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ORIGINAL ARTICLE

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Immune response to E7 protein of human papillomavirus type 16 anchored on the cell surface

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Abstract To target the E7 protein of human papilloma virus 16 to the cell surface, a fusion gene was constructed. It encodes the signal peptide, part of the immunoglobulin (IgG)-like domain, the transmembrane anchor of vaccinia virus (VV) hemagglutinin (HA), and the complete E7-coding sequence. The fusion gene was expressed under the HA late promoter by a recombinant VV, designated VV-E7-HA. The E7-HA protein was displayed on the surface of cells infected with the recombinant virus and was more stable than unmodified E7. The biological properties of the VV-E7-HA virus were compared with those of a VV-E7 virus that expressed the unmodified E7 and with a VV expressing the Sig-E7-LAMP fusion protein. While the first two of these recombinants were based on VV strain Praha, the third was derived from the WR strain of VV. Infection of mice with the VV-E7-HA virus induced the formation of E7-specific antibodies with the predominance of the IgG2a isotype, whereas the other two viruses did not induce the formation of E7-specific antibodies. Unlike the other two viruses, VV-E7-HA did not induce a response of cytotoxic T lymphocytes or Th1 cells and did not protect mice against the growth of E7-expressing tumors. Thus, VV-E7-HA induced a differently polarized immune response to the E7 protein than the other two viruses.

Keywords Antibody · Cellular immunity · E7 · Hemagglutinin · HPV16 · Protein targeting · Vaccinia virus

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Introduction

Recombinant vaccinia virus (VV) vectors have been used to induce immune responses to many different antigens derived from viruses, bacteria, parasites and mammals (for a review, see [23]). It has been shown that the anchoring of an antigen on the surface of cells infected with a recombinant virus encoding this antigen can increase its immunogenicity. For example, appending the transmembrane domain of membrane immunoglobulin (IgG1) to the carboxy terminus of a secreted *Plasmodium* antigen results in a change in the subcellular location of the S-antigen and increases the immune response to the otherwise weak immunogen [18]. Enhanced immune responses have been observed after fusing the MSA1 signal and anchor sequences with malaria merozoite surface antigen 1 [32]. Similarly, the addition of the membrane anchor to the C terminus of rotavirus VP7 glycoprotein has enhanced the immunogenicity of this protein when expressed by recombinant VV [2].

It has been demonstrated that the hemagglutinin (HA; A56R gene) of VV is not required for infection and replication of this virus [10]. The substitution of the external IgG-like domain of HA (AA 34–103) with a single-chain antibody results in the synthesis of a fusion protein which is exposed on the envelope of extracellular virus (EEV) and on the surface of virus-infected cells. The specificity (anti-ErbB2) of the single-chain antibody is retained, and the surface of EEV can bind the corresponding antigen [7].

An etiological association between human papillomavirus type 16 (HPV16) infection and cervical neoplasia has been firmly established [22]. The viral E7

oncoprotein, which is localized in the nucleus and cytoplasm of HPV16-infected and transformed cells, represents an attractive target for therapeutic vaccines.

In this study we report on our attempts to increase the immunogenicity of the E7 protein by changing its subcellular location via fusing it to the transmembrane sequence of VV HA. The immunogenicity of the recombinant VV expressing this fusion protein has been compared with that of two other VV-E7 recombinants.

Material and methods

Plasmid vectors

Plasmid pRS3 was prepared as follows. An internal portion of the VV HA, between its 64th and 275th amino-acid residues, was replaced by the entire E7 polypeptide. The E7 gene of HPV16 was amplified with the oligonucleotide primers E7–1: 5'-GCTGACGTCATGCATGGAGATACACC-3' and E7–2: 5'-AGACCAAGGTTTCTGAGAACAGATGGG-3' by PCR, with plasmid pEA16E7 (prepared by Ingrid Jochmus, Heidelberg, Germany) as the DNA template. The resulting 300-bp *Aat*II-StyI fragment containing the E7 gene was inserted between the *Aat*II and *Sty*I sites of plasmid pHA. To ensure translation into the complete E7-HA fusion protein in frame, the *Sty*I ends were ligated as blunt ends. The resulting plasmid was denoted pHA-E7. Plasmid pHA was prepared by cleaving the G fragment of a *Sal*I genomic library of VV strain Praha with restriction enzymes *Hin*dIII and *Sal*I, and then cloning the 1,796-bp fragment obtained which contained the HA gene, into pUC18. To obtain a plasmid that would allow "transient dominant selection" of recombinant viruses, plasmid pHA-E7 was cleaved with *Bam*HI and ligated with a *Bam*HI fragment, which contains the β-galactosidase gene of *Escherichia coli* and the 7.5-promoter of VV. The resulting plasmid was denoted pRS3. Plasmid pH5-E7 was prepared by amplifying the E7 gene with primers E7-B: 5'-ATAGGATCCCTGTAATCATGCATG-GAG-3' and E7-E: 5'-GGCGAATTCGATTATGGTTTCTGA-GAACAG-3' by PCR, using plasmid pEAE7 as the DNA template. The amplified 325-bp fragment was cleaved with *Bam*HI and *Eco*RI and ligated with plasmid pSC59-H5 cut with the same enzymes. Plasmid pSC59-H5 had been prepared by insertion of the VV H5 promoter into plasmid pSC59 (obtained through the courtesy of B. Moss, Bethesda, Md.). The 167-bp fragment containing the H5 promoter [8] had been prepared by PCR with primer H5–1: 5'-GCCAGATCTGACACTGTCTTTATTCTATACTTAAAAA-GTGAAAATAAATAC-3' and primer H5–2:5'- GCTGTCGACG-AGCTCCTAGGATCCTATTTACGATACAAACTTAACGG-ATATCG-3', with the H fragment of the *Hin*dIII VV genomic library used as the DNA template. The resulting fragment was cleaved with *Bgl*II and *Sal*I, and was ligated with pSC59 cut with *Bam*HI and *Sal*I.

