**Objective:** To test whether an E7 peptide/CpG vaccine is effective in preventing and treating human papillomavirus–positive tumors in a murine model.

**Intervention:** First, an E7 peptide/CpG vaccine was administered systemically on days −14 and −7, and tumor cells were injected subcutaneously on day 0. Second, tumor cells were injected on day 0, and vaccine was administered on days 7, 14, and 21.

**Main Outcome Measures:** Tumor size was measured 3 times per week. A tetramer assay was used to assess the presence of activated, E7-specific lymphocytes in spleen and tumor cells harvested from mice treated with a similar vaccination regimen.

**Results:** In the prophylactic study, 75% of mice injected with E7 peptide/CpG resisted tumor formation. In the therapeutic setting, tumors initially regressed and experienced delayed progression when compared with controls. Survival rates improved in E7/CpG-vaccinated mice. Tetramer analysis detected increased numbers of activated, E7-specific lymphocytes in the spleens and tumors of animals treated with the experimental vaccine when compared with controls.

**Conclusion:** The use of CpG motifs as an adjunct to peptide-based immunotherapy has potential impact on the treatment of human papillomavirus–associated cancers.

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**HEAD AND NECK SQUAMOUS cell carcinoma (HNSCC) presents a significant international health problem. In 2002, more than 500 000 new cases were diagnosed, with more than 250 000 deaths.** In the United States alone, there was an estimated 28 260 new cases diagnosed in 2004, with 7230 deaths. Human papillomavirus (HPV) infection has been implicated in a large number of these tumors (approximately 15%-23% of all HNSCCs and >50% of oropharyngeal squamous cell carcinomas). Despite technological advances in treatment, 5-year survival rates for HPV-associated HNSCC remain poor, emphasizing the need for new therapeutic strategies.

Tumor immunotherapy, which stimulates the host immune system to either prevent tumor formation or treat existing tumors, has the potential to become a valuable therapeutic modality for HPV-associated tumors. Human papillomavirus–transformed cells express 2 HPV oncoproteins, E6 and E7, which are critical to the induction and maintenance of cellular transformation. Because E7 can be recognized as foreign by the host immune system, it is an ideal target for immunotherapy. Thus far, several prophylactic vaccine strategies have had success in clinical trials, and therapeutic vaccines have successfully treated tumors in preclinical models. Among the most successful therapeutic vaccines in preclinical animal models are live recombinant vaccines packaged in viral or bacterial vectors, such as Listeria, Salmonella, and bacillus Calmette-Guerin. Our laboratory has previously demonstrated the ability to treat HPV-16 E7-transformed tumors with recombinant bacterial vaccines.

One of the reasons bacterial vaccines generate a potent immune response is the presence of unmethylated CpG motifs in bacterial DNA. CpG motifs are a specific DNA sequence pattern of a CG dinucleotide flanked by two 5’ purines (or a 5’ purine and thymine) and two 3’ pyrimidines. These motifs serve as a “danger signal” to vertebrate immune systems, activating B cells and plasmacytoid dendritic cells and inducing a Th1-like pattern of cytokine production and maturation of antigen-presenting cells.
Because of these properties, CpG oligodeoxynucleotides (ODNs) have been used as immune adjuvants in many immunotherapeutic studies, including some that target HPV-16 E7.\textsuperscript{17–20} In most of these studies, the entire E7 protein was used as a cancer vaccine, with CpG ODN used as an adjuvant.\textsuperscript{17,18,20} In another study, a 35-amino acid peptide sequence was used to target E7-expressing tumors.\textsuperscript{19}

In the present study, we questioned whether the HLA-restricted 9-amino acid epitope of E7 would be effective in preventing and/or treating HPV-16 E7-transformed tumors when administered with CpG ODN. We found that systemic administration of the H-2b–restricted immunodominant E7 epitope (RAHYNIVTF) with CpG ODN can generate a sufficient immune response to completely prevent tumor growth when given prophylactically and that it can significantly affect established tumor growth as well. In addition, tetramer studies show that the E7 peptide/CpG ODN vaccine induces the production of effector CD8\textsuperscript{+} T cells, which are specific to E7.

