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Human Papillomavirus Type 16 L1 Capsomeres Induce L1-Specific Cytotoxic T Lymphocytes and Tumor Regression in C57BL/6 Mice†

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We analyzed capsomeres of human papillomavirus type 16 (HPV16) consisting of the L1 major structural protein for their ability to trigger a cytotoxic T-cell (CTL) response. To this end, we immunized C57BL/6 mice and used the $L1_{165-173}$ peptide for ex vivo restimulation of splenocytes prior to analysis (^{51}Cr release assay and enzyme-linked immunospot assay [ELISPOT]). This peptide was identified in this study as a D^b-restricted naturally processed CTL epitope by HPV16 L1 sequence analysis, major histocompatibility complex class I binding, and ^{51}Cr release assays following immunization of C57BL/6 mice with HPV16 L1 virus-like particles (VLPs). HPV16 L1 capsomeres were obtained by purification of HPV16 L1 lacking 10 N-terminal amino acids after expression in *Escherichia coli* as a glutathione *S*-transferase fusion protein (GST-HPV16 L1ΔN10). Sedimentation analysis revealed that the majority of the purified protein consisted of pentameric capsomeres, and assembled particles were not observed in minor contaminating higher-molecular-weight material. Subcutaneous (s.c.) as well as intranasal immunization of C57BL/6 mice with HPV16 L1 capsomeres triggered an L1-specific CTL response in a dose-dependent manner as measured by ELISPOT and ^{51}Cr release assay. Significant reduction of contaminating bacterial endotoxin (lipopolysaccharide) from the capsomere preparation did not diminish the immunogenicity. Antibody responses (serum and vaginal) were less robust under the experimental conditions employed. In addition, s.c. vaccination with HPV16 L1 capsomeres induced regression of established tumors expressing L1 determinants (C3 tumor cells). Our data demonstrate that capsomeres are potent inducers of CTL responses similar to completely assembled $T=7$ VLPs. This result is of potential relevance for the development of (combined prophylactic and therapeutic) HPV-specific vaccines, since capsomeres can be produced easily and also can be modified to incorporate heterologous sequences such as early HPV proteins.

Infection by certain (high-risk) human papillomaviruses (HPVs) is the most significant risk factor in the development of malignant tumors of the anogenital tract. Cervical cancer is the most common tumor in this group, particularly in developing countries where the majority of the almost half a million annual worldwide cases occur (34). Virus-like particles (VLPs) obtained by expression of the HPV L1 capsid protein using recombinant vectors (41) are the most advanced candidates for a prophylactic vaccine, which aims to prevent initial virus infection and thus hopefully subsequent premalignant lesions (CIN) and ultimately cancer (3). VLPs were shown to protect against challenge with infectious virions in animal papillomavirus models (e.g., the canine oral papillomaviruses in beagles) (3, 46). This protection was dependent upon neutralizing antibodies against conformational L1 epitopes (4). VLP-based vaccines focusing on the most frequent cancer-associated HPV types (i.e., HPV type 16 [HPV16]and HPV18) are now in clinical trials (16, 19). Initial results indicate safety and immunogenicity of the vaccines (16). The first data on the efficacy of HPV16 VLPs were published recently (19).

HPV vaccines are particularly needed in developing countries, and subcapsid particles (pentameric capsomeres) may be an economically advantageous alternative to VLPs especially in medically underfunded areas, since they can be readily purified after expression in *Escherichia coli* (6, 23, 51) and are very stable (27, 33). Immunization with papillomavirus L1 capsomeres triggers neutralizing antibodies (13, 38, 50) and protects against experimental challenge in the canine oral papillomavirus model (50). It is unclear, however, whether capsomeres also induce virus-specific cytotoxic T cells (CTLs). HPV16 L1-specific T-cell epitopes have not yet been described, and there is only circumstantial evidence for the induction of L1-specific CTLs. Immunization of mice with HPV16 L1 VLPs was shown to prevent the growth of L1-expressing C3 tumor cells (9) or to activate T cells as demonstrated by their proliferative capacity and gamma interferon (IFN-γ) secretion when exposed to complete particles in vitro

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(10). L1-specific CTLs are not considered to be of particular relevance in prevention or therapy of HPV-related diseases. However, chimeric VLPs (CVLPs) containing sequences of the early virus E7 protein (e.g., CVLPΔCE7$_{1-55}$ [30]) may be of use for both prophylaxis (induction of neutralizing antibodies) and HPV-specific therapy (induction of E7-specific CTLs) (3, 8, 14). Because incorporation of long peptide sequences into the L1 protein prevents the formation of CVLPs yet is compatible with the capsomere structure (30), it is important to determine whether capsomeres are as immunogenic as VLPs.

Another challenge for the design of vaccination strategies for resource-poor settings is the development of noninvasive (needleless) delivery of the vaccine. Infection by cervical cancer-related HPVs occurs at mucosal surfaces, so local induction of antibodies in cervical secretions (e.g., via intranasal [i.n.] immunization) is a reasonable option. Induction of humoral and T-cell immune responses following i.n. application of VLPs was demonstrated earlier (1, 10), yet the use of HPV capsomeres has not yet been described. Here we show that subcutaneous (s.c.) or nasal immunization of C57BL/6 mice with HPV16 L1 capsomere particles induces L1-specific antibodies and CTL responses that were measured by different in vitro assays (specific for the newly identified L1-derived CTL epitope ^{165}AGVDNRECI^{173}) and in an in vivo model (regression of L1-positive tumor cells).

MATERIALS AND METHODS

Cell lines and culture conditions. All cell lines used were of C57BL/6 origin (H2b). RMA cells, a Rauscher virus-induced thymoma, and the RMA-derived transporter associated with antigen processing (TAP)-deficient mutant cell line RMA-S as well as the embryonic stem cell line B6 (25) were cultured in RPMI medium supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.01 mM β-mercaptoethanol. C3 cells derived from embryonic mouse cells transfected with the HPV16 genome (12) were cultured in RMA medium with 0.1 mg of kanamycin per ml and 0.8 mg of G418 per ml.

Generation of HPV16 L1 VLPs. HPV16 L1 VLPs and CVLPs consisting of a C-terminally truncated L1 protein (HPV16 L1ΔC) fused to the first 55 amino acids (aa) of oncoprotein E7 (L1ΔC E7$_{1-55}$ CVLP) were generated as described previously (30).

Preparation of HPV16 L1 capsomeres. A plasmid for the expression of HPV16 L1 lacking 10 N-terminal residues fused to glutathione *S*-transferase (GST) was cloned from a modified pGEX plasmid described earlier (42, 43). To remove the C-terminal simian virus 40 tag sequence, the plasmid was cleaved by *Eco*RI/*Sal*I and the C-terminal *Eco*RI/*Sal*I fragment of the corresponding GST16L1 wild-type vector was inserted.

E. coli BL21 cells transformed with this resulting pGEX plasmid (pGEX16L1ΔN10) were grown at room temperature in Luria-Bertani medium containing 1 mM ampicillin. At an optical density at 600 nm (OD$_{600}$) of 0.3, recombinant protein expression was induced by adding 0.25 mM isopropyl-β-D-thio-galactoside (IPTG) to the medium. The GST-L1 fusion protein was purified essentially by the method of Chen et al. (7). The bacteria were harvested 15 h after induction by centrifugation. Pelleted bacteria were resuspended in buffer L (40 mM Tris [pH 8.0], 200 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol) supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) and lysed using a high-pressure homogenizer (Avestin, Ottawa, Canada). ATP and $MgCl_2$ were added to final concentrations of 2 and 5 mM, respectively (for complete folding of L1 by bacterial chaperones and cofactors). After a 1-h incubation at room temperature, 3.5 M urea (final concentration) was slowly added to the lysate, and incubation continued for 2 h (to dissociate GroEL from L1). The lysate was then dialyzed against buffer L (18 h at 4°C, three buffer exchanges) and cleared by centrifugation at 25,000 × *g* for 1 h.

