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Detection of Neutralizing Antibodies against Human Papillomaviruses (HPV) by Inhibition of Gene Transfer Mediated by HPV Pseudovirions

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The goal of this study was to develop a human papillomavirus (HPV) neutralization assay using HPV pseudovirions generated in vitro. For this purpose, gene transfer efficiency of HPV virus-like particles (VLPs) was improved by using direct interaction between a reporter plasmid and the VLPs. Electron microscopic observation of the interaction between DNA molecules and VLPs revealed that VLPs always interact with a single DNA molecule and that VLPs bind to the end of linearized DNA molecules. An 100-fold improvement in the gene transfer was obtained by simple interaction between a linearized DNA molecule and VLPs. Moreover, direct interaction methods offer the possibility of transferring plasmids a size higher than that of the papillomavirus genome. The approach that we developed to generate HPV-16 and HPV-31 pseudovirions proved to be suitable for testing neutralizing antibodies in human sera both after immunization and after natural infection.

Numerous serological studies have demonstrated that infection with genital human papillomaviruses (HPVs) is followed by a serologic immune response to the viral capsid proteins. Anti-virus-like particle (VLP) antibodies are rarely detected in patients with transient HPV DNA detection (4) but are associated with persistence of DNA detection (8, 9). This immune response persists for many years, is in large part HPV type specific, and is directed against conformational epitopes (6, 7, 27, 38, 39).

Immunization with HPV VLPs generates predominantly type-specific neutralizing antibodies (26, 27). The first test developed for testing neutralizing antibodies was based on the mouse xenograft system (2, 5, 21). However, the number of HPV types that have been grown successfully in this model is very limited, and the technique is time-consuming. The second means to measure neutralizing antibodies is to generate pseudovirions and to measure the in vitro inhibition of gene transfer and expression due to the pseudovirions. Several ways of producing pseudovirions have been developed (30, 35, 37), some of which are easily applicable to a number of HPV types.

Papillomavirions are constituted of an icosahedral capsid which encapsidates a closed, circular, double-stranded DNA of about 8 kbp. These particles are 50 to 55 nm in diameter and are constituted of major L1 and minor L2 structural proteins (10, 20). The L1 protein of HPVs can self-assemble into VLPs (14, 18, 19, 23, 29, 42). Moreover, it has been shown that HPV VLPs composed of L1 or L1 and L2 have the ability to package irrelevant plasmid DNA in cellular (27, 30) and acellular (11, 16, 35) systems. The pseudovirions obtained have the ability to transfer the plasmid DNA into cells, where the reporter gene is expressed.

Entry of these artificial gene delivery vehicles into cells is dependent on the interaction between VLPs and the cell surface. Two putative cell receptors have recently been identified for HPVs. The first is α6 integrin (12, 24), which interacts with an as yet unknown L1 region, and the second is constituted of the cell surface glycosaminoglycans, which interact with the carboxyl-terminal portion of HPV L1 (15).

We previously demonstrated the possibility of packaging DNA into HPV VLPs in vitro to form pseudovirions and that pseudoinfection could be inhibited by anti-VLP antibodies (35). However, the detection of neutralizing antibodies was of limited sensitivity, and such tests could not be used to detect neutralizing antibodies in human sera. In this study, we have increased the level of gene transfer by using a new method of VLP-DNA complex formation and developed a sensitive test to detect the minute amount of neutralizing HPV antibodies that is present after natural infection.

MATERIALS AND METHODS

Plasmids. Three different-sized plasmids coding for luciferase were used: a 9.7-kbp plasmid (pTG11033; Transgene, Strasbourg, France), a 7.1-kbp plasmid (pCMV-Luc; Clontech, Ozyme, Montigny le Bretonneux, France), and a 9.1-kbp plasmid (pCMV-Luc with addition of a 2-kbp DNA sequence). All plasmids were used as circular or linear DNA. The plasmids were linearized with *Eco*RI (7.1 and 9.1 kbp) or *Eco*RV (9.7 kbp) restriction enzyme. The dephosphorylation of linear DNA was achieved by addition of calf intestine phosphatase (Promega).

