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## Defined Flanking Spacers and Enhanced Proteolysis Is Essential for Eradication of Established Tumors by an Epitope String DNA Vaccine<sup>1</sup> ✓

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# Defined Flanking Spacers and Enhanced Proteolysis Is Essential for Eradication of Established Tumors by an Epitope String DNA Vaccine<sup>1</sup>

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Loss of immunogenic epitopes by tumors has urged the development of vaccines against multiple epitopes. Recombinant DNA technologies have opened the possibility to develop multiepitope vaccines in a relatively rapid and efficient way. We have constructed four naked DNA-based multiepitope vaccines, containing CTL, Th cell, and B cell epitopes of the human papillomavirus type 16. Here we show that gene gun-mediated vaccination with an epitope-based DNA vaccine protects 100% of the vaccinated mice against a lethal tumor challenge. The addition of spacers between the epitopes was crucial for the epitope-induced tumor protection, as the same DNA construct without spacers was significantly less effective and only protected 50% of the mice. When tested for therapeutic potential, only the epitope construct with defined spacers significantly reduced the size of established tumors, but failed to induce tumor regression. Only after targeting the vaccine-encoded protein to the protein degradation pathway by linking it to ubiquitin, the vaccine-induced T cell-mediated eradication of 100% of 7-day established tumors in mice. The finding that defined flanking sequences around epitopes and protein targeting dramatically increased the efficacy of epitope string DNA vaccines against established tumors will be of importance for the further development of multiepitope DNA vaccines toward clinical application. *The Journal of Immunology*, 2001, 166: 5366–5373.

Infection with genital human papillomavirus (HPV)<sup>3</sup> is a global problem for several reasons. HPV infection is sexually transmitted, and infection rates as high as 46% in sexually active women have been reported (1). High risk HPV is detected in 99.7% of cervical cancers (2), the second most common cancer in women worldwide (3), and is considered necessary for induction of this malignancy (4). However, no vaccine is available to prevent and treat HPV-linked diseases, including cervical cancer. Increased incidence of HPV-induced lesions in immunosuppressed patients and the spontaneous regression of some precancerous cervical lesions indicate a potential role of the immune system against cervical cancer (5). Thus, immunotherapy against a virus-induced malignancy such as cervical cancer is a reasonable option. The development of increasingly sophisticated vaccination methods

has opened the possibility for new HPV vaccines. The likelihood of success in cancer immunotherapy is determined by the efficacy of the therapy in inducing an immune response against the tumor rejection Ag(s) (6). Many tumors escape immunological destruction by down-regulation or loss of immunogenic epitopes. Therefore, it is generally accepted that vaccination with multiple epitopes will be advantageous over single epitope-based vaccines (7–9). DNA-based vaccination opens the possibility to combine epitopes of different proteins into one vaccine (10, 11).

A DNA-based vaccine would be a good candidate for immunization and induction of a protective immune response against viruses and virus-induced tumors. However, for the high risk papillomaviruses involved in cervical cancer, the use of viral DNA for vaccination harbors the danger of transformation, as the HPV genome contains two oncogenes, E6 and E7 (12, 13). The expression of these two oncoproteins is necessary not only for transformation, but also for maintenance of the oncogenic phenotype. Therefore, E6 and E7 are present in all cervical carcinoma cells and are the proteins of choice to target for immunotherapy. The fact that epitopes of the most abundantly expressed type HPV16 E7 have been mapped for several MHC haplotypes in both mice and humans (14–17) opens the possibility to develop epitope-specific vaccination strategies against this HPV type.

To develop an effective vaccination strategy against HPV16-induced tumors we have constructed and studied several naked DNA-based multiepitope vaccines containing CTL, Th cell, and B cell epitopes of HPV16. The in vivo efficacy of the epitope string constructs in a mouse tumor model was determined by tumor rejection induced by an immune response against the HPV16 E7 H2-D<sup>b</sup> epitope (RAHYNIVTF) (17).

It is known that residues flanking a MHC class I epitope strongly influence its liberation efficiency by the proteasome system (18, 19). The expression and immunogenicity of epitopes in a

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<sup>3</sup> Abbreviations used in this paper: HPV, human papillomavirus; DC, dendritic cell; S+, string construct with spacers; S-, string construct without spacers; S+/Ub, string construct with spacers and ubiquitin; S-/Ub, string construct without spacers with ubiquitin; ELISPOT, enzyme-linked immunospot.

multiepitope vaccine suggested that spacers between epitopes are not necessary to obtain CTL responses (20). However, data on the impact of flanking epitope sequences on the *in vivo* induction of therapeutic anti-tumor immunity are not available. Here, vaccination with two similar epitope string DNA constructs that only differ in the presence or the absence of spacers between the epitopes enabled us to determine the influence of defined epitope flanking sequences on induction of CTLs and subsequent protection and therapeutic uses against tumors in the widely used HPV16 murine tumor model C3 (17).

