DNA vaccines based on chimeric potyvirus-like particles carrying HPV16 E7 peptide (aa 44-60)

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Abstract. Vaccine strategies for the treatment of human papillomavirus-induced cervical cancer are based mainly on the human papillomavirus 16 E7 (HPV16 E7) oncoprotein. The immunogenicity of the E7 gene has been enhanced by its fusion to many different genes. Here, we linked a short sequence coding for the E7 peptide (aa 44-60) containing immunodominant epitopes for B and T cells to the 3' end of the gene coding for the whole coat protein (CP) of the potyvirus, potato virus A (PVA), and its deleted form (CPdel) with a short C-terminal deletion of 5 amino acids (LGVKG). CP-E7 and CPdel-E7 fusion proteins, just like CP alone, spontaneously assembled into virus-like particles in both procaryotic and eucaryotic cells. The CP-E7 and CPdel-E7 fusion genes induced slightly stronger E7-specific cytotoxic T-lymphocyte responses than the whole E7 gene, although they were still lower than those elicited by the previously constructed fusion gene, Sig/E7GGG/LAMP-1. The E7- and CP-specific antibody responses were not detected in mice vaccinated with CP-E7 and CPdel-E7 fusion genes. The CP-E7 and CPdel-E7 fusion genes protected mice against the development of tumors induced by TC-1 cells producing the E7 antigen and were also effective in the therapeutic setting, i.e. when the vaccination was performed after tumor cell administration. Their antitumor effect was comparable to those of the whole E7 gene and Sig/E7GGG/LAMP-1 fusion gene. There was no relevant difference between immune responses elicited by CP-E7 and CPdel-E7 DNA vaccination.

Introduction

Human papillomaviruses (HPVs) are the causative agent of cervical cancer. HPV16 is the most prevalent type of HPV associated with cervical cancer, detected in about 50 percent of cervical cancer. Therefore, it is the most important target for development of both prophylactic and therapeutic anti-HPV vaccines. Preventive immunization is based on the structural L1 and L2 proteins of HPV and should result in the induction of neutralizing antibodies. The L1 monomers assemble spontaneously into virus-like particles and enter the mammalian cells through specific receptors (1). Vaccination with L1 virus-like particles has been shown to be very effective, generating 100% protection against persistent HPV infection (2). Therapeutic vaccination against HPV is aimed at eliciting cellular immune responses to viral E7 and/or E6 oncoproteins that are the only viral proteins constitutively expressed in cells of cervical cancer. The therapeutic vaccines against HPV-associated cancer have appeared promising, but there is still a need for improvement (3). Different strategies have been utilized, including the fusion of the HPV16 E7 protein or the E7-derived peptides to proteins forming virus-like particles (4-6).

VLPs (virus-like particles) are known to induce not only potent antibody responses but also strong cell-mediated responses (6,7). The nature of the induced immune responses against L1 VLPs has been shown to vary with different delivery systems. Monkeys immunized intramuscularly with plasmid DNA or a replicon incompetent adenoviral vector expressing HPV16 L1 developed strong Th1/Tc1 responses, potent humoral responses and only weak neutralizing antibodies, while immunization with HPV16 L1 VLPs led to a potent humoral response with high levels of neutralizing antibodies and a strong L1-specific Th2 response (8). VLPs alone have not proved very efficient at inducing cytotoxic T-lymphocyte (CTL) responses but have become a very powerful vaccine when applied together with adjuvants activating antigen-presenting cells (7). The delivery of DNA vaccines by a gene gun is considered to have such an adjuvant effect. Mild damage caused to the skin by the gold particles may act as maturation and stimulation signals for) Langerhans cells and dermal dendritic cells (9).

PVA is a filamentous RNA virus which belongs to the genus Potyvirus (family Potyviridae), the largest group of plant viruses. Potyvirus particles are flexible rods, 680-900 nm long and 11-15 nm wide, consisting of more than 2000 copies of a coat protein subunit and one single-stranded RNA genome (10). The potyvirus CP is a multifunctional protein

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which plays a role in virus transmission by aphids (11), virus movement in plants (12) and virion formation. Three regions can be distinguished in the potyviral CP protein. The central core region involves 214-217 amino acids (aa) and is considered to be responsible for particle integrity. Both the N-terminal (29-94 aa) and C-terminal (18-20 aa) regions are surface exposed and are not required for virion assembly (13,14). The expression of potyvirus CP (Johnsongrass mosaic virus, JGMV) in bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*), insect cells or mammalian cell culture has been demonstrated to result in the formation of potyvirus-like particles (PVLPs) (14,15). It has been shown that self-assembly of the particles is not impaired, even by the insertion of a foreign 26 kD protein into the JGMV CP (14,15). Such chimeric PVLPs have been highly immunogenic, even in the absence of any adjuvant (14,16).

The aims of this study were to investigate the ability of DNA vaccines, coding for the PVA CP protein associated with HPV16 E7 peptide (aa 44-60), to elicit E7-specific immune responses and to compare their efficacy with those of the E7 gene and the previously prepared Sig/E7GGG/LAMP-1 fusion gene (17). Furthermore, we assessed the influence of C-terminal deletion and fusion to the E7 peptide (aa 44-60) on the CP's ability to assemble into VLPs in procaryotic and eucaryotic cells.

Materials and methods

Animals. Female C57BL/6 mice, aged 6-8 weeks (H-2^b; Charles River, Germany), were used in the immunization experiments. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals.