Viruses and cells

Vaccinia virus clone P13, generated from the Sevac VARIE smallpox vaccine (strain Praha) [16], was used as the parental virus for the construction of recombinants. The recombinant viruses were grown in human-embryo diploid cells (LEP) or monkey-kidney cells (CV-1). Thymidine kinase-deficient (TK^-) RAT 2 rat cells [30] were used for the selection of TK^- VV recombinants. HPV16 E6E7-expressing TC-1 cells, derived from C57BL/6 mice [19], were kindly provided by T.C. Wu (Baltimore, Md.). All cells were cultivated in modified E-MEM (EPL) medium containing bovine serum growth-active proteins, but no complete serum [21]. The VV recombinants used for immunization experiments were grown in chorioallantoic membranes of 11-day-old chicken embryos and were partially purified by the modified method of Joklik [13, 17].

Construction of VV recombinants

Vaccinia virus recombination and selection of TK- recombinants were performed by standard procedures [24]. VV-HA-E7 was prepared using plasmid pRS3. Recombinant viruses produced by a single crossing over were selected by their co-expression of β-galactosidase [4]. "Blue" virus was plaque-purified. After the second plaque purification, "colorless plaque" viruses were isolated and recombinants with double crossing over were identified as carriers of the E7 insert by dot-blot hybridization and PCR. Expression of the E7 fusion protein and absence of HA in selected virus clones were confirmed by immunoblotting using VV-specific antisera. The E7-HA fusion gene was expressed from the late promoter of HA. A schematic view of the protein produced by VV-E7-HA is shown in Fig. 1.

A VV expressing the unmodified E7 protein was prepared by recombination with plasmid pH5-E7. The E7-coding sequence was inserted into the thymidine kinase (TK) gene, and its expression was controlled by the H5 early-late promoter. The third virus used in this study, VV-SigE7LAMP, expresses a fusion molecule consisting of the E7 protein with signal and a transmembrane sequence of the lysosome-associated membrane protein (LAMP1). This recombinant virus, originally prepared with the WR strain, was obtained through the courtesy of T.C. Wu (Baltimore, Md.) [19]. The viruses VV-pS2S (TK^-) and WR-pS2S (TK^-), which express the middle envelope protein (preS2 + S) of hepatitis B virus, had been prepared using plasmid pM3 [15]. The VV-gE (HA^-, TK^+) virus had also been prepared previously [17].

Peptides

The synthesis of peptides $E7–1_{(1–20)}$, $E7–2_{(10–30)}$, $E7–3_{(20–40)}$, $E7–4_{(30–50)}$ and $E7–5_{(40–60)}$ derived from the sequence of HPV16 E7 used in ELISA has been described earlier [14]. The peptide HPV16 $E7_{(49–57)}$ (RAHYNIVTF) [6] was used for the production of MHCI tetramer; the peptide $E7_{(49–57)}$ and E7 peptide-8Q $_{(44–62)}$ (QAEPDRAHYNIVTFCCKCD) [28] were used for restimulation of splenocyte bulk cultures and in ELISPOT.

MS2E7 protein

MS2E7 protein was produced in *Escherichia coli* transformed with plasmid pEX 8mer-HPV16 E7 and purified according to Jochmus-Kudielka et al. [12]. MS2E7 molecule contains E7 protein (encoded by HPV16 nt 585–855) fused to the first 100 amino acids of the bacteriophage MS2 polymerase.

Fig. 1 Schematic diagram showing structure of E7-HA fusion protein. Indicated amino-acid positions refer to the wild-type HA sequence. HA-Sig: HA signal peptide; HA-Ig-domain: part of the IgG-like domain; HA-TM: transmembrane domain; E7: full-length E7 polypeptide (98 AA)

Antibodies

Rabbit antiserum against E7, which was used in immunoblot and immunofluorescence tests, was prepared by immunization with four i.m. doses, each containing 2.5 mg MS2E7 fusion protein [12] in Freund's adjuvant. The first dose was administered in complete adjuvant, the subsequent three doses in incomplete adjuvant. Mouse anti-MS2E7 serum was prepared by the same procedure. Each dose contained 0.5 mg protein.

Preparation of H-2D^b/E7$_{(49–57)}$ tetramers

Major histocompatibility complex (MHC)-I tetramers were prepared as described by Altmann et al. [1]. In brief, heavy and light chains were expressed separately in *E. coli* and used in form of inclusion bodies for folding reaction. The mouse H-2D^b (MHC-I heavy chain), human β2-microglobulin (light chain) and E7$_{(49–57)}$ peptide were folded in vitro to preform MHC-1 monomers. After concentrating the reaction mixtures and buffer exchange, the preformed monomers were subjected to enzymatic biotinylation by BirA biotin synthetase, and then purified with S300 column chromatography and Mono Q ion exchange column chromatography. Tetramers were obtained by mixing the biotinylated protein complex with streptavidin-R–phycoerythrin conjugate (Molecular Probes) at a molar ratio of 4:1.

Immunoblotting

Infected cell cultures were washed twice with PBS and lysed on ice with RIPA buffer (1% NP40, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0) for 30 min. Cell lysates were clarified by centrifugation at 19,000 *g* for 10 min at 4°C. Supernatants were mixed with Laemmli buffer containing 2-mercaptoethanol. Samples were heated for 5 min at 95°C. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 15% gel. The proteins separated were then transferred onto a nitrocellulose membrane using a semi-dry electrophoretic blotting method. Membrane, preincubated with 10% skimmed milk in phosphate-buffered saline (PBS) for 1 h, was incubated with E7 specific rabbit antiserum diluted 1:1,000 in PBS containing 5% milk at 4°C overnight. After being washed (PBS–0.2% Tween, five times for 5 min) the membrane was incubated for 1 h with goat anti-rabbit IgG labeled with horseradish peroxidase (HRP; Sigma) diluted 1:5,000 in PBS–5% milk. Finally, the membrane was washed again as above, developed by ECL (Amersham) and exposed to autoradiography film.