Vaccination with epitope string naked plasmid DNA has been found to be far less effective than virus-mediated introduction of the same epitope string (21). However, specific targeting of epitope-based DNA vaccines to several cellular compartments was shown to increase protection against a lethal viral challenge (21–23). Here we show for the first time that intradermal vaccination with an epitope string naked DNA construct using a gene gun can induce immune responses that prevent tumor growth after vaccination, but, most importantly, cure well-established tumors. The finding that the unique combination of defined flanking sequences around epitopes and specific protein targeting opens the possibility to treat established tumors with epitope string DNA vaccines will be of importance for further development of these vaccines toward clinical application.

## Materials and Methods

### Cells and mice

The HPV16-induced tumor cell line C3 and the T cell clone F $\alpha$ C3 were cultured as originally described (24). WEHI 164 cells (ATCC CRL-1751, American Type Culture Collection, Manassas, VA) JY cells and RD cells (ATCC CCL-136) were cultured in RPMI or IMDM (BioWhittaker, Walkersville, MD), respectively; supplemented with 10% heat-inactivated FCS (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine, 100  $\mu$ g/ml kanamycin, and 50 mM 2-ME.

Six-week-old, specific pathogen-free, female C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) and housed at the Loyola University animal facility under conventional conditions.

### T cell line 7E7

7E7 was generated from the PBMC of an HLA-A\*0201-positive patient with stage 2 carcinoma of the cervix. A modification of the method previously described by Lalvani et al. (25) was used for *in vitro* stimulation. Briefly, on day 0,  $10^7$  cells were pulsed with 100  $\mu$ M of the HPV16 E6<sub>29–38</sub> peptide for 1 h at 37°C. The cells were subsequently diluted to  $2 \times 10^6$ /ml in T cell medium and plated at 1 ml/well into a 24-well plate with 20 ng/ml IL-7 (Genzyme, Cambridge, MA). IL-2 (20 U/ml; Perkin-Elmer/Cetus, Emeryville, CA) was added to the cultures on day 3. On days 7 and 14 the cultures were restimulated with thawed, autologous peptide-pulsed, irradiated PBMC at an effector:stimulator ratio of 2:1. On day 3 after restimulation 20 U of IL-2 was added to the cultures. After demonstration of HPV16 E6<sub>29–38</sub> specificity on day 21, the line was cloned by limiting dilution at one cell per well. This resulted in a putative clone 7E7 that was 100% CD8<sup>+</sup> and recognized both HPV16 E6<sub>29–38</sub>-pulsed and vaccinia-HPV-infected target cells. 7E7 was propagated in an Ag-independent manner by weekly stimulations with pooled allogeneic irradiated PBMC feeders and PHA. Since there is no formal proof of clonality, 7E7 is referred to as a CTL line.

### Vaccine construction

The selection of epitopes represented in the current DNA vaccines (Fig. 1A) is based on the previous identification of HPV16 epitopes for HLA-A\*0201 and HLA-A\*2401 (15). The epitope composition of all four DNA constructs is the same, and the difference between the constructs is the addition of Ala-Ala-Tyr (AAY) spacers between the epitopes (S+ and S+/Ub) and the addition at the carboxyl terminus of an ubiquitin molecule (S+/Ub and S–/Ub). The constructs S+ and S– were entirely made using synthetic oligonucleotides (Loyola Macromolecular Facility) that covered the 5' and 3' ends of the construct and overlapped 30 bp in the middle. The encompassed single-stranded regions were filled in using 2.5 U of PFU polymerase (Stratagene, La Jolla, CA) in the following PCR reaction: one cycle for 4 min at 94°C, 25 cycles for 2 min at 94°C, plus 4 min at 72°C.

The dsDNA was amplified with 5' and 3' primers in a standard PCR reaction using PFU polymerase. The PCR product was cloned into the *EcoRV* site of pZero (Invitrogen, Carlsbad, CA) and subcloned using *KpnI*-*NotI* (S+) or *XbaI*-*BamHI* (S–) into the mammalian expression vector used for vaccination. To construct the S+/Ub- and S–/Ub-containing vectors, S+ and S– were amplified by a standard PCR, using a primer to delete the 3' TAA stop codon from the sequence and add a 5' *XbaI* and a 3' *NotI* restriction site. Both PCR products were cloned into the backbone vector using *XbaI*-*NotI*. The pCMV-Ub F1/2 containing the ubiquitin A76 DNA sequence (21) (obtained from Dr. J. Lindsay Whitton, The Scripps Research Institute, La Jolla, CA) was digested with *NotI* to release the ubiquitin A76 DNA. This *NotI* fragment was cloned into the *NotI* site 3' of the epitope string sequence, and after checking the orientation, sequence analysis revealed the in-frame fusion of ubiquitin to both epitope string sequences in the mammalian expression vector. The DNA used for vaccination was grown in large quantities and purified by Althea Technologies (San Diego, CA). All DNA samples were RNA free and endotoxin free by *Limulus* amoebocyte lysate testing and had an OD<sub>260</sub>:OD<sub>280</sub> ratio >1.9.

