Human Papillomavirus Type 16 Pseudovirions with Few Point Mutations in L1 Major Capsid Protein FG Loop Could Escape Actual or Future Vaccination for Potential Use in Gene Therapy

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Abstract HPV prophylactic vaccination based on VLPs was implemented 7 years ago and has now shown a high degree of efficiency to reduce HPV-induced lesions. Moreover, it was shown that HPV-derived virus-like particles or pseudovirions could be used as gene therapy vectors. As a consequence, characterization of the antigenic structure of HPV capsids is crucial for designing future HPV vaccines with better or broader efficacy and for the design of HPV-derived gene therapy vectors with reduced immunogenicity or vaccination escaping. In this study, we have generated 10 HPV16 FG loop L1 protein mutants and analyzed their ability to self-assemble into VLP, their immunogenicity, and their ability to transduce cells when used as pseudovirions. Most of the mutants had lost their ability to transduce cells at the exception of two chimeric HPV16/31 L1 protein FG loop mutants. Sera from mice immunized with HPV16 L1 wt VLPs very weakly neutralized pseudovirions derived from these two HPV16/31 L1 protein FG loop mutants. These findings suggest that only a few point substitutions within the FG loop are sufficient to generate a new serotype escaping vaccination. As

a consequence, derived pseudovirions might be suitable as gene therapy vectors in vaccinated subjects.

Background

Human Papillomaviruses (HPVs) are small non-enveloped viruses with a double-stranded DNA genome of about 8 kb encapsidated in a structure consisting of 72 capsomers. The viral capsid is composed of the major capsid protein, L1, and the minor capsid protein, L2. Immunization with L1 protein self-assembled into virus-like particles (VLPs) confers type-restricted protection [1–4]. As observed with most vaccines, production of neutralizing antibodies in HPV immunization has appeared to be a key part of the protective immune response, and serum antibodies are, therefore, the most likely effector mechanism. The antibody response is mainly generated against conformational epitopes present on the outer VLP surface that are responsible for neutralizing antibody production [5]. Understanding the antigenic structure of HPV capsids is crucial for the design of future HPV vaccines with better or broader efficacy and HPV-derived gene vectors [6–9] with reduced immunogenicity. One prerequisite for generating such vectors is a greater understanding of viral determinants provoking a neutralizing immune response, in order to design vectors in which the conformational epitopes responsible for the production of neutralizing antibodies are deleted or modified. This strategy was previously used for the production of Adenovirus 5 vectors with genetic exchange of the hypervariable regions of the hexon that

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Table 1 Primers used for chimeric HPV16/31 L1 FG loop mutant genes production

Primer name	Sequence 5′ → 3′
5′HPV16 L1	CCAGATCTATGTCTCTTTGGCTGCCTAGTGAGGC
3′HPV16/31 L1 260–270	CACTTTCACCAACAGTACCACTCCTATTAAAAAAATGTC
3′HPV16/31 L1 273–288/290	TGCAGTAGACCCAGAGCCTTTAATGTATAAATCTGTTGG
3′HPV16 L1	CCAGATCTTTACAGCTTACGTTTTTTGCGTTTAG
5′HPV16/31 L1 260–270	CATTTTTTTAATAGGAGTGGTACTGTTGGTGAAAGTGTACC
5′HPV16/31 L1 273–288	GGCTCTGGGTCTACTGCAACATTAGCCAATTCAAATTAT
5′HPV16/31 L1 273–290	GGCTCTGGGTCTACTGCAACATTAGCCAATTCAACATAT
3′HPV16/31 L1 260–288/290	GTATAAATCTGTTGGTACACTTTCACCAACAGTACCACTCCTATTAAAAAAATG
5′HPV16/31 L1 260–288/290	GAAAGTGTACCAACAGATTTATACATTAAAGGCTCTGGGTCTACTGCAAC

