Deletion of a major neutralizing epitope of human papillomavirus type 16 virus-like particles

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Human papillomavirus type 16 (HPV-16) is a major cause of human cancer. Effective prophylactic vaccines are based on type-specific neutralizing antibodies. A major neutralizing epitope has been defined by the monoclonal antibody H16.V5. To investigate the importance of this epitope for overall immunogenicity of HPV-16, HPV-16 virus-like particles devoid of the H16.V5 epitope were engineered by site-directed mutagenesis of ten non-conserved, surface-exposed residues. Removal of the H16.V5-defined epitope had only a marginal effect on antigenic reactivity with antibodies in sera from infected subjects, but affected immunogenicity in experimental immunization of mice, with reduced induction of both antibody responses and CTL responses.

INTRODUCTION

Human papillomaviruses (HPVs) cause a wide variety of benign and pre-malignant epithelial tumours. Of the more than 100 different types of HPV that have been characterized, approximately two dozen specifically infect genital and oral mucosa (de Villiers et al., 2004). HPVs are the most common of the sexually transmitted diseases. A small number of genital infections with high-risk HPV types will progress to invasive cervical cancer (de Villiers et al., 2004). HPV has also been established as a cause of cancer of the penis, vulva, anus, vagina and oropharynx (Munger et al., 2004). Carcinoma of the cervix is one of the most prevalent cancers in women worldwide and is associated with HPV in more than 98 % of cases (Bosch & de Sanjose, 2003). HPV vaccine development is therefore a prime priority for preventative cancer research today.

HPV capsids consist of 72 capsomers, each containing five copies of the HPV major capsid protein, L1 (Chen et al., 2000). The capsid proteins, when expressed, self-assemble to form virus-like particles (VLPs), i.e. particles that are morphologically indistinguishable from the authentic virions but lack the potentially oncogenic viral genome (Kirnbauer et al., 1992). These VLPs elicit high titres of systemic neutralizing antibodies in several animal model systems (e.g. rabbits, cows and rhesus monkeys), even when injected in the absence of an adjuvant (Roden et al., 2004). The neutralizing antibodies primarily recognize conformational L1 epitopes displayed on intact VLPs or virions (Chen et al., 2000). It has been shown previously that most antibodies in human sera that are reactive with intact HPV-16 capsids can be blocked by a single monoclonal antibody (mAb), H16.V5 (Wang et al., 1997). H16.V5 binds to a conformational epitope and reacts with all known HPV-16 isolates (Rodent al., 1997; White et al., 1999). Therefore, it is used as a reference mAb for characterization of VLPs and epitope retrieval (Carter et al., 2003, 2006; Wang et al., 2003). H16.V5 is also used in inhibition-based HPV serology assays that have been used in major HPV vaccination trials (Brown et al., 2004; Fife et al., 2004; Koutsy et al., 2002; Shank-Retzlaff et al., 2005; Villa et al., 2006).

Vaccination with VLPs has been shown to be highly efficient for protection against subsequent challenge with both cutaneous and mucosal papillomaviruses, as well as for protection against natural HPV infection in human trials (Harper et al., 2004; Koutsy et al., 2002). The neutralizing antibodies are in principle restricted to the virus type used for immunization, indicating the presence of a strongly immunodominant type-specific epitope (Christensen et al., 2001; Slupetzky et al., 2001; Wang et al., 1997; White et al., 1999). As well as being highly efficient at eliciting a high-titre neutralizing antibody response, HPV VLPs are also highly efficient at eliciting a cytotoxic T lymphocyte (CTL) response (De Bruijn et al., 1998; Dupuy et al., 1997; Revaz et al., 2001; Rudolf et al., 1999). VLP-based vaccines have been found to be highly efficacious in preventing and treating transplantable cancers in several mouse models, in spite of the fact that immunization is made with an exogenous protein (De Bruijn et al., 1998; Revaz et al., 2001). The high antibody immunogenicity appears to be due, at least in part, to the
neutralizing epitope defined by H16.V5 is important is an
H16.V5. Improved knowledge of whether the type-specific
characterize the immunogenic and antigenic properties of
VLPs to bind H16.V5 mAb. We did not seek to elucidate
In this study, our primary aim was to delete the ability of
problem with pre-existing neutralizing antibodies.
from HPV VLPs is one possible solution to overcome the
(Ruedl (Da Silva et al., 2001), although another report in a
hepatitis B VLP system found that pre-existing neutralizing
antibodies had only minimal effect on CTL responses
(Ruedl et al., 2005). Removal of the neutralizing epitope(s)
from HPV VLPs is one possible solution to overcome the
problem with pre-existing neutralizing antibodies.

In this study, our primary aim was to delete the ability of
VLPs to bind H16.V5 mAb. We did not seek to elucidate
which residues were H16.V5 contact residues but aimed to
characterize the immunogenic and antigenic properties of
HPV-16 L1 VLPs engineered to lack the ability to bind
H16.V5. Improved knowledge of whether the type-specific
neutralizing epitope defined by H16.V5 is important is an
issue of fundamental interest for HPV vaccination.