Production and purification of VLPs. HPV-31 VLPs were expressed in Sf21 cells infected with a recombinant baculovirus encoding the HPV-31 L1 open reading frame and purified according to previously described procedures (34). As a negative control, hepatitis B virus core VLPs were expressed using a recombinant baculovirus encoding the first 144 amino acids of the hepatitis B virus core

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TABLE 1. Gene transfer according to VLP-DNA interaction method and plasmid conformation

pCMV-luc	Method	Luciferase expression (cps/well)			
		No VLPs	HBcΔ VLPs	HPV-16 VLPs	HPV-31 VLPs
Circular	Disassembly-reassembly	15		83	120
	Osmotic shock	14		626	1,252
	Direct interaction	15	18	2,210	4,345
Linear	Disassembly-reassembly	20		287	870
	Osmotic shock	19		1,064	4,606
	Direct interaction	17	19	3,639	12,053

gene (HBcΔ) (36). Production and purification procedures were performed as above.

VLP-DNA complex formation. Three methods were used for complex formation: disassembly-reassembly, osmotic shock, and direct interaction. Disassembly and reassembly of VLPs were performed according to a previously described procedure (11, 35). VLP-DNA complexes were also obtained by osmotic shock according to the method described by Barr et al. (1) for polyomavirus, with some modifications. Ten micrograms of VLPs and 1 μg of DNA were mixed in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.01 mM $CaCl_2$. After 10 min at 37°C, the mixture was subjected to osmotic shock by dilution in 350 μl of distilled water and incubated for 20 min at 37°C.

In the direct interaction method, 10 μg of VLPs and 1 μg of DNA were mixed in 40 μl of 150 mM NaCl and incubated for 30 min at room temperature.

HBcΔ VLPs and bovine serum albumin (BSA) (Sigma) were used as negative controls, and Lipofectamine (Life Technologies) was used as a positive control.

Protection of DNA from DNase. To evaluate the amounts of protected plasmid DNA, the experiments were performed using 10 μg of VLPs and 1 μg of plasmid corresponding to a positive-negative charge ratio of 10:1. After formation, VLP-DNA complexes were treated with Benzonase (Merck, Darmstadt, Germany) as previously described (11). The amount of DNA was quantified using Molecular Analyst software (Bio-Rad, Ivry/Seine, France), and results are expressed as the percentage of Benzonase-protected DNA.

DNA retardation assay. One microgram of plasmid was incubated for 30 min at 25°C in 84 mM NaCl (pH 6) with an amount of VLP corresponding to a VLP-DNA charge ratio ranging from 1:1 to 10:1. Plasmid DNA (1 μg) and increasing amounts of VLPs were each diluted in 10 μl of 50 mM NaCl and then mixed. After 30 min, samples were electrophorezed through a 1% agarose gel in 1× TAE (Tris-acetate-EDTA) buffer.

Electron microscopy. VLP-DNA complexes (charge ratio, 10:1) were dropped onto carbon-coated grids. After 1 min, the excess material was discarded by contact with filter paper. Before drying, the preparations were positively stained by washing the grids with 2 drops of 1.5% aqueous uranyl acetate. After 1 min, the uranyl acetate was removed and the grids were washed with MilliQ-grade water. Grids were observed in dark-field mode at ×25,000 nominal magnification with a JEOL 1200 EX electron microscope. The results are expressed as the ratio of the number of VLPs bound to DNA to the total number of VLPs observed in three randomly chosen fields.

Transfection experiments. Cos-7 cells grown in monolayers in Dulbecco's modified Eagle's medium (DMEM)/Glutamax (Life Technologies) supplemented with 10% fetal calf serum (FCS), 100 IU of penicillin, and 100 μg of streptomycin per ml were seeded in 96-well plates (Nunc; Life Technologies). After washing with serum-free medium, pCMV-luc/VLPs diluted in 50 μl of culture medium were added to each well. After 1 h of incubation at 37°C, the complexes were removed, and 50 μl of DMEM/Glutamax supplemented with 10% FCS was added. Cells were then incubated for 48 h at 37°C. Luciferase gene expression was measured by luminescence assay (luciferase reporter gene assay with constant light signal; Roche Molecular Biochemical, Meylan, France). The luminescence was integrated over 10 s (Victor^2; Wallac, Perkin Elmer), and results were expressed as counts per second (cps) per well.

Detection of neutralizing antibodies. Neutralization of the pseudovirions with anti-VLP antibodies was investigated by inhibition of gene transfer. Anti-VLP antibodies diluted 1:1,600 or human serum diluted 1:20 in phosphate-buffered saline (PBS) was added to pseudovirions and incubated at 37°C for 30 min before addition to the cells. Cells were then incubated for 48 h at 37°C, and luciferase gene expression was measured by luminescence assay. Results were expressed as the percentage of inhibition of luciferase activity. Neutralizing antibodies were considered to be present for inhibition greater than 80%.