Immunofluorescence

CV1 cells ($4{\cdot}10^3$) were grown in 16-well chamber slides (Nunc). Confluent monolayers were infected with recombinant VV at MOI 0.1. After overnight incubation, infected cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and washed with PBS 3×5 min. Then 0.1% Triton X-100 was added for 2 min and cells were washed with PBS 3×5 min and blocked with 10% skimmed milk in PBS for 1 h. Next, rabbit E7 antiserum diluted 1:50 in 5% milk–PBS was added for 1 h at room temperature. The monolayers were washed as above and incubated with a FITC-conjugated swine anti-rabbit secondary antibody (diluted 1:200) at room temperature for 1 h. Finally the cells were washed with PBS, overlaid with 50% glycerol in PBS, and examined with a Nikon 600 Eclipse microscope.

The expression of E7 on the surface of infected cells was examined by FACS analysis. Briefly, monolayers of CV1 cells in Petri dishes were infected at MOI 0.2. After overnight incubation, when the CPE in all cultures was about 50%, cells were harvested by trypsinization and resuspended in FACS buffer (PBS, 3% bovine serum albumin (BSA), 0.1% NaN_3). The cells were incubated with rabbit E7 antiserum diluted 1:50 in FACS buffer for 1 h at 0°C, then washed three times with the same buffer and incubated with a FITC-conjugated swine anti-rabbit secondary antibody (diluted 1:200 in FACS buffer) for 1 h at 0°C. Samples were analyzed on a Becton Dickinson FACScan instrument using WinMDI 2.8 software.

Subcellular fractionation

Preparation of subcellular fractions was performed as described by Jin et al. [11]. Briefly, CV-1 cells ($8{\cdot}10^6$) were infected with viruses at MOI 2 at 37°C. After 1 h, unadsorbed virus was removed and cells washed with medium were incubated for another 8 h. The cells were scraped into medium, centrifuged, washed once with PBS and resuspended in 1 ml of hypotonic buffer (42 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, pH 7.4) with protease inhibitor cocktail (Sigma) diluted 1:100 and incubated on ice for 15 min. Cells were then passed through a 29-G needle six times; and cell disruption was verified under a microscope. The extract was centrifuged at 200 *g* for 10 min at 4°C, and sediment containing the nuclear fraction was further purified (see below). The supernatant was centrifuged at 10,000 *g* for 10 min at 4°C to separate the heavy membrane fraction and then at 150,000 *g* for 90 min to collect the light membrane fraction. The remaining supernatant was used as the cytosolic fraction. Nucleus purification was performed according to the published protocol [9]. To nuclear fraction resuspended in 4 ml of sucrose buffer I [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–Cl, pH 8.0, 1 mM DTT, 0.5% (v/v) NP-40], another 4 ml of sucrose buffer II (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–Cl, pH 8.0, 1 mM DTT) were added and thoroughly mixed. The extracts were layered over 4.4 ml of sucrose buffer II in Beckman polyallomer SW 40.1 tubes and centrifuged at 30,000 *g* for 45 min at 4°C to prepare sediment containing nuclei. The sediments were resuspended in 100 µl of Laemmli buffer containing 2-mercaptoethanol. 0.8 ml of cytosolic fraction was mixed with 200 µl of 5×Laemmli buffer. All samples were denatured in boiling water for 3 min, separated on 12% SDS–PAGE and analyzed by immunoblot.

Immunization of mice

Six-week-old inbred mice, strain C57BL/6 (H-2^b; Charles River) were injected intraperitoneally with 0.5 ml PBS containing 10^7 PFU of recombinant VVs. All experiments on laboratory animals were conducted maintaining the principles of the Czech law 246/92 Sb. on "Breeding and Utilization of Experimental Animals."

Restimulation of splenocytes in vitro

HPV16E7-specific lymfocyte bulk cultures were generated from splenocytes obtained from immunized mice 12 days after virus inoculation. In brief, mouse spleens were homogenized with cell dissociation (Sigma) in complete RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco), $5{\times}10^{-5}$ M 2-mercaptoethanol, 1.5 mM glutamine, and antibiotics. Lymphocytes were separated on Histopaque-1083 (Sigma) and cultivated at a concentration of $5{\times}10^6$ cells/ml in complete RPMI 1640 with addition of 0.3 µg or 15 µg E7$_{(49–57)}$ peptide or 1 µg (8Q) peptide for 6 days.

Tetramer-staining and FACS analysis

Splenocytes were used for analysis immediately after their isolation or after 6-day in vitro restimulation. Lymphocytes for tetramer-staining were adjusted onto $2{\times}10^7$ cells per ml with FACS buffer (PBS supplemented with 2% FBS and 10 mM sodium azide), incubated with 2 µl of rat anti-mouse CD16/CD32 (Fc-block; Pharmingen; 0.05 mg/ml) for 20 min on ice, washed and an aliquot of $2{\times}10^6$ cells was resuspended in 80 µl of ice-cold FACS buffer. The samples were stained on ice each with 20 µl of a mixture of 2 µl of tetramer–PE and 2 µl of rat anti-mouse CD8a-FITC antibody (Pharmingen; stock concentration 0.5 mg/ml) in FACS buffer for at least 1 h in the dark. The lymphocytes were washed and resuspended

in 200 µl of FACS buffer. The stained cells were analyzed on a FACScan instrument, using CellQuest software (Becton Dickinson).