For purification, the cleared lysate was loaded onto a glutathione-Sepharose column with a peristaltic pump. The column was washed first with buffer L containing 2.3 M urea and then with buffer L with 1 mM dithiothreitol, and then the column was incubated overnight at 4°C with thrombin protease to cleave L1 from GST. Subsequently, the cleaved L1 protein was eluted with buffer L, and thrombin was inactivated by addition of 1 mM phenylmethylsulfonyl fluoride and removed by gel filtration (Sephacryl S-300). For further experiments, this material was used without a concentration step.

The lipopolysaccharide (LPS) content within the capsomere preparation was measured by the *Limulus* amoebocyte lysate test (22) (E-Toxate; Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's recommendations. LPS was removed by polymyxin B (29) (Sigma-Aldrich).

Sucrose gradient sedimentation. Five micrograms of HPV16 L1ΔN10 protein and/or 100 ng of VLPs was layered onto 4.8 ml of a linear 5 to 30% sucrose gradient and centrifuged at 31,000 rpm for 16 h in an SW60 Ti rotor (Beckman, Palo Alto, Calif.) at 10°C. Fractions (0.3 ml each) were collected from the top, the pellet was resuspended in 0.3 ml of phosphate-buffered saline (PBS), and the sucrose concentration in the fractions was determined using a refractometer. The fractions were assayed for HPV16 L1 by immunoblotting with the anti-HPV16 L1 monoclonal antibody CamVir-1 (PharMingen, Heidelberg, Germany). Bovine serum albumin (4.3S; Sigma-Aldrich) and catalase (11S; Sigma) were used for sedimentation markers.

Generation of MVA-HPV16 L1ΔC and infection of murine cell lines with recombinant MVA. For construction of recombinant modified vaccinia virus Ankara (MVA), the HPV16 L1ΔC gene (30) was excised from pCEP4HPV16L1ΔC (kindly provided by A. Burger, MediGene AG, Martinsried, Germany) by digestion with *Kpn*I, followed by T4 DNA polymerase treatment, and a final digestion with *Not*I. The insert was cloned into the MVA transfer plasmid pIIIdHR-sP (44) to place the L1ΔC target gene under the transcriptional control of a strong synthetic vaccinia virus-specific promoter. The resulting vector plasmid, pIIIdHR-sP-HPV16L1ΔC, was used for the generation of recombinant MVA following the methodology of transient host range selection (44). Briefly, BHK-21 cell monolayers in six-well tissue culture plates (Corning, New York, N.Y.) were infected with 0.01 infectious unit of MVA (cloned isolate F6 after 582 passages on chicken embryo fibroblasts) per cell, and 90 min after infection, the cells were transfected with 1.5 μg of plasmid DNA per well using FuGENE 6 transfection reagent (Roche) as recommended by the manufacturer. Forty-eight hours after infection, cells were harvested and processed to release virus material for selective passage on RK-13 cell monolayers. Recombinant MVA-HPV16L1ΔC was detected upon formation of typical cell foci in RK-13 cell monolayers that do not support productive growth of parental MVA. After three rounds of plaque purification in RK-13 cells, MVA-HPV16L1ΔC was passaged in BHK-21 cells to remove the selectable marker gene K1L. The results of PCR analysis of viral DNA suggested that MVA-HPV16L1ΔC was stable genetically and that there was no nonrecombinant MVA (data not shown). The production of recombinant L1 protein by MVA-HPV16L1ΔC was confirmed by Western blot analysis of lysates from infected BHK-21 cells (data nor shown). Stocks of MVA-HPV16L1ΔC were prepared in BHK-21 cells, and the virus titers on CEF cells were determined using vaccinia virus-specific immunostaining. The viruses were aliquoted and stored at −80°C. Subconfluent cell layers of B6 and EL4 cells were infected (multiplicity of infection of 5) with MVA-HPV16L1ΔC or MVA-F6 (control infection) for 1 h. Cells were washed and cultured for 16 h before use as stimulator cells or as targets.

Preparation of protein extracts and Western blots. Sixteen to 96 h after infection, protein extracts were separated in sodium dodecyl sulfate–12% polyacrylamide gels and transferred to nitrocellulose membranes (Du Pont, Bad Homburg, Germany) using a semidry blotter (Bio-Rad, Munich, Germany), and then the membrane was incubated with L1-specific antibody CamVir-1. Bands were detected by enhanced chemiluminescence (Du Pont) after incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (Dianova, Hamburg, Germany).

Peptide binding assays. Binding of high-pressure liquid chromatography-purified synthetic peptides (kindly provided by S. Stevanovic [University of Tübingen] and R. Pipkorn [DKFZ, Heidelberg, Germany]) to K^b and D^b molecules on the surfaces of RMA-S cells was assayed in duplicate by incubation of 2 × 10^5 RMA-S cells per well of a 96-well U-bottom microtiter plate together with titrated concentrations (1.5 to 100 μg/ml) of synthetic peptides overnight at 37°C in serum-free culture medium preparing two wells for each sample. Stabilization of D^b and K^b molecules was tested by addition of 100 μl of culture supernatant of the hybridomas B22.249 (20) and E3-25 (11), respectively (both kindly provided by G. Hämmerling [DKFZ]). After incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody, stained cells were analyzed by flow cytometry in a fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany). Living gates were defined on cells negatively stained with propidium iodide. As a measure of major histocompatibility complex class I (MHC-I) expression levels, the average mean fluorescence intensities for the duplicate wells were determined (36).

Immunization of mice and generation of CTL lines. C57BL/6 mice were injected s.c. with 10 μg of VLPs (in 100 μl of PBS) (for the immunization and for the booster doses [12 days after immunization]) or with different quantities of capsomeres as indicated. Control animals received the same volume of PBS only. Ten to 14 days after the booster doses, 2×10^7 to 4×10^7 spleen cells were cocultured together with 2×10^6 inactivated B6 cells infected with MVA-HPV16L1ΔC or RMA-S cells pulsed with a 10 μM concentration of the L1-derived synthetic peptide AGVDNRECI ($L1_{165-173}$) in minimal essential medium supplemented with 10% FCS, glutamine, penicillin and streptomycin, and β-mercaptoethanol (CTL medium). For long-term culture and restimulation, CTLs were propagated in CTL medium supplemented with 5% (vol/vol) supernatant of rat spleen cell cultures (that had been induced by concanavalin A) and with 25 mM methyl-α-mannopyranoside and were distributed at various cell concentrations into 24-well plates (Corning) together with 5×10^6 irradiated (33 Gy) syngeneic spleen cells and 1×10^5 irradiated (100 Gy) stimulator cells per well as previously described (40).

^{51}Cr release assay. CTL activity was analyzed 5 days after in vitro restimulation by a ^{51}Cr release assay or an enzyme-linked immunospot assay (ELISPOT). In some experiments, ELISPOTs were performed ex vivo at the day of splenectomy. In ^{51}Cr release assays, 5,000 or 10,000 target cells labeled with 50 mCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C were incubated together with titrated numbers of effector cells in 200 μl per well of a 96-well plate (Corning) for 4 h. Then, 50 μl of supernatant was removed from each well, and released radioactivity was measured in a Microbeta counter (Wallac, Turku, Finland). Specific lysis was calculated according to the following formula: percent specific lysis = [(cpm of the sample − spontaneous release)/(total release − spontaneous release)] × 100, where total release and spontaneous release are measured in counts per minute (cpm). An assay was scored positive if the specific lysis of a sample was at least 10% above the specific lysis of the control.