### Stable transfectants

Ten million human JY cells were resuspended in 350  $\mu$ l of IMDM without FCS and transfected with 10  $\mu$ g of DNA (vector alone, S+/Ub, or S–/Ub) in a 0.4-cm cuvette using the Bio-Rad electroporator at 0.25 kV, 950  $\mu$ F, and  $\infty$  resistance. After one wash cells were incubated for 24 h in IMDM and 10% FCS and selected on 2 mg/ml G418 until stable transfectants were obtained. The cloned transfectants were tested for expression of the construct by RT-PCR before use in cytotoxicity assays.

### Tetramer staining

H2-D<sup>b</sup> tetramers labeled with PE and containing the HPV16E7 peptide RAHYNIVTF were obtained from the National Institute of Allergy and Infectious Disease Tetramer Facility (Atlanta, GA). One million splenocytes were incubated for 1 h with 20  $\mu$ l of 1/100 diluted tetramer and 1/100 diluted anti-CD8-FITC Ab (PharMingen, San Diego, CA) in PBS/0.5% BSA. After the incubation cells were washed twice in PBS/0.5% BSA, and expression levels of specific TCRs and CD8 molecules on the surface of  $5 \times 10^4$  splenocytes were determined by FACS analysis.

### TNF- $\alpha$ assay

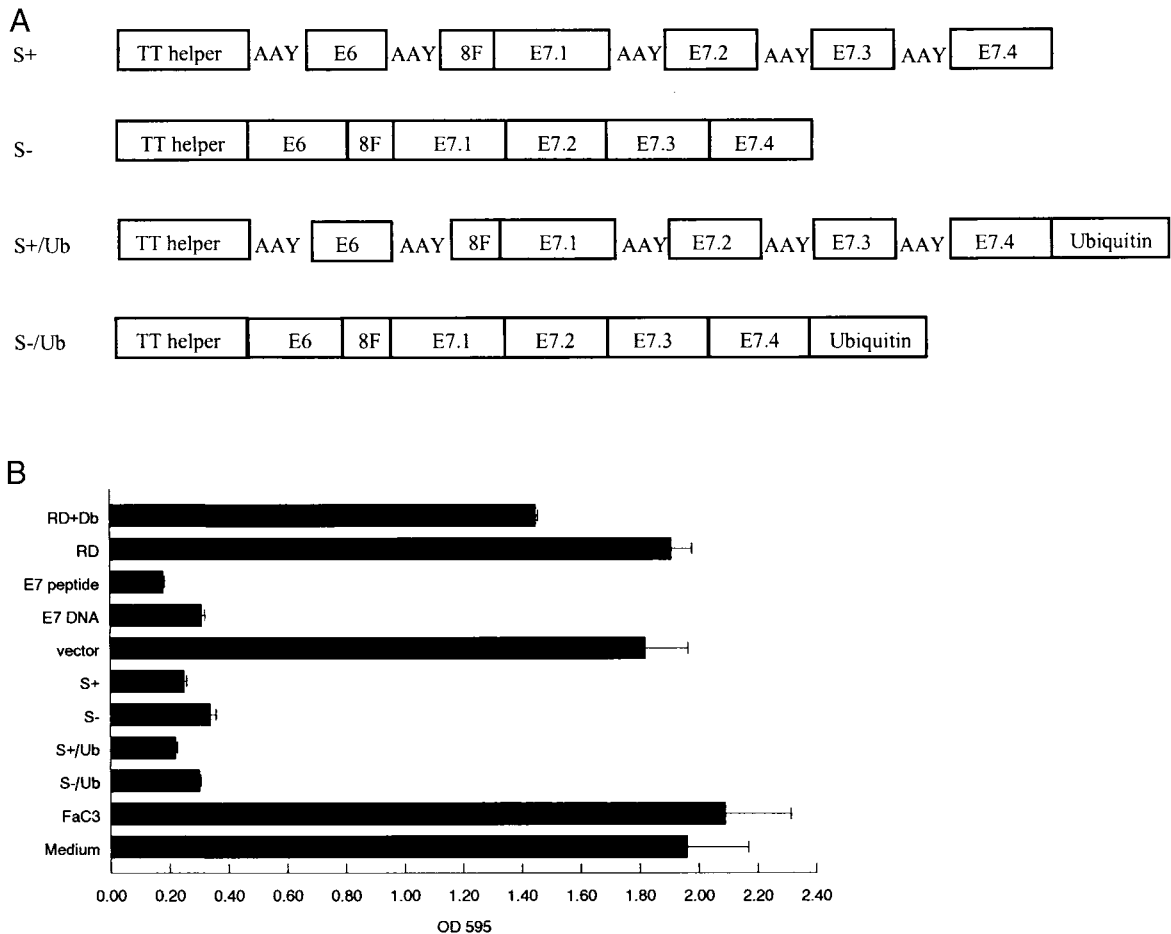
Semiconfluent human rhabdomyosarcoma RD cells (ATCC CCL-136) were trypsinized and plated in six-well tissue culture plates (Costar, Cambridge, MA) at  $5 \times 10^6$  cells/well in 2 ml of standard culture medium. After 2–4 h when the cells were attached, they were washed in OptiMem serum-free medium (Life Technologies, Gaithersburg, MD) and transfected overnight with 2.5  $\mu$ g of H-2D<sup>b</sup> in pCDNA3 (Invitrogen) using Lipofectamine (Life Technologies) according to the manufacturer's instructions. After 24 h the transfected RD cells or stable transfected JY cells were harvested and incubated overnight at 37°C with the E7-specific murine T cell clone F $\alpha$ C3 or HPV16 E6 (TIHDILECV)-specific human T cell line 7E7 at a ratio of 10:1. After 16 h 50  $\mu$ l of the supernatants were harvested and tested for the presence of TNF- $\alpha$  in a WEHI/MTT assay (26). Different concentrations of mouse or human TNF- $\alpha$  were used as positive controls. The OD was determined at 595 nm.