Cell lines. The efficacy of DNA vaccines was evaluated using TC-1 tumor cells (18) (kindly provided by T.C. Wu, Johns Hopkins University, Baltimore, MD), prepared by transformation of primary C57BL/6 mouse lung cells with HPV16 E6/E7 oncogenes and activated *H-ras*. To verify the expression of the E7 and CP proteins from constructed plasmids, mouse NIH 3T3 fibroblasts and human embryonic kidney 293T cells (kindly provided by J.A. Kleinschmidt, DKFZ, Heidelberg, Germany) were transfected and production of proteins was determined in cell lysates. All cells were grown in Dulbecco's Modified Eagle's medium (PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Plasmids. The constructions of pBSC, pBSC/E7 (19), pBSC/E7GGGLAMP (17) and pMPM4Ω/PVA-CP (20) were described previously. Two fusion genes coding for the HPV16 E7-derived peptide (aa 44-60) fused to the C-terminus of full-length PVA CP (CP-E7) or PVA CP shortened by 5 aa (CPdel-E7) were prepared by two sequential PCR reactions modifying the 3' end of the PVA CP gene by primers with long overhangs coding for the E7 epitope. For the first PCR reaction, the upstream primer, 5'-AAAACCATGGAAGCCG GAACTCTTGATGC-3', with the start codon ATG containing *Nco*I restriction site (underlined) on its 5' end was used. The downstream primers coding for the junction of CP and E7 were 5'-TGTTGTAGTGGGCGCGGTCCGGCTCGGCCTGAAC CCCCTTCACGCCTAGAAGGT-3' for the CP-E7 gene and 5'-GTTGTAGTGGGCGCGGTCCGGCTCGGCCTGAACA AGGTGATGCATGTTGCGATTAAC-3' for the CPdel-E7 gene. In the second PCR reaction, we used the same primers for construction of both the CP-E7 and the CPdel-E7 fusion genes, the upstream primer that had been used in the first PCR reaction and the downstream primer coding for the C-terminus of E7 peptide, 5'-AAAAGATCTACTTGCAGCAG AAGGTCACGATGTTGTAGTGGGCGCGGTCCGGCTC-3', which contained the *Bgl*II restriction site (underlined). The PCR products were cloned into the *Nco*I and *Bgl*II restriction sites of pUC57T/A and pMPM4Ω.

PVA CP, PVA CP-E7 and PVA CPdel-E7 genes were cut from pMPM4Ω plasmids by *Nco*I and *Sal*I restriction enzymes and cloned to the corresponding sites in the pUC131 plasmid (kindly provided by J.A. Kleinschmidt). Finally, these three genes were excised from pUC131 by *Xho*I and *Sal*I restriction enzymes and cloned to the *Xho*I site in the pBSC plasmid. The accuracy of generated pBSC/CP, pBSC/CP-E7 and pBSC/CPdel-E7 was confirmed by DNA sequencing.

The plasmids were propagated in *Escherichia coli* XL1-blue or DH5α strains cultured in Luria-Bertani broth with 100 μg/ml ampicillin added, and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

In-gel digestion of proteins for detection of E7 peptide by MALDI. For in-gel digestion with trypsin, a method adapted from Shevchenko *et al* (21) was used. The gel was washed with water (two times for 10 min) and then the band of interest was excised and cut into 1-mm cubes. The gel particles were washed three times with 0.1 M NH_4HCO_3/acetonitrile 1:1 (v/v) for 15 min with a volume roughly equal to 3-fold the gel volume. All remaining liquid was removed and the gel pieces were covered with 100% acetonitrile. Acetonitrile was removed after 5 min and the gel was rehydrated with 0.1 M NH_4HCO_3 and an equal volume of acetonitrile was added. After 15 min, all liquid was removed and the gel particles were vacuum dried. Then reduction with dithiothreitol (DTT) followed by alkylation with iodoacetamide (IAA) was performed and the samples were freeze dried. The gel pieces were covered with trypsin solution from bovine pancreas (10 μg/ml) in 50 mM NH_4HCO_3 and incubated at 37˚C overnight. The peptides were extracted from the gel by addition of a volume of 25 mM NH_4HCO_3, followed by the same volume of acetonitrile, and sonicated for 10 min. The supernatant was recovered, 100 μl of 30% acetonitrile with 0.1% trifluoro acetic acid (TFA) was added to the gel particles and sonicated again for 15 min. This step was repeated with 50% acetonitrile containing 0.1% TFA. The extracts were pooled and vacuum dried. The samples were purified on ZipTip$_{C18}$ (Millipore) prior to MALDI-TOF mass spectrometry.

Matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry. Analyte (tryptic digest) (1 μl) was mixed with 3 μl of matrix solution prepared as follows: 10 mg of DHB (2,5-dihydroxybenzoic acid, Sigma) were dissolved in 1 ml of 30% acetonitrile/0.1% TFA (1:2, v/v). The mixture was spotted on a MALDI target plate. The peptide mixture for external calibration was purchased from

Bruker, Germany. A mass spectrometer BIFLEX IV (Bruker) with reflector was used for analyses. Spectra of the peptide mixtures were recorded in the reflector mode at a laser wavelength of 337 nm. Peptide mass maps were searched against theoretically derived maps of investigated proteins.

Detection of CP proteins by immunoblotting. 293T cells were grown on 6-cm dishes and transfected with 15 μg plasmids by modified calcium-phosphate precipitation (22). After two days, the cells were collected and spun at 1200 rpm for 5 min at 4˚C. The cell pellets were washed with 1 ml ice-cold PBS (23) and resuspended in 100 μl of lysis buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 2 mM EDTA, 100 mM Tris-HCl, pH 8) (24). Cell lysates were then passed five times through a 21-G needle and centrifuged at 13000 rpm for 3 min at 4˚C. The last two steps (i.e. passing through a needle and centrifugation) were repeated. The cell lysates were mixed 1:1 with 0.02% bromphenol blue solution and denaturated by incubation at 98˚C for 3 min. Proteins were further separated by 10% SDS-PAGE, electroblotted onto a polyvinylidene difluoride (PVDF) membrane and incubated with mouse anti-CP monoclonal antibody, clone PVA 634 (25) and secondary peroxidase-labelled anti-mouse IgG antibodies (Amersham Biosciences, Little Chalfont, UK). The membranes were stained using the ECL Plus kit (Amersham Biosciences).