Immune sera against HPV-16 and 31 VLPs were obtained by subcutaneous immunization of five mice with each of the VLP preparations. Eight- to 10-week-old female BALB/c mice (IFFA Credo, St. Germain l'Arbresle, France) received three doses of 5 μg of HPV VLPs combined with aluminum hydroxide as the adjuvant at 7-day intervals, followed by a booster dose 14 days later. Blood samples were obtained 2 weeks after the last injection by retroorbital puncture. Anti-VLP antibodies were titered by enzyme-linked immunosorbent assay (ELISA). Neutralization was also investigated with CamVir-1 (Pharmingen, Newcastle, England) and H16.V5 and H16.B20 monoclonal antibodies (7).

Serum samples. Selected sera obtained from cervical cancer patients and controls from Colombia were investigated for HPV-16 neutralizing antibodies. Twenty cervical cancer patients were selected for the presence of HPV-16 DNA in cervical scrapes, and half of them were anti-HPV 16 VLP-positive on ELISA (34). A group of 20 HPV DNA-negative women with normal cervical cytology were included in the analysis. Half of these women were anti-HPV-16 VLP positive and half of them were negative. These women are a subset of the women nested within a large ongoing prospective study on cervical neoplasia and genital HPV infections conducted at the Gynecological Outpatient Clinic of the Liga Colombiana de Lucha contra el Cancer in Bogotà. These women were included in the study as age-matched control subjects without evidence of ongoing HPV infection and were selected to match the age distribution of the cancer cases by 5-year age groups.

Moreover, sera from 20 children aged 6 to 12 months were obtained at the pediatric clinic of the La Misericordia Hospital in Bogotà and used as negative controls for the detection of neutralizing antibodies. The Ethics Committee of the National Cancer Institute of Colombia approved the study design.

To verify the specificity of the neutralization test, anti-HPV antibodies were used, including anti-VLP antibodies obtained by immunization of 10-week-old mice with recombinant HPV-16 or HPV-31 VLPs and three anti-HPV-16 monoclonal antibodies: CamVir 1, H16.V5, and H16.B20.

RESULTS

Investigation of the efficiency of three VLP-DNA interaction methods for gene transfer. As reported previously for HPV-16 VLPs, HPV-31 VLPs can be disassembled and reassembled in the presence of a reporter plasmid and transduce Cos-7 cells, resulting in expression of the packaged reporter gene (Table 1). In order to improve the efficiency of gene transfer by HPV VLPs, we investigated two other methods of complexing a 7.1-kbp plasmid coding for luciferase with HPV VLPs. The results indicated greater efficiency of direct interaction, with luciferase expression increasing from 1.2×10^2 cps with the disassembly-reassembly technique to 1.2×10^3 with osmotic shock and 4.3×10^3 cps by direct interaction.

In order to determine whether plasmid conformation has an effect on gene delivery, experiments were also performed with linear plasmid. Direct interaction showed the highest luciferase expression compared to the other methods, with a value of 1.2×10^4 cps. It must be noted that increased gene transfer was observed when the plasmid was linearized, whatever the method used (Table 1). Gene transfer with Lipofectamine induced luciferase activity of 1.2×10^4 cps with circular DNA and 3.8×10^2 cps with linear DNA. No gene transfer was observed in the absence of HPV VLPs or with HBcΔ VLPs.

In order to confirm the specificity of the gene transfer, VLP-DNA complexes obtained by direct interaction were incubated with anti-VLP antibodies before Cos-7 cell transfection. Incubation with anti-HPV-31 VLP antibodies totally abolished gene transfer with HPV-31 pseudovirions, but did not significantly reduce (5%) gene transfer with HPV-16 pseudovirions. Accordingly, incubation of pseudovirions with anti-HPV-16 VLP antibodies reduced gene transfer of HPV-16 pseudovirions and HPV-31 pseudovirions by 90 and 19%, respectively.