METHODS

Software for visualization of HPV-16 L1 protein and space model
building. The programs used for visualizing the L1 protein structure
were GCG Wisconsin, Swiss PDBViewer, RasMol, WHAT IF, ANTIGENIC
and ProtScale. The atomic coordinates of an L1 monomer from Chen
et al. (2000) were used.

Selection of residues for mutagenesis. Probable surface exposure
of residues was evaluated through visual studies of the L1 pentamer in
RasMol and Swiss PDBViewer. Antigenicity predictions were done
using GCG Wisconsin, ProtScale and WHAT IF software. The variability
of residues among HPV types was determined by comparing L1
sequences of different HPV types using the 1997 human papilloma-
ivirus sequence database (http://hpv-web.lanl.gov).

Construction of H16.V5 epitope-depleted HPV-16 VLPs. A trun-
cated, chemically synthesized HPV-16 L1 gene lacking the C-terminal
34 residues was purchased from Interactiva Biotechnologie GmbH.
The gene was codon-adapted for expression in Escherichia coli.
Truncations of up to 30 C-terminal residues have been reported to
have little or no effect on the stability or solubility of the expressed
protein, whereas C-terminal deletions longer than 30 residues had
been reported to render the protein unstable and extremely sensitive
to proteases (Chen et al., 2000). Therefore, an additional 14 aa
segment, codon-adapted for expression in E. coli, was ligated to the
construct, creating an HPV-16 L1 gene lacking only 20 residues in the
C terminus.

All mutations in the truncated HPV-16 L1 sequence were performed using a QuickChange Multi Site-directed Mutagenesis kit (Stratagene).
All constructs were sequenced to confirm the mutations. Constructs
were then cloned into the BamHI/Nool sites in the expression vector
pVL1393 (Pharmingen) using standard molecular biological cloning
techniques.

Generation of truncated ovalbumin–L1 HPV-16 constructs. Oligo-
nucleotides encoding the ovalbumin CTL epitope (SIINFEKL) and T
helper (Th) epitope (TETWSSNVMEERK) were purchased from
Shafer-N. These peptides fit the binding motifs for the murine MHC I
molecules K\textsuperscript{b} and D\textsuperscript{b} and MHC II. The oligonucleotides were cloned
into the C terminus (nt 1445–1558) of the truncated HPV-16 L1
major capsid protein lacking the C-terminal 20 aa using standard
molecular biological techniques.

Generation of recombinant baculoviruses. Spodoptera frugiperda
(Sf9) cells were grown at 27 °C in SF-900 II medium (Life Techno-
lologies) supplemented with 4 % fetal calf serum (FCS) and 2 mM
glutamine. Five micrograms of baculovirus transfer plasmid (pVL1393;
Pharmingen) containing truncated wild-type HPV-16 L1, H16.V5
epitope-depleted HPV-16 L1, wild-type HPV-16 L1–ovalbumin or
H16.V5 epitope-depleted HPV-16 L1–ovalbumin was used to
transfect Sf9 cells, together with 0.5 μg linearized Baculo-Gold
DNA (Pharmingen). The pVL1393 baculovirus transfer vector contains
the complete polyhedrin gene locus of Autographa californica
multiple nucleopolyhedrovirus cloned into the pUC8 vector, but
lacks the polyhedrin gene-coding region. After plaque purification of recombinant clones, high-titre recombinant virus was generated.
Cultures of High Five insect cells were infected with recombinant
high-titre baculovirus at an m.o.i. of 10. After 72 h at 27 °C, cells
were harvested (Christensen et al., 1994; Le Cann et al., 1994;
Touze et al., 1998).

Purification of HPV VLPs. Infected insect cells were harvested,
pelleted and resuspended in 10 ml PBS (pH 6.0) containing 0.5 %
NP-40. After 30 min incubation on ice, the cells were sonicated four
times for 40 s each at 60 % maximal power (Virsonic 300; VirTis). Lysates
were loaded onto a 40 % (w/v) sucrose cushion and centrifuged for 3 h at 125 000 g at 4 °C. Pellets were resuspended in 3 ml PBS containing 32 %
CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2} and
5 mM HEPES, and sonicated for 3 s at 60 % maximal power. The
suspension was then placed in 13.4 ml Beckman Quickeal
seals. Samples were centrifuged for 20 h at 150 000 g at 4 °C. Gradients
were fractionated by puncturing the tubes at the top and bottom with
a 21-gauge needle and ten drops of each fraction were analysed. The
gradient fraction densities were determined by refractometry. Positive
fractions were dialysed against PBS containing 0.5 M NaCl, 5 mM
HEPES, 1 mM MgCl\textsubscript{2} and 1 mM CaCl\textsubscript{2}.

