**In vitro gene transfer using human papillomavirus-like particles**

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**ABSTRACT**

Recombinant papillomavirus-like particles have recently been shown to be highly effective for the prevention of papillomavirus infections and associated tumors, and a virus-like particle-based vaccine against the most prevalent HPV causing genital infection in humans will be developed in the near future. Another use of these virus-like particles may lie in gene therapy and DNA immunization. We report here that human papillomavirus-like particles composed of the major capsid protein (L1) of HPV-16 are able to package unrelated plasmid DNA *in vitro* and then to deliver this foreign DNA to eukaryotic cells with the subsequent expression of the encoded gene. The results indicate higher gene transfer than with DNA alone or with liposome. Virus-like particles are a very promising vehicle for delivering genetic material into target cells. Moreover, the preparation of the gene transfer vehicle is relatively easy.

**INTRODUCTION**

The efficacy, specificity and safety of the vector systems used for gene transfer are important factors in view of their applications in humans, including gene therapy and DNA immunization. The current techniques for plasmid-based transfer into eukaryotic cells are liposomes (1,2), which could contain viral fusion peptides or whole viruses in view to facilitate the release of DNA into the cytoplasm (3,4). The use of DNA-coated microparticles or the use of a non-viral gene delivery system such as the high-mobility-group 1 (HMGI) chromosomal protein (5), the α2-macroglobulin (6), and peptides were also investigated (7). Alternative means for gene targeting within cells have been developed based on the concept of receptor targeting (8,9). The efficacy of such a delivery system has been enhanced by the addition of or coupling to viruses or virus proteins (10–12). Viruses are in fact a natural and most effective vector system for gene transfer (13). Adenoviruses (14–16), adeno-associated viruses (16–18), Epstein–Barr virus (19) and retroviruses (20–22) have been engineered to carry therapeutic genes and to deliver their own genomes into target cells. Moreover, a tissue-specific gene transfer could be obtained through the cell specificity of the viral cell-receptors (23). However, there are problems associated with the use of viral vectors such as the potential production of replication-competent viruses if a recombination event occurs in the target cells, inactivation of the vectors by preexisting neutralizing antibodies to these viruses in the host and the immunological response induced by the virus (24,25).

Over the past 10 years, it has been shown that the structural protein(s) of many viruses have the ability to self-assemble into virus-like particles (VLPs) when expressed in eukaryotic or prokaryotic expression systems and papillomavirus VLPs from different genotypes have already been obtained (26). Papillomaviruses are members of the *papovaviridae* family. They are non-enveloped icosahedral DNA viruses which infect humans as well as a variety of animals. The papillomavirus virion capsid consists of two proteins, L1 and L2. L1 is the major capsid protein and when expressed in eukaryotic expression systems is able to self-assemble into virus-like particles (VLPs) (27,28). The L2 protein has been reported to be involved in DNA binding and encapsidation (29). HPV VLPs have a receptor for numerous cells including red blood cells (30–33). The results indicate that HPV VLPs bind to a wide range of cell types, the highest level of binding being observed with epithelial and mesenchymal cells. Binding to cells has recently been suggested to be through an α6-integrin receptor (34).

In the present study, we have built a synthetic gene vector system using recombinant pseudo-viruses composed of the major capsid protein of type 16 papillomavirus self-assembled into virus-like particles. We report here that such particles are able to package unrelated plasmid DNA *in vitro*, and could therefore be used to deliver foreign DNA into a variety of cells with the subsequent expression of the encoded gene.

**MATERIALS AND METHODS**

**In vitro expression of papillomavirus-like particles**

HPV-16 VLPs were obtained by expression of the HPV-16 L1 gene in the baculovirus expression system as previously described (35). Four days post-infection, *Spodoptera frugiperda* (Sf21) cells infected with the recombinant baculovirus were harvested and lysed by 0.5% NP-40 in PBS for 15 min at room temperature (RT). The nuclear fraction was recovered by centrifugation (10 000 g, 15 min, 4°C) and sonicated by three bursts of 15 s at maximum power...