As reported previously, papillomavirus capsids have 8 kbp of capacity for DNA encapsidation. We therefore investigated the capacity of gene transfer by using a plasmid larger than the HPV genome (9.1 or 9.7 kbp). No protection from DNase treatment was observed with the 9.7-kbp plasmid by the disassembly-reassembly method, but 45% protection was achieved with a 7.1-kbp plasmid. DNA protection by osmotic shock was observed with the 9.7- and 7.1-kbp plasmids, with values of 51 and 53%, respectively, and by the direct interaction method, with values of 70 and 67%, respectively.

Using the direct interaction method, luciferase activity was 1.2×10^4 and 5.7×10^4 cps with the 7.1-kbp and 9.1-kbp linearized plasmids, respectively.

We also investigated the formation of HPV-31 VLP-plasmid complexes by monitoring their electrophoretic mobility as a function of VLP-DNA charge ratios by retardation assay on an agarose gel. The electrophoretic mobility of DNA was completely abolished for a charge ratio (positive/negative) of 10:1. The lack of migration suggests the formation of a large complex between VLPs and DNA. In all subsequent experiments, a charge ratio of 10:1 was used. The effects of pH and NaCl and $CaCl_2$ concentrations on VLP-DNA complexes in gene transfer were investigated. The gene transfer, measured as luciferase expression after direct interaction of pCMV-luc with HPV-31 VLPs, increased from 4.9×10^1 to 1.6×10^3 cps/well when the pH rose from 3 to 6 and then decreased to 4.3×10^2 cps at pH 7.

Gene transfer was maximal when complexes were formed in the presence of 84 mM NaCl at pH 6, with luciferase activity of 4.4×10^3 cps, and then decreased slowly with higher NaCl concentrations, to reach 5.1×10^2 cps with 340 mM NaCl.

In order to find the optimal concentration of calcium ions for efficient gene transfer into Cos-7 cells using pCMV-luc, VLP-DNA complex formation was performed in 84 mM NaCl (pH 6) with increasing $CaCl_2$ concentrations. The highest gene transfer was observed when complexes were formed in the absence of $CaCl_2$. Increasing the $CaCl_2$ concentration in the VLP-DNA complex buffer induced inhibition of gene transfer, which reached a plateau of around one third of the original luciferase expression for $CaCl_2$ concentrations higher than 10 μM.

When the ideal conditions for VLP-DNA interaction were used (84 mM NaCl and pH 6), gene delivery was increased 100-fold (1.2×10^2 to 1.2×10^4 cps/well) compared to the original method, consisting of packaging of the DNA after disassembly and reassembly of HPV VLPs.

Analysis of VLP-DNA complex by electron microscopy. We investigated the formation of VLP-DNA complexes by electron microscopy after mixing VLPs with circular or linearized 7.1- and 9.7-kbp plasmids at a positive-negative charge ratio of 10:1. HPV VLPs exhibited a very high affinity for circular 7.1-kbp DNA molecules, since 93% of HPV VLPs were observed in contact with DNA molecules (Fig. 1a and b). By comparison, only 3% of HBcΔ VLPs were observed to bind to DNA. Similar results were observed with a 9.7-kbp plasmid. It must be noted that HPV VLPs always bound to only one DNA molecule and that the DNA molecules appeared be 40 to 70% smaller than DNA molecules alone (Fig. 1c).

With the linearized plasmids of 7.1 and 9.7 kbp, VLPs were observed to be associated with 80 and 95% of the DNA molecules, respectively. Moreover, the VLPs were mainly located at the end of the DNA chain (Fig. 1d). In addition, the size of the DNA chain was decreased by 50% (Fig. 1d) to 90% (Fig. 1e) compared to DNA molecules not associated with HPV VLPs (Fig. 1f). As it was possible that the VLPs interacted with the phosphate present at the end of the linearized DNA, the experiments were also performed using dephosphorylated linear DNA. No significant reduction in gene transfer was detected after removal of the phosphate (2.6×10^3 cps versus 2.4×10^3 cps), and the VLPs always bound to the end of DNA molecules, as observed by electron microscopy.

Detection of neutralizing antibodies in immunized mice and in naturally infected women. Neutralization of gene transfer by anti-HPV L1 antibodies was investigated with HPV-16 and HPV-31 pseudovirions generated by the direct interaction method using a linearized plasmid. All tests were done at least twice in duplicate. Neutralization was not observed with two monoclonal antibodies (HPV 16.B20 and Camvir1) which bind to linear epitopes. With monoclonal antibody HPV16.V5, directed against a conformational epitope of HPV-16, a neutralization titer of 6,400 was observed, but no neutralization was observed with HPV-31 pseudovirions at a dilution of 1:25 (Table 2).