Western blot analysis. Reduced VLP samples were run on NuPage 4–
12 % Bis-Tris gels (Invitrogen) and blotted onto nitrocellulose
membrane (Invitrogen). Membranes were blocked overnight with 5 %
non-fat dried milk and incubated with anti-HPV-16 L1 monospecific
antipeptide antibodies (L1-16A or L1-30A; Dillner et al., 1991).
Following incubation with horseradish peroxidase-conjugated anti-
rabbit IgG (Santa Cruz Biotecnologies), membranes were developed with the ECL Plus system (Amersham Biosciences).

As a positive control, purified baculovirus-expressed VLPs of HPV-16
(obtained from Dr John T. Schiller, National Cancer Institute,
Bethesda, USA) were used.

Mouse immunization with VLPs. Female C57BL/6 mice (8 to 12
weeks old; Taconic M&B) were injected subcutaneously with 10 μg

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truncated wild-type, H16.V5 epitope-depleted, wild-type HPV-16 L1 ovalbumin or ovalbumin–H16.V5 epitope-depleted HPV-16 VLPs administered with an equal volume of complete Freund’s adjuvant (CFA). A group of control mice was injected with PBS/CFA only. Each group consisted of five mice. The mice were boosted on days 14 and 28 with VLPs in solution with incomplete Freund’s adjuvant (IFA). The mice were bled through the vena saphena on days 27, 35 and 42.

ELISA. ELISA plates were coated overnight at 4 °C with baculovirus-produced VLPs (H16.V5-epitope depleted or wild-type) in cold PBS (pH 7.2). Disrupted VLPs generated through incubation at room temperature for 4 h in carbonate buffer (pH 9.6) were used as a negative control. After washing of the plates four times with PBS/0.5 % Tween 20 (PBS-T), they were blocked with PBS supplemented with 10 % horse serum (10 % HS-PBS) at room temperature for 1 h. mAb H16.V5 or mAb D9 (kindly provided by Dr Neil Christensen, Pennsylvania State University College of Medicine, Hershey, USA) diluted in 10 % HS-PBS was incubated for 1 h at room temperature. Fifty microlitres of sheep anti-mouse IgG horseradish peroxidase conjugate (diluted 1 : 2000 in 10 % HS-PBS; Amersham Biosciences) was added to the ELISA plates after washing four times with PBS-T. The plates were incubated for 1 h at room temperature and washed four times with PBS-T. 3.3',5,5'-Tetramethylbenzidine substrate (100 μl per well; PharMingen) was added and allowed to react for 15 min. The reaction was stopped by the addition of 100 μl 1 M HCl per well and the absorbance was read at 450 nm. In the case of ELISAs using serum from immunized mice, the serum was added to the wells at dilutions ranging from 1 : 100 to 1 : 218 700 (diluted in 10 % HS-PBS) and incubated for 1 h. After washing, bound antibody was detected using sheep anti-mouse IgG horseradish peroxidase conjugate (diluted 1 : 2000 in 10 % HS-PBS; Amersham Biosciences).

For ELISAs using human serum, the serum was incubated for 2 h at room temperature. After washing, a mouse monoclonal anti-human IgG (γ-chain-specific) antibody (diluted 1 : 800 in 10 % HS-PBS; EuroDiagnostica) was added and allowed to react for 90 min at room temperature. Anti-mouse IgG horseradish peroxidase conjugate (diluted 1 : 2000 in 10 % HS-PBS; Amersham Biosciences) was used as secondary antibody.

CTL assay. Female C57BL/6 mice (8 to 12 weeks old; Taconic M&B) were injected subcutaneously with 10 μg wild-type, H16.V5 epitope-depleted, wild-type HPV-16 L1 ovalbumin or ovalbumin–H16.V5 epitope-depleted HPV-16 VLPs administered with an equal volume of IFA. One group of control mice was injected with PBS/IFA only and one group with 100 μg of the ovalbumin H2-K1-restricted CTL epitope and Th peptide. Each group consisted of four mice. The mice were boosted on day 14 with VLPs in solution with IFA. Three weeks after the booster immunization, mice were sacrificed by cervical dislocation and spleen cells were isolated. After incubation in nylon wool columns for 1 h at 37 °C and 5 % CO2, enriched T cells were washed through the column with complete cell culture medium (RPMI 1640 plus 10 % FCS, 1 mM sodium pyruvate, 5 μM 2-mercaptoethanol, 10 mM HEPEs, 0.1 mg gentamicin ml−1). Cells were cultured for 5 days at 37 °C and 5 % CO2 in complete cell culture medium. Interleukin (IL)-2 (Chiron) was added to the wells on day 3. On day 5, erythrocytes, dead cells and debris were removed by centrifugation with Lympholyte M (Cedarlane Laboratories). Specific cytolytic activity was determined by a 51Cr-release assay. Target mouse ascites lymphoma lymphoblast EL-4 cells (H-2b) were labelled with 51Cr (100 μCi per well) for 1 h at 37 °C and washed three times. Half of the EL-4 cells were further loaded with ovalbumin peptides. Target cells (2500 cells per well) were then incubated with effector cells at different effector/target ratios in V-bottomed 96-well microtitre plates for 6 h at 37 °C. Supernatant was collected and 51Cr release was quantified using a gamma counter. Specific lysis was calculated using the formula: [(experimental release—the spontaneous release)/(maximum release—the spontaneous release)] × 100. Spontaneous release was determined in control microcultures containing 51Cr-labelled target cells in culture medium with no effector cells. Maximum release was determined by lysing 51Cr-labelled target cells with 0.5 % NP-40. As controls, lymphocytes from mice immunized with PBS and lymphocytes from mice immunized with the ovalbumin CTL epitope/Th peptide were analysed in parallel with those from VLP-immunized mice.