allow evasion of pre-existing humoral immunity [10]. The marketed HPV vaccines are composed of major capsid L1 VLP of HPV16/18 or HPV6/11/16/18. HPV vaccinal immunity is more and more present and will increase difficulty for use of HPV as gene therapy vector in the future. Currently, only young women are vaccinated but vaccination of men is going on in some countries since it can prevent penile, anal, and anogenital warts; and it could prevent oropharynx cancers and the transmission of HPV to their sexual partners. Moreover, future highly multivalent HPV vaccines are under development. A L1 VLP multivalent-based vaccine containing nine HPV Types (HPV 6/11/16/18/31/33/45/52/58) [7], and a pan-HPV L2 base vaccine using minor capsid protein L2, which contain cross-neutralizing epitope provoking cross-neutralizing antibodies against all HPV type [11–13]. Thus, to develop gene therapy vectors escaping current or future HPV vaccination we aimed to develop pseudovirions derived from HPV 16 L1 mutant without the minor capsid L2 protein. These HPV-derived gene therapy vectors could be used in all types of vaccinated people as they could escape L1 vaccination (current vaccine and future nine HPV-type multivalent vaccines) with the FG loop mutation and they could escape pan-HPV L2 base vaccination as they do not contain L2 protein.

Methods

Production of Chimeric HPV16/31 L1 FG Loop Mutant Genes

HPV16/31 L1 260–270, HPV16/31 L1 273–288, and HPV16/31 L1 273–290 mutant chimeric genes were obtained by mutagenesis of HPV16 L1 gene [14] using a two-step PCR protocol. In the first step, L1 N-terminus coding fragments 5′HPV16/31 L1 260-270 and 5′HPV16/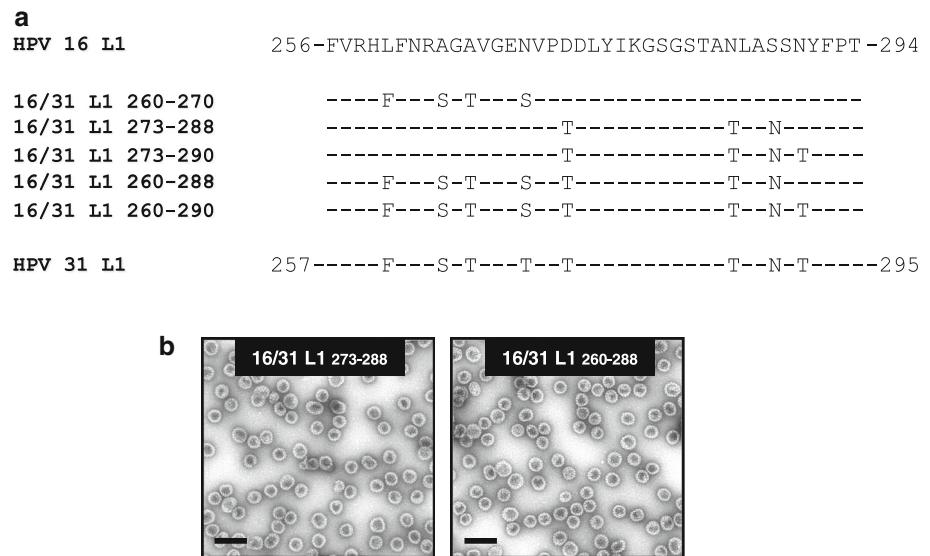31 L1 273–288/290 were generated using HPV16 L1 wt sequence as template, 5′HPV16 L1 with 3′HPV16/31 L1 260–270 or 3′HPV16/31 L1 273-288/290 as primers, respectively (Table 1). L1 C-terminus coding fragments 3′HPV16/31 L1 260-270, 3′HPV16/31 L1 273–288, and 3′HPV16/31 L1 273–290 were amplified using HPV16 L1 gene as template; 3′HPV16 L1 with 5′HPV16/31 L1 260–270 or 5′HPV16/31 L1 273-288 or 5′HPV16/31 L1 273–290 as primers, respectively (Table 1). HPV16/31 L1 260–270, HPV16/31 L1 273–288, and HPV16/31 L1 273–290 mutant genes were obtained in an assembly step using 5′HPV16 L1 and 3′HPV16 L1 primers and the respective overlapping N- and C-terminus coding fragments.