### METHODS

#### MICE

The C57BL/6 mice were 6 to 8 weeks old and were purchased from Charles River Laboratories (Wilmington, Mass). All animal experiments were performed under approved protocols from the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania, Philadelphia.

#### CELL LINES

The TC-1 cell line (provided by T. C. Wu, PhD, Johns Hopkins University, Baltimore, Md) was derived from C57BL/6 mice lung epithelial cells.\textsuperscript{9} The cells were immortalized with HPV-16 E6 and E7 and transformed with the c-H-ras oncogene. They express both E6 and E7 and are highly tumorigenic.\textsuperscript{9} This cell line was grown in culture with Roswell Park Memorial Institute (RPMI) 1640, 10% fetal calf serum, 2mM L-glutamine, 100 U/mL of penicillin G sodium, 100 µg/mL of streptomycin, 100µM nonessential amino acids, and 1mM sodium pyruvate at 37°C and 10% carbon dioxide.

#### PEPTIDE VACCINATIONS

The H-2b–restricted E7 epitope (RAHYNIVTF) and latent membrane protein 1 (LMP-1) (YLLEMLWRL) peptides were synthesized by Suzanna Horvath, PhD, Biopolymer Synthesis Center, California Institute of Technology, Pasadena. Purity was determined by reverse-phase high-performance liquid chromatography and was found to be greater than 95% pure.

The ODNs, CpG-containing ODN 1826 (5’-TCC ATG ACG TTC CTG ACG TT-3’) and non-CpG–containing ODN 1982 (5’-TCC AGG ACT TCT CTC AGG TT-3’), were synthesized by Invitrogen Corp, Carlsbad, Calif. These were brought to a concentration of 100 µg/µL in phosphate-buffered saline and stored at −20°C.

#### IN VIVO TUMOR GROWTH EXPERIMENTS

**Prophylactic Vaccination**

The 6- to 8-week-old C57BL/6 mice were divided into 5 groups of 4 mice and received intraperitoneal injections as follows:

- **Group 1:** E7 peptide and ODN 1826 (a CpG-containing ODN).
- **Group 2:** E7 peptide alone.
- **Group 3:** Control peptide (LMP-1) with ODN 1826.
- **Group 4:** E7 peptide with ODN 1982 (a non-CpG–containing ODN).
- **Group 5:** Unvaccinated naive group.

On the day of vaccination, the peptides (100 µg/dose) were solubilized in a minimal amount of dimethyl sulfoxide (<1% of final vaccine volume). These were mixed with ODNs and brought to a final concentration of 100 µg per dose of peptide and 20 µg per dose of ODN in 200 µL of phosphate-buffered saline and kept on ice until injection.

For the prophylactic experiments, vaccines were administered twice on days −14 and −7. On day 0, the mice were challenged with 5 × 10\textsuperscript{5} TC-1 cells injected subcutaneously into the left flank, and the tumors were measured every 2 or 3 days thereafter.

**Therapeutic Vaccination**

The C57BL/6 mice were divided into 5 groups of 8 mice. On day 0, all mice were injected subcutaneously with 5 × 10\textsuperscript{5} TC-1 cells in the left flank. On day 7, tumors were palpable in most mice (mice with no palpable tumor were put into the naive group). On days 7, 14, and 21, the groups of mice were injected intraperitoneally with the peptide vaccinations described previously.

Tumors were measured 2 to 3 times per week with digital calipers spanning the shortest and longest surface diameters. The mean of the 2 diameters was then calculated and used in further analysis. For comparison of tumor diameters, the means and standard errors were calculated for each treatment group at day 28, and statistical significance was calculated using the unpaired t test. P <.05 was considered significant.

The mice were euthanized if the tumor diameter reached 20 mm or if they showed signs of distress (eg, cachexia, ulcerated tumors, or diminished ability to ambulate) in accordance with the IACUC protocols. Tumor measurements at each time point are shown for surviving mice only. Statistical comparison of survival rates was performed using the log-rank test.