### DNA-gold bullet preparation and gene gun-mediated delivery

The Helios gene gun system (Bio-Rad, Hercules, CA) was used for intradermal gene delivery. Bullets containing 2  $\mu$ g of DNA/shot were generated according to the manufacturer's protocols. Briefly, 100  $\mu$ g of DNA was precipitated on 25 mg of 1- $\mu$ m gold particles in the presence of 100  $\mu$ l of 0.05 M spermidine (Sigma, St. Louis, MO) using 100  $\mu$ l of 1 M CaCl<sub>2</sub>/preparation. The gold was washed three times with 1 ml of 100% 200 proof ethanol (Aldrich, Milwaukee, WI) and was resuspended in 3 ml of 0.1 mg/ml polyvinylpyrrolidone in 100% ethanol. The gold was then loaded into the tubing using the tubing prep station (Bio-Rad), and the gold loaded tubing was cut into 0.5-inch pieces to load into the cartridges. The bullet-containing cartridges were loaded into the gene gun and delivered into the mouse dermis at a helium pressure of 450 psi.

### Tumor protection experiments

Mice were anesthetized by *i.p.* injection of 2.4 mg of ketamine (Abbott Laboratories, Chicago, IL) mixed in 80  $\mu$ l of PBS with 0.48 mg of xylazine (Sigma). The abdominal area was shaved, and the DNA was delivered into the epidermis. This procedure was repeated 2 wk after the first DNA delivery, and 2 wk after the second vaccination mice were challenged *s.c.* with  $0.5 \times 10^6$  C3 tumor cells in 100  $\mu$ l of HBSS (Sigma). After tumor cell injection the tumor development in individual mice was monitored two or



**FIGURE 1.** A, Schematic presentation of the four epitope string DNA vaccines. Epitopes include: TT, tetanus toxin universal helper peptide (aa 830–843); E6, HLA-A\*0201 peptide of HPV16 E6 (aa 29–38); 8F, a B cell epitope of HPV16E7 (52); E7.1, HLA-A201 peptide of HPV16 E7 (aa 11–20); E7.2, H2-D<sup>b</sup> + HLA-A24 peptide + helper peptide of HPV16 E7 (aa 48–57); E7.3, HLA-A201 peptide of HPV16 E7 (aa 82–90); and E7.4, HLA-A201 peptide of HPV16 E7 (aa 86–93). B, TNF- $\alpha$  assay to determine expression and processing of the DNA vaccines. TNF- $\alpha$  secretion on activation of the E7 peptide-specific FaC3 CTL clone on recognition of the E7 peptide on RD cells transfected with H2-D<sup>b</sup>. OD ( $\pm$ SD) from the MTT assay for the different targets are plotted on the x-axis and are the equivalent of TNF- $\alpha$  production of the activated T cells. Low values represent specific recognition by the T cell clone, whereas target cells not recognized have a high OD (no TNF- $\alpha$  production).

three times a week. C3 cells were derived from a large batch of C3 tumor cells of the same passage, tested for tumor formation, and frozen in liquid nitrogen. For every tumor injection a vial from this batch was thawed and grown according to a standard procedure for 10 days to obtain the required number of tumor cells. On the day of injection 80% confluent cell cultures were trypsinized and washed three times with HBSS. Cells were concentrated at  $5 \times 10^6$ /ml, and 100  $\mu$ l was injected s.c. in the left flank of the mice.

#### Tumor therapy

Mice in therapy experiments received a tumor s.c. injection with  $0.5 \times 10^6$ /ml of C3 cells in 100  $\mu$ l of HBSS at either 2 or 7 days before DNA introduction. On day 2 after inoculation no tumors were palpable, but on day 7 all mice in the therapy experiments had developed a palpable tumor. The intradermal DNA administrations were repeated at 7 and 14 days after the first treatment, and during this time the tumor sizes in the mice were recorded two or three times a week.