Detection of CP proteins by immunofluorescence. NIH 3T3 cells were grown on slides in 24-well plates and transfected with 4 μg of plasmids by modified calcium-phosphate precipitation (22). Two days after transfection, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 containing 5 μg/ml DAPI for 3 min. The CP protein was stained with mouse anti-CP monoclonal antibody, clone PVA 634 (25), and secondary anti-mouse IgG antibodies labeled with FITC (Sigma).

Electron microscopy of potyvirus-like particles produced in procaryotic cells. Purified recombinant PVA-CP proteins were prepared from *E. coli* cells transformed with pMPM4Ω-derived plasmids by ultracentrifugation on a CsCl or sucrose gradient (20). The samples were applied directly to the carbonated microscopic grids and negatively stained with uranyl acetate (26).

Electron microscopy of potyvirus-like particles in eucaryotic cells. 293T cells grown on slides in 24-well plates were transfected with 4 μg plasmids by modified calcium-phosphate precipitation (22). Two days after transfection, the slides were flat embedded in LR white resin. In brief, cells were rinsed with Sörensen buffer (SB; 0.1M Na/K phosphate buffer, pH 7.3), fixed with 3% formaldehyde and 0.1% glutaraldehyde in SB, dehydrated through an ethanol series of increasing concentration, embedded in LR white resin and polymerized at 4˚C for two days. Sections (80-nm-thick) were contrasted with uranyl acetate and observed with a FEI Morgagni electron microscope operating at 80 kV.

Preparation of gene gun cartridges. Plasmid DNA was coated onto 1-μm gold particles (Bio-Rad, Hercules, CA) as described previously (19). Each cartridge contained 1 μg of DNA coated onto 0.5 mg of gold particles.

Stimulation of splenocytes for in vitro assays. For *in vitro* assays, mice (3 per group) were vaccinated using a gene gun with 1 μg of plasmid DNA into the shaven abdomen, at a discharge pressure of 400 psi, and boosted with the same dose two weeks later. Splenocytes from vaccinated mice were isolated two weeks after the second vaccination and restimulated for five or six days with 0.001 μg/ml E7-specific H-2D^b CTL epitope, RAHYNIVTF (aa 49-57) (27), or with 10 μg/ml E7-derived peptide, QAEPDRAHYNIVTFCCKCD (aa 44-62), carrying H-2D^b CTL epitope, B cell epitope and T helper cell epitope (28). Control splenocytes were incubated without peptides.

ELISPOT assay. The ELISPOT assay described by Miyahira *et al* (29) and Murali-Krishna *et al* (30) was modified to detect HPV16 E7-specific T cells. The 96-well filtration plates (Millipore Corp., Bedford, MA) were coated with 10 μg/ml rat anti-mouse IFN-γ or IL-4 antibody (BD Biosciences Pharmingen, San Diego, CA) in 50 μl of PBS. After overnight incubation at 4˚C, the wells were washed and then blocked with culture medium containing 10% fetal calf serum. Different concentrations of either non-restimulated or restimulated splenocytes from each vaccinated group of mice, starting from $1x10^6$/well, were added to the wells. Cells were incubated at 37˚C for 24 h either with or without 0.001 μg/ml $E7_{49-57}$ peptide or 10 μg/ml $E7_{44-62}$ peptide. The plates were washed and incubated with 5 μg/ml biotinylated anti-IFN-γ or anti-IL-4 antibody (BD Pharmingen) in 50 μl of PBS at 4˚C overnight. After washing, the avidin-horseradish peroxidase conjugate (BD Pharmingen) was added and the plates were incubated for 2 h at room temperature. After washing, spots were developed by adding 50 μl of 0.5 mg/ml aminoethylcarbazole solution (Fermentas Inc., Hanover, MD) and 0.03% H_2O_2 and incubated at room temperature for 1 h. The spots were counted using a dissecting microscope.

Tetramer staining. E7-specific $CD8^+$ CTLs were detected by the tetramer-staining assay in lymphocyte bulk cultures, restimulated *in vitro* for 6 days with HPV16 $E7_{49-57}$ peptide, using the R-phycoerythrin labeled H-2D^b/$E7_{49-57}$ tetramer reagent, as described previously (31). The stained cells were analyzed on a FACScan instrument, using Cell Quest software (Becton-Dickinson).

Detection of anti-E7 specific antibodies by GST capture ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (32,33). Ninety-six-well plates (Dynatech, Chantilly, VA) were coated overnight at 4˚C with 200 ng/well of glutathione casein in 50 mM carbonate buffer, pH 9.6. Thereafter, the wells were incubated for 1 h at 37˚C with 100 μl of blocking buffer (0.2% casein in PBS with 0.05% Tween-20) and washed 3 times in washing buffer (PBS with 0.05% Tween-20). The cleared lysate from *E. coli* expressing the glutathion-S-transferase (GST)-E7-tag protein diluted in blocking buffer to 25 μg/100 μl was added to each well for 1 h at 37˚C. Unbound material was washed away 5 times in washing buffer. Mouse sera assayed for anti-E7 antibodies were diluted 1:50 in blocking buffer containing 0.25 μg/μl total lysate proteins from the GST-tag-transformed *E. coli* (to block reactivities of the sera with contaminating

E. coli proteins) and incubated for 1 h at 37˚C. After washing the plates 5 times, bound mouse antibodies were detected by sheep anti-mouse IgG antibodies conjugated to horseradish peroxidase (Amersham Biosciences) diluted 1:2000 in blocking buffer for 1 h at 37˚C. The plates were washed 5 times and stained with 100 μl of 10 μg/ml tetramethylbenzidine (Sigma) and 0.003% H_2O_2 for 5-10 min. The reaction was stopped by adding 50 μl of 1 M sulfuric acid and the absorbance was measured at 450 nm.

