for increased intercellular spreading of Ag and enhanced Ag-specific CD8+ T cell activity leading to potent antitumor effects in vivo. This promising approach may facilitate future vaccine development for the control of cancers and infectious diseases.

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