### ACTIVATED AND ANTIGEN-SPECIFIC CD8\textsuperscript{+} T CELLS IN TUMORS AND SPLEENS

The C57BL/6 mice were divided into 5 groups of 3 mice. On day 0, the experimental group of mice was injected subcutaneously with 1 × 10\textsuperscript{5} TC-1 cells in the left flank. On days 7 and 14, mice were injected intraperitoneally with 100 µg per dose of E7 peptide and 20 µg per dose of ODN 1826 in phosphate-buffered saline. The same controls were used as for the experiments described previously. On day 21, the mice were euthanized and tumors and spleens harvested. Tumors were digested with collagenase P (2 mg/mL) (Roche Applied Science, Indianapolis, Ind) and DNase (1 mg/mL) (Sigma Chemical Co, St Louis, Mo) for 1 hour at 37°C. Cells were isolated from the tumor digests using a 100-µm cell strainer (BD Biosciences Pharmingen, San Diego, Calif). Three-color flow cytometry for CD8\textsuperscript{+} and non-CpG–containing antibodies; BD Biosciences Pharmingen, C57BL/6 (allophycocyanin-conjugated antibody; BD Biosciences Pharmingen), CD62L (fluorescein isothiocyanate–conjugated antibody; BD Biosciences Pharmingen), and the tetramer of the H-2D\textsuperscript{b}–restricted immunodominant E7 epitope in the C57BL/6 mouse (RAHYNIVTF) was performed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences Pharmingen). The E7/Db tetramer was made by the National Institute of Allergy and...
Infectious Diseases Tetramer Core Facility at Emory University, Atlanta, Ga, through the National Institutes of Health AIDS Research and Reference Reagent Program. The E7/Db tetramer contains 4 class I molecules, bound to an avidin core, with the immunodominant epitope loaded on each molecule. This complex is conjugated to a fluorochrome and binds the T-cell receptor of T cells specific for the epitope. Cells were analyzed by comparing tetramer-positive, CD8⁺/H11001, CD62Llo cells within the spleen and the tumor generated by the 4 vaccines. Statistical analysis comparing the percentages of activated and antigen-specific T cells between the groups was performed using the t test.

RESULTS

PREVENTION OF E7-TRANSFORMED TUMOR GROWTH

To determine whether the growth of HPV-16 E7–transformed tumors can be prevented with an H-2b–restricted peptide/CpG ODN vaccine, 20 C57BL/6 mice were divided into 5 groups. The first group was vaccinated with 100 µg of the E7 peptide RAHNITYF with 20 µg of bioactive CpG ODN 1826. The second group received 100 µg of E7 peptide alone; the third group, 100 µg of irrelevant peptide YLLEMLWRL (termed LMP-1) with 20 µg of ODN 1826; and the fourth group, E7 peptide with the non-CpG ODN 1982. The fifth group remained unvaccinated. Two vaccinations were administered intraperitoneally at weekly intervals. One week after the second dose, 5 × 10⁴ cells from the HPV-16 E7–transformed syngeneic cell line TC-1 was injected into the left flank of the mice.

While the control vaccines did not have a statistically significant effect on tumor growth, 3 of the 4 mice treated with E7 peptide/CpG ODN 1826 did not develop tumors during the course of the experiment (Figure 1A). The fourth mouse of the experimental group developed a tumor in week 4. The average tumor size of the E7/CpG ODN 1826 group was significantly smaller compared with all the control groups (P=.02, t test; Figure 1B). All control and naive mice developed large tumors and were killed by week 6. This experiment is representative of 4 separate experiments. Survival curves (Figure 1C) demonstrate that mice vaccinated with E7 peptide with CpG ODN 1826 have improved survival over mice in any of the control groups (P=.01, log-rank test).