Detection of anti-CP specific antibodies by DAS-ELISA. For the detection of PVA CP, the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was employed using buffers described by Clark and Adams (34) as reported by Filigarova *et al* (35). The PVA CP expressed in *E. coli* and also in tobacco leaves infected with PVA isolate Lichte Industrie (kindly provided by Dr Dedic, Potato Research Institute, Havlickuv Brod, Czech Republic) was used as an antigen.

Tumor protection experiment. Mice (eight per group) were twice vaccinated using a gene gun with 1 μg of plasmids at a two-week interval. Two weeks after the second vaccination, mice were subcutaneously challenged into the back with $3x10^4$ TC-1 cells suspended in 0.15 ml PBS and then monitored twice a week for tumor growth.

Tumor therapeutic experiment. Mice (eight per group) were subcutaneously inoculated into the back with $3x10^4$ TC-1 tumor cells suspended in 0.15 ml PBS. Three and ten days later, mice were given 1 μg of plasmids using a gene gun. The mice were monitored twice a week for tumor growth.

Statistical analysis. Tumor formation after DNA immunization was analyzed by the log-rank test using Prism 3 software (GraphPad Software Inc., San Diego, CA, USA). A difference between groups was considered significant if P<0.05.

CP protein
1 MEAGTLDAGETPAQKSEDRK
21 KEGEGNSSKAVAVKDKDVDL
41 GTAGTHSVPRLKSMTSKLTL
61 PMLKGKSVVNLDHLLSYKPK
81 QVDLSNARATHEQFQNWYDG
101 VMASYELEKSSMEIILNGFM
121 VWCIENGTSPDINGVWTMMD
141 DEEQVSYPLKPMLDHAKPSL
161 RQIMRHFSALAEAYIEMRSR
181 EKPYMPRYGLQCNLRDQSLA
201 RYAFDFYEITATTPVRAKEA
221 HLQMKAAALKNSNTNMFGLD
241 GNVTTSEEDTERHTATDVNR
261 NMHHLLGVKGV

The C terminus (aa 261-288) of the CP-E7 protein
261 NMHHLLGVKGV QAEPDRAHYNIVTFCCK

The C terminus (aa 261-283) of the CPdel-E7 protein
261 NMHHLV QAEPDRAHYNIVTFCCK

Figure 1. Amino acid sequence of CP protein and C-termini of CP-derived fusion proteins. The E7-derived peptide is underlined.

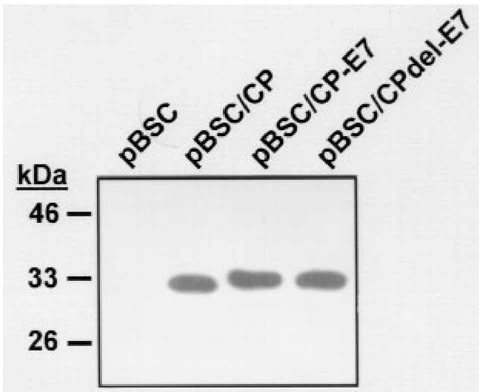

Figure 2. Immunoblotting detection of the CP antigen in 293T cells transfected with pBSC-derived plasmids. Proteins separated by 10% SDS-PAGE were transferred onto a PVDF membrane, detected by the CP-specific antibody (PVA 634) and visualized by ECL. The experiment was repeated with similar results.

Results

Generation of pBSC/CP, pBSC/CP-E7 and pBSC/CPdel-E7 DNA vaccines and detection of protein expression by immunoblotting. By modifications of the 3' end of the CP gene, we generated two fusion genes linking the sequence coding for the HPV16 E7-derived peptide (aa 44-60) to the whole CP gene (CP-E7) or to the CP gene with a short C-terminal deletion (CPdel-E7) as described in Materials and methods (Fig. 1). The shortened form of CP was used because we were afraid that the fusion of the E7 peptide to the whole CP could disturb the formation of VLPs. The fusion genes and the CP gene alone were cloned into the mammalian expression plasmid, pBSC, downstream of its immediate early cytomegalovirus promoter. The expression of the CP antigen was detected in transfected 293T cells by immunoblotting. The levels of expression of CP, CP-E7 and CPdel-E7 proteins were comparable (Fig. 2). We observed small differences in the mobility of proteins depending on the size. The presence of the E7-derived peptide in the CP-E7 and CPdel-E7 proteins was further confirmed by MALDI mass spectrometry revealing the presence of 1.298 kD peptide corresponding to a C-terminal fragment of E7 peptide (AHYNIVTFCCK) generated after trypsin digestion of CP-E7 and CPdel-E7 proteins (data not shown).