ELISPOT. MultiScreen HA sterile plates (96-well plates) (MAHAS4510; Millipore, Eschborn, Germany) were equilibrated with PBS and coated with 200 ng of anti-mouse IFN-γ capture antibody (clone R4-6A2; PharMingen) in 100 μl of PBS overnight at 4°C (controls were processed in the absence of antibody). After blocking for 2 h with 100 μl of medium (RPMI, 5% FCS, and penicillin or streptomycin) at 37°C, splenocytes were seeded into three wells in serial twofold dilutions from 200,000 to 25,000 cells per well in 100 μl of medium. For each three wells, one was left untreated (negative control), one received 200 ng of pokeweed mitogen (Sigma) in 2 μl of PBS (positive control), and one received 0.2 μmol of $L1_{165-173}$ peptide in 2 μl of PBS (test sample). The plates were incubated for 16 to 20 h at 37°C. Cells were removed by washing the plates six times with PBS containing 0.05% Tween 20 and once with PBS alone. Following the addition of 100 μl of PBS containing 200 ng of biotinylated anti-mouse IFN-γ antibody (clone XMG1.2; PharMingen) per well, plates were kept at 4°C overnight. The wells were washed three times with PBS and filled with 100 μl of streptavidin-alkaline phosphatase (PharMingen) (diluted 1:1,000) in PBS. After 2 h at room temperature, wells were washed three times with PBS and developed for 1 to 10 min with 100 μl of 5-bromo-4-chloro-3-indolylphosphate (BCIP)/Nitro Blue Tetrazolium liquid substrate system (Sigma). The reaction was stopped by rinsing the wells with ice-cold water. Spots were quantified in an ELISPOT reader (Zeiss-Vison C; Zeiss, Oberkochen, Germany). Wells with medium instead of T cells were run in parallel as background controls. Counts of the negative-control wells were subtracted from the samples. A sample was scored positive if it reached a value at least twice as high as the value for any of the control animals.

Tumor regression experiments. C57BL/6 mice received 0.5×10^6 C3 cells (kindly provided by J. ter Schegget, Leiden University Medical Center) in 100 μl of PBS s.c. in the right flank. For subsequent immunization (14 to 21 days later), the animals received 10 μg of capsomeres or PBS s.c. into the tail base. Tumor size (measured with a ruler) was determined 1 week after immunization and subsequently every 4 days until mice had to be sacrificed. The spleens of control mice (with strong tumor growth) and of mice showing partial or total regression were removed, and splenocytes were cultured as described above. CTL activity was analyzed by ELISPOT. Tumor sizes of the mice within a group were calculated as arithmetic means with standard error of the means (SEMs).

Nasal immunization. Mice were immunized i.n. with 5 or 10 μg of capsomeres with or without 5 μg of cholera toxin subunit B (CTB; Sigma-Aldrich) as an adjuvant (total of 12 μl per nostril) under short-term (methoxyfluorane) or long-lasting anesthesia (xylazine hydrochloride plus ketamine hydrochlorine). Control mice received the same volume of PBS. Mice were given one (day 14 after immunization) or two (days 10 and 20 after immunization) booster doses. The animals were sacrificed 12 days after the single booster dose or 10 days after the second booster dose, spleens were removed, and blood was taken by heart puncture. Vaginal lavage specimens were obtained using 200 μl of PBS on 5 consecutive days (covering the complete murine menstruation cycle [31] and pooled for analysis) immediately prior to termination of the experiment.

HPV ELISA. Thirty nanograms of VLPs diluted in PBS (100 μl) was bound per well of round-bottom enzyme-linked immunosorbent assay (ELISA) plates (Becton Dickinson) by incubating at 37°C for 1 h. Incubated wells containing PBS were used as a negative control. Plates were washed three times with PBS containing 0.05% Tween 20 and incubated for 1 h at 37°C with 100 μl of milk buffer (5% milk powder and 0.05% Tween 20 in PBS) per well. After the wells were washed three times with PBS, serum (diluted 1:10 in milk buffer) or vaginal wash (concentrated 10:1 in a Speed Vac [Bachhofer, Reutlingen, Germany]) specimens were added to three wells in a total volume of 50 μl per well and incubated for 1 h at 37°C. Samples were removed and washed three times with PBS. To detect IgA and IgG, HRP-conjugated goat anti-mouse IgA and HRP-conjugated rabbit anti-mouse IgG (heavy and light chains) (Zymed, San Francisco, Calif.) diluted 1:3,000 were used. After incubation for 1 h at 37°C and three washes with PBS, substrate (200 μg of tetramethylbenzidine per ml in a solution of 0.1 M Na acetate [pH 6.0] and 0.03% H_2O_2) was added, the reaction was stopped with 1 M H_2SO_4, and the plates were measured in an ELISA reader at 450 nm. Samples were given positive scores when the OD_{450} reached values equal to or above the positive-control values (polyclonal anti-HPV16 VLP mouse serum used at a dilution of 1:100).

RESULTS

The aim of this study was to investigate the CTL response of HPV16 L1 capsomeres following immunization of C57BL/6 mice. It was previously shown that immunization with L1 VLPs protects the mice from outgrowth of tumorigenic C3 cells and that the protection was mediated by $CD8^+$ T cells (9). However, the L1-specific CTL epitopes involved were not identified.

Screening HPV16 L1 for potential CTL epitopes. To define epitopes restricted by the H2b haplotype, we first screened the primary structure of L1 for sequences fitting the allele-specific peptide binding motifs of the murine MHC-I molecules K^b and D^b. Using the SYFPEITHI database (36), 10 octameric peptides fitting the K^b-specific peptide binding motif and 5 nonameric peptides showing the binding motif specific for D^b were found (Table 1). Of the potential K^b-binding peptides, five peptides showed a score of 20 or greater for binding to the K^b molecule, which was close to the score of 25 of the K^b-restricted control epitope SIINFEKL ($OVA_{257-264}$). Within the D^b system, one peptide ($L1_{165-173}$) showed an extraordinarily high score of 29, approaching the score of the natural D^b-restricted CTL epitope of the influenza virus matrix protein ($NP_{366-374}$), whereas the scores of the remaining potential D^b-binding peptides ranged between 19 and 23 (Table 1).

Next we performed peptide binding assays to analyze the binding affinities of the candidate peptides listed in Table 1. None of the peptides comprising the K^b-specific binding motifs, except the control peptide SIINFEKL, efficiently stabilized expression of K^b molecules on the surfaces of RMA-S cells (Fig. 1A). However, of the peptides with the D^b-specific binding motif, $L1_{165-173}$ (AGVDNRECI) at a concentration of 25 μg/ml induced maximal stabilization of D^b expression and a level of expression similar to that of the control peptide $NP_{366-374}$ (Fig. 1B). These results demonstrate that of the 15 candidate peptides showing allele-specific peptide binding motifs for either K^b or D^b, only one peptide had a binding affinity similar to that of a natural CTL epitope.

Identification of a HPV16 L1-derived CTL epitope. To test whether the high-affinity D^b-binding peptide $L1_{165-173}$ also represents a naturally processed L1-specific CTL epitope in vivo, we performed immunization experiments on C57BL/6 mice.