Hyperimmune mouse sera were obtained by immunizing 8- to 10-week-old mice with HBcΔ, HPV-16, and HPV-31 VLPs produced in insect cells. No neutralization was observed with the anti-HBc antibody. Anti-HPV-16 VLP antibodies had an autologous neutralizing titer of 3,200 but also inhibited HPV-31 pseudovirions at a titer of 100. Similarly, anti-HPV-31 antibodies neutralized HPV-31 pseudovirions at a titer of 12,800 but also neutralized HPV-16 pseudovirions up to a 1:50 dilution.

Due to the high sensitivity of the test, we also investigated the possibility of detecting HPV-16 neutralizing antibodies in human sera after natural infection (Table 3). No HPV-16 neutralizing antibodies were detected in 20 6- to 12-month-old children. Among 25 selected HPV DNA-negative women from the general population of Colombia, neutralizing antibodies were not detected whether they were positive or negative for anti-VLP antibodies. Among 31 selected women with invasive cervical cancer, 12 (39%) had evidence of neutralizing antibodies. These antibodies were not detected in those without anti-HPV-16 VLP antibodies and were present in 67% of those with anti-HPV-16 VLP antibodies.

DISCUSSION

One aim of this study was to improve HPV VLP-mediated gene delivery. Thus, in addition to the previously described VLP disassembly-reassembly method, two other procedures were investigated to form VLP-DNA complexes, osmotic

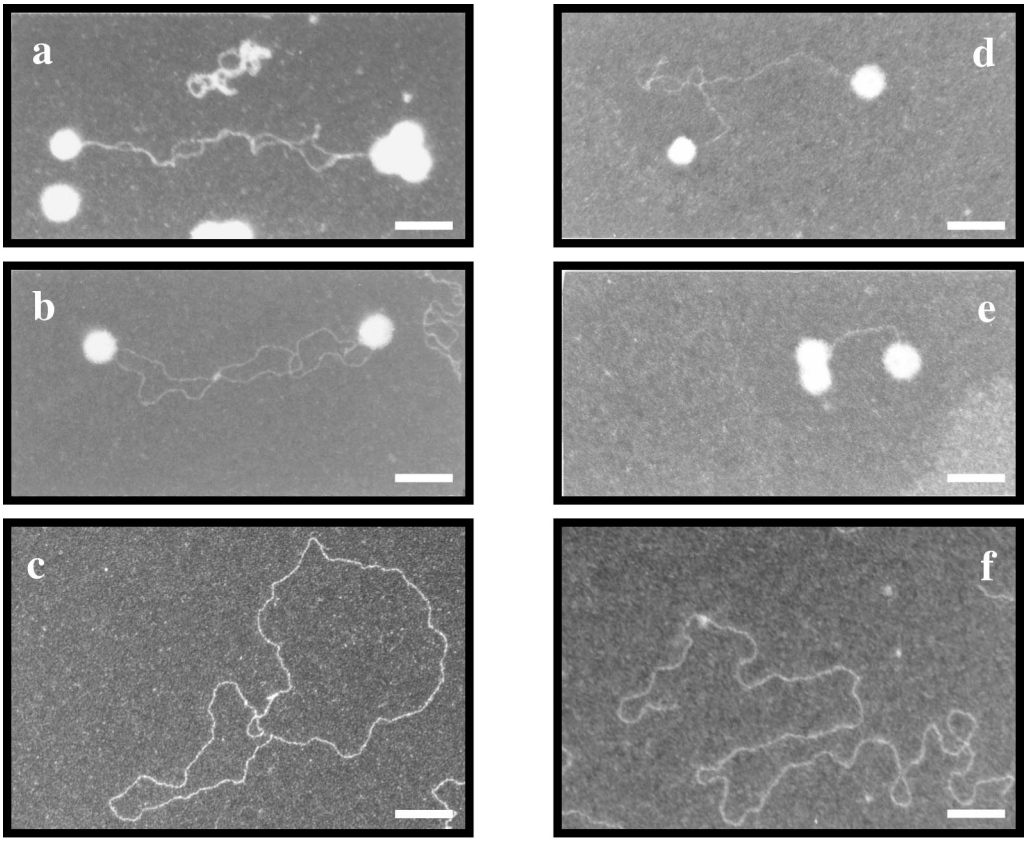

FIG. 1. Electron micrographs of interactions observed between HPV-31 VLPs and heterologous DNA under circular (a and b) and linear forms (d and e) Bar, 100 nm. Bottom row, control DNA circular (c) and linear (f) molecules.