Detection of cellular location of CP proteins by immunofluorescence and visualization of PVA VLPs produced in procaryotic and eucaryotic cells by electron microsopy. Transfection of NIH 3T3 cells and subsequent immunofluorescent staining were used to determine the cellular localization of the CP protein and CP-fusion proteins. The cells transfected with pBSC/CP, pBSC/CP-E7 or pBSC/CPdel-E7 showed cytoplasmic localization of the CP antigen (Fig. 3). In some cells, CP protein formed spindle-shaped aggregates, while aggregates of CP-E7 and CPdel-E7 proteins showed spherical morphology. In non-transfected cells no specific staining was detected (data not shown).

Electron microscopy of purified recombinant CP, CP-E7 and CPdel-E7 proteins obtained by centrifugation in a CsCl density gradient or on a sucrose cushion revealed the formation of VLPs in *E. coli* cells transformed with pMPM4Ω-derived plasmids (Fig. 4). This was the evidence that CP monomers

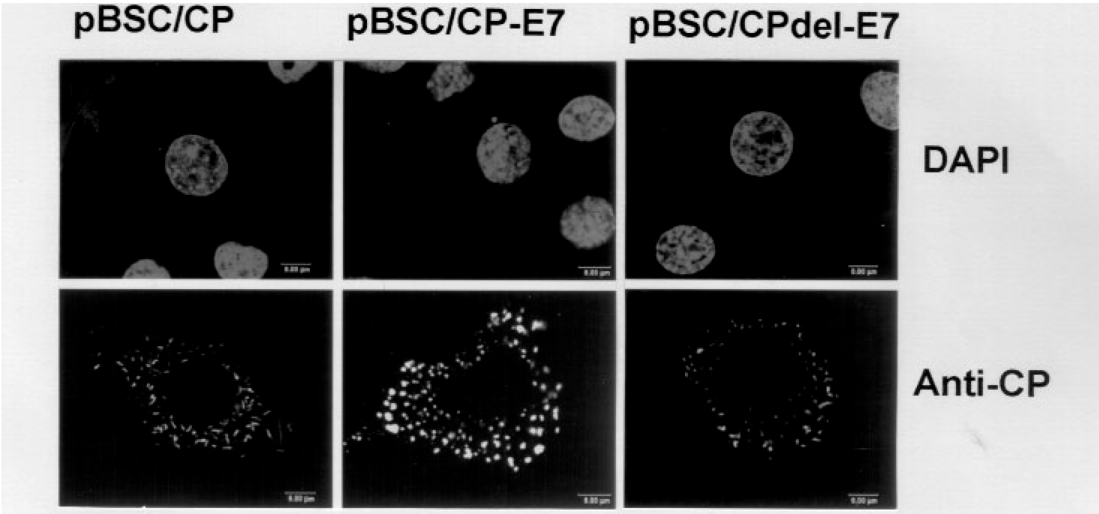

Figure 3. Immunofluorescence detection of the CP antigen in NIH 3T3 cells transfected with pBSC-derived plasmids. The paraformaldehyde-fixed cells were stained with anti-CP monoclonal antibody (PVA 634) and DAPI 2 days after transfection and examined by a confocal microscope. The scale bars represent 8 μm.

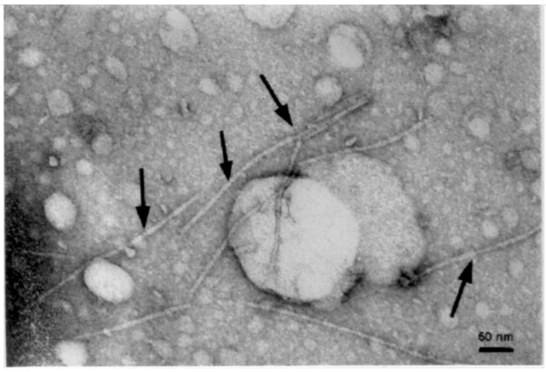

Figure 4. Transmission electron micrograph of potyvirus-like particles produced in procaryotic cells. PVA CP VLPs were purified from *E. coli* by centrifugation in CsCl density gradient. The arrows indicate VLPs. The scale bar represents 50 nm.

of PVA, when expressed in a heterologous host (*E. coli*), self-assembled to form VLPs. The morphology of CP-E7 and CPdel-E7 VLPs did not differ from that of CP VLPs, shown in Fig. 4.

Electron microscopy of 293T cells transfected with pBSC/CP, pBSC/CP-E7 or pBSC/CPdel-E7 revealed the formation of aggregates of VLPs (Fig. 5). While non-modified CP VLPs were aggregated into parallel straight bundles, aggregates of modified CP-E7 VLPs kept globular formation as the individual threads were curved. The CPdel-E7 VLPs also stuck to each other; the aggregates were similar in morphology to both CP and CP-E7 VLPs.

Vaccination with pBSC/CP-E7 or pBSC/CPdel-E7 DNA vaccines enhanced an E7-specific $CD8^+$ T cell-mediated immune response. We performed tetramer assays to detect the $E7_{49-57}$ peptide-loaded H-$2D^b$ tetramer positive $CD8^+$ T cells and ELISPOT assays to assess numbers of E7-specific IFN-γ- or IL-4-secreting cells in splenocytes from mice vaccinated with the set of DNA vaccines that were further investigated for their abilities to induce anti-E7 antibodies, to protect mice