ELISPOT assay

Ninety-six well plates MAHA 45 (Millipore) were coated with 5 µg/ml of the anti-mouse IFNγ monoclonal antibody R4–6A2 (Pharmingen) or anti-mouse IL4 monoclonal antibody BVD4–1D11 (Pharmingen) in 0.1 M Na–phosphate buffer (pH 9.0) at room temperature overnight. The antibody-coated plates were washed four times with PBS and blocked with RPMI 1640 with 10% FBS for 1 h at room temperature. 100 µl of in vitro restimulated splenocytes in culture medium were added to the wells and incubated for 20 h at 37°C in 5% CO_2 in the presence or absence of peptides. Wells were washed three times with PBS and three times with PBS containing 0.05% Tween 20, followed by overnight incubation at 4°C with 2 µg/ml of the biotinylated anti-mouse IFNγ monoclonal antibody XMG 1.2 (Pharmingen) or the biotinylated anti-mouse IL4 monoclonal antibody BVD6–2462 (Pharmingen) in PBS, respectively. The wells were washed with PBS, 0.05% Tween 20. Avidin–horseradish peroxidase (HRP) conjugate (Pharmingen) diluted 1:1,000 in PBS, 0.05% Tween 20 was added to the wells for 3 h at 37°C. After washing the plates with PBS, the spots were stained with 3-amino-9-ethyl carbazol.

Serological tests for E7 antibodies

Sera of immunized mice were individually tested by ELISA for the presence of specific anti-E7 antibody. Wells of microtiter plates (Maxisorb, Nunc, Denmark) were coated with 2 µg of the oligopeptides E7–1 to E7–5 in 100 µl carbonate buffer, pH 9.6, at 37°C. Unbound antigen was removed and free potential binding sites were blocked with 1% bovine serum albumin (BSA). After threefold washing, the wells were incubated with 100 µl of 1:25 dilution of serum for 1 h. The plates were then repeatedly washed and 100 µl of 1:2,000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Sigma, USA) was added for 1 h. The plates were washed again, stained with *o*-phenylene-diamine and absorbance at 492 nm was measured. Control sera known to be positive and negative were tested on each plate. Antibody isotypes were determined in plates coated with the E7–1 peptide, using HRP-conjugated rat anti-mouse IgG2a or IgG1 (Pharmingen) diluted 1:500.

Results

The VV-E7-HA recombinant virus expresses the E7 polypeptide fused into VV hemagglutinin

The VV-E7-HA construct was designed to express the E7-HA fusion protein. This protein contained the N-terminal portion of the VV hemagglutinin polypeptide chain including the signal sequence and part of the IgG-like domain (AA 1–62), the entire HPV16 E7 sequence (98 AA) in place of the internal part of the hemagglutinin polypeptide chain (211 AA), and the C-terminal portion of hemagglutinin including the transmembrane anchor (AA 273–315) (Fig. 1).

E7-HA is a glycoprotein targeted to the cell surface

In vitro expression of the E7-HA protein

To determine whether the VV recombinants induced synthesis of the E7 protein in CV1 cells, lysates of infected cells were analyzed by immunoblotting using E7-specific antibody. The lysates analyzed in Fig. 2A were prepared from cells harvested 18 h after infection. Fusion proteins produced by VV-E7-HA virus were found in two main bands of m.w. 33 and 35 kDa (lane 3) and in two minor bands of smaller size that probably represented fragments of the longer polypeptides. All polypeptides must have been glycosylated, because neither of the bands was found if the infected cells were kept in the presence of tunicamycin (5 µg/ml). The m.w. of the unglycosylated E7-HA polypeptide was about 30 kDa (lane 4). The upward shifts of 5 and 3 kDa might indicate the presence of one or two carbohydrate residues. With the use of the PCGENE program, two potential N-linked glycosylation sites were indeed predicted in the E7-HA protein: in the 34th position of HA and in the 29th AA of E7. The VV-E7 virus produced a 19 kDa E7 protein (lane 5), which was of the same size as that of the E7 protein produced in cells transfected with expression plasmid pBK-E7 or in Caski cells (not shown). The size of the E7 protein was not influenced by the addition of tunicamycin; however, the latter apparently reduced the amount of E7 produced (lane 6). A faint E7 band was discernible in lane 6 after longer film exposure (not shown). The

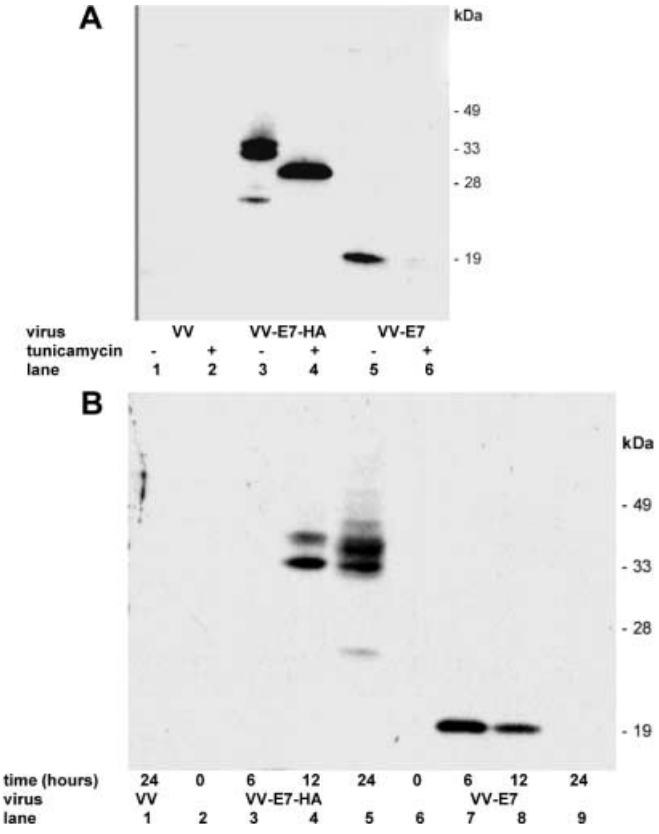

Fig. 2 Immunoblot analysis of E7 proteins expressed by recombinant VV. **A** CV1 cells were infected with parental VV, VV-E7-HA or VV-E7, cultivated in the absence or presence of tunicamycin of 5 µg/ml and harvested after 18 h. **B** Cells were harvested at different times after infection. The samples were analyzed on SDS–15% PAGE and by immunoblot using E7-specific rabbit antiserum