The larger loop mutants HPV16/31 L1 260–288 and HPV16/31 L1 260–290 were obtained using a three-step PCR protocol. In the first step, L1 N-terminus coding fragment 5′HPV16/31 L1 260–288/290 was generated using HPV16 L1 [14] gene as template and 5′HPV16 L1 and 3′HPV16/31 L1 260–288/290 as primers (Table 1). L1 C-terminus coding fragments 3′HPV16/31 L1 260–288 and 3′HPV16/31 L1 260–290 were obtained using 3′ fragments 3′HPV16/31 L1 273-288 or 3′HPV16/31 L1 273-290 (see above) as template, 3′HPV16 L1 and 5′HPV16/31 L1 260–288/290 as primers (Table 1). HPV16/31 L1 260–288 and HPV16/31 L1 260–290 mutant genes were obtained in the third assembly step using 5′HPV16 L1 and 3′HPV16 L1 primers and the respective overlapping N- and C-terminus fragments. Sequences of HPV16 L1 mutants are presented in Fig. 1a.

Generation of Recombinant Baculoviruses and Purification of L1 VLPs

The Bac-to-Bac baculovirus expression system (Invitrogen, Fisher-Scientific, Illkirch, France) was used for expression of the HPV L1 proteins in *Spodoptera frugiperda* cells (*Sf*21, Invitrogen). Recombinant baculoviruses encoding HPV16/31 L1 protein FG loop mutants were generated

Fig. 1 a FG loop sequence of the HPV16/31 L1 mutants expressed. Nomenclature refers to positions in the HPV16 L1 sequence. **b** Purified chimeric HPV16/31 L1 proteins self-assembled into VLPs. VLPs were negatively stained and observed by transmission electron microscopy at 50,000 magnification (*bar* represents 100 nm)


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a
HPV 16 L1          256-FVRHLFNRAGAVGENVPDDLYIKGSGSTANLASSNYFPT-294

16/31 L1 260-270       ----F---S-T---S------------------------
16/31 L1 273-288       ------------------T-----------T--N------
16/31 L1 273-290       ------------------T-----------T--N-T----
16/31 L1 260-288       ----F---S-T---S---T-----------T--N------
16/31 L1 260-290       ----F---S-T---S--T-----------T--N-T----

HPV 31 L1          257-----F---S-T---T--T-----------T--N-T-----295
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b

16/31 L1 273-288

16/31 L1 260-288

according to the manufacturer's instructions. Production of HPV16/31 L1 protein FG loop mutant genes and the corresponding primers are described in the addition file. Baculoviruses encoding HPV16 L1 and HPV31 L1 wild-type genes [15, 16]; HPV16 L1 HBc 266/267 and HPV16 L1 HBc 282/283 genes; and HPV16 L1 270A, HPV16 L1 285A, and HPV16 L1 270/285A genes were generated as described previously [17, 18].

VLPs were produced in *Sf*21 insect cells and purified from the nucleus of the cells by isopycnic centrifugation on CsCl gradient as described previously [16]. CsCl gradient fractions were investigated for density by refractometry and for the presence of L1 proteins by electrophoresis in 10 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Coomassie blue staining. L1-Positive fractions were pooled, diluted in PBS, and pelleted in a Beckman SW28 rotor (3 h, 28,000 rpm, 4 °C). After centrifugation, VLPs were resuspended in 0.15 M NaCl and sonicated by one 5 s burst at 60 % maximum power. Total protein content was determined using Qubit^{TM} Fluorometric Quantitation Protein (Invitrogen). Semi-quantification of L1 protein was determined by signal integration using ImageJ software (NIH, http://rsbweb.nih.gov/ij/download.html) of Western blotting results using anti-L1 antibody CAMVIR-1 (BD Biosciences, Le Pont de Claix, France) which recognizes a linear epitope located at position 204–210 aa in the EF loop of the HPV16 L1 protein [19]. Relative amounts of L1 protein were then recorded by comparison with HPV16 L1 wt Western blotting signal integration. The data presented are the means of three determinations.

Production of VLPs was analyzed by electron microscopy. The preparations were applied to carbon-coated grids and negatively stained with 1.5 % uranyl acetate and observed at ×50,000 nominal magnification with a JEOL 1010 electron microscope. VLP production was semi-quantified by determination of the mean number of particles observed per field (calculated from three to six micrographs). Relative amounts of VLPs were expressed as a ratio of mutant to wt VLPs.