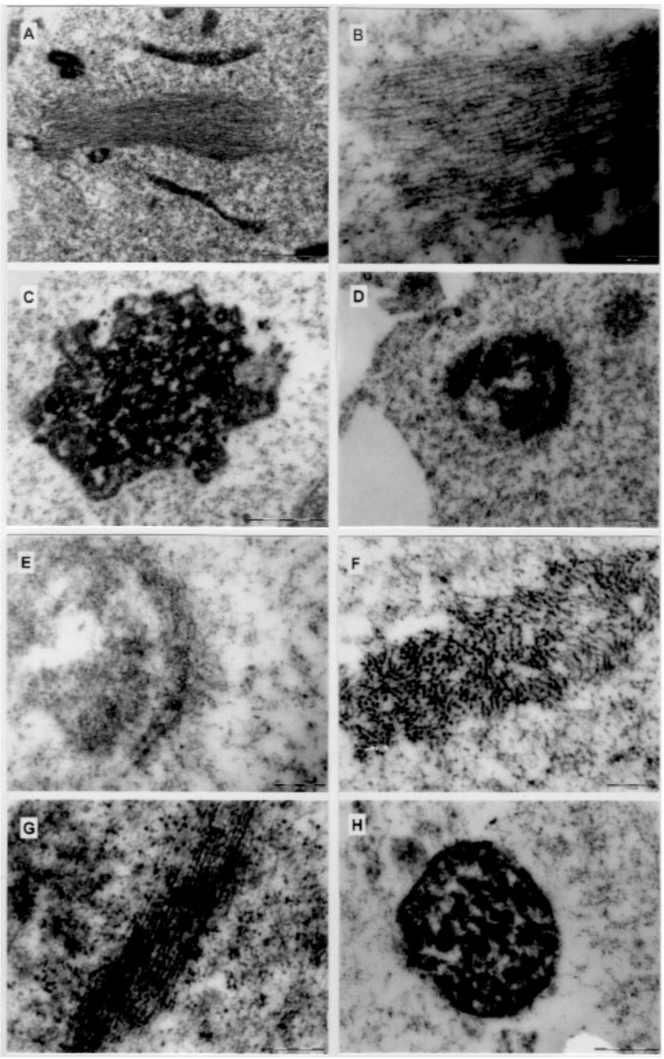

Figure 5. Transmission electron micrographs of potyvirus-like particles in mammalian cells. The 293T cells were transfected with pBSC-derived plasmids and examined by electron microscopy 2 days later. Aggregates of VLPs of PVA CP (A, B), CP-E7 (C-E) and CPdel-E7 (F-H) were found in cells. The scale bars represent 1 μm (A,C), 500 nm (D, H) or 200 nm (B, E-G).

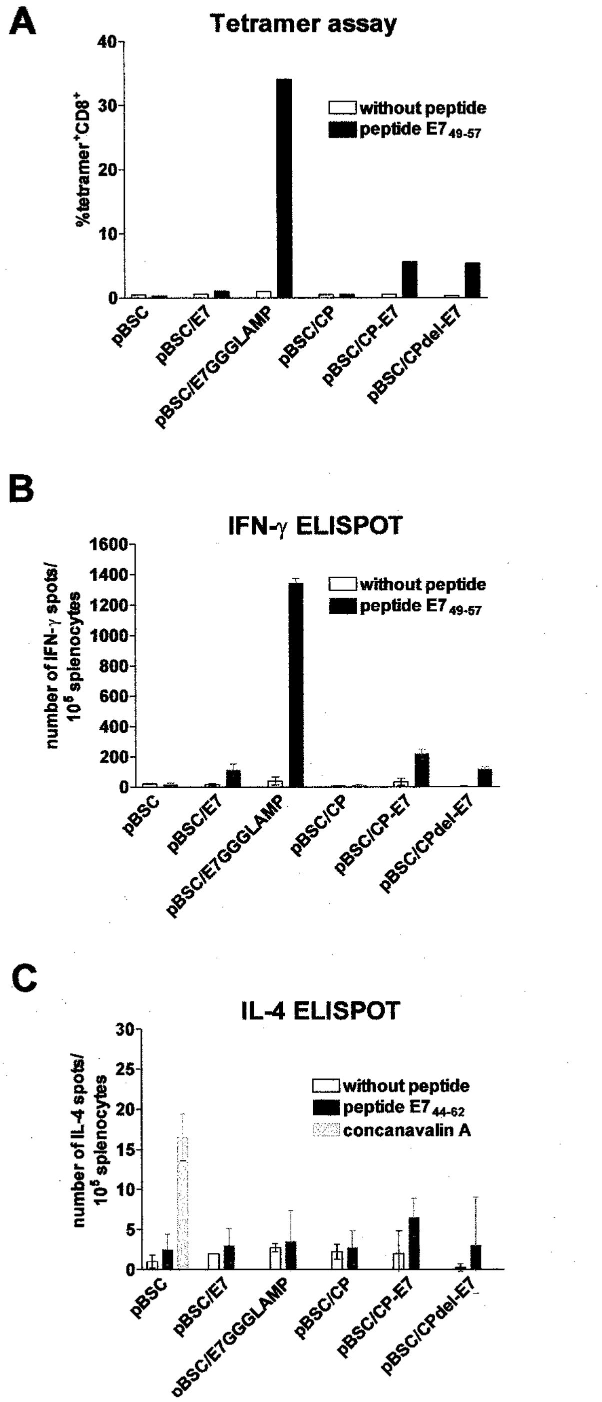

Figure 6. Detection of immune responses by tetramer staining and ELISPOT assays. Mice (n=3) were immunized two times with 1 μg of plasmids at a 2-week interval. Splenocytes were isolated two weeks after the second vaccination and incubated five days for ELISPOT assay or six days for tetramer staining with or without E7-derived peptides. $E7_{49-57}$ peptide-loaded H-$2D^b$ tetramer-positive $CD8^+$ cells were assessed by tetramer assay (A). IFN-γ-producing E7-specific $CD8^+$ lymphocytes were detected after the stimulation of splenocytes with $E7_{49-57}$ peptide (B). IL-4-secreting cells were detected in splenocytes incubated with $E7_{44-62}$ peptide (C). Splenocytes stimulated with concanavalin A were used as a positive control in the ELISPOT assay detecting IL-4 positive cells.