**Secreted alkaline phosphatase pseudovirus neutralization assay.** HPV-16 and bovine papillomavirus 1 (BPV-1) pseudovirions were kindly provided by Dr John T. Schiller. 293TT cells were plated in 96-well tissue culture-treated flat-bottomed plates (Corning Costar) at 30 000 cells per well in 100 μl neutralization buffer (DMEM without phenol red, plus 10 % heat-inactivated FBS, 1 % penicillin/streptomycin/Fungizone and 10 mM HEPES (Invitrogen)). Pseudovirions were diluted 10 000-fold. At these dilutions, target cells typically generated enough secreted alkaline phosphatase (SEAP) for an output reading of 30–70 relative light units (RLU). Diluted pseudovirions were plated in 96-well, untreated, polystyrene plates (Nalge Nunc), included with 20 μl serum diluted 1 : 50, 1 : 100 and 1 : 200 and placed on ice for 1 h. BPV-1 pseudovirions were used as a specificity control and diluted 1 : 12 000. The 100 μl pseudovirion–antibody mixture was transferred on to the pre-plated cells and incubated for 68–72 h. At the end of the incubation, 50 μl supernatant was harvested and clarified at 1500 g for 5 min. The SEAP content in the clarified supernatant was determined using a Phospho-Light Chemiluminescent Reporter Gene Assay System for Detection of Secreted Human Placental Alkaline Phosphatase kit (Applied Biosystems), as directed by the manufacturer, with 15 μl clarified supernatant. Ten minutes after the substrate was added, samples were read in white ChromaluX HB flat-bottomed microplates (Dymex) for 0.20 s per well using a Wallac Victor 1420 Multilabel counter. Serum neutralization titres were defined as the reciprocal of the highest dilution that caused at least a 50 % reduction in SEAP activity. A serum was considered to be positive for neutralization in the HPV-16 assay if it was neutralizing (SEAP production inhibition of >50 %) at a dilution at least 4-fold higher than the titre observed in the BPV-1 neutralization assay.

**RESULTS**

**Identification of residues targeted for mutagenesis.**

The C-terminal truncated HPV-16 L1 major capsid protein lacking 20 aa (GenBank accession no. AF393502.1; Protein Data Bank entry 1DZL) was analysed using 3D structural analysis for identification of putative antigenic structures.

The main criteria for selection of residues for mutation were as follows. (i) Amino acids were to be surface exposed in the 3D models, as illustrated in Fig. 1. In addition, surface probability was estimated using hydrophilicity/hydrophobicity plots (Hopp-Woods) (Table 1). (ii) Amino acids were to be variable among immunologically distinct but phylogenetically closely related HPV types. The selected amino acids were found to be variable among closely related HPV types (Table 1). (iii) Amino acids were to be directed outwards and to have little or no interaction with neighbouring amino acids (as exemplified in Fig. 1). (iv) Mutation of the selected amino acids should have no effect on the 3D structure of the HPV pentamer.
Three lines of investigation were used to investigate the effect of the mutations on the 3D structure. (i) The effect of the mutations on the 3D structure was computer simulated using the Swiss PDBViewer software: none of the mutations seemed to affect the 3D structure. (ii) Gradient fraction densities were determined by refractometry and only protein-containing fractions with the correct density were used in the experiments. (iii) Conformational-dependent mAbs (H16.E70 and H16.V5) were tested for their ability to bind wild-type and mutant VLPs. The conformational-dependent mAb H16.E70 was also found to bind VLPs in which the H16.V5 epitope had been deleted.