Determination L1 VLP Antigenicity

Microplate wells (Maxisorp, Nunc) were coated with HPV16 L1, HPV31 L1, or with each HPV16 L1 mutant protein in PBS (200 ng/well). After incubation at 4 °C overnight and two washes with PBS–Tween 20 (0.1 %), wells were saturated with PBS supplemented with 1 % FCS for 1 h at 37 °C. Duplicate wells (two tests and one control) were incubated with monoclonal antibodies (MAbs) diluted in PBS 5X–Tween (1 %)–FCS (10 %) for 1 h at 37 °C. The different MAbs used were the H16.V5 MAb that recognized an epitope located on the L1 FG loop [15, 20, 21] and 13 type-specific conformationally dependent HPV31-neutralizing MAbs [22, 23]. MAb dilutions were adjusted to give 1 optical density unit against type-specific HPV VLPs. After four washes, peroxidase-conjugated goat anti-mouse IgG (Fc specific) (Sigma, Saint Quentin Fallavier, France) diluted 1:1,000 in PBS–Tween (1 %)–FCS (10 %) was added to the wells and incubated for 1 h at 37 °C. After four washes, 0.4 mg/ml *o*-phenylene-diamine and 0.03 % hydrogen peroxide in 25 mM sodium citrate and 50 mM Na2HPO4 were added. After 30 min, the reaction was stopped with 4 N H2SO4 and absorbance was read at 492 nm. The data presented are the means of four determinations. Results are expressed as relative binding defined as the reactivity of one Mab to mutant VLPs divided by the Mab reactivity observed with wt type-specific VLPs.

Determination of transduction capacity
of pseudovirions

Pseudovirions were produced by the previously published disassembly–reassembly method [24] with some modifications [25]. L1 VLPs (100 μg) were incubated in 50 mM Tris–HCl buffer (pH 7.5) containing 20 mM DTT and 1 mM EGTA for 30 min at room temperature. At this stage, 10 μg of pGL3-luc (Promega, Charbonnières-les-Bains, France) was added to the disrupted VLPs. The preparation was then diluted with increasing concentrations of CaCl$_2$ (up to a final concentration of 5 mM) in the presence of 10 nM ZnCl$_2$. Pseudovirions were then dialyzed overnight against PBS 1 × and stored at 4 °C before use.

COS-7 cells (10^4/well, ATCC CRL-1651) were seeded in 96-well plates (TPP, ATGC). After 24 h incubation at 37 °C, cells were washed twice before addition of pseudovirions. Pseudovirions serially diluted threefold from 1:3 to 1:7,290 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) were added to the wells, plates were incubated for 3 h at 37 °C, and then 100 μl of complete DMEM (DMEM supplemented with 10 % FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin) was added. After incubation for 48 h at 37 °C, infectivity was scored by measuring the luciferase expressed by transfected cells using the Firefly Luciferase assay kit (Interchim, Montlucon, France) and luciferase expression was quantified using a Multiskan microplate luminometer (Thermo-Fisher Scientific, Courtaboeuf, France). The data presented are the means of 3 determinations performed in duplicate. Results are expressed as relative transduction defined as the transduction of luciferase gene of mutant pseudovirions by the transduction observed with wt pseudovirions.

Immunogenicity of VLP Mutants in Mice

Six-week-old female BALB/c mice (CERJ Janvier, Le Genest St Isle, France) were sub-cutaneous immunized on days 0, 7, and 21 with 10 μg of HPV16 L1 VLPs, HPV 31 L1 VLPs, HPV16/31 L1 260-288 VLPs, or HPV16/31 L1 273-288 VLPs without adjuvant in 0.15 M NaCl (10 mice per group). Two weeks after the last injection, serum samples were collected and stored at −20 °C. All animal procedures were performed according to the approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals, and experiments were approved by the regional animal ethics committee (Approval ID CL2006-047, Comité Régional d'Ethique en matière d'Expérimentaion Animale Centre-Limousin). Neutralizing antibodies were determined by inhibition of luciferase gene transfer using the different pseudovirions. For this purpose, COS-7 cells (10^4/well)