#### Enzyme-linked immunospot (ELISPOT) assay

The ELISPOT assay was used to detect peptide-specific T cells after stimulation with the synthetic HPV16 E7 CTL peptide RAHYNIVTF (17). Multiscreen HA plates (Millipore, Bedford, MA) were coated with 5  $\mu$ g/ml anti-IFN- $\gamma$  Ab (PharMingen) at 4°C. The next day the plates were washed with PBS/0.5% Tween 20 (Sigma) and blocked with culture medium. Splenocytes were applied at  $1 \times 10^6$  and  $2 \times 10^5$  cells/well in medium containing 25 IU of IL-2 and 10  $\mu$ g/ml of E7 peptide. After 40 h incubation at 37°C and 5% CO<sub>2</sub>, plates were washed with PBS/Tween and incubated

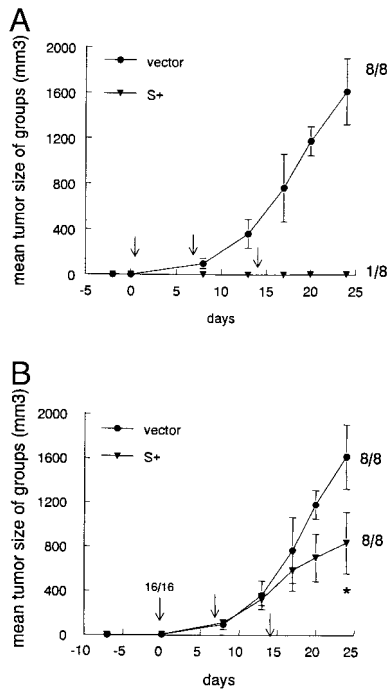
for 2 h at room temperature with 2.5  $\mu$ g/ml of biotinylated anti-IFN- $\gamma$  Abs. After washing with PBS/Tween, 1.25  $\mu$ g/ml of avidin-alkaline phosphatase (Sigma) was added to the wells in 50  $\mu$ l of PBS and incubated for 2 h at room temperature. This assay was performed using 50  $\mu$ l of alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Promega, Madison, WI) in alkaline phosphatase buffer for 15 min. Adding tap water stopped the reaction, and the plates were allowed to dry before counting the individual spots, representing T cells activated by the E7 peptide.

## Results

### Tumor protection after vaccination is increased with spacers between epitopes

The different DNA epitope constructs (Fig. 1A) intended for in vivo studies were tested for processing and presentation of the dominant murine epitope in a colorimetric TNF- $\alpha$  assay (Fig. 1B). Recognition of target cells induced TNF- $\alpha$  release by the T cells (FaC3), resulting in a low OD in the MTT assay. The RD-D<sup>b</sup> target cells were transfected with E7.2 peptide (RAHYNIVTF) encoding string epitope DNA, HPV16 E7 DNA, or loaded with E7.2 synthetic peptide (positive control). The untransfected RD cells, medium of targets not incubated with T cells, and supernatant of FaC3 T cells incubated without targets (FaC3) served as negative controls. All cells transfected with E7.2 peptide encoding



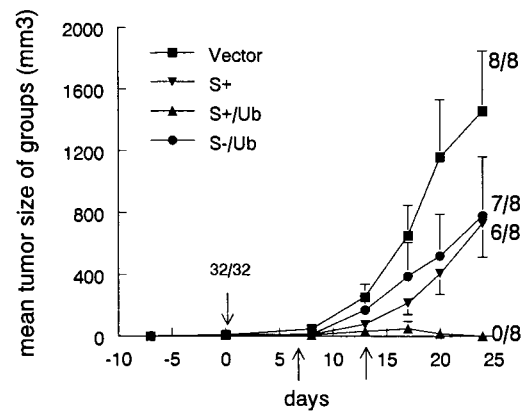


**FIGURE 3.** A, Tumor therapy against tumors started 2 days after tumor inoculation. Two groups of eight mice received either the empty vector DNA or the S+ DNA coding for the epitope string plus spacers (S- is not effective in therapy). Arrows indicate the treatment days (0, 7, and 14), and the mean tumor sizes of the groups (cubic millimeters)  $\pm$  SE are depicted on the y-axis. All mice treated with S+ rejected the tumor, and all controls developed a tumor. Values are representative of two independent experiments with similar results. B, Treatment of 7-day established tumors. Mean tumor sizes  $\pm$  SE in mice bearing 7-day established tumors and treated with S+ on days 0, 7, and 14. On the first treatment day all mice ( $n = 16$ ) had palpable tumor mass. Tumors continued to grow in all mice treated with the DNA vaccine. Mice were sacrificed on day 24 due to extensive tumor masses. Despite the persistence of tumors in all mice, treatment with the string construct significantly slowed tumor growth (\*,  $p = 0.04$ , by Student's  $t$  test). Values are representative of two independent experiments with similar results.

eradicated their 7-day established tumor masses. These data indicate that targeting the DNA encoded protein to the proteasome is crucial for therapeutic treatment of the established tumors ( $p = 0.0002$ , S+ vs S+/Ub). Similar to the effect on protection, addition of defined spacers around the epitopes significantly improved the final therapeutic outcome ( $p = 0.03$ , S-/Ub vs S+/Ub).