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(Vibra-cell, Bioblock Scientific, Strasbourg, France). The nuclear lysate was loaded onto CsCl, centrifuged to equilibrium in a Beckman SW 28 rotor (20 h, 27 000 r.p.m., 4°C) and harvested in 1.5 ml fractions. Fractions were checked for density and HPV reactivity by ELISA as previously described (36). The positive fractions in the ELISA with density ranging from 1.27 to 1.29 were pooled, diluted in PBS and subjected to ultracentrifugation in a SW 28 rotor (3 h, 28 000 r.p.m., 4°C). The pellet was resuspended in 0.15 M NaCl. Protein content was determined using the MicroBCA Pierce assay. From Coomassie blue staining of the VLPs preparations electrophoresed in SDS-polyacrylamide gels and calculation of the VLPs quantities from the proportion of DNA protected from Benzonaze treatment, it was estimated that the HPV-16 VLPs represent only 50% of the protein content of the VLP preparation used in the following experiments.

**Plasmids**

Two plasmids carrying different reporter genes were used. pGreenLantern plasmid (Life Technologies, Ergany, France) is a 5 kbp plasmid which contains the gene coding for the green fluorescent protein (GFP) from *Aequoria victoria* under the control of the cytomegalovirus promoter. pCMV-β plasmid (Clontech, Ozyme, Montigny le Bretonneux) is a 7.2 kbp plasmid which contains the gene coding for the *Escherichia coli* β-galactosidase. pBlueBacIII (Invitrogen, San Diego, USA), a 10.3 kbp plasmid, was also used in encapsidation studies.

**Disruption of HPV-16 VLPs and refolding**

Disassembly and reassembly of the recombinant HPV-16 VLPs were performed according to a procedure adapted from the procedure described by Colomar et al. (37) for SV40 virions. Briefly, 5 µg of purified HPV-16 VLPs (Theoretically 1.5 × 10^11 VLPs) was incubated in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EGTA and 20 mM DTT in a final volume of 50 µl at room temperature for 30 min. At this step, 1 µg of one of the two expression plasmids in 50 mM Tris–HCl buffer (pH 7.5) and 150 mM NaCl was added to the disrupted VLPs. The preparation was then diluted with increasing concentrations of CaCl2 up to a final concentration of 5 mM and incubated at 20°C for 1 h. The refolded VLPs were treated with 10 IU Benzonase (Merck, Darmstadt, Germany) for 1 h at 20°C to verify whether the DNA plasmid was packaged into the VLPs and not adsorbed on their surface.

In order to evaluate the amounts of packaged plasmid DNA, the encapsidation experiments were performed using 25 µg of VLPs and 5 µg of plasmid. After refolding and Benzonase treatment, the mixture was incubated in the presence of 3% SDS and 1 mg/ml of proteinase K (Appligene, Illkirch, France) for 2 h at 56°C. The same experiments, without Benzonase treatment, were conducted as controls and to evaluate the yield of DNA purification. Plasmid DNA was then phenol-extracted and ethanol-precipitated. Purified DNA was digested using EcoRI or BamHI restriction enzyme for pCMV-β or pGreenLantern plasmid and pBlueBacIII, respectively. Linearized plasmids were diluted by 2-fold dilutions in TE and electrophoresed in 1% agarose gels. Relative amounts of packaged plasmid was determined by densitometry using a Gel Doc 1000 imager and Molecular Analyst® software (BioRad, Hercules, USA).

**Density analysis of refolded VLPs**

Refolded VLPs in the presence and absence of plasmid DNA were loaded onto CsCl, centrifuged to equilibrium in a Beckman SW 41Ti rotor (20 h, 39 500 r.p.m., 4°C) and harvested in 0.4 ml fractions. The density of each fraction was determined with an Abbe refractometer and HPV VLPs were detected by ELISA as previously described (36). Fractions containing VLPs were dialysed against PBS, and plasmid DNA was extracted as before.