We substituted large amino acids in the protein with small residues and expected an effect because of the chemical change. The amino acids Gly, Ser and Ala are uncharged and relatively small amino acids and are predicted to be less antigenic. Ala is weakly hydrophobic and smaller than any other amino acid except Gly. Ala substitution is not expected to have major effects on secondary structure, but will remove any chemically significant side-chain interactions. Mutation to Gly was only performed in regions predicted to have no secondary structure. At position 285, different HPV types contain different residues, although hydrophilicity is maintained. Therefore, we opted for Ser substitution at this residue. At position 348, many amino acids were hydrophobic and we therefore chose Ala substitution. Thus, the following amino acid substitutions were made (Table 1): Asn-56→Gly, Asn-138→Gly, Asn-270→Gly, Asn-285→Ser, Ile-348→Ala, Ser-349→Gly, Thr-350→Gly, Ser-351→Gly, Glu-352→Gly and Thr-353→Gly.

Fig. 1. (a) Structure of the HPV-16 L1\(_{20-474}\) monomer. The FG loop is coloured in orange, the HI loop in green, the DE loop in magenta, the EF loop in red and the BC loop in yellow. Amino acids that were selected to be mutated are space-filled and coloured: aa 56, yellow; aa 138, magenta; aa 270, blue; aa 285, orange; aa 348–353, green. (b) Side view of the structure of the HPV-16 L1 pentamer. (c) Top view of the structure of the HPV-16 L1 pentamer. Amino acids that were selected to be mutated are space-filled and coloured as in (a). The graphics were created by using RasMol software.
Characterization of purified VLPs

VLPs were isolated from insect cells and purified. Fractions were analysed by Western blotting using anti-HPV-16 L1 antipeptide antibody L1-16A or L1-30A (Dillner et al., 1991). The wild-type and H16.V5 epitope-depleted truncated HPV-16 L1 showed bands co-migrating with the HPV-16 L1 control (Fig. 2). The wild-type and H16.V5 epitope-depleted truncated HPV-16 L1 with ovalbumin was slightly larger. Some lower molecular mass bands, presumably degradation products, were also seen (Fig. 2). The truncated HPV-16 L1 migrated similarly to the HPV-16 control. The reason for this is not known but it is possible that there were residual secondary structures affecting mobility.

L1-positive fractions were adsorbed onto carbon-coated grids, stained with uranyl formate and examined by transmission electron microscopy (Fig. 3). The VLPs exhibited a mean diameter of 50–60 nm and were present as defined particles with little or no aggregation. Smaller particles and partially assembled structures were also seen.

Verification of H16.V5 epitope depletion and assembly by ELISA

To verify the assembly of wild-type particles and H16.V5 epitope depletion of mutant particles, mAbs H16.V5 (against the major neutralizing epitope, reacting with intact VLPs of all HPV-16 strains), H16.E70 (another HPV-16 neutralizing mAb that reacts with only some strains of HPV-16) (Roden et al., 1997) and D9 (which binds to disrupted HPV-16 VLPs) were reacted with the different VLPs. The truncated wild-type HPV-16 L1 VLPs and the H16.V5 epitope-depleted HPV-16 bound strongly to H16.E70.

Table 1. Variability, surface probability, antigenic index and hydrophilicity of the amino acids selected for mutation

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Amino acid position</th>
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<tr>
<td></td>
<td>56</td>
</tr>
<tr>
<td>Surface probability*</td>
<td>5.90</td>
</tr>
<tr>
<td>Antigenic index†</td>
<td>1.70</td>
</tr>
<tr>
<td>Hydrophilicity‡</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Amino acid found in§:

- HPV-16
- HPV-33
- HPV-35
- HPV-58
- HPV-31
- HPV-52
- HPV-6b
- HPV-11
- HPV-18
- HPV-45
- HPV-51
- HPV-53
- HPV-56

*Surface probability was calculated by using Wisconsin GCG software according to the formula of Emini et al. (1985).
†Antigenic index is a measure of the probability that a region is antigenic. It is calculated by summing several weighted measures of secondary structure. Wisconsin GCG software was used. The method is according to Jameson & Wolf (1988).
‡Hydrophilicity is calculated according to the algorithm of Hope & Woods (1981).
§Superscript letters indicate the species: a, 9; b, 10; c, 7; d, 5; e, 6. All belong to phylogenetic genus Alpha.
Immunization of C57BL/6 mice with truncated wild-type and H16.V5 epitope-depleted HPV-16 L1 VLPs with and without ovalbumin and analysis of immune sera

In order to investigate the antibody induction of mutant particles, four groups of C57BL/6 mice were immunized subcutaneously with 10 μg of the different VLPs in PBS/CFA. Control mice were injected with PBS/CFA only. Each group consisted of five mice. The mice were boosted on days 14 and 28 with VLPs in IFA and antibody levels were measured by ELISA (Fig. 4). Antibody titres against wild-type VLPs from mice immunized with H16.V5 epitope-depleted truncated VLPs were more than a logarithm lower than from mice immunized with wild-type truncated VLPs.

We concluded that the H16.V5 epitope-depleted HPV-16 L1 VLPs were considerably less immunogenic than wild-type HPV-16 L1 VLPs.