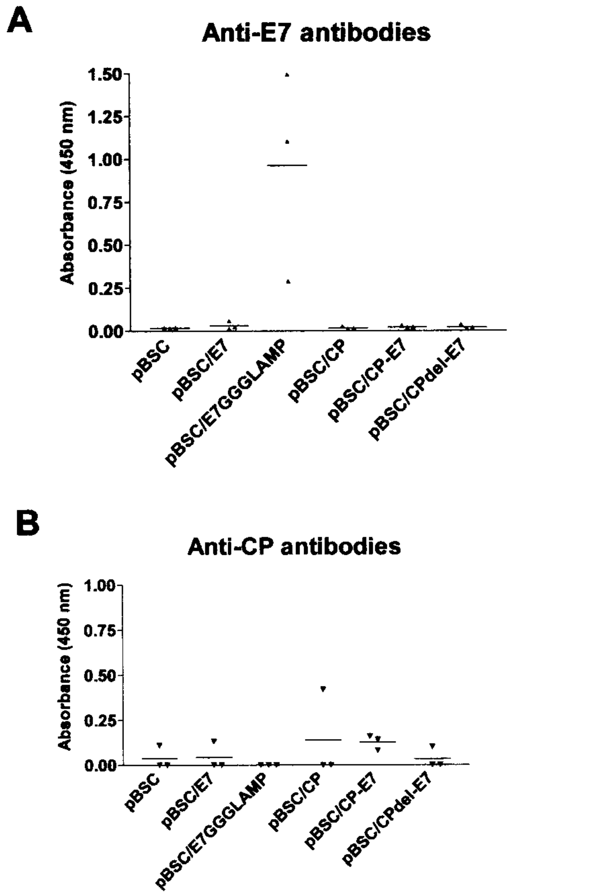

Figure 7. Detection of humoral immune responses. Mice (n=3) were immunized two times with 1 μg of plasmids. E7-specific (A) and CP-specific (B) antibodies were determined by ELISA two weeks after the last immunization in sera diluted 1:50 (A) or 1:100 (B).

against the growth of TC-1-induced tumors and cure TC-1-induced tumors.

For the tetramer assay, splenocytes were restimulated by 6-day-incubation with the $E7_{49-57}$ peptide. About 5% of restimulated splenocytes from mice vaccinated with pBSC/CP-E7 or pBSC/CPdel-E7 were both CD8 and tetramer positive, the corresponding figures for pBSC/E7-vaccinated and pBSC/E7GGGLAMP-vaccinated mice were 0.5 and 33%, respectively. No E7-specific responses were detected in mice immunized with either pBSC or pBSC/CP. The results of a representative experiment are shown in Fig. 6A.

Furthermore, we investigated the ability of the tested DNA vaccines to induce E7-specific IFN-γ-secreting splenocytes by ELISPOT assay after 5-day restimulation with the $E7_{49-57}$ peptide (Fig. 6B). About 200 and 100 E7-specific $CD8^+$ T cells were detected per 105 splenocytes isolated from the pBSC/CP-E7-vaccinated and pBSC/CPdel-E7-vaccinated mice, respectively. These counts were comparable to that of E7-specific $CD8^+$ T cells detected in pBSC/E7-vaccinated mice (about $100/10^5$), but were approximately ten times lower than that in pBSC/E7GGGLAMP-vaccinated mice (about 1300/105). No E7-specific splenocytes were revealed in mice vaccinated with either pBSC or pBSC/CP.

We did not detect any E7-specific IL-4-secreting splenocytes by ELISPOT assay. Numbers of IL-4-secreting splenocytes did not exceed 12 per 10^5 regardless of whether or not they had been restimulated with the $E7_{44-62}$ peptide and, thus, neither E7-specific responses nor relevant differences among the tested groups could be observed (Fig. 6C). As a positive control, splenocytes stimulated with concanavalin A were used.

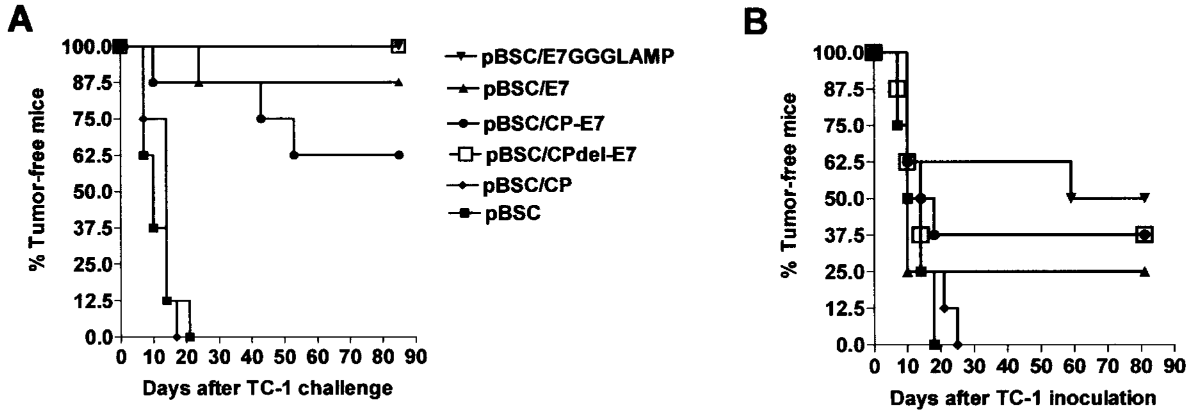

Figure 8. DNA immunization against TC-1 tumor cells. Mice (n=8) were either immunized two times at a two-week interval and challenged with 3x10^4 mouse TC-1 tumor cells two weeks after the last vaccination (A) or inoculated with 3x10^4 mouse TC-1 tumor cells and immunized on days 3 and 10 (B). Both experiments were repeated with similar results.