**Electron microscopy**

Samples were applied to carbon-coated copper grids and negatively stained with 1.5% uranyl acetate. Photographs were taken at 50 000× nominal magnification and 80 kV acceleration in a JEOL 1010 electron microscope.

**Transfection/infection experiments**

Cells grown in monolayers in DMEM/Glutamax (Life Technologies) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin were seeded in 6-well plates (Nunc, Life Technologies) and grown to 80% of confluence (5 × 10^5 cells/well). Cells were washed twice with DMEM/Glutamax. pCMV-β-VLPs or pGreenLantern-VLPs diluted in 1 ml of culture medium were added to wells and allowed to incubate for 4 h at 37°C. At this step, the VLPs were removed and 3 ml of DMEM/Glutamax supplemented with 10% FCS was added. As a positive control, cells were transfected using the same amount of DNA (1 µg) with 10 µl of Lipofectamine (Life Technologies) according to the manufacturer’s instructions. After 4 h incubation at 37°C, 2 ml of culture medium containing 10% FCS was added. The cells were then incubated for 48 h at 37°C. At this step, cells were investigated for GFP or β-galactosidase activity.

For neutralization experiments, the artificial viruses were preincubated for 30 min at 37°C with PBS containing anti-HPV16-VLPs mouse antiserum diluted from 1/100 to 1/100 000. The anti-VLP titer of such sera is 64 000 as determined by ELISA. The control serum was obtained by immunization of mice with 5 µl of recombinant hepatitis B core particles (HBc) produced in insect cells and adsorbed onto Al(OH)3 as adjuvant (unpublished data). As second control, particles were incubated with target cells in the presence of 10% FCS.

**Cell lines**

Nine cell lines were used for these experiments. There were four human cell lines comprising MRC5 (lung), HeLa (cervix), HuH-7 (liver) and CaCo2 (colon). The NIH 3T3 (embryo) cells were of mouse origin and four other animal cell lines comprising Vero cells (monkey kidney), Cos-7 (monkey kidney), MDCK (dog kidney) and CHO (Chinese hamster ovary) were used.

**Detection of reporter gene activity**

*In situ* β-galactosidase activity determination was performed as described by Müller et al. (36). Quantitative determination of β-galactosidase produced in transfected cell lines was assessed by a β-gal ELISA (Boehringer Mannheim, Dassel, Germany) according to the manufacturer’s recommendations.

For the determination of GFP activity, the number of fluorescent and non-fluorescent cells was determined using FACSscan analysis to measure GFP protein expression. For this purpose, cells were...
treated with trypsin, centrifuged and resuspended in 1 ml culture medium. Cells were analyzed by cell sorting using a FACSsort (Becton-Dickinson, Mountain View, CA, USA). Alternatively, cells were directly observed with a fluorescence microscope and photographed.

RESULTS

In vitro disassembly–reassembly of papillomavirus-like particles

HPV-16 VLPs were produced in Sf21 insect cells using a recombinant baculovirus. VLPs were purified by isopycnic banding in CsCl (35). CsCl gradient fractions containing VLPs were pooled, diluted in PBS and then ultracentrifuged. The pellet was then resuspended in a buffer containing EGTA and DTT and incubated at room temperature for 30 min. Figure 1 shows that in these conditions VLPs (Fig. 1a) were completely disaggregated into structures resembling capsomers (Fig. 1b). Plasmid DNA was then added and the preparation was diluted in a buffer containing 5 mM CaCl₂ and 1% DMSO in order to refold the VLPs (Fig. 1c). Around 75% of the L1 proteins seemed to re-assemble into VLPs in these conditions.