were seeded in 96-well plates. After 24 h incubation at 37 °C, cells were washed twice before addition of the pseudovirion/sera mixture. The amount of pseudovirions was adjusted to obtain a relative luciferase activity of 0.2 relative light unit (RLU). Fifty microliters of diluted pseudovirions were mixed with 50 μl of mouse sera diluted by twofold dilution in DMEM (Invitrogen) to obtain final serum dilutions of 1:12.5 to 1:25,600. After 1 h incubation at 37 °C, the mixture was added to the wells and plates were incubated for 3 h at 37 °C. Then, 100 μl of complete DMEM were added, and the luciferase gene expression was measured after incubation for 48 h at 37 °C as described above. Neutralization titers were defined as the reciprocal of the highest dilution of mouse sera that induced at least 50 % reduction in luciferase activity. Geometric mean titers were calculated for each group. Animals without detectable neutralizing antibodies were assigned a titer of 1 for GMT calculation.

Statistical Methods

Antibodies neutralization titers were compared using the Wilcoxon signed-rank test, a non-parametric paired difference statistical test. Antibodies neutralization titers obtained with homologous HPV type used for mice immunization were compared one by one with antibodies neutralization titers obtained with other pseudovirions. Statistical tests were two-tailed, with a 5 % type I error, and were performed using the XLStat-Life software (Addinsoft, France).

Results

Expression and Characterization of HPV-16 L1 Protein Mutants

The production of HPV16 L1 wild type (wt) and HPV31 L1 wt of two HPV16 L1 mutants with insertion of the hepatitis B core (HBc) DPASRE motif at positions 266/267 and 282/283, and of three HPV16 L1 mutants with amino acid substitution at position 270 and/or 285 has been described previously [18, 19]. The other five HPV16/31 chimeric L1 protein mutants (HPV16/31 L1 260–270, HPV16/31 L1 273–288, HPV16/31 L1 273–290, HPV16/31 L1 260–288, and HPV16/31 L1 260–290) have amino acid substitution in the HPV16 FG loop sequence by the corresponding HPV31 FG loop sequence (Fig. 1a).

The L1 protein yield, as determined by western blotting using CamVir-1 antibody, was similar to that observed with HPV16 wt L1 protein for most of the 10 FG loop mutants, with the exception of the three constructs with point mutations at position 270 and/or 285 and for the

Table 2 Relative production of L1 protein and VLPs with the different L1 mutants, and analysis of their reactivity with H16.V5 MAb and their capacity to transduce cells (all data were given relatively to data observed with HPV16 L1 wt protein)

| L1 protein	Relative amount of L1 protein	Relative amount of VLPs	HPV16.V5 reactivity	Cell transduction[a]
16 L1 wt	100	100	100	100
16 L1 HBc 266/267	107	96	2	0
16 L1 HBc 283/284	120	111	4	0
16 L1 270A	25	18	100	–
16 L1 285A	13	13	100	–
16 L1 270/285A	6	5	40	–
16/31 L1 260–270	1	0	–	–
16/31 L1 273–288	105	86	6	93
16/31 L1 273–290	100	0	0	–
16/31 L1 260–288	116	82	0	50
16/31 L1 260–290	100	0	0	–
31 L1 wt	103	105	0	98

[a] Gene transfer capacity was only investigated in L1 mutants with high production of L1 protein (>25 % of the L1 wt) and with VLP production

–: not tested

chimeric HPV16/31 L1 260-270 mutant (Table 2). VLPs were thus not detected, or only at reduced levels for these four mutants. High levels of well-conformed VLPs were observed for 4 of the other 6 mutants, two with insertion of the HBc motif in positions 266/267 and 282/283, as described previously [18], and two of the chimeric mutants corresponding to insertion of HPV31 FG loop sequences (273–288 and 260–288) within the HPV16 L1 backbone (Fig. 1b; Table 2).

The reactivity of the different L1 mutant VLPs was investigated by ELISA using the H16.V5 neutralizing MAb. Most of the mutants had reduced reactivity (Table 2) suggesting that FG loop mutations lead to modification of the immunodominant neutralizing epitope recognized by the H16.V5 MAb. In addition, no or weak reactivity (less than 1/10 of the reactivity with wt HPV31, data not shown) was observed with 13 type-specific HPV31 neutralizing MAbs.