Analysis of human HPV-16 serum reactivity with truncated wild-type and H16.V5 epitope-depleted HPV-16 L1 VLPs

To evaluate the serological reactivity of the H16.V5 epitope-depleted VLPs, a panel of human serum samples from 49 women testing positive for HPV-16 DNA in PCRs of cervical samples (positive controls) and 11 women reporting no sexual experience (negative controls) (Andersson-Ellstrom et al., 1996) were tested for reactivity with control full-length HPV-16 VLPs and truncated wild-type and H16.V5 epitope-depleted HPV-16 VLPs. All VLPs were specifically reactive with the positive control panel (Table 3). Comparison with HPV-16 neutralization data found that antibody reactivities increased with increasing neutralization titres (Table 3). Reactivity with the H16.V5 epitope-depleted VLP was only marginally reduced (Table 3). The serological reactivity of the truncated H16.V5 epitope-depleted VLPs correlated well with the reactivity with similar VLPs containing the H16.V5 epitope (Table 3). The serological reactivity to full-length HPV-16 VLPs also correlated well with the reactivity to truncated HPV-16 VLPs (r = 0.94). The correlation between serological reactivity to full-length HPV-16 VLPs and truncated H16.V5 epitope-depleted HPV-16 L1 VLPs was r = 0.86. Thus, we found that the serological reactivity of H16.V5 epitope-depleted VLPs correlated well with the reactivities of similar VLPs.

Table 2. Reactivity in ELISA of the engineered L1 VLPs with defined monoclonal antibodies

Results are means of absorbance values at 450 nm of three independent ELISAs. NT, Not tested.

<table>
<thead>
<tr>
<th>Antigen: HPV 16 VLPs containing:</th>
<th>mAb H16.V5 (major neutralizing)*</th>
<th>mAb H16.E70 (neutralizing)†</th>
<th>mAb H16.D9 (epitope on disrupted VLPs)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact VLPs</td>
<td>Disrupted VLPs</td>
<td>Intact VLPs</td>
</tr>
<tr>
<td>H16.V5 epitope C-terminal 20 residues Ovalbumin epitope</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>− − −</td>
<td>0.107</td>
<td>0.144</td>
<td>2.931</td>
</tr>
<tr>
<td>− − +</td>
<td>0.100</td>
<td>0.127</td>
<td>NT</td>
</tr>
<tr>
<td>+ − −</td>
<td>1.747</td>
<td>0.124</td>
<td>2.788</td>
</tr>
<tr>
<td>+ − +</td>
<td>0.661</td>
<td>0.155</td>
<td>NT</td>
</tr>
<tr>
<td>+ + −</td>
<td>2.515</td>
<td>0.042</td>
<td>3.121</td>
</tr>
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</table>

*H16.V5 antibody reacts with the immunodominant type-specific epitope exposed on correctly assembled VLPs only. Upon immunization with VLPs, the vast majority of neutralizing antibodies generated can be blocked by the H16.V5 antibody.
†H16.E70 antibody is neutralizing, but most antibodies to VLPs do not react with this epitope.
‡D9 antibody reacts with an epitope exposed on disrupted VLPs but not on intact VLPs.
containing the H16.V5 epitope. The detectable ELISA absorbances were only moderately reduced for the H16.V5 epitope-depleted VLPs.

**Analysis of mouse HPV-16 wild-type and H16.V5 epitope-depleted serum neutralizing ability**

To investigate whether the wild-type and H16.V5 epitope-depleted VLPs were immunogenic for eliciting neutralizing antibodies in mice, groups of five mice each were immunized and their sera tested in a pseudovirion neutralization assay. Serum was considered to be positive for neutralizing antibodies in mice, groups of five mice each were immunized with VLPs lacking the H16.V5 epitope and C terminus; EA: HPV-16 VLPs lacking the C terminus; PBS: I: HPV-16 VLPs lacking the C terminus; EA: HPV-16 VLPs. & I: HPV-16 VLPs containing the H16.V5 epitope. The detectable ELISA absorbances were only moderately reduced for the H16.V5 epitope-depleted VLPs.

**Ability of H16.V5 epitope-depleted VLPs to induce an efficient CTL response**

To test whether H16.V5 epitope-depleted VLPs were able to elicit a CTL response effectively, C57BL/6 mice were immunized subcutaneously with the different VLPs or with PBS/IFA only (negative control) or with the ovalbumin CTL epitope and Th peptide (positive control). Three weeks after the booster immunization of mice and subsequent culturing of the spleen cells, the cytolytic activity against syngeneic EL-4 cells loaded with the ovalbumin CTL epitope peptide was analysed in a ⁵¹Cr-release assay (Fig. 5). The CTL reactivity of the mice immunized with the VLPs containing the ovalbumin epitope was rather low and mice immunized with the corresponding H16.V5 epitope-depleted VLPs had an even lower cytolytic response (Fig. 5).