Density shift of VLPs after DNA encapsidation

After reassembly in the presence of pGreenLantern, VLPs were centrifuged to equilibrium on CsCl gradient. Detection of HPV-16 VLPs by ELISA in the different fractions of the gradient revealed two peaks corresponding to densities of 1.28 and 1.31 g/cm³ respectively (Fig. 2A). Refolded VLPs in the absence of plasmid DNA gave only one peak at a density of 1.28 g/cm³ (Fig. 2B). Quantitative analysis indicated that the heavy VLPs represented 40% of total VLPs. Plasmid DNA was detected in the two fractions corresponding to the high density VLPs but not in the three fractions corresponding to the low density VLPs.

Figure 1. Purified HPV-16 virus-like particles (a). HPV capsomers obtained after treatment of VLPs with EGTA and DTT (b). HPV VLPs refolded after reassembly of the capsomer-like structures in the presence of CaCl₂, DMSO and DNA (c). Bars represent 50 nm.

Figure 2. Density in CsCl gradient of refolded VLPs. (A) Refolding in the presence of pGreenLantern. pGreenLantern DNA was searched in fractions containing VLPs. (B) Refolding without any plasmid DNA. ○, CsCl density plot; ◆, HPV-16 VLPs (ELISA OD).
Plasmid DNA encapsidation according to plasmid size

The efficiency of plasmid DNA encapsidation into VLPs was investigated according to plasmid size. We studied the encapsidation of pGreenLantern, pCMV-β and pBlueBacIII of 5, 7.2 and 10.3 kbp, respectively. The amount of plasmid DNA packaged into VLPs (i.e. protected from Benzonase hydrolysis) was compared to the total amount of DNA in the preparation (i.e., non-Benzonase treated VLPs) (Fig. 3). Benzonase treatment of each of the three plasmids used gave rise to undetectable DNA fragments by agarose gel electrophoresis and ethidium bromide staining (data not shown). The results indicated that both pGreenLantern and pCMV-β were protected from Benzonase degradation but this was not the case for pBlueBacIII. Densitometric analysis of electrophoresis profile indicated that 36% of pGreenLantern and 19% of pCMV-β were encapsidated.

In vitro transfer of plasmid DNA via papillomavirus VLPs

Initially, HeLa cells were studied for their sensitivity to pGreenLantern-VLPs. The fluorescence of the GFP produced in the transfected cells was analyzed by FACS analysis. Seventy per cent of HeLa cells transfected by the pGreenLantern plasmid packaged into the VLPs were fluorescent (Table 1). By comparison, no fluorescent cells were observed when the cells were incubated with the plasmid DNA alone or with the plasmid plus the L1 protein assembled into capsomers (Fig. 1b), and 40% of the cells were fluorescent when treated with pGreenLantern plasmid and Lipofectamine. The specificity of the transfer by VLPs was further demonstrated by the fact that Benzonase treatment did not affect the transfer. Moreover, free capsomers are not able to act as vector for gene transfer (Table 1).

The transfer of pCMV-β plasmid was also investigated (Fig. 4). This plasmid was transferred in a proportion of the same order as that evaluated by FACSScan analysis for pGreenLantern (data not shown). The β-galactosidase expression was measured by an ELISA in the cell lysate. The results (Fig. 5) indicate that the expression level correlates with the amount of pCMV-β-VLPs used. An increase in the expression level of β-galactosidase of ∼100 was observed between concentrations of VLP vector of 1 and 10 µg.

Neutralization of VLPs mediated transfection by antisera to HPV-16 VLPs

Serial dilutions of a mouse antiserum directed against HPV-16 VLPs, obtained by immunisation with recombinant VLPs, were incubated with refolded VLPs prior to incubation with Cos-7 cells. As shown in Figure 6, this antiserum completely inhibited the pCMV-β expression at dilutions from 1/100 to 1/10 000. Only 14.5% of gene expression was observed at a dilution of 1/50 000, and at a dilution of 1/100 000 the expression was equivalent to the expression observed without addition of anti-VLPs. This inhibition was specific since sera (at a dilution 1/100) obtained by immunisation of mice with recombinant HBe VLPs did not neutralize plasmid DNA transfer. In addition, incubation in the presence of fetal calf serum did not affect gene transfer. These results indicate a neutralising titer comprised between 50 000 and 100 000 for this mouse sera with an anti-VLP end-point titer of 64 000.