kinetics of production of the E7-HA glycoprotein and the unmodified E7 in cells infected with the respective recombinant viruses appeared to be different (Fig. 2B); E7-HA was detected at 12 h after infection and its amount was higher at 24 h (lanes 4 and 5), whereas the E7 molecule was detected already at 6 h (lane 7) after infection and thereafter its amount kept decreasing; a low amount of E7 present in cells at 24 h after infection (lane 9) was only visible on autoradiograms after a longer exposure time (not shown). Cells infected with non-recombinant VV did not express any E7-specific bands (Fig. 2A and B; lanes 1 and 2 and 1, respectively).

Subcellular location of the E7 antigen

The presence of the E7 antigen inside permeabilized cells was detected by immunofluorescent staining with specific antibodies (Fig. 3A). The cell infection with VVE7-HA was accompanied by formation of large syncytia. Their presence is typical of cultures infected with HA^- mutants [10]. The E7 antigen was found at the nuclear membranes and in cytoplasmic structures of VV-E7-HA-infected cells (a). Unmodified E7 expressed by VV-E7 was mainly present in the nuclei of infected cells (b). Cells infected with parental VV exhibited some fluorescence of very low intensity (c). E7 antigen on the surface of non-permeabilized infected cells was determined by flow cytometry. Fluorescence of high intensity was found on the surface of cells infected with VV-E7-HA only, whereas the FACS profile of VV-E7 infected cells showed no difference from cells infected with parental VV (Fig. 3B). To determine the amounts of E7 proteins in subcellular fractions, CV1 cells, infected with recombinant VVs for 8 h, were lysed in hypotonic solution and fractionated by centrifugation into nuclear,