REGRESSION OF E7-TRANSFORMED TUMORS

To determine whether peptide vaccination with CpG ODN can affect the growth of established tumors, 5 × 10⁴ TC-1 cells were used to establish tumors in the left flank of 40 C57BL/6 mice. On days 7, 14, and 21, the mice were treated with E7 peptide/CpG ODN 1826 or a control vaccine.

The E7 peptide/CpG ODN 1826 group experienced an early tumor regression, with average tumor sizes decreasing from 7.7 to 5.6 mm from week 2 to week 3, while tumors in other groups continued to grow from 10.5 to 12.3 mm. The difference in average tumor size between the experimental and control groups was statistically significant on day 21 and thereafter (P<.001, t test). These results are representative of 2 separate experiments (Figure 2A). Survival was also improved in the mice treated with E7 peptide/CpG ODN 1826 (Figure 2B).
INDUCTION OF ACTIVATED CD8+ T CELLS WITHIN TUMORS

To analyze tumor-infiltrating lymphocytes, the TC-1 cell line was used to establish tumors in the left flank of C57BL/6 mice. On days 7 and 14, mice were treated with E7 peptide/CpG ODN 1826 or a control vaccine (control groups were the same as in the prophylactic experiment). On day 21, the mice were euthanized and the tumors harvested. Single-cell suspensions were made, and the tumor cells were stained with anti-CD8 and anti-CD62L antibodies. CD62L is a cell surface marker, which is down-regulated on activated effector T lymphocytes.21

Through the use of fluorescence-activated cell sorter analysis, it was determined that E7/CpG ODN 1826 vaccinated mice have the highest percentage of activated CD8+ lymphocytes within the tumors. The difference was statistically significant compared with all other groups (t test, P=.03). Of note, mice vaccinated with the irrelevant peptide LMP-1 with CpG ODN 1826 also had an increased number of lymphocytes compared with mice vaccinated with E7 alone, vaccinated with E7 peptide/non-CpG ODN 1982, and unvaccinated mice (P=.03) (Figure 3).

INDUCTION OF ACTIVATED E7-SPECIFIC T CELLS WITHIN TUMORS

To determine whether the E7 peptide/CpG ODN 1826 vaccine was able to induce E7-specific tumor-infiltrating lymphocytes, a tetramer assay was performed. Tumors were harvested from vaccinated mice on day 21 and stained with antibodies to CD62L and CD8 and with the E7/Db tetramer. The mean±SE for the percentages of E7 tetramer-positive, CD62Llo, CD8+ lymphocytes are depicted in Figure 4. These data are from 1 experiment with 3 mice per group and are representative of 2 separate experiments. An increased percentage of tetramer-positive, activated tumor-infiltrating lymphocytes was seen in mice vaccinated with E7/CpG ODN 1826. Although the increase was substantial and consistent, statistical significance was not achieved in these experiments (t test, P=.14).
Experiments are depicted in Figure 4. Compared with the control groups, E7 peptide with CpG oligodeoxynucleotide (ODN) 1826 induces a higher percentage of E7-specific lymphocytes within the tumors. Five groups of C57BL/6 mice were challenged with TC-1 cells and vaccinated with the indicated vaccines on days 7, 14, and 21. On day 28, the mice were euthanized and the tumors harvested. Single-cell suspensions were made, and the tumor cells were stained with anti-CD8 and anti-CD62L antibodies and with E7/D\(^{0}\) tetramers. Cells were analyzed by fluorescence-activated cell sorting with multicolor staining. Data are expressed as percentage of CD8\(^{-}\), CD62L\(^{hi}\) cells that are E7 tetramer positive. These data are representative of 2 similar experiments. The mean percentage of E7-specific lymphocytes in E7/CpG ODN 1826–vaccinated mice was higher compared with each of the control groups, although not to a statistically significant degree (P=1.4, t test; n=3 per group).