#### Spacers and ubiquitin enhance CTL priming

Vaccination with MHC class I-restricted T cell epitopes aims at induction of CTL that recognize the specific peptides on the tumor cells. The induction of CTLs by the different DNA-based vaccinations was tested by ELISPOT assay and tetramer analysis. The number of E7-specific CTLs was established in splenocytes of vaccinated mice by stimulation with the synthetic E7 CTL peptide. As depicted in Fig. 5A, all DNA vaccines induced specific CTL responses that were statistically different from those in mice vaccinated with the empty backbone vector (except for S-). In addition, the CTL precursor frequencies for most vaccinations were significantly different and reflected the differences observed in the vaccination and therapeutic studies with these constructs. Both addition of ubiquitin and spacing of the epitopes significantly increased precursor frequencies. Tetramer analysis with H2-D<sup>p</sup>-E7 peptide complexes together with CD8 staining confirmed that the DNA



**FIGURE 4.** Effect of ubiquitination on therapy of established tumors. Mean tumor size  $\pm$  SE in the groups during therapy of 7-day established C3 tumors. Four groups of eight mice were injected with C3 tumor cells 7 days before the start of the treatments. All mice ( $n = 32$ ) had palpable tumor at the first treatment (indicated by arrows) with 2  $\mu$ g of DNA on day 0. The number of tumor-bearing mice per group on day 24 is indicated. There was a significant difference in tumor size on day 24 between the therapy with the vector alone and the string construct with spacers and ubiquitin ( $p = 0.0002$ , S+ vs S+/Ub) and spacers ( $p = 0.03$ , S-/Ub vs S+/Ub) to the string construct significantly increased the therapeutic potential, as determined by tumor-free mice (by Fisher's exact test). This is a representative graph of two identical experiments.

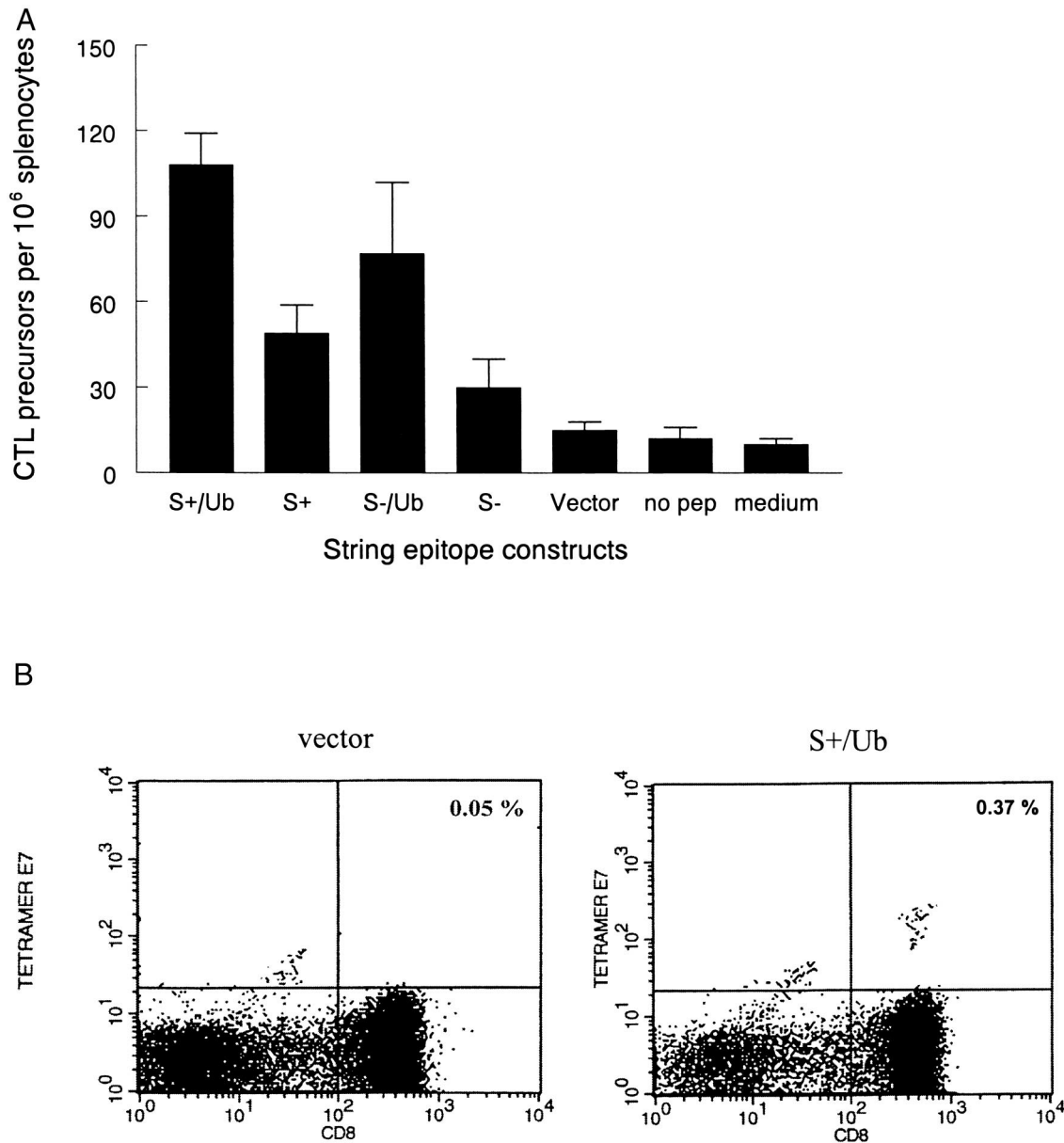
vaccines induced specific CTLs against the murine E7 epitope in the vaccine (Fig. 5B).