TABLE 1. HPV16 L1 peptides fitting the binding motifs for the murine MHC-I molecules K^b and D^b

MHC-I molecule	Peptide no.[a]	Peptide designation[b]	Sequence	Score[c]
K^b	1	$L1_{1–8}$	MQVTFIYI	21
	2	$L1_{3–10}$	VTFIYILV	17
	3	$L1_{20–27}$	VYHIFFQM	17
	4	$L1_{237–234}$	VHTGFGAM	16
	5	$L1_{232–239}$	GAMDFTTL	24
	6	$L1_{257–263}$	KYPDYIKM	20
	7	$L1_{269–276}$	GDSFFYL	20
	8	$L1_{352–359}$	GNQLFVTV	18
	9	$L1_{440–447}$	LEDTYRFV	18
	10	$L1_{468–475}$	KKYTFWEV	22
	11	*$OVA_{257–264}$*	*SIINFEKL*	*25*
D^b	12	$L1_{15–23}$	ENDVNVYHI	22
	13	$L1_{78–86}$	IKKPNNNKI	19
	14	$L1_{79–87}$	KKPNNNKIL	23
	15	$L1_{165–173}$	AGVDNRECI	29
	16	$L1_{417–425}$	IHSMNSTIL	21
	17	*$NP_{366–374}$*	*ASNENMETM*	*30*

[a] Peptides 1 to 11 are octamers, and peptides 12 to 17 are nonamers.
[b] $OVA_{257–264}$ (chicken ovalbumin) and $NP_{366–374}$ (influenza virus matrix protein) are naturally K^b- and D^b-restricted CTL control epitopes, respectively, and are shown in italic type.
[c] Score for binding to the K^b or D^b molecule was according to the SYFPEITHI data base developed by Rammensee et al. (36).

Since syngeneic L1-expressing cell lines suitable for selective restimulation of L1-specific CTLs in culture are not available, we infected B6 cells with an MVA vector expressing HPV16 L1 (MVA-HPV16L1ΔC) that was generated as described in Materials and Methods. By Western blot analysis, expression of the L1 protein of the expected size of about 49 kDa was detected in the MVA-HPV16L1ΔC-infected cells but not in cells infected with nonrecombinant MVA (Fig. 2). The presence of L1 protein in MVA-HPV16L1ΔC-infected B6 cells for at least 4 days suggested that coculture experiments should permit successful restimulation of L1-specific CTLs in vitro.

In a subsequent immunization experiment, C57BL/6 mice were injected with 20 μg of L1ΔC $E7_{1-55}$ CVLPs (30) ($n = 3$) or with PBS ($n = 2$) and sacrificed 2 weeks later to establish spleen cell cultures. After three rounds of in vitro restimulation with B6 cells infected with MVA-HPV16L1ΔC, the spleen cells were analyzed by a ^{51}Cr release assay. Splenocytes of all three immunized animals recognized RMA-S target cells pulsed with peptide $L1_{165-173}$, whereas none of the mock-immunized animals showed a cytotoxic response specific for this peptide (Fig. 3). Furthermore, splenocytes of mice immunized with L1 VLPs or L1ΔC $E7_{1-55}$ CVLPs lysed MVA-HPV16L1-infected B6 cells after repeated rounds of in vitro restimulation with RMA-S cells pulsed with peptide $L1_{165-173}$ (data not shown). These data demonstrate that the $L1_{165-173}$ peptide is endogenously processed and presented by MVA-HPV16L1ΔC-infected B6 cells and thus leads to efficient restimulation of L1-specific CTLs.

In subsequent immunization experiments, stimulation of splenocytes ex vivo (for ELISPOT) or restimulation after in vitro culture (^{51}Cr release assay and ELISPOT) was performed with the $L1_{165-173}$ peptide.

Characterization of the HPV16 L1ΔN10 protein. Next we investigated whether complete capsids with a $T=7$ geometry

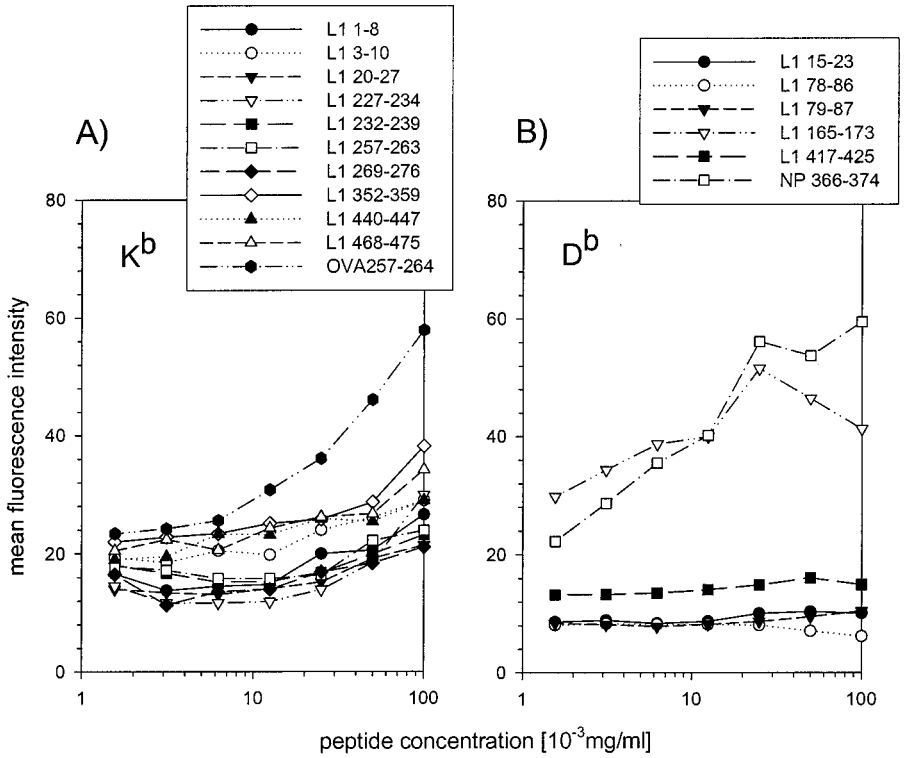


FIG. 1. Binding of HPV16 L1 peptides to K^b (A) or D^b (B) molecules on RMA-S cells. HPV16 L1 peptides are shown in Table 1. Stability of the MHC molecules was measured by fluorescence-activated cell sorting analysis. Note that only the D^b-specific peptide $L1_{165-173}$ (in panel B) induces stability similar to that of the naturally processed epitope ($NP_{366-374}$).

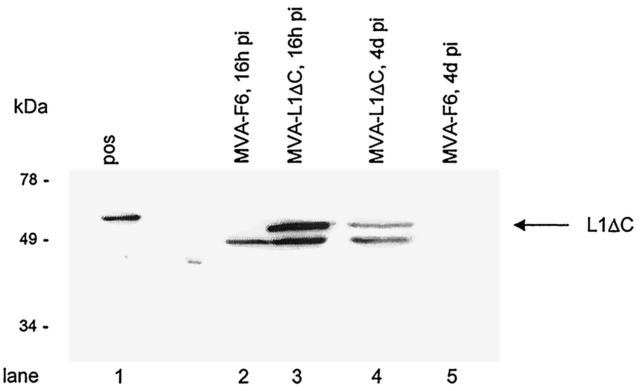


FIG. 2. Expression of HPV16 L1 in B6 cells after infection with recombinant HPV16 L1 MVA (MVA-L1ΔC). Western blot analysis of protein extracts of cells (infected with MVA-L1ΔC or MVA-F6 [nonrecombinant]) obtained 16 h and 4 days (4d) postinfection (pi). The specificity of the monoclonal antibody was confirmed by detection of L1 in purified VLPs obtained from recombinant baculovirus (pos). The slight difference in molecular mass between L1 protein obtained from VLPs and from recombinant MVA is due to the 34-aa C-terminal deletion in the latter (L1ΔC). The lower ca. 45-kDa band represents a cross-reacting cellular or vaccinia virus protein occurring with a different kinetics after infection by recombinant and nonrecombinant vaccinia virus (not visible 4 days after MVA-F6 infection).