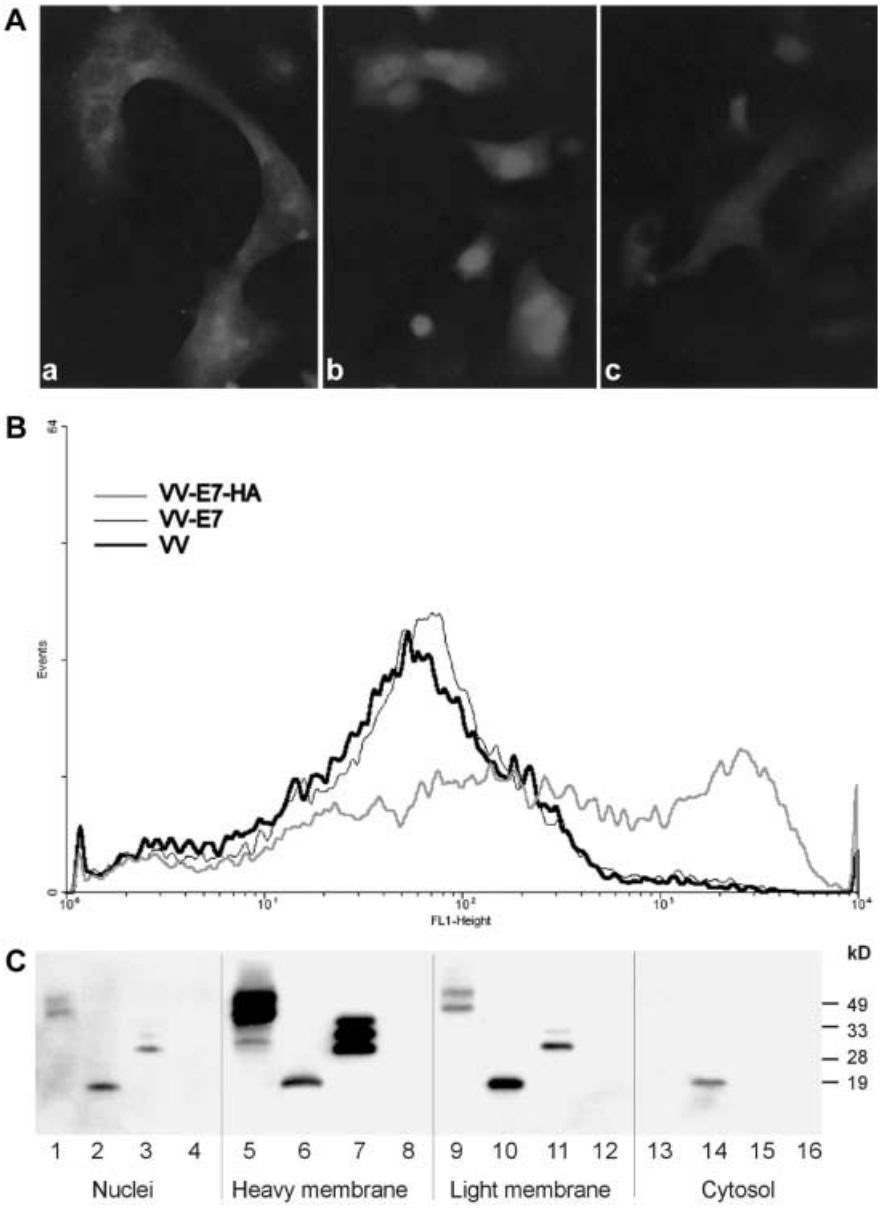

Fig. 3 Location of E7 proteins in CV1 cells after infection with recombinant VVs. **A** Immunofluorescence of E7 antigen in cells infected with: (a) VV-E7-HA; (b) VV-E7; or (c) VV. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and subsequently stained with E7 specific antibodies. **B** Detection of E7 on the surface of cells infected with VV-E7-HA (*grey line*), VV-E7 (*hair line*) or parental VV (*thick line*) by cytofluorometry. **C** Location of recombinant E7 proteins in subcellular fractions by immunoblot. The cells were infected with VV-E7-HA (*lanes 1, 5, 9, 13*), VV-E7 (*lanes 2, 6, 10, 14*), WR-SigE7LAMP (*lanes 3, 7, 11, 15*), parental VV (*lanes 4, 8, 12, 16*) and fractionated. Subcellular fractions were analyzed on SDS–12% PAGE and by immunoblot using E7 specific rabbit antiserum

heavy membrane, light membrane and cytosolic fractions. The amounts of E7 proteins in these fractions were analyzed by immunoblot (Fig. 3C). Most HA-E7 and E7-LAMP proteins were found in the heavy membrane fraction (lane 5 and 7, respectively) which included the cellular, mitochondrial and lysozomal membranes. Nuclear (lanes 1 and 3, respectively) and light membrane fractions (lanes 9 and 11, respectively) contained only low amounts of these proteins. Neither E7-HA nor E7-LAMP proteins were detected in the cytosolic fraction (lanes 13 and 15, respectively). Non-modified E7 protein predominated in the light membrane fraction (lane 10), though a substantial amount of E7 was found in all the other subcellular fractions (lanes 2, 6 and 14). The presence of E7 in membrane fractions could be explained by its capacity to bind to viral DNA, which is abundant in areas in the cytoplasm where VV maturation occurs. In agreement with published data, non-modified E7 was found in the nuclear and cytosolic fractions (lanes 2 and 14).

The E7-HA fusion protein induces an antibody response but not cell-mediated immunity

To determine how the fusion of the E7 protein with VV HA influenced the immunogenic properties of E7, we immunized C57BL/6 mice with one dose of 10^7 PFU of VV-E7-HA or VV-E7 virus by intraperitoneal route. Besides using these viruses prepared in our laboratory, we employed the WR-SigE7LAMP virus, which had been shown to induce a cell-mediated response and protection against tumor growth. This virus was included as positive control in immunity tests in spite of the fact that it was prepared from another parent virus strain. After the vaccinations, both humoral and cellular immune responses were examined in individual mice.

Induction of E7-specific antibodies

Groups of eight mice each were killed 6 weeks after virus inoculation. Their sera were individually examined for the presence of anti-E7 and anti-VV antibodies. The animals immunized with VV-E7-HA produced antibodies reactive with synthetic oligopeptides derived from the E7 amino-acid sequences 1–20 and 40–60 (Fig. 4A). Antibodies specific for the amino-acid sequence 20–40 were not detected in mouse sera (not shown). E7-Specific antibodies were not detected in sera of mice immunized with the VV-E7, WR-SigE7LAMP, or control viruses. To characterize the antibody response to VV-E7-HA more closely, the isotypes of anti-E7 IgGs were determined. Anti-E7 IgG2 prevailed in mice inoculated with VV-E7-HA, whereas the IgG1 isotype predominated after immunization with the purified MS2E7 fusion protein (Fig. 4B). All recombinant viruses induced anti-VV antibodies in all of the mice inoculated; however, the responses elicited by VV-E7-HA and WR-SigE7LAMP

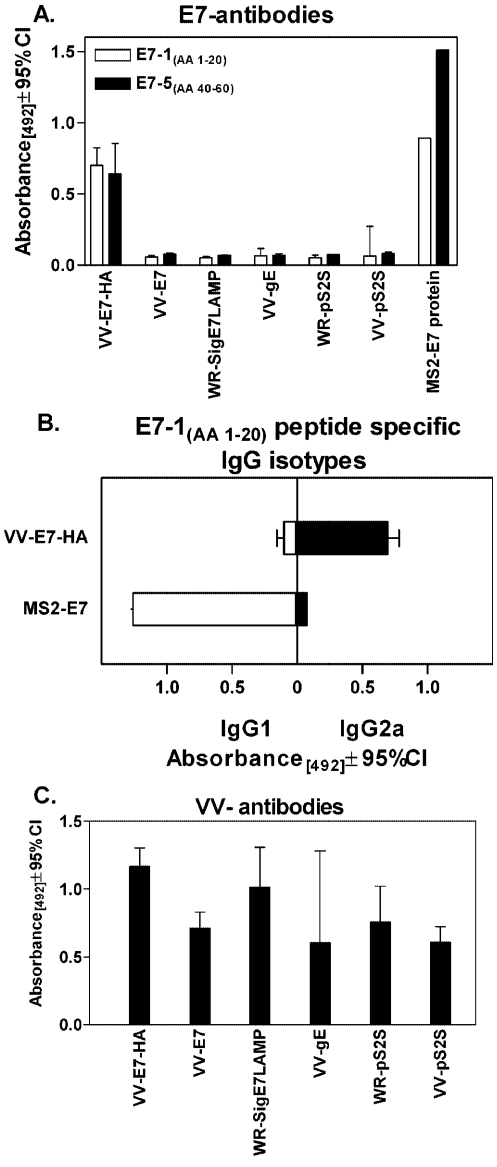

Fig. 4 E7-specific antibody response of mice inoculated with recombinant VVs. **A** Individual sera of six immunized mice were assayed by ELISA for antibodies specific for E7 oligopeptides E7-1 and E7-2. **B** Anti-E7-1 specific IgG1 and IgG2a isotypes were determined in individual positive sera of animals inoculated with VV-E7-HA. Anti MS2-E7 was a pool of sera of mice immunized with recombinant protein. **C** The same mouse sera as in **A** were examined for the presence of VV-specific antibodies

were the highest. The similar anti-VV antibody responses to these two recombinants suggest that the viruses multiplied at a similar rate, and that consequently the differences in anti-E7 antibody response to them cannot be ascribed to a difference in their replication.