#### Processing of HLA-A2 epitopes

To determine whether epitopes other than the murine E7 were also processed and presented in HLA-A2, we developed stable transfectants of S+/Ub, S-/Ub, and the empty vector. These stable transfectants were tested for recognition of the HPV16E6 HLA-A2-restricted epitope 29–38 by an E6 peptide-specific CTL line (Fig. 6). Upon incubation of the JY transfectants with the CTLs, the TNF- $\alpha$  secretion of the T cells was determined in a colorimetric Wehi assay. The results from this assay indicate that in contrast to the JY cells with the empty vector, JY transfectants with S+/Ub and S-/Ub were recognized by the E6-specific CTLs. In addition, the cells transfected with the construct containing AAY flanking sequences around the HLA-A2 epitope were better recognized than the cells transfected with the construct lacking these flanking sequences.

#### Discussion

After the initial observation that injection of DNA could induce Ag-specific immune responses (27), vaccination with DNA has been explored and successfully applied by many investigators and in different fields (28, 29). Although the DNA vaccination approach seems to be safe, DNA vaccination with oncogenes still harbors the risk of transformation for the cells that receive and express the oncogenes (30, 31). Peptide- or RNA-based vaccines (24, 32, 33) have no transformation risks. However, RNA vaccines are degradation sensitive and require ex vivo loading of DCs, which is more elaborate and expensive than DNA vaccination and may decrease reproducibility. Therefore and because epitopes of HPV16 have been identified for many HLA and H-2 haplotypes, we have selected a DNA-based string epitope approach. As the induction of a Th cell response may be important for the clinical outcome (34), a tetanus toxin universal helper epitope (35, 36) and

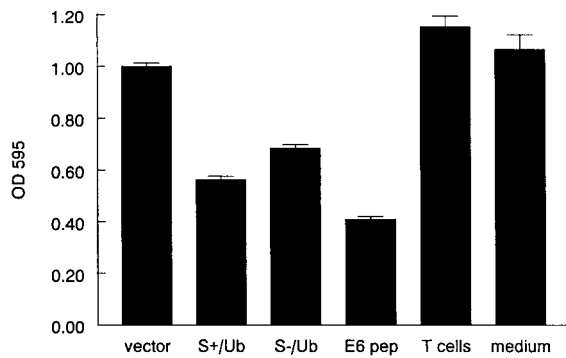


**FIGURE 5.** A, CTL induction by different DNA vaccines. CTL precursor frequency in splenocytes after two intradermal vaccinations with 2  $\mu$ g of DNA, 2 wk apart. Splenocytes were stimulated with the HPV16E7 CTL peptide RAHYNIVTF. The number of E7 peptide (pep)-specific CTL per  $10^6$  splenocytes ( $\pm$ SD), determined by ELISPOT, after different vaccinations is depicted on the y-axis. ANOVA with multiple comparisons indicated significantly different precursor frequencies between all DNA vaccinations except for S+ vs S- and S- vs vector. B, Tetramer analysis on splenocytes from mice vaccinated with the empty vector or with the S+/Ub DNA. Splenocytes were stained with CD8-FITC and H2-Db-RAHYNIVTF-PE and analyzed by FACS. The *upper right quadrant* indicates the cells that are CD8 positive and bind to the tetramer-peptide complex. All cells that bind the tetramer-peptide complex are CD8<sup>+</sup>.

an HPV16 E7 helper epitope (37) were introduced. However, proliferation responses against both helper peptides were undetectable in vaccinated mice (data not shown). This may indicate that the induction of a Th response in this model is not required for the induction of a CTL response. It has been shown that direct delivery of DNA on gold particles with the gene gun into skin DCs will activate the DCs and induce migration to the draining lymph nodes (38). These activated DCs can induce CTL responses, which may omit the necessity for CD4 Th cell activation. To test this we have vaccinated a group of eight CD4 knockout mice and eight regular C57BL/6 mice and challenged these mice after two vaccinations with C3 tumor cells. All eight naive control mice developed a tumor, and both groups of CD4 knockout and vaccinated C57BL/6 mice remained 100% tumor free (data not shown). These data con-

firm that for tumor protection after gene gun vaccination in this model the presence of CD4 cells is dispensable.