shock and direct interaction. The highest level of gene transfer was observed when complexes were formed by direct interaction between the plasmid DNA and VLPs. Moreover, it must be noted that DNA protection from Benzonase degradation could be obtained with a plasmid of 9.7 kbp, a size exceeding the size of the HPV genome, in contrast to what is achieved by packaging the DNA into VLPs after disassembly and reassembly. Accordingly, high levels of gene transfer could be obtained with a 9.1-kbp plasmid. This offers an additional advantage to the direct interaction method, allowing larger genes of interest to be transfected.

In addition, a 10-fold increase in gene transfer efficiency was observed with linearized plasmid compared to circular plasmid. In contrast, Kreiss et al. (22) observed that gene transfer is higher with circular DNA than linear DNA when using lipoplexes. We observed similar results when using Lipofectamine, for which circular DNA yielded the highest levels of gene transfer. It thus appears that the topology of the transfected DNA molecules determines the level of gene expression and depends on the vehicle used.

As pH, ionic strength, and Ca^{2+} concentration are important factors in the assembly process of the capsids of many viruses, including polyomavirus and papillomavirus (3, 25, 31, 32), the influences of these factors on VLP-DNA complex formation were investigated. When the interaction was performed at pH 6 with a positive-negative charge ratio of 10:1 in a buffer containing 84 mM NaCl and with a linearized plasmid, a level of luciferase activity corresponding to 3.4×10^4 cps/mg of protein was observed. This is 100- to 200-fold higher than the level observed using the disassembly-reassembly method.

Electron microscopy analysis confirmed that HPV L1 VLPs exhibit a very high affinity for both circular and linear DNA molecules. This interaction results in shortening of DNA molecules, which was particularly evident with linearized plasmids. This suggests partial packaging of the DNA or DNA condensation. With circular DNA, the VLPs bind along their entire length. The interaction and possible encapsidation take place at both ends of the molecule with linearized DNA. It could be suggested that the binding of the VLPs to the end of linear DNA molecules is due to the presence of a free phosphate group at the end of the linearized molecule. However, phos-

TABLE 2. HPV neutralizing titer of anti-L1 antibodies

Anti-VLPs or MAb	Neutralizing antibody titer	
	HPV-16	HPV-31
Anti-HPV-16 VLPs	3,200	100
Anti-HPV-31 VLPs	50	12,800
Anti-HBcΔ VLPs	<25	<25
H16.V5 MAb	6,400	<25
H16.B20 MAb	<25	<25
CamVir -1 MAb	<25	<25

TABLE 3. Detection of HPV-16 neutralizing antibodies in human sera

Group	Anti-VLP result	No. with neutralizing antibodies to HPV-16/total (%)
General population		0/45 (0.0)
Children (6–12 months old)	Negative	0/20 (0.0)
HPV DNA-negative women	Negative	0/15 (0.0)
	Positive	0/10 (0.0)
Cervical cancer patients		12/31 (38.7)
HPV-16 DNA-positive women	Negative	0/8 (0.0)
	Positive	11/17 (64.7)
HPV DNA-negative women	Negative	0/5 (0.0)
	Positive	1/1 (100)

phate groups seem not to be involved in this interaction, since phosphatase treatment did not reduce the gene transfer or the binding of the VLP to the end of the DNA molecule (data not shown).

These results are similar to those described by Stokrova et al. (33) and Forstova (13) for the packaging of DNA by osmotic shock using polyomavirus VLPs. The similarities suggest that in the osmotic shock technique, the gene transfer is more probably due to the direct interaction between DNA and VLPs rather than packaging of the DNA molecule. With HPV-31 VLPs, we observed that gene transfer was lower with osmotic shock than by direct interaction. This result might be due to the conditions of the osmotic shock, since we demonstrated the significance of NaCl concentration and pH in the VLP-DNA interaction.