The ability of the different L1 mutants to transduce cells was investigated by producing the corresponding pseudovirions
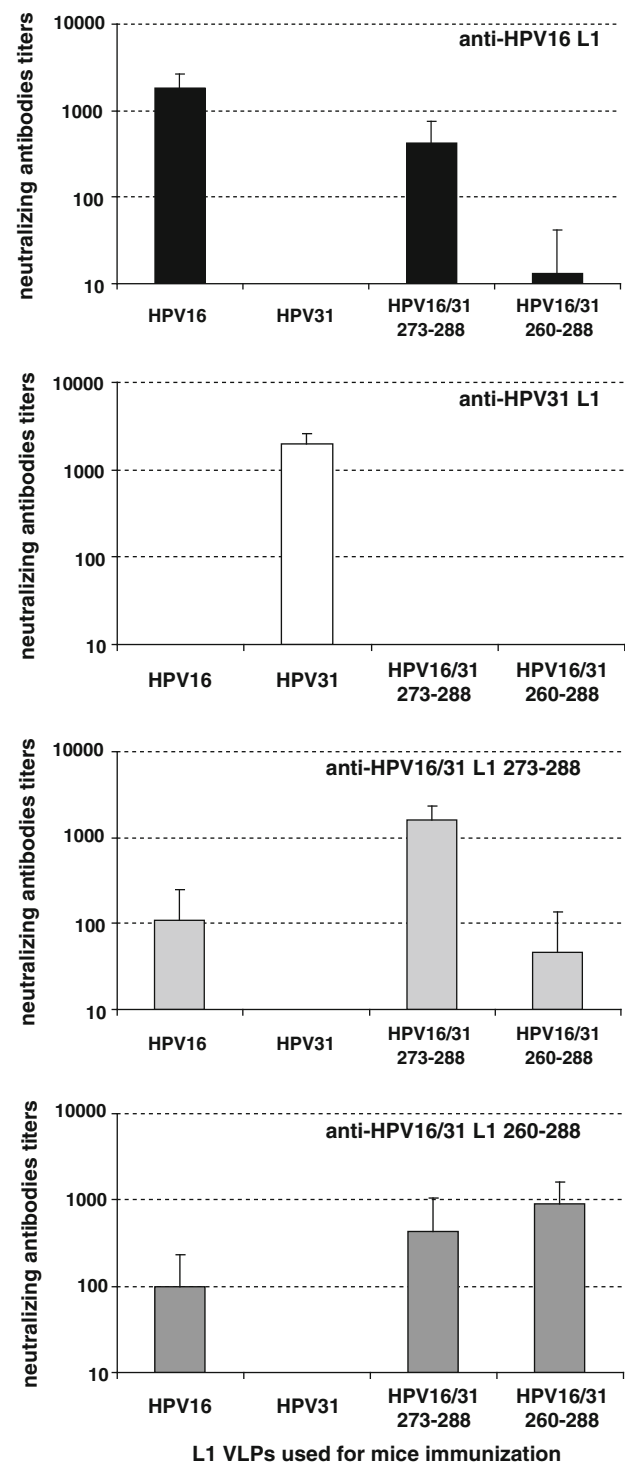
Fig. 2 Neutralizing immune response to HPV16, HPV31, and HPV16/31 chimeric mutant VLPs in mice. Inhibition greater than 50 % was considered positive. Values are the average geometric mean ± SD of the determination of neutralizing titers performed in triplicate in 10 mice per group. Pseudovirions used to detect the corresponding (*filled square*) anti-HPV16 L1, (*open square*) anti-HPV31 L1, (*grey square*) anti-HPV16/31 273-288 L1, and (*dark grey square*) anti-HPV16/31 260-288 L1 in mice immunized with HPV16 L1, HPV31 L1, HPV16/31 273-288 L1, or HPV16/31 260-288

encoding luciferase. All pseudovirion mutants lost their ability to transduce cells, with the exception of the two chimeric HPV16/31 L1 FG loop substitution mutants (HPV16/31 L1 273–288 and HPV16/31 L1 260–288) with similar luciferase expression levels to those observed with HPV16 and HPV31 wt pseudovirions (Table 2).