Most reports on epitope string DNA vaccination have employed virus-based approaches to introduce the DNA constructs into the host. The disadvantage of virus-based delivery methods is the pre-existing or virus-induced immunity against viral components that may hamper the effects of subsequent vaccinations (39, 40). Delivery of naked DNA will allow vaccination repeats as often as necessary and with any DNA-based vaccination regimen available. However, it was shown that in contrast to introduction of DNA using vaccinia virus, the injection of naked epitope string DNA constructs was not always effective (7, 21, 41). Our data indicate that naked DNA encoding tumor-specific epitopes can induce specific CTL (Fig. 5), can protect 100% of mice against a tumorigenic



**FIGURE 6.** Processing of HLA-A2-restricted epitope. Recognition of stable transfected human HLA-A2-positive JY cells with S+/Ub or S-/Ub by an HPV16E6-specific human HLA-A2-restricted CTL line. In a colorimetric Wehi assay, the TNF- $\alpha$  production of the T cells on incubation with the transfectants was determined in triplicate samples. JY cells exogenously loaded with the HPV16E6 peptide (pep) served as a positive control for T cell specificity, whereas vector-transfected JY cells, T cells alone, and medium represent the negative controls. Values are representative of two independent experiments.

tumor challenge and can be effective against 2-day tumors (Figs. 2 and 3A). However, the same epitope string DNA vaccine was not effective against well-established tumors (Fig. 3B) and required further improvement.

The possibility of targeting proteins to a cellular compartment to increase epitope processing, MHC loading, or Th cell response induction is another advantage of the use of DNA-based vaccines that has been employed by several investigators to optimize vaccination (21–23). Rodriguez et al. have shown that adding ubiquitin to the epitope DNA vaccine to target it to the protein degradation pathway increased CTL precursor frequencies (21). We confirmed the induction of a CTL response and increased CTL precursor frequencies for our string DNA vaccines containing ubiquitin at the carboxyl terminus. Moreover, to our knowledge this is the first report to show complete eradication of well-established tumors using a naked DNA epitope string vaccine and the crucial role of ubiquitin for therapeutic results in tumor-bearing mice.

Flanking sequences are important for the accurate processing of epitopes, but may occasionally also prevent Ag processing (42, 43). Where some results have indicated a preference for natural flanking sequences around the epitopes for proteasome processing (44), others have shown that flanking of epitopes with alanine increased the epitope processing and recognition by T cells (45, 46). The role of epitope flanking sequences in the *in vivo* induction of anti-tumor immunity is unknown. In string constructs the epitopes are separate entities into artificial proteins without natural flanking sequences. Therefore, it is of particular importance to study the role of flanking sequences in epitope string DNA vaccines. As predictions on the impact of flanking sequences based on the published data were impossible, two identical vaccines with and without defined spacers between epitopes were constructed. This provided the opportunity to study the role of flanking sequences in the induction of protective and therapeutic immune responses against tumors *in vivo*. Selection of the spacer sequence is based on the following considerations. The proteasome prefers cleavage behind hydrophobic and basic residues, i.e., residues that in the majority of haplotypes represent the C-terminal anchor residues of HLA epitopes. Therefore, the efficient generation of the correct C-terminus is in most cases an intrinsic property of the proteasome (47). In contrast, proteasomal processing of the epitope's N-terminus is less well defined. In consequence, epitope generation efficiency is

strongly influenced by the efficiency of the N-terminal cleavage site usage by the proteasome. Based on the analysis of a large number of epitopes, the spacer residues AAY were determined to support epitope generation (18, 48). The two alanine residues flanking the C-terminal will support C-terminal cleavage of the epitope without negatively influencing the N-terminal cleavage of the adjacent epitope. The tyrosine residue was selected to introduce a strong N-terminal cleavage site adjacent to N-terminal residues of the E7 epitopes.

The observation by Thomson et al. (20) that unnatural flanking sequences in an epitope string DNA construct do not prevent processing of the epitopes is correct and is confirmed by this and other studies (7, 22). However, our data indicate that the addition of adequate spacers between the epitopes facilitates the processing of peptides from the epitope string, resulting in increased CTL precursor frequencies and significantly improved vaccination and therapy of tumor-bearing mice. In addition to the processing of a murine epitope we showed improved processing and presentation of a human HLA-A2-restricted epitope from the vaccine. This indicates that the results obtained in the murine models are not restricted to murine cells, but are also observed for other epitopes expressed in human cells and are therefore relevant for the induction of an immune response in humans.

Peptide vaccination with the epitopes included in the current epitope string DNA vaccine has been tested in clinical trials in end-stage cervical cancer patients and patients with early cervical lesions (CIN III). Despite CTL induction in some patients, vaccination of end-stage patients was clinically ineffective (49, 50), probably due to the immunocompromised state of these patients (50). However, when the same peptides were used in patients with CIN III lesions, 12 of 18 patients cleared the human papillomavirus from their cervical smears (51). This indicates that peptides encoded in the current epitope string DNA vaccine are clinically relevant, and that patients with early cervical lesions are preferred for the effective treatment with immunotherapeutic HPV vaccines against cervical cancer.

In conclusion, we have obtained proof that the combined addition of defined spacers and ubiquitin to an epitope string naked DNA vaccine significantly increased its *in vivo* efficacy. This applies to both prevention of tumor establishment and treatment of established tumors in our model. These data suggest that similar immunization strategies may be used in patients for the treatment of early cancers or the eradication of minimal residual disease.

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