(360 L1 molecules, 72 pentameric capsomeres) are required to activate L1-specific CTL in vivo or whether incompletely assembled particles (pentameric capsomeres) are sufficient to induce a similar effect. We expressed GST-L1ΔN10 (HPV16 L1 protein in which 10 aa of the N terminus had been deleted was fused to GST) in *E. coli* and purified it using glutathione-Sepharose chromatography and thrombin-mediated elution by the method of Chen et al. (6, 7). The L1ΔN10 protein oligomerizes into pentameric capsomeres, and high salt concentrations combined with low pH favor the further assembly of these pentamers into $T=1$ capsids (6). The L1 protein to be used for immunization was characterized by sucrose gradient sedimentation and size exclusion fast protein liquid chromatography. Analysis of individual gradient fractions by Western blot analysis (Fig. 4) and L1-specific capture ELISA (data not shown) (30) revealed that the majority of the L1-specifc material sedimented at approximately 11S, consistent with the position of pentamers (50). In addition, there was Western blot-reactive material of higher S values (Fig. 4). By analyzing different amounts of the individual fractions by Western blotting (data not shown), we estimated that the proportion of fast-sedimenting material accounted for less than 10% of the total. Comparison of the sedimentation properties of VLPs prepared from recombinant baculovirus either run in a parallel tube (Fig. 4) or added to the L1ΔN10 preparation (not shown) showed that full-size capsids ($T=7$) mostly pellet under these centrifugation conditions (Fig. 4). From these data, we concluded that we have obtained pentameric capsomeres together with a small fraction of aggregated material of less well-defined structure. This material was used for the subsequent immunization experiments. As reported earlier (6), L1ΔN10 does not assemble into complete $T=7$ capsids. Results obtained by fast protein liquid chromatographic analysis of our material (data not shown) were consistent with this interpretation. Although a slight heterogeneity was present in the HPV16 L1ΔN10 preparation (Fig. 4), we will refer to this material as capsomeres.

Immunization with capsomeres induces L1-specific CTLs and antibodies. We measured the in vivo priming of HPV16 L1-specific T cells by ELISPOT and ^{51}Cr release assay following immunization with capsomeres as described in Materials and Methods. Confirming the observations in another study (28), we obtained consistent results with both assays. ELISPOT proved to be more sensitive. In most instances, ELISPOT gave positive results after ex vivo testing of the spleen cells of the immunized mice, whereas repeated rounds of restimulation were needed to yield a positive result in the ^{51}Cr release assay (28).

Immunization of C57BL/6 mice (two doses given s.c.) with 10, 5, 1, or 0.1 μg of capsomeres induced an L1-specific CTL response in a dose-dependent manner: in three independent experiments, 11 of 16 mice gave positive results by an ELI-

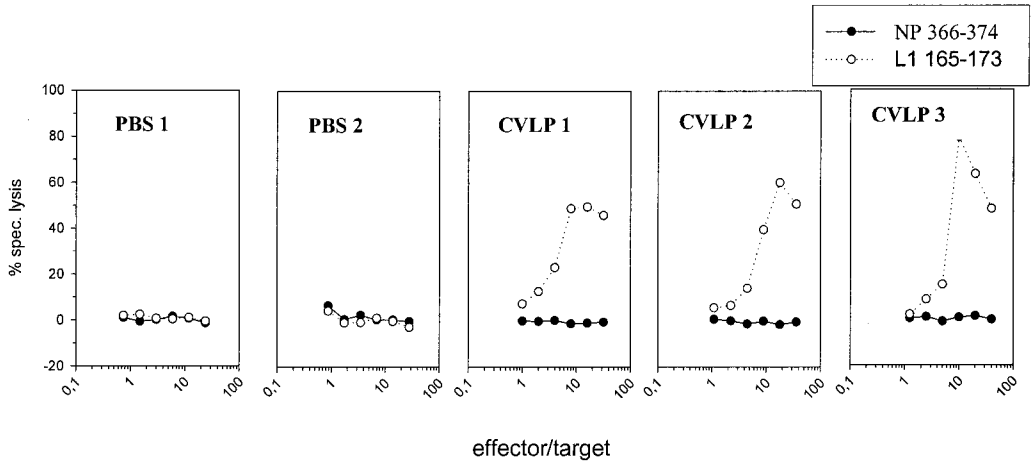


FIG. 3. CTL activity against HPV16 $L1_{165-173}$. Splenocytes of C57/B6 mice immunized with buffer (PBS 1 and PBS 2) or HPV16ΔCE7$_{1-55}$ CVLPs (30) (CVLP 1 to 3) were restimulated with MVA-L1ΔC-infected syngeneic cells (B6) and exposed to RMA-S cells loaded with peptide $L1_{165-173}$ or $NP_{366-372}$ (negative control), and ^{51}chromium was measured in the supernatant. The percentage of specific lysis is shown on the *y* axes.

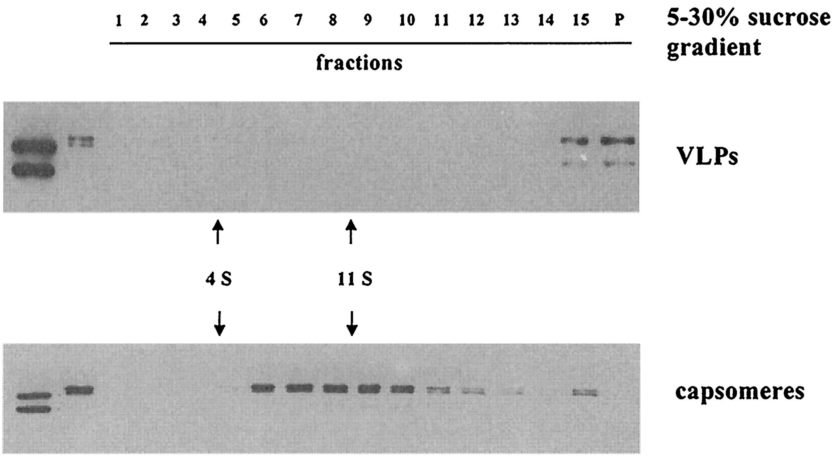


FIG. 4. Size distribution of the HPV16 L1ΔN10 protein. Purified VLPs (top blot) or L1ΔN10 protein (capsomeres [bottom blot]) was sedimented through a linear sucrose gradient, and 30-μl portions of individual fractions (collected from top to bottom of the tube) were analyzed by Western blotting using an L1-specific monoclonal antibody. The starting materials are shown at the left in both blots. VLPs used in this experiment consist of the C-terminally truncated L1 (HPV16 L1ΔC) (30), so the L1 molecules are slightly smaller than those in the capsomeres and show two bands due to partial degradation. VLPs are found in the bottom fractions and in the pellet (P). Sedimentation of the bulk of HPV16 L1ΔN10 protein is consistent with pentameric capsomeres. The positions of the marker proteins bovine serum albumin (4S) and catalase (11S) analyzed in parallel tubes are indicated.

SPOT and ^{51}Cr release assay (both mice in the 10-μg group, all six mice in the 5-μg group, three of four mice in the 1-μg group, and none of the four mice in the 0.1-μg group). Eight mice immunized twice with PBS gave negative results in the ex vivo analysis by ELISPOT (0 to 2 IFN-γ-secreting T cells/10^5 splenocytes), and seven of eight mice also gave negative results after three rounds of in vitro restimulation (ELISPOT and ^{51}Cr release assay). The average numbers of IFN-γ-secreting T cells of the mice in one group are listed in Table 2. In one of the immunization experiments, we included a group of four mice that received two doses of 10 μg of baculovirus-expressed HPV16 L1 VLPs. Three animals gave positive results by ELISPOT ex vivo and ^{51}Cr release assay after two rounds of in vitro restimulation. In a direct comparison, the average number of activated T cells was similar to that after two immunizations with 5 μg of capsomeres (Table 2). To test for a possible adjuvant effect of *E. coli*-derived endotoxin present in the capsomere preparation (0.002 ng of LPS per μg of L1 protein), we repeated the immunization after removal of the LPS. After treatment with polymyxin B (29), LPS was not detected under the standard assay conditions employed (<0.0002 ng/μg of L1 protein). Mice (five per group) received 10 μg of capsomeres before and after polymyxin B treatment. ELISPOT analysis revealed a similar reactivity of all mice (Table 2). From these data, we conclude that the contaminating LPS has a negligible (if any) effect on the capsomere-induced T-cell response.