HPV16/31 Mutants are Distinct Serotypes from HPV16 and HPV31 and the Corresponding Pseudovirions Escape HPV16 Vaccination

To better identify the epitopes present on the chimeric HPV16/31 L1 273–288 and HPV16/31 L1 260–288 VLPs, BALB/c mice were immunized subcutaneously, with these two mutants or with HPV16 or HPV31 wt VLPs. After three injections without adjuvant, neutralizing antibodies were detected using the 4 corresponding L1 pseudovirions.

Sera from mice immunized with HPV31 L1 wt VLPs neutralize neither the two mutant pseudovirions ($p = 0.002$) nor HPV16 L1 wt pseudovirions ($p = 0.002$) (Fig. 2). However, sera from mice immunized with HPV16 L1 wt VLPs very weakly neutralized HPV16/31 L1 273–288 pseudovirions (GMT = 110) and HPV16/31 L1 260–288 pseudovirions (GMT = 100). This was around 18 times weaker than the GMT observed against the homologous type (HPV16) (GMT = 1838, $p = 0.002$) (Fig. 2). Mice immunized with HPV16/31 L1 273–288 VLPs evidenced homologous neutralizing antibodies (GMT = 1,600) and their sera also neutralized HPV16 L1wt and HPV16/31 L1 260–288 pseudovirions but with a fourfold reduced level of neutralizing antibodies (GMT = 425 and 429, respectively, $p = 0.004$). Mice immunized with the HPV16/31 L1 260–288 mutant evidenced no or very low levels of neutralizing antibodies against HPV16 and HPV31 pseudovirions (GMT = 13 and <10, respectively, $p = 0.002$). However, the homologous neutralizing immune response of this mutant was as effective as the homologous neutralizing responses observed in mice immunized with HPV16 or HPV31 wt VLPs. In addition, anti-HPV16/31 L1 260–288 antibodies neutralized the HPV16/31 L1 273–288 pseudovirions with a GMT of 46 (around 1/20 of the GMT observed against homologous pseudovirions, $p = 0.21$).

Discussion

Most of the different HPV16 L1 FG loop mutants we generated evidenced reduced reactivity with HPV16-neutralizing MAbs. In addition, most of them lost their ability to transduce genes although most assembled into VLPs, suggesting that HPV viruses containing such L1 mutations are not infectious. Two chimeric HPV16/31 mutants, HPV16/31 273–288 and HPV16/31 260–288, retained the ability to transduce cells and lost their reactivity to a monoclonal antibody directed against the immunodominant epitope of HPV16. Mice immunized with these two VLP mutants also had a reduced ability to induce neutralizing antibodies against HPV16 and HPV31 pseudovirions, but a strong neutralizing immune response was observed against pseudovirions obtained with these two L1 mutants. These findings also confirmed that the FG loop of HPV16 contained the major neutralizing epitope and suggested that D273T, N285T, and S288N mutations within this loop are together sufficient to generate a new serotype.

HPV pseudovirions are of great interest for their potential use as vectors for gene therapy against cancer [8, 24] or as efficient DNA delivery vectors for DNA vaccine [6, 7, 9, 13, 25]. That is the reason why HPV-derived gene vectors development escaping current and future vaccines is a matter because it is concomitant with HPV vaccine expansion and improvement.