Cell-mediated immunity

In testing the E7-specific cellular response to the different viruses, groups of two mice were used. Each spleen

was processed separately. Immunization with either of the E7-expressing viruses resulted in a primary response undetectable by ELISPOT or the tetramer assay in freshly isolated splenocytes (not shown). However, when the splenocytes were restimulated in vitro by E7 peptides for 6 days, IFNγ- and IL4-secreting splenocytes were detected (Fig. 5A and B, respectively). The binding of the $2D^b/E7_{(49–57)}$ tetramer by $CD8^+$ lymphocytes (Fig. 5C) was also found. Specific responses in IFNγ-ELISPOT and the tetramer assay were detected in mice immunized with VV-E7 or WR-SigE7LAMP but not with VV-E7-HA or the control virus (VV). In contrast, immunization with VV-E7-HA but not with VV-E7 or WR-SigE7LAMP resulted in the presence of IL4-secreting splenocytes. Similar results were obtained in three independent experiments.

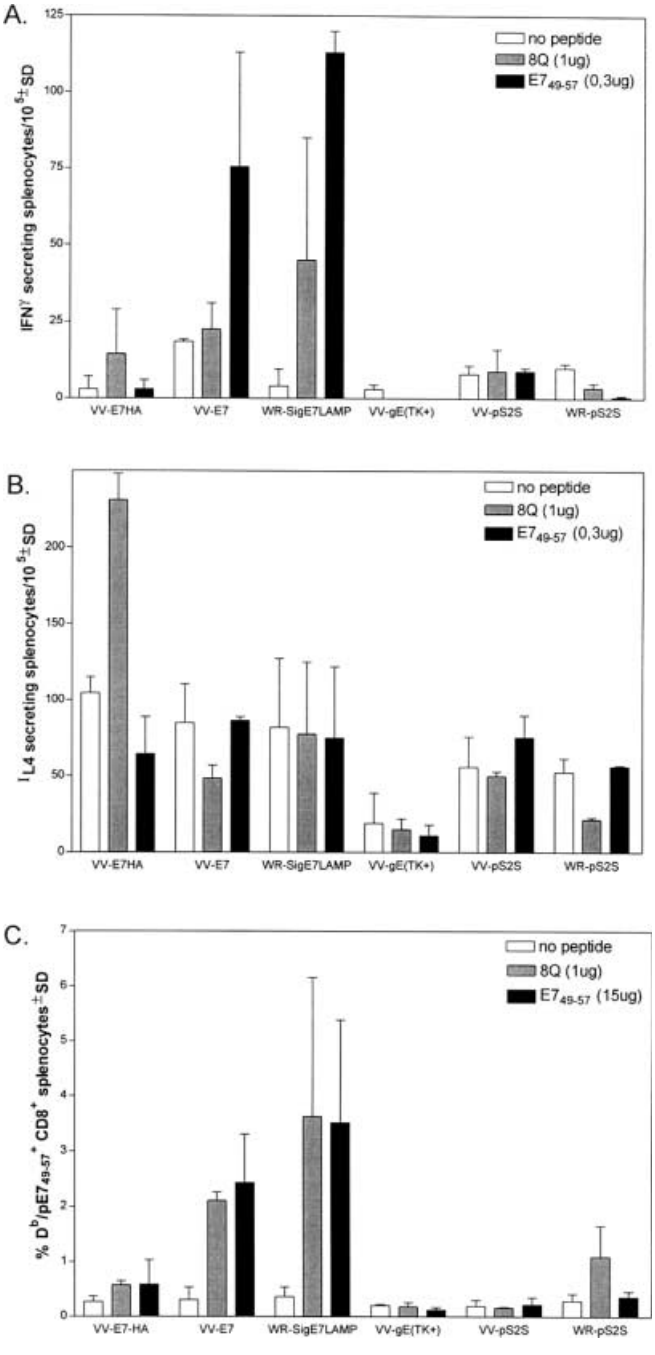

Fig. 5 Cell-mediated response of mice inoculated with recombinant VVs. Splenocytes isolated 12 days after virus inoculation were restimulated with indicated oligopeptides for 6 days and then analyzed by ELISPOT for cytokine production. Frequency of E7 specific cells secreting: **A** IFNγ, **B** IL4. **C** Frequency of $D^b/pE7_{(49–57)}{}^+$ $CD8^+$ cells among restimulated splenocytes detected by flow cytometry

Vaccination with VV-E7 but not with VV-E7-HA protects against E7-expressing tumor challenge

In order to determine whether the recombinant E7-expressing viruses induced antitumor immunity, vaccinated mice (eight per group) were challenged with a dose of $2{\cdot}10^4$ TC-1 cells/animal. Statistically significant protection against tumor growth was found after immunization with VV-E7 or WR-SigE7LAMP. Vaccination with VV-E7-HA, similarly to control viruses, did not induce any significant delay of tumor growth as compared with a group of animals inoculated with PBS only (Fig. 6A). Comparison of the growing tumors by size (Fig. 6B) showed that their mean size in mice vaccinated with VV-E7-HA and in animals given no VV (PBS controls) was similar. The inoculation of control viruses (VV-gE, VV-pS2S or WR-pS2S) enhanced the growth of tumors, which was in line with our previous unpublished results.