Table 1. Specific gene transfer using pGreenLantern plasmid packaged into HPV-16 VLPs

<table>
<thead>
<tr>
<th>HeLa cells transfected with</th>
<th>Gene transfect (µg)</th>
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<tr>
<td>5 µg of VLP-pGreenLantern</td>
<td>70</td>
</tr>
<tr>
<td>5 µg of VLP-pGreenLantern, Benzonase treated</td>
<td>70</td>
</tr>
<tr>
<td>5 µg of capsomers + pGreenLantern</td>
<td>0</td>
</tr>
<tr>
<td>pGreenLantern + Lipofectamine</td>
<td>40</td>
</tr>
<tr>
<td>pGreenLantern alone</td>
<td>0</td>
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As many cells have been reported to have a surface receptor to papillomavirus, we investigated nine cell lines for their capacity to be transfected by the pCMV-β plasmid packaged into HPV16-VLPs. The results (Table 2) show that all cell lines could be efficiently transfected by this vector and that the β-gal expression level obtained was generally 3–4 times higher than that observed after transfection of the same cells by lipofection, except for HuH-7 hepatocytes which are transfected 10 times more efficiently using liposome than VLPs. For other cell lines the level of expression of β-galactosidase varied from 102 pg/ml in Vero cells to 3142 pg/ml in Cos-7 cells.

DISCUSSION

We report here that recombinant HPV-16 L1 virus-like particles, obtained by expression of the major capsid protein of type 16 human papillomavirus in insect cells, can encapsidate very efficiently a plasmid carrying either a gene for the GFP or β-galactosidase during in vitro disassembly–reassembly of VLPs. Introduction of the plasmid into a variety of cells by these pseudovirions or artificial viruses is easily identified by the accumulation of the gene product, and the number of cells carrying the gene are linearly dependent on the HPV-16 VLP concentration in the transfer assay. The host range of such vectors seems to be large, including human and non-human primate, rodent and dog cells. A recent study assigns the role of papillomavirus cellular receptor to the α6-β4 and α6-β1 integrins (34). These heterodimeric glycoproteins are distributed in epithelial cells (38) and mesenchymal tissues (39), respectively. However, the α6 subunit has a more limited tissue distribution than most integrins. The results of transfection by means of VLPs confirm those of VLP binding to cells obtained by others.
and indicate that HPV VLPs could bind to cells of epithelial and mesenchymal origin. In addition, our results show that HPV VLPs could bind to three cell lines (MRC5, CaCo2 and Vero) other than those previously tested. However, very low β-galactosidase expression was observed with HuH-7 hepatocyte cells, for which Volpers et al. (40) reported binding of VLPs, indicating the presence of α6 integrin at the surface of these cells. Our results would suggest that internalisation of VLPs by cells was not as efficient with HuH-7 cells as with the other cell lines investigated.

The evidence for encapsidation of the plasmid rather than association between the plasmid and the L1 protein was based on Benzonase treatment resistance of VLP-mediated transfer of DNA into cells. However, we have evidenced that the size of the plasmid directly influences the rate of encapsidation. One limitation of the gene-transfer method reported here seems to be the length of the information to be transferred. It is generally accepted that a virus could at most encapsidate DNA of the same length as its own genome. Papillomavirus, with a theoretical 8 kbp capacity of encapsidation and a capsid which is easily obtained by the self-assembly of only one protein, is a good candidate for the construction of artificial viruses for gene transfer. In contrast with results obtained by others (41, 42), our findings indicate that HPV L2 protein is not required for efficient encapsidation of DNA.