Our result suggests that a few point mutations within the FG loop of HPV16 and HPV31 might lead to the generation of a new serotype that escapes neutralization by anti-HPV16 antibodies in a mouse model. To our knowledge, it is the first time that HPV pseudovirion mutants produced in vitro are still capable to transduce cells and, moreover, lost their reactivity to monoclonal antibody directed against the immunodominant epitope of the HPV backbone. Some mutants have been developed by point mutation in human papillomavirus type 16 L1 hypervariable surface-exposed FG loops and had a reduced reactivity against H16.V5 [26]. Moreover, mice immunized with these mutants develop antibodies that could not neutralize HPV16 wt pseudovirions. However, despite the fact that these mutations abrogated the ability to induce neutralizing and cross-neutralizing antibodies against HPV16 wt, these mutants lost their capacity to incorporate the L2 protein in the capsid and to encapsidate pseudogenomes using 293TT cellular pseudovirions production [27]. In other words, some exposed amino acids residues are important for neutralizing antibodies rising and can be removed to obtain less immunogenic VLPs but at the same time are strictly necessary to obtain effective pseudovirions with the cellular pseudovirions production method. Using disassembly–reassembly method allowed us to produce less immunogenic effective pseudovirions and showed that the disassembly–reassembly method is of interest in a goal of gene therapy. Our chimeric HPV16/31 mutants, HPV16/31 273–288 and HPV16/31 260–288, could be used to develop HPV vectors, and they potentially have the advantage of not being neutralized by pre-existing neutralizing antibodies due to infection or due to immunization with current or future multivalent HPV vaccines.

Whereas these pseudovirions are only composed of L1 protein by disassembly–reassembly method [28] and not

composed of L1 and L2 proteins by cellular assembly method [29], they have still the capacity of gene transfer. This is of great interest because future pan-HPV vaccines could be a pan-HPV L2 base vaccine using minor capsid protein L2, containing cross-neutralizing epitope [11–13]. Although cellular assembly method leads to higher production of pseudovirions with greater cell transduction, L2 protein is required for this production [29–32] and so these HPV gene vectors could not be used in people who will be vaccinated with future pan-HPV L2 base vaccine. The use of L1-only pseudovirions produced by disassembly–reassembly method must also overcome two major issues for use in human therapy. L1/L2 pseudovirions generated in mammalian cells cannot be used for gene therapy drug development because of the impossibility to control the DNA encapsidation and fragment of cellular DNA can be packaged. Whereas the disassembly–reassembly method allows the use of DNAse to eliminate all foreign DNA before adding the plasmid of interest. Another point is that the minor capsid protein L2 contains cross-neutralization-provoking cross-neutralizing antibodies against all HPV types when L2 is injected as L2 protein or polypeptide [11–13]. L2 protein can even lead to development of cross-reactive and even cross-neutralizing antibodies in the context of L1/L2 VLPs or L1/L2 pseudovirions [13]. Thus, for use in gene therapy, with vector re-administration because of transient expression of the gene, HPV-derived vectors should not incorporate L2 proteins but only L1.

In the case of a multivalent base HPV vaccine containing nine HPV Types (HPV 6/11/16/18/31/33/45/52/58) [33], our chimeric HPV16/31 mutants, HPV16/31 273–288 and HPV16/31 260-288, could be used as they are distinct serotypes escaping neutralization by anti-HPV16 antibodies. It is well known that L1 also can give rise to cross-reactive antibodies but unlike L2 protein cross-neutralizing antibodies are not pan-HPV but are raised against related HPV type. For future papillomavirus VLP-derived gene vectors design, these mutants vectors could be derived from not related HPV type included in the actual or future L1 HPV vaccine nine HPV types to avoid totally cross-neutralizing antibodies as it already works well with our 16/31 pseudovirions. Moreover, Faust and Dillner have shown that point mutation in human papillomavirus type 16 L1 hypervariable surface-exposed loops leads to abrogation of the ability to induce cross-neutralizing antibodies [27]. Taken together with our results, we could suppose that our 16/31 pseudovirions could not induce cross-neutralizing antibodies. Another possibility could be the use of animal papillomavirus L1 pseudovirions. However, only few animal papillomavirus pseudovirions have been produced and their transduction capacity were only analysed using human embryonic 293TT cells [34, 35]. Furthermore, their capacity to transduce human cells has not been compared to human papillomavirus pseudovirions. In fact, this strategy will not overcome the problem of neutralizing antibodies production due to vector re-administration for use in gene therapy. For these purposes, panel of chimeric HPV pseudovirions should be developed.

Our work shows that it is possible to develop chimeric L1 FG loop pseudovirions between two HPV types by few point mutations. As a proof of concept, our strategy could be used between many HPV types to produce a panel of chimeric HPV pseudovirions for gene therapy that could escape induced immunity due to the actual or future vaccination or to vector re-administration.

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