Discussion

In the present study we changed the subcellular location of the E7 protein expressed by human papillomavirus type 16 (HPV16) to the surface of infected cells, intending thereby to increase the immunogenicity of this tumor antigen. For this purpose, a recombinant VV with the E7 gene fused into the transmembrane sequence of the VV HA gene was prepared. The E7-HA fusion protein, as shown by immunofluorescence and subcellular fractionation, is found in the heavy membrane fraction and is localized on the surface of VV-E7-HA infected cells. Our approach was motivated by a previous report by Galmiche et al. [7], but our method of preparation of recombinant viruses differed in certain details. We inserted the E7 DNA fragment in a different region of HA and removed part of the HA chain. Consequently, our virus was HA^-. Still, the construct induced a high expression of the fusion protein on the cell surface. We were interested in finding out whether this targeting of the antigen would influence its immunogenicity, as reported previously by Andrew et al. and Langford et al. [2, 18]. In these authors' experiments, the cell-surface anchoring of antigen expressed by recombinant VV increased both the cellular and humoral immune responses to foreign antigen. Moreover, we presumed that vaccination with the E7-HA fusion

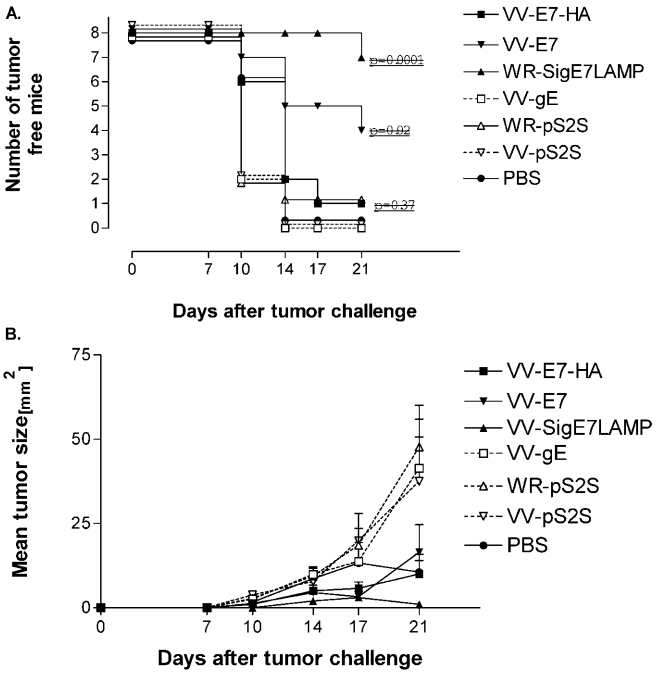

Fig. 6 In vivo tumor protection and growth of tumors in mice inoculated with recombinant VVs. Eleven days after virus inoculation groups of mice were challenged with $2{\cdot}10^4$ of TC1 tumor cells administered s.c. *Graph A* shows numbers of tumor-free animals; *graph B* demonstrates the mean size of growing tumors >1 mm^2. The results indicate that VV-E7-HA was not able to protect mice from the growth of E7 expressing tumor. Immunization with VV-E7 induced protection of a portion of animals. WR-SigE7LAMP was included as a positive control virus that is able to induce full protection of animals. The in vivo protection test was repeated three times with similar results. Statistical significance was determined using the log-rank test. All groups were compared with the group that received PBS only

protein expressed under the HA (A56R) late promoter might protect mice against challenge with HPV16-induced-tumor cells. Bronte et al. [3] have shown that vaccination with viruses expressing tumor-associated antigen under late promoters mediates protection against tumor growth, albeit a combination of early/late promoters was more effective.

In our model, immunization with VV-E7-HA provided no protection against the growth of E7-expressing tumors. Analysis of the immune responses showed that E7-HA did not induce a response of $CD8^+$ T lymphocytes or Th1 cells, which are the effectors of protection against HPV-induced tumor [6]. On the other hand, our VV-E7-HA recombinant was a good inducer of anti-E7 antibodies, as well as of Th2 cells, the producers of IL4. An analysis of the IgG isotypes revealed a Th1-like response, with predominance of the IgG2a isotype. Presumably, the switch to IgG2a might be related to an increased production of IFNγ as a result of the infection with VV and is not a consequence of the E7-specific Th1 response [20]. We demonstrated that the fusion of E7 with the signal peptide and transmembrane domain of HA increased the stability of the E7 protein. Unmodified E7 is a short-lived molecule [25] and its rapid degradation by the ubiquitine-proteasome pathway may contribute to its ability to induce CTLs [29]. However, the increased stability of E7-HA cannot wholly explain its different immunogenicity, because WB analysis indicated that the SigE7LAMP fusion protein accumulated in recombinant VV-infected cells at similar levels to E7-HA (not shown).

The predominance of the antibody response over CTL might be ascribed to the concurrent activity of several factors. It is possible that soon after the inoculation of VV-E7-HA, the infected cells were able to present E7 on their surface mainly to B cells but not to T lymphocytes, because the expression of MHC molecules might at this time (late phase) be downregulated owing to the viral infection [31]. Activated B cells soon start to play the role of antigen-presenting cells, and their interaction with T cells results in a proliferation of Th2 cells. Under these conditions the activation of type 1 helper lymphocytes by cross-priming in secondary lymphoid organs might be suppressed by an already established response of Th2 lymphocytes [26]. The viruses VV-E7 and VV-E7-HA induced in mice opposite polarized type 1 and type 2 responses, characterized by proliferation of IFNγ- and IL4-producing lymphocytes, respectively. It is well known that IL4 has the potential to modulate the function of cytotoxic and IFNγ-producing T cells, and that polarization of the immune response is usually established soon after exposure to antigen and is sustained by immunological memory. Since polarization of the immune response is a critical parameter for the outcome of virus infection [27] and protective antitumor immunity [5], we plan to use the recombinants described in this paper for a study of the detrimental effect of the type 2 response on protective immunity to HPV-associated tumors.

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