Figure 4. Expression of the GFP or β-galactosidase in cells transfected by pGreenLantern and pCMV-βgal plasmids in the presence of Lipofectamine or encapsidated into papillomavirus VLPs.

(30,34,40)
However, two recent publications indicated that L1 alone had the capacity to interact with DNA (43,44) and thus it could be speculated that the encapsidation of foreign DNA is at least partially due to the DNA binding property of L1 protein.

From the results we estimated that between 1/3 and 1/6 of HPV-16 VLPs contained a plasmid, proportions which correlate with the number of VLPs found in the heavy fraction of CsCl gradients. These proportions have to be compared with only 1/25,000 reported by Unckell et al (43) for HPV-33 VLPs expressed in Cos-7 cells containing multiple copies of a marker plasmid. Assuming that VLPs have 72 capsomers, each capsomer being a pentamer of L1 and that VLPs represent only 50% of the protein content of the preparation, it was calculated that 150 VLPs per cell were used for the transfection experiments. From the efficiency of pCMV-β transfection (Fig. 5) in HeLa cells it could be estimated that 6000 VLPs per cell are necessary to observe β-galactosidase expression in comparison to the 5 × 10^7 reported by Unckell et al (43). If it is assumed that 1 out 6 VLPs contain the plasmid, it could be calculated that 1 among 1000 full VLPs succeeds in transfecting the pCMV-β plasmid into HeLa cells.

### Table 2. β-Galactosidase expression in different cell lines after transfection with 5 μg of pCMV-β VLPs

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>β-galactosidase expressed (pg/ml)</th>
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<tr>
<td></td>
<td>Lipofection</td>
</tr>
<tr>
<td>MRC5</td>
<td>79</td>
</tr>
<tr>
<td>HeLa</td>
<td>234</td>
</tr>
<tr>
<td>CaCo2</td>
<td>320</td>
</tr>
<tr>
<td>HuH-7</td>
<td>437</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>65</td>
</tr>
<tr>
<td>Cos-7</td>
<td>487</td>
</tr>
<tr>
<td>Vero</td>
<td>41</td>
</tr>
<tr>
<td>MDCK</td>
<td>35</td>
</tr>
<tr>
<td>CHO</td>
<td>43</td>
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Gene transfer was specifically inhibited by preincubation of VLPs by anti-VLP antisera, in confirmation of the results of neutralization assays obtained with HPV-33 or HPV-16 virion pseudotypes generated in cell culture (42,43). Thus, the HPV pseudovirions described here are also a useful system to detect the anti-papillomavirus neutralizing antibodies. Using VLPs from the most frequent genital HPVs as immunogens, HPV vaccination trials in man are planned in the near future in order to prevent both HPV infection and related cancers. The development of a standardized in vitro neutralization assay using HPV pseudovirions would be a great help in monitoring the immune response to the vaccine and for comparison of the potency of different vaccine preparations or vaccine doses.

The use of virus-like particles for gene transfer has some advantages over the use of recombinant viruses. The production of such pseudovirions is easier than with usual virus vectors and there is less chance of inducing neutralizing antibodies. Moreover, they are safer because no recombination with human papillomavirus DNA is possible, since they do not contain a papillomavirus DNA sequence. With papillomavirus, only low levels of anti-VLP antibodies have been evidenced in humans (45). However, there is no evidence that such antibodies are neutralizing. If the presence of preexisting neutralizing antibodies impairs the use of HPV-16 VLPs, other VLPs and in particular VLPs derived from non-human viruses should be produced for such gene transfer and their applications in humans.

In conclusion, VLPs are highly promising vehicles for delivering genetic material into target cells. They are easy to prepare, transduce cells without disturbing cellular physiology and the efficacy of transduction is determined by the susceptibility of the target cell to vector penetration.

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