**DISCUSSION**

Knowledge of the antigenic and immunogenic structure of papillomaviruses is important for the design of prophylactic vaccines and may be important for preparation and quality control of different batches of VLPs to be used for vaccination. New insights may also further the use of VLPs as immunotherapeutic vectors. For example, it may enable the design of VLPs that are not neutralized by neutralizing antibodies to the immunodominant type-specific epitope (naturally occurring or induced by prior immunization).

Here, we successfully designed a strategy for the removal of the major type-specific conformational neutralizing epitope of HPV-16 (as defined by the H16.V5 antibody). Substitution of ten surface-exposed, non-conserved residues from the four major surface-exposed loops resulted in VLPs with barely detectable reactivity with mAb H16.V5.

The outer surface of each L1 pentamer has five broad pockets, created by the hypervariable loops. These are all putative receptor pockets (Chen et al., 2000). Regions within the FG and HI loops have already been proposed as receptor sites for H16.V5 antibody, as shown by the fact that hybrid VLPs with the FG and HI loops transplanted into the HPV-11 L1 protein gained significant binding of H16.V5 antibody (Christensen et al., 2001). H16.V5 antibody also binds to hybrid VLPs containing only the FG loop, but it binds more strongly to hybrid VLPs that contain both FG and HI loops (Christensen et al., 2001), suggesting that the FG loop contains the predominant epitope recognized by H16.V5, but that it requires the HI loop to maintain conformational stability. Our constructs that did not bind H16.V5 contained only two mutations within the FG loop (aa 270 and 285). Christensen et al. (2001) was able to transfer H16.V5 binding by transferring the FG loop; therefore, it is possible that aa 270 and/or 285 are part of the H16.V5-binding site. A single amino acid substitution (F50L) has been reported previously to remove H16.V5 binding (White et al., 1999). However, F50L VLPs are not able to fold correctly and F50 is
predicted to be a buried residue in the particle (Carter et al., 2003). We chose not to study the effect of mutating this amino acid, because we aimed to mutate possible contact residues only and specifically avoided residue changes affecting the 3D structure of VLPs.

We found that a truncated form of HPV-16 lacking the last 20 aa in the C terminus (with or without the H16.V5 epitope) self-assembled into VLPs of similar appearance to full-length VLPs by electron microscopy. Constructs containing an insert with the ovalbumin epitope formed VLPs of the correct size, but also smaller, more irregular, VLPs. Thus, neither H16.V5 epitope deletion nor C-terminal truncation seemed to affect assembly of VLPs. Deletions of up to 30 C-terminal residues has been reported previously to have little effect on the stability of the expressed protein (Chen et al., 2000). However, it has also been reported that truncations of the C terminus may affect VLP formation and that stability may depend not only on the length of the insert but also on the nature of the inserted gene (Liu et al., 2002).

We found that antibody titres to HPV-16 VLPs from mice immunized with H16.V5 epitope-depleted HPV-16 VLPs were at least a logarithm lower than those from mice immunized with similar VLPs containing the H16.V5 epitope. In addition, serum samples from mice immunized with wild-type VLPs were found to be neutralizing in a pseudovirion neutralization assay, in contrast to serum samples from mice immunized with VLPs lacking the H16.V5 epitope. Therefore, we concluded that the H16.V5 epitope-depleted HPV-16 L1 VLPs were considerably less immunogenic than similar VLPs containing this epitope.

We tested neutralization capacity of sera in a pseudovirion neutralization assay and found that sera of H16.V5-depleted VLPs found a high correlation with reactivity with similar VLPs containing the H16.V5 epitope. In addition, serum samples from mice immunized with similar VLPs containing the H16.V5 epitope were at least a logarithm lower than those from mice immunized with H16.V5 epitope-depleted HPV-16 VLPs.

Table 3. Reactivity in ELISA of engineered HPV-16 VLPs with antibodies in human serum and comparison with their neutralization titres

<table>
<thead>
<tr>
<th>Antigen: HPV 16 VLPs containing:</th>
<th>Serum samples from unexposed women [n=11; direct ELISA A450 (1:30)]*</th>
<th>Serum samples from exposed women [n=49; direct ELISA A450 (1:30)]†</th>
<th>Neutralization activity of serum samples from exposed women (n=49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H16.V5 C-terminal epitope 20 residues</td>
<td>Mean A450 Positive (%)</td>
<td>Mean A450 Positive (%)</td>
<td>Negative Positive Low‡ High‡</td>
</tr>
<tr>
<td>+ +</td>
<td>0.033 0</td>
<td>0.359 71.4</td>
<td>0.237 47.3 0.322 85.7 0.536 87.5</td>
</tr>
<tr>
<td>+ −</td>
<td>0.063 9</td>
<td>0.307 67.3</td>
<td>0.218 42.1 0.292 71.4 0.428 81.3</td>
</tr>
<tr>
<td>− +</td>
<td>0.018 0</td>
<td>0.240 48.9</td>
<td>0.139 47.3 0.250 42.9 0.324 62.5</td>
</tr>
</tbody>
</table>

*Serum samples from HPV-16 DNA-positive- (n=49) and from HPV-16-unexposed (n=11) women were selected from previous serological studies (Andersson-Ellström et al., 1996) and tested in ELISAs using VLPs with and without the H16.V5 epitope or the C-terminal 20 residues.