The L1-specific humoral immune response was also measured: serum IgG antibodies were detected by VLP-specific ELISA in 10 of 16 mice, again in a dose-dependent manner (two of two mice, five of six mice, three of four mice, and none of the four mice in the groups given 10, 5, 1, and 0.1 μg, respectively). The ODs of the positive samples ranged from 0.3 to 1.8 (mean ± SEM, 0.90 ± 0.17), whereas the mean OD of the preimmunization sera was 0.21 ± 0.04. The scores of 13 of the 16 mice immunized with HPV16 L1 capsomeres examined by ELISA and T-cell assay agreed (9 positive, 4 negative). Of the three remaining animals, two were CTL positive and antibody negative and one was CTL negative but antibody positive.

These data demonstrate that s.c. immunization of C57BL/6 mice with HPV16 L1 capsomeres or with $T=7$ VLPs induces a D^b-restricted CTL response. Our results also confirm observations made by others, i.e., that L1-specific antibodies can be

TABLE 2. HPV16 L1-specific T-cell response in C57BL/6 mice measured by ELISPOT after s.c. immunization of different amounts of HPV16 L1 capsomeres or VLPs[a]

Expt and antigen	No. of mice	Mean no. of IFN-γ-positive T cells/10^4 splenocytes ± SEM
Expt 1		
Capsomeres		
10 μg	2 }	1.1 ± 0.4
5 μg	2 }	
1 μg	4	0.6 ± 0.2
0.1 μg	4	0.0 ± 0.0
None (control)	4	0.1 ± 0.1
Expt 2		
VLPs (10 μg)	4	5.8 ± 3.4
Capsomeres (5 μg)	4	8.5 ± 3.0
None (control)	4	0.8 ± 0.8
Expt 3		
Capsomeres (10 μg)	4	7.4 ± 0.6
Capsomeres (10 μg) with LPS removed	4	10.8 ± 1.0
None (control)	4	0.6 ± 0.2

[a] Data from three different independent experiments are shown. In experiments 1 and 3, the IFN-γ-positive T cells were counted after ex vivo analysis of splenocytes without in vitro restimulation. In experiment 1, due to the small number of animals ($n = 2$), IFN-γ-positive T cells of groups of mice given 10 and 5 μg of capsomeres were pooled. In experiment 2, the IFN-γ-positive T cells were counted after one in vitro restimulation. SEM, standard error of the means. In experiment 3, LPS was removed from the capsomere preparation by polymyxin B treatment (see "Immunization with capsomeres induces L1-specific CTLs and antibodies").

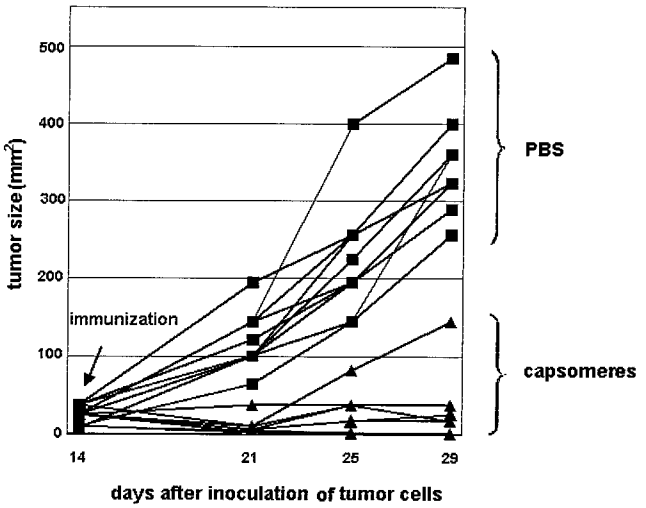


FIG. 5. Growth of C3 tumors in C57BL/6 mice after s.c. immunization with HPV16 L1 capsomeres. Mice received tumor cells and were immunized with 10 μg of capsomeres or with PBS when the tumors reached an average size of 25 mm^2. Surface tumor size was measured and is indicated for the individual mice over time. Because of the size of tumors in the control group (PBS), the experiment was terminated at day 29.

raised by immunization with capsomeres of human and animal papillomaviruses (13, 50).

To test for L1-specific activity in vivo, we performed a tumor rejection experiment using C3 cells, which were established by transformation with the complete HPV16 genome (12). In an earlier study, it was demonstrated that immunization with HPV16 L1 VLPs inhibited the growth of C3 in mice, suggesting that the L1 protein is expressed, although biochemically only L1-specific transcripts were detected (9). A total of 5×10^5 cells per animal were inoculated into mice ($n = 20$) (Fig. 5). When the tumors had reached a mean size of 25 ± 3 mm^2 at day 14, we injected 10 mice with 10 μg of HPV16 L1 capsomeres and 10 mice with PBS. At day 29 postinoculation, the tumors of all control mice had grown to a mean size of 345 ± 20 mm^2, and the experiment was terminated. In one of the capsomere-immunized mice, the tumor was slightly larger. In six of the capsomere-immunized mice, the tumor remained the same size as at the day of immunization (partial responders), whereas in three animals no tumors were detectable at the site of inoculation (complete responders). The mean size of tumors in the capsomere treatment group was 32 ± 13 mm^2. The results are shown in Fig. 5. Similar results were obtained in a second experiment (data not shown).

After completion of the first tumor regression experiment (Fig. 5), the T-cell responses were measured by ELISPOT in eight mice (two mice of the control group and six mice of the capsomere treatment group, i.e., four partial and two complete responders). Whereas no reactivity was found in the control group, four of the six capsomere-immunized mice reacted positively (two of four of the partial responders and the two complete responders [data not shown]). We concluded from this result that an $L1_{165\text{-}173}$-specific T-cell response correlates with capsomere-induced regression of C3 cells in C57BL/6 mice. However, the data also indicate that in addition to $L1_{165\text{-}173}$, other D^b- or K^b-restricted epitope(s) within the HPV16 L1 protein are involved in tumor rejection (see Discussion).

Next we measured the immune response after i.n. immunization with capsomeres. Twenty mice were inoculated two or three times with 5 or 10 μg of capsomeres with or without CTB adjuvant (Table 3). All were positive by ELISPOT and ^{51}Cr release assays, albeit to a lesser degree than after s.c. immunization (Fig. 6). Serum IgG but not IgA antibodies were detected in 8 of 20 animals (mean OD, 0.52; SEM = 0.14 versus 0.13; SEM of 0.02 for the preimmune sera). We found IgG antibodies in vaginal lavage specimens for 4 of 20 mice (mean OD of 0.35 and SEM = 0.02 versus mean OD of 0.17 and SEM = 0.02 for the lavage specimens prior to immunization); only one of these four mice was also positive by serum ELISA. Vaginal IgA at an extremely low concentration (OD of 0.7 obtained after the immunoglobulins were concentrated) was detected in one animal.