Stokrova et al. (33) concluded that the length of apparently packaged DNA after osmotic shock as observed by electron microscopy is in agreement with the percentage of DNA protected from DNase degradation. Similar results were observed with HPV-31 VLPs, since the reduction in size was 40 to 70% and DNA protection was 67 to 70%. However, we did not observe any reduction in gene transfer efficiency after DNase treatment of the complexes. If protection was due to partial packaging of the DNA, a reduction in gene transfer would be observed after DNase treatment. We therefore suggest that the reduction in plasmid size was due to compaction of the DNA molecule after interaction with the VLPs. This DNA compaction could result in modifications making the molecule inaccessible to DNase degradation.

On the other hand, our results obtained with papillomavirus and those observed for polyomavirus (33) also suggest that modification of the VLP conformation may be the consequence of the interaction of the VLPs with one DNA molecule. Such interaction might prevent further DNA binding, since, in contrast to what was expected, we never observed two or more DNA molecules attached to one VLP, even if HPV VLPs contained 360 L1 able to bind to DNA.

The high level of gene transfer obtained by the direct interaction of VLPs with a linearized plasmid makes detection easier and more sensitive to neutralizing antibodies. The neutralizing antibody test that we have developed seems to be highly specific, since the monoclonal antibodies directed against the major neutralizing epitope of HPV-16 did not neutralize HPV-31 pseudovirions. However, when mice are immunized against HPV-16 or HPV-31 VLPs, they develop high titers of autologous antibodies and low titers of cross-neutralizing antibodies. This suggests the existence of a common neutralization epitope in the L1 protein of HPV-16 and HPV-31, in addition to the common neutralization epitope recently identified in L2 protein (17, 28). The existence of a common neutralizing epitope in these two L1 proteins is not surprising, since HPV-16 and HPV-31 show high homology, with 83% amino acid sequence identity.

The high sensitivity of the test makes possible the detection of neutralizing antibodies in human sera after natural infection. This is the first report of the detection of such antibodies after natural genital infection, and this finding suggests that this test is more sensitive than others developed for the detection of neutralizing antibodies (38, 40). The test seems to be specific, since neutralizing antibodies were not detected in children or in HPV DNA-negative, anti-HPV-16 VLP-negative women. However, neutralizing antibodies were not detected in anti-VLP-positive, HPV DNA-negative women from the general population who could be considered individuals who have recovered from infection.

In order to verify that the neutralization was due to anti-VLP antibodies, immunoglobulin G (IgG) from three positive serum samples was purified by protein A-Sepharose affinity chromatography. The purified IgG fractions were all found to neutralize HPV-16 pseudoinfection.

Among women with invasive cancer, an increased proportion of neutralizing antibodies were detected among both anti-VLP-positive and -negative women, with the highest frequency observed among cancer patients with evidence of both HPV-16 DNA and anti-HPV-16 VLP antibodies. These findings correlate with the detection of anti-VLP antibodies, their frequency of detection being increased in patients with the highest grade of lesions (8, 9). The detection of HPV-16-neutralizing antibodies seems to be linked to the detection of HPV-16 DNA and the presence of anti-HPV-16 VLPs in these patients. These findings also suggested that anti-VLP antibodies detected in HPV DNA-negative individuals from the general population are not neutralizing. These results could also be due to the fact that neutralizing antibodies were only a fraction of the anti-VLP antibodies or eventually to the fact that the neutralizing antibody test was less sensitive than the anti-VLP test.

In addition, we also investigated neutralizing antibodies in human plasma samples. All plasma samples investigated induced neutralization of the pseudovirions. Considering that the plasma samples were obtained in heparanized tubes, it was suspected that the small quantity of heparin present in the tubes was sufficient to inhibit gene transfer, since it is known that heparin blocks the fixation of VLPs to cells (14). To test this hypothesis, we injected PBS buffer containing 2% BSA in a heparanized tube. The PBS-BSA did not neutralize HPV-16 pseudovirions. However, the same preparation completely inhibited gene transfer after being in contact with the heparanized tube. This indicates that plasma could not be used for the detection of HPV-neutralizing antibodies.

In conclusion, a 100-fold improvement in gene transfer was obtained by simple interaction between a linearized DNA molecule and VLPs. Moreover, the direct interaction method of-

fers the possibility of transferring plasmids larger than the papillomavirus genome. These results confirm the potential of artificial viruses based on recombinant papillomavirus VLPs for in vitro and in vivo DNA delivery purposes. Moreover, the approach that we developed to generate HPV-16 and HPV-31 pseudovirions proved to be suitable for testing neutralizing antibodies in human sera, both after immunization and after natural infection.

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