†The cut-off point was set at 0.178, which corresponds to the cut-off used in previous studies (X. Wang, J. T. Schiller, M. Lehtinen & J. Dillner, unpublished data).

‡Sera neutralizing at a 1:50 dilution, but not at a 1:100 dilution, were classified as low-neutralizing serum, and those neutralizing at a 1:100 or higher dilution as high-neutralizing serum.

Fig. 5. Ability of engineered VLPs to induce cellular immune responses in mice. Specific lysis of MHC I-expressing ovalbumin epitope-loaded EL-4 cells by splenocytes from mice immunized with: ▼, ovalbumin synthetic peptide with the CTL epitope and Th epitope; ○, HPV-16 VLPs lacking the C terminus, with ovalbumin; △, HPV-16 VLPs lacking the H16.V5 epitope and C terminus, with ovalbumin; ■, HPV-16 VLPs lacking the C terminus; ●, HPV-16 VLPs lacking the H16.V5 epitope and C terminus. Specific lysis was determined as: [(experimental release−spontaneous release)/(maximum release−spontaneous release)]×100. Differences in specific lysis are shown, obtained by subtracting the lysis of EL-4 targets without peptide. Engineered VLPs had a low immunogenicity in inducing CTLs in C57BL/6 mice.
75% of HPV-16-infected patient sera from binding to HPV-16 virions (Wang et al., 1997), but our current study found that it was possible to separate the H16.V5 epitope from the immunodominant type-specific epitope recognized by human sera. This suggests that the H16.V5-defined epitope is not the most important epitope during natural HPV infection.

The mAb H16.E70 also neutralizes HPV-16 and is directed against a conformational epitope with the variable residue at 282 being critical (Roden et al., 1997). H16.E70 inhibits the reactivity of human sera to HPV-16 VLPs to a much lesser extent than H16.V5 (Wang et al., 1997). The fact that H16.E70 only recognizes some strains of HPV-16 whereas human sera and the H16.V5 antibody appear to react with all viral variants of HPV-16 (Roden et al., 1997; White et al., 1999) implies that the human serum-reactive epitope is distinct from the epitope recognized by H16.E70. In line with this concept, the mutated L1 VLPs that we produced had strongly reduced H16.V5 antibody binding, but binding by H16.E70 was not measurably affected.

We tested whether H16.V5 epitope-depleted VLPs containing ovalbumin effectively elicited a CTL response. A desirable property of VLP-based vehicles is to be able to generate an immunological response towards an introduced peptide, without obstruction from type-specific neutralizing antibodies directed towards the carrier itself. We found that VLPs with the C-terminal 20 aa replaced by an ovalbumin epitope had only low immunogenicity in a CTL assay, which was reduced further for VLPs lacking the H16.V5 epitope. The C-terminal insertion of the ovalbumin epitope appeared to affect the conformation and stability of the VLPs, as evidenced by somewhat reduced H16.V5 binding. Glycosaminoglycans (heparin, heparan sulphate, etc.) are candidate primary receptors for HPV VLPs (Drobi et al., 2003; Giroglou et al., 2001). Modis et al. (2002) have described the structure of the C terminus and suggested that large parts of it are surface exposed and antigenic. Joyce et al. (1999) have suggested that the C-terminal 15 aa are responsible for the interaction with cells through heparan sulphate and identified a conserved region within the last 15 aa of most HPVs that could conceivably be (part of) a heparin-binding motif. Enzymic removal of the C terminus of HPV-11 completely abolished binding to heparin (Joyce et al., 1999), but a similar experiment with HPV-33 did not affect binding (Giroglou et al., 2001). Reduced binding to the HPV receptor means reduced binding to antigen-presenting cells and is therefore likely to affect the ability to induce an effective CTL response. Possibly, the C-terminal truncation could affect the ability to bind to and infect cells. It would be interesting to explore whether alternative strategies to insert epitopes into VLPs that leave the VLP C terminus intact would result in a more effective CTL induction.

In conclusion, we have designed a strategy that can successfully remove a major type-specific neutralizing epitope from HPV-16 VLPs. Our removal of the H16.V5-defined epitope appeared to affect the ability to elicit antibodies in immunized experimental mice, but had a marginal effect on antigenic reactivity with antibodies in sera from infected subjects, suggesting that the H16.V5 epitope and the epitope recognized by human sera can be separated, suggesting that H16.V5 does not define a major immunodominant epitope on HPV-16.

These findings may have important implications for the continued design of VLP vaccines, as well as for monitoring of functional antigenicity of VLP preparations and serological assays for measuring VLP antibody responses.

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