Vaccination with the pBSC/CP-E7 or pBSC/CPdel-E7 DNA vaccines did not induce production of either anti-E7 or anti-CP antibodies. ELISA was used to quantitate anti-E7 and anti-CP antibodies in sera of vaccinated mice (Fig. 7). Blood samples for ELISPOT assay were obtained simultaneously with splenocytes from the same mice. No anti-E7 or anti-CP antibodies were detected in sera of mice vaccinated with pBSC or pBSC-derived plasmids containing CP-E7, CPdel-E7 or E7 genes. The pBSC/E7GGGLAMP plasmid induced the production of anti-E7 antibodies in all three mice. One out of three mice immunized with the pBSC/CP plasmid was positive for anti-CP antibodies.

Vaccination with the pBSC/CP-E7 or pBSC/CPdel-E7 DNA vaccines protected mice against the growth of E7-expressing TC-1 cells. To determine whether vaccination with the CP-E7 or CPdel-E7 fusion genes protects mice against E7-expressing tumors, animals (8 per group) were immunized twice with plasmids by the gene gun and then challenged with TC-1 cells (Fig. 8A). All mice that received control plasmids pBSC or pBSC/CP developed tumors within 21 days. In contrast, all mice immunized with pBSC/CPdel-E7 and 5 out of 8 (62.5%) mice immunized with pBSC/CP-E7 remained tumor-free for 85 days after TC-1 challenge ($P<0.01$). Thus, both CP-E7 and CPdel-E7 generated protection against the formation of TC-1-induced tumors, comparably to E7 (87.5%) or Sig/E7GGG/LAMP-1 (100%).

The potential of DNA vaccines was also assessed by therapeutic immunization that was initiated three days after TC-1 inoculation (Fig. 8B). While all mice in control groups vaccinated with pBSC or pBSC/CP plasmids developed tumors within 25 days, 37.5% (3/8) of mice immunized with the pBSC/CP-E7 or pBSC/CPdel-E7 plasmids remained tumor-free for 80 days after inoculation of tumor cells. Both CP-E7 and CPdel-E7 cured mice with a similar efficacy to E7 (25%) or Sig/E7GGG/LAMP-1 (50%).

Discussion

In this study, we linked the sequence coding for the HPV16 E7 peptide (aa 44-60) to the PVA CP gene, which could be helpful for the production of the E7 peptide in plants. Before the preparation of plant vaccines, we investigated the immunogenicity of fusion genes by DNA vaccination. We supposed that a DNA vaccine based on the virus-like particles expressing immunogenic epitopes could elicit a strong CTL response. Firstly, we confirmed the expression of the fusion proteins by immunoblotting and MALDI and demonstrated the formation of VLPs in procaryotic and eucaryotic cells by transmission electron microscopy. We investigated the immunogenicity of the products of the fusion genes by DNA vaccination with a gene gun on the mouse model using TC-1 cells for induction of tumors. The fusion genes significantly protected mice against the growth of TC-1-induced tumors and cured a portion of animals with pre-inoculated TC-1 cells. The antitumor effects of these fusion genes were comparable to those of the full-length E7 gene or Sig/E7GGG/LAMP-1 fusion gene. However, the other E7-fusion genes containing full-length E7 have been shown to induce more potent antitumor effects (36,37).

The chimeric CP monomers of JGMV potyvirus containing a decapeptide replacing 16 out of the 18 aa of the C-terminal surface-exposed region have been reported to retain the ability to assemble into hybrid VLPs in procaryotic as well as eucaryotic cells (14,15). In another study, the chimeric JGMV CP carrying the 98-amino-acid sequence replacing 14 amino acids at the C terminus produced in *E. coli* formed chimeric VLPs (16). Similarly, we found that the PVA CP monomers either alone or carrying the E7 peptide (aa 44-60) on their C-termini or modified C-termini with a short deletion (5 aa) could assemble into VLPs in both procaryotic and eucaryotic cells.

The model of three-dimensional structure of intact PVA particles has revealed that the N- and C- terminal regions are exposed on the surface of VLPs (38) just like in another potyvirus - JGMV (14). The JGMV VLPs carrying peptides of up to 27 aa have been shown to generate formations of parallel-laying VLPs in cells (15,16). In this study, we observed that the 'threads' of PVA CP VLPs aggregated into parallel bundles, while PVA CP-E7 VLPs formed spherical aggregates. The formation of both types of aggregates was observed with CPdel-E7 VLPs. These data suggested that the expression of the E7 epitope on the surface of PVA VLPs disturbed the laying of one VLP to another, resulting in the formation of morphologically different aggregates.

The induction of protective immunity against tumor development and low levels of antibody responses has been shown in the A31 lymphoma model for the DNA vaccine delivered by injection, comprising the fusion gene coding for the coat protein of potexvirus, linked to the modified idiotype, scFv (4). We found that the DNA vaccine coding for PVA CP, fused to the immunodominant E7 epitope and delivered by a gene gun, induced protection against the development of tumors expressing E7, but neither anti-E7 nor anti-CP antibodies were detected. The difference may result from a variation in the routes of vaccine delivery and/or the nature of both the E7-derived antigen and the CP.

In summary, we showed the potential of fusion genes linking the sequence coding for the HPV16 E7 immunodominant epitope to the PVA CP gene to induce E7-specific cellular immune responses in the mouse model.

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