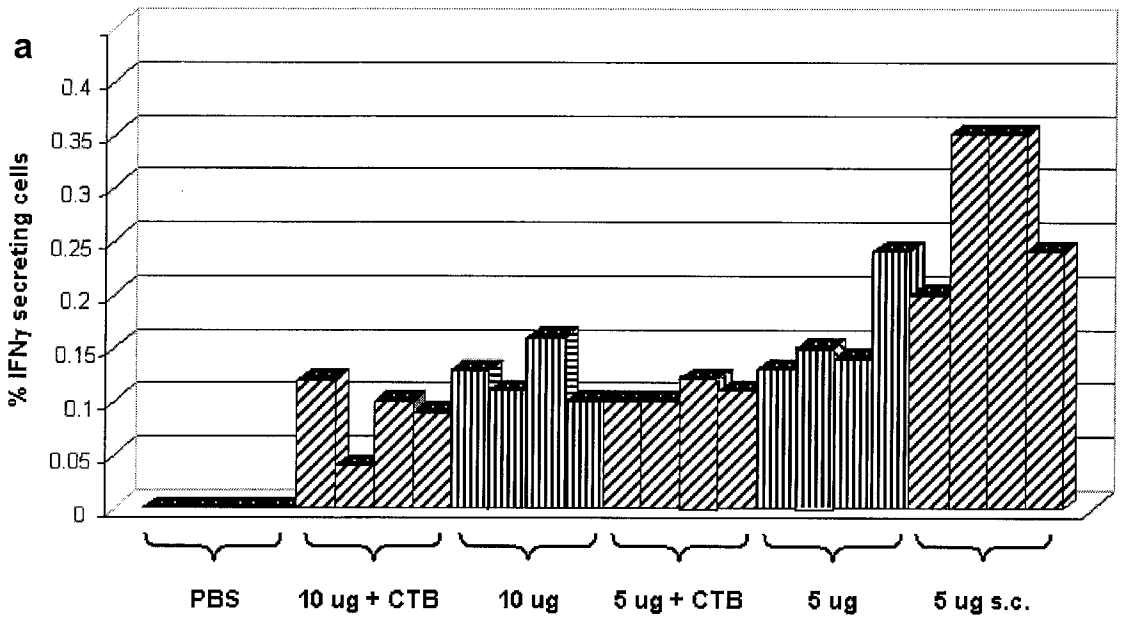
DISCUSSION

Immunization with VLPs of different human and animal papillomaviruses generated by expression of the L1 protein with or without the minor structural protein L2 has proven to be very efficient in inducing L1-specific neutralizing antibodies

TABLE 3. i.n. immunization of C57BL/6 mice ($n = 20$) with HPV16 L1 capsomeres (summary of two independent experiments)

Immunization scheme[a]	No. of mice						
	Total	Positive by ELISPOT[b]	Positive by ^{51}Cr release assay[b]	Serum IgG positive[c]	Serum IgA positive[c]	Vaginal IgG positive[c]	Vaginal IgA positive[c]
Three 10-μg doses							
With CTB	4	4	4	2	0	0	1
Without CTB	4	4	4	2	0	0	0
Three 5-μg doses							
With CTB	4	4	4	0	0	3	0
Without CTB	8[d]	8[d]	8[d]	4	0	1	0

[a] Mice were immunized with two or three 10- or 5-μg doses of capsomeres with or without 5 μg of CTB.
[b] As for s.c. immunization (see text) ELISPOT showed a positive reaction of the splenocytes ex vivo or required fewer rounds of restimulation for a positive score than the ^{51}Cr release assay.
[c] Only one mouse gave a positive result in more than one of these assays (serum IgG and vaginal IgA).
[d] In one experiment encompassing four of these eight mice, the mice were given only two doses of capsomeres.



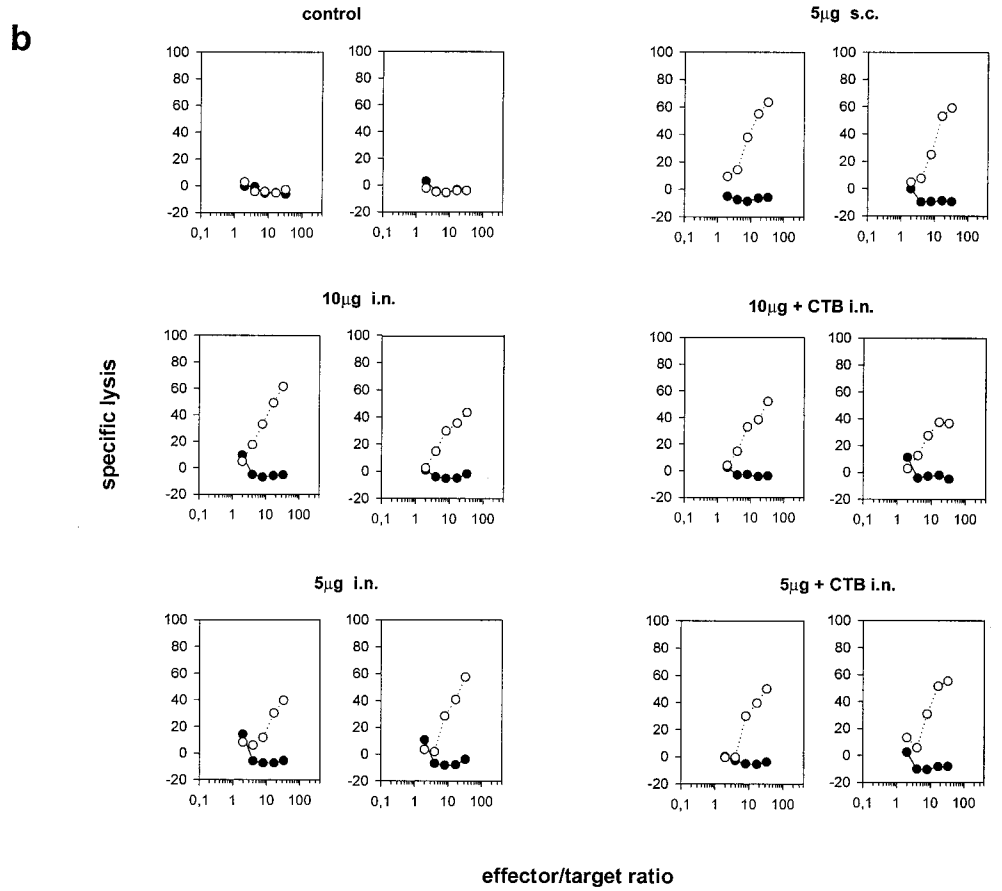


FIG. 6. CTL responses in C57BL/6 mice after immunization (i.n. or s.c.) with HPV16 capsomeres. Mice (four in each group) were immunized i.n. with PBS or HPV16 L1 capsomeres (5 or 10 μg) with or without CTB or s.c. with capsomeres (5 μg). (a) ELISPOT after two in vitro restimulations of the splenocytes. Each bar represents the number of activated T cells from one mouse. The mice were immunized i.n., except for one group that received the capsomeres s.c. The numbers (means ± SEMs) of IFN-γ-expressing T cells/10^4 splenocytes for the groups follow: 0.0 ± 0.0 for the PBS group (control), 8.8 ± 1.7 for the group given 10 μg of capsomeres and CTB, 12.5 ± 1.3 for the group given 10 μg of capsomeres, 10.8 ± 0.5 for the group given 5 μg of capsomeres and CTB, 16.5 ± 2.5 for the group given 5 μg of capsomeres, and 28.5 ± 3.8 for the group given 5 μg of capsomeres s.c. (b) ^{51}Cr release assay after four in vitro restimulations. Specific lysis is shown against RMA-S cells (black circles) and RMA-S cells loaded with peptide $L1_{165-173}$ (open circles). The two individual mice shown in the graphs in panel b correspond to the mice in panel a as follows (counting from left to right for each group shown in panel a): control (PBS), mouse 1 and 3; 5μg s.c., mouse 2 and 3; 10μg i.n., mouse 1 and 3; 10μg + CTB i.n., mouse 1 and 4; 5μg i.n., mouse 1 and 2; 5μg + CTB i.n., mouse 2 and 3.