Figure 4. Compared with the control groups, E7 peptide with CpG oligodeoxynucleotide (ODN) 1826 induces a higher percentage of E7-specific lymphocytes within the tumors. Five groups of C57BL/6 mice were challenged with TC-1 cells and vaccinated with the indicated vaccines on days 7, 14, and 21. On day 28, the mice were euthanized and the tumors harvested. Single-cell suspensions were made, and the tumor cells were stained with anti-CD8 and anti-CD62L antibodies and with E7/D\(^{0}\) tetramers. Cells were analyzed by fluorescence-activated cell sorting with multicolor staining. Data are expressed as percentage of CD8\(^{-}\), CD62L\(^{hi}\) cells that are E7 tetramer positive. These data are representative of 2 similar experiments. The mean percentage of E7-specific lymphocytes in E7/CpG ODN 1826–vaccinated mice was higher compared with each of the control groups (P=.007, t test; n=3 per group).

In this study, we describe a cancer vaccine strategy that entails an HPV-16 E7 peptide epitope combined with a CpG motif–containing ODN (ODN 1826). In a murine model, this vaccine is capable of inducing E7-specific CD8\(^{+}\) T lymphocytes, preventing tumor formation, and causing initial regression followed by delayed growth of E7-expressing tumors when given therapeutically. Neither E7 peptide alone nor ODN 1826 given with an unrelated peptide was as effective.

Peptide-based vaccine strategies have several attractive features. They are nontoxic, noninfectious, inexpensive, and relatively easy to produce. There are several disadvantages, including the need to customize each vaccine to a patient’s particular HLA subtype and their rapid degradation. Another key disadvantage is their lack of immunogenicity. In fact, peptide vaccinations are capable of inducing tolerance in certain experimental systems. Thus, adjuvants such as the oil-based incomplete Freund's adjuvant and saponin-based QS-21 have been used in clinical trials with some success. Although refinement in the formulations of these compounds has minimized their toxic effects, there are some advantages of using CpG motifs as alternate immune adjuvants. Similar to the peptides, their low cost and ease of construction makes them attractive for large-scale clinical trials. Perhaps more importantly, however, CpG motifs specifically stimulate dendritic cells through toll-like receptor 9, which ultimately results in the production of Th1 cytokines such as interleukin 12 and interferon 15. CpG motifs also up-regulate the expression of costimulatory molecules on the dendritic cell surface. These molecules include CD80, CD86, and CD40. The culmination of these events is the enhancement of a cell-mediated immune response, a vital component of successful antitumor vaccine efficacy.

In our study, the administration of CpG motifs even without the E7 peptide still resulted in more activated tumor-infiltrating lymphocytes (Figure 3). However, these activated lymphocytes had no effect on actual tumor progression. This demonstrates the need for antigen specificity in this system. While most HNSCCs do not express E7, other antigens may be effective when used in conjunction with CpG ODN owing to its potency as an immune adjuvant. For example, future clinical trials could use tumor extract in combination with CpG ODNs in an attempt to prevent recurrences after conventional treatment. This vaccine regimen may be able to prevent recurrences after conventional treatment. This vaccine regimen may be able to break immunological tolerance and raise an immune response to yet unidentified tumor antigens.

Although the vaccinations were able to prevent tumor formation and slow the growth of established tu-
mors, they were not able to completely eradicate established tumors. In the other similar experimental systems, therapeutic vaccinations were effective in eradicating tumors in some mice. In these experiments, either E7 protein or a longer E7 peptide was used so that there was antigen-specific stimulation of both CD4+ and CD8+ lymphocytes. In our system, only the CD8+ cells are stimulated. This raises the question of whether lack of CD4 help may play a role in our inability to cure tumors. While our results are encouraging, further studies are required to determine the need for CD4 help in therapeutic vaccinations, and adjustments are needed to optimize the vaccine strategy. Future experiments may include the addition of CD4 help in the form of endogenous cytokines or stimulating anti-CD40 antibodies.

In summary, we have demonstrated efficacy of a cytotoxic T-lymphocyte epitope vaccination in preventing and slowing the growth of tumors transformed by HPV-16 E7. An E7 peptide/CpG motif–containing ODN vaccine has the potential to be a valuable adjuvant therapy for HPV-associated head and neck tumors in the future.

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