even in the absence of an adjuvant. Neutralizing activity has been demonstrated by in vitro infection assays using pseudovirions or infectious particles (2, 5, 16, 18, 35, 49) and by protection against experimental challenge in animal models (4, 50). Initial data from a human trial suggest that immunization also protects against infection and disease following natural exposure to HPV16 (19). On the other hand, VLPs are extremely potent inducers of a CTL response. This effect was first demonstrated when mice immunized with CVLPs containing sequences of HPV16 E7 linked to either the L1 or L2 protein (15, 30) were shown to elicit E7-specific CTLs (40) and suppressed the growth of E7-positive syngeneic tumor cells in C57BL/6 mice (15, 40). In another study, $CD8^+$ T-cell-dependent protection against C3 tumors was shown by immunization with HPV16 L1 (or L1 and L2) VLPs, but the specificity of the CTLs was not further characterized (9). Since immunization was successful without the addition of adjuvant, it was assumed that HPV particles bind to and are taken up by antigen-presenting cells. In fact, this mechanism was confirmed in two independent studies demonstrating that VLPs activate dendritic cells and trigger the presentation of CTL epitopes, thus leading to the activation of $CD8^+$ cells (21, 39). Since immunogenicity was also demonstrated for other VLP-linked antigens (24, 32), papillomavirus particles are considered suitable carriers for alien proteins, which by themselves are unable to induce an MHC-I-restricted immune response. The restrictions of the CVLPs available at this time are the small number of heterologous molecules per particle in the case of L2 fusions (at full occupancy yielding 1/30 of the molarity of the L1 molecules [15]) or the limited size of acceptable heterologous sequences in the case of the L1 fusions (up to 60 aa of the HPV16 E7 protein [30]). In earlier studies, we had found that inserting longer stretches (i.e., the complete 98-aa HPV16 E7 molecule) into a C-terminally truncated HPV16 L1 molecule was still compatible with formation of capsomeres (30). With respect to a combined prophylactic and therapeutic vaccine inducing both neutralizing antibodies and E7-specific CTLs (17, 40), we hypothesized that such chimeric capsomeres would be a more valuable therapeutic immunogen, as they provide a larger set of alien epitopes. In this study, we demonstrated that HPV16 L1 capsomeres induce not only antibodies (as shown previously [13, 38, 50]) but also CTLs (Table 2 and Fig. 5 and 6). Capsomeres and VLPs stimulated similar numbers of T cells as measured by ELISPOT (Table 2), yet a definitive statement about efficacy can be made only when more-detailed side-by-side titrations have been performed. In a first round of immunization with HPV16 $L1/E7_{1-98}$ chimeric capsomeres, we also obtained E7-specific (together with L1-specific) T cells in C57BL/6 mice (unpublished data). These experiments will be extended towards the development of a capsomere-based therapeutic vaccine.

Endotoxins of gram-negative bacteria have been shown to exert a variety of effects on the innate immune system through binding to members of the Toll-like receptor (TLR) family (26, 47). Since the HPV L1 protein used in this study is produced in *E. coli*, it is conceivable that the immunogenic properties of the HPV16 L1 capsomeres are dependent upon contaminating LPS. However, treatment by polymyxin B reduced the LPS content to a concentration that could not be detected by the *Limulus* amoebocyte lysate test (22) (<0.002 ng per μg of HPV16 L1 protein) yet did not alter the ability of capsomeres to induce an L1-specific T-cell response. Further, the largest amount applied in our experiments (0.02 ng of LPS per mouse) is 10^6 times below the concentration typically used to activate the mouse immune system in vivo (26). Therefore, we assume that an adjuvant effect of LPS is unlikely to play a role in our experiments. This hypothesis can be verified by using the appropriate TLR knockout mice for immunization.

We demonstrated the potential of capsomeres for therapeutic vaccination in vivo by the unequivocal effect against C3 cells in tumor regression experiments (tumor challenge before immunization; Fig. 5); published data with HPV16 L1 VLPs had shown efficacy in the same tumor model in the less-stringent tumor protection protocol (tumor challenge after immunization) (9). Tumor experiments with chimeric capsomeres containing the complete HPV16 E7 protein that aim at the induction of E7-specific CTLs will be initiated soon.

In this proof-of-principle study, we tested L1 capsomeres for induction of CTL activity, aware that L1-specific T cells will likely have no therapeutic effect against persistently virus-infected cells. To perform CTL analyses, we first identified an H2b-restricted T-cell epitope (see text and Fig. 1 to 3). The predictions made by sequence analysis using the SYFPEITHI database (36) proved valuable in our case, as only the nonameric peptide $L1_{165-173}$ exhibiting a very high score by the algorithm was positive in the MHC binding experiments (Fig. 1); in subsequent experiments, $L1_{165-173}$ turned out to be a bona fide CTL epitope. However, we have evidence that for tumor regression, additional CTL epitope(s) may be relevant, as only four of six mice responding to the immunization (Fig. 5) reacted in the $L1_{165-173}$-specific assays. In addition, preliminary data obtained by immunization with irradiated C3 tumor cells (300 Gy) point at the induction of a CTL response against another (decameric) L1 epitope. It was identified when we extended the SYFPEITHI database screening to the potential D^b-binding decameric peptides that, compared to nonamers, are less likely to represent bona fide CTL epitopes (37). Experiments are under way to confirm the roles of the putative novel epitope and the nonameric $L1_{165-173}$ epitope in rejection of C3 cells. In particular, in the case of the $L1_{165-173}$ negative responder mice, we also have to consider a putative role of the innate immune system that plays a role in controlling the natural course of papillomavirus infections through induction of cytokines (for reviews, see references 45 and 48). The induction of an adaptive versus innate immune response is delayed; hence, if the former is responsible for the antitumor effect, the tumors are expected to continue to grow initially after immunization. Due to our experimental protocol (first check of the mice was 7 days after immunization; Fig. 5), we have no information about the growth characteristics immediately after vaccination. In future experiments, we will investigate the aspect of innate immunity in more detail.

From our own analysis (Fig. 4) as well as from earlier data (6), we can exclude the possibility that our preparation of HPV16 L1ΔN10 protein used for immunization contained fully developed ($T=7$) particles consisting of 360 L1 molecules. It also appears unlikely that the small fraction of faster-sedimenting material (Fig. 4) contains incomplete ($T=1$) particles assembled from 60 L1 molecules, since assembly into that form requires high levels of salt and low pH (6). Such conditions

were not used during preparation of the L1ΔN10 protein, and we did not detect particles by electron microscopy (data not shown). However, the possibility that conditions favoring assembly existed at any step during the procedure of immunization cannot be completely excluded.

Induction of antibodies by papillomavirus capsomeres has been published previously following intraperitoneal, intramuscular, or intradermal injection (13, 38, 50). Here we report for the first time the development of an HPV L1-specific humoral immune response following i.n. immunization with capsomeres. It is unclear, however, whether the nasal mucosa is actually the inducer site in our experiments. As previously suggested for HPV16 VLPs (1), induction of the mucosal immune response requires deposition of the antigen in the lungs, which most likely occurred in our experiments since the mice had been anesthetized during immunization (see Materials and Methods). Since we did not perform serum titrations in our ELISAs, no comparisons of the efficacy of s.c. and i.n. immunizations and also between this and other studies can be made.

We found immunoglobulins in vaginal lavage specimens in only a fraction of the immunized animals, independent of the presence of the mucosal adjuvant CTB (see above and Table 3). Because we were evaluating a CTL response, we used C57BL/6 mice, whereas BALB/c mice are often used if the experiment aims to measure antibody production (1, 13). We will investigate whether stronger responses can be achieved utilizing a different mouse strain and/or a modified immunization protocol. In fact, induction of L1-specific neutralizing antibodies along with CTLs directed against early proteins is required for a combined prophylactic and therapeutic vaccine (8, 17).

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