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NHPV16 VLP Vaccine Induces Human Antibodies That Neutralize Divergent Variants of HPV16

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Genital HPV genotypes are generally distinct serotypes, but whether variants within a genotype can represent serologic subtypes is unclear. In this study we used serum from human volunteers vaccinated with HPV16 L1 VLPs from variant 114K, to examine cross-neutralization of variants from each of the five major phylogenetic branches of HPV16. Recombinant Semliki Forest virus-derived pseudovirions for each variant were generated and combined with serum from vaccines, and the mixture was monitored for infectivity in a standard C127 cell focal transformation assay. Sera from all 10 VLP-immunized individuals had neutralizing activity against each of the variant pseudovirions. For each of the sera, variant titers differed by only fourfold or less from the median titer. Therefore, from a vaccine perspective, HPV16 variants belong to a single serotype. Vaccination with HPV16 114K L1 VLPs generates antibodies that should confer a similar degree of protection against all known phylogenetic branches of HPV16.

Key Words: neutralization; HPV16; variants; pseudovirions; vaccine; VLP; serotypes; papillomavirus; immunoprophylaxis; clinical trial.

INTRODUCTION

Cervical cancer is one of the most frequent causes of cancer deaths in women worldwide (Pisani *et al.,* 1999), and human papillomavirus (HPV) has been implicated as the major etiological agent for this disease (IARC, 1995). Therefore a vaccine that can prevent transmission of the virus, and thereby prevent cervical cancer, is a desirable goal. Prior to human vaccine trials with HPV, animal vaccine studies using animal papillomaviruses showed that parenteral immunization with virus-like particles (VLPs) composed of the L1 major capsid protein is effective in protecting animals against experimental challenge with a homologous genotype, but not a heterologous genotype, of virus (Breitburd *et al.,* 1995; Christensen *et al.,* 1996b; Kirnbauer *et al.,* 1996; Suzich *et al.,* 1995). The L1 VLPs possess the immunodominant neutralization epitopes of the virus, and passive transfer studies with immune serum and immune IgG strongly suggest that protection is mediated primarily by neutralizing antibodies (Breitburd *et al.,* 1995; Suzich *et al.,* 1995). Immunization of animals with HPV L1 VLPs also induces high titers of *in vitro* neutralizing antibodies (Lowe *et al.,* 1997; Roden *et al.,* 1997; Smith *et al.,* 1995; Unckell *et al.,* 1997; White *et al.,* 1998). The encouraging results in animals led to the initiation of human clinical trials of vaccines that may prevent transmission of HPV (Hines *et al.,* 1998; McNeil, 1997; Schiller, 1999; Sherman *et al.,* 1998).

In humans, several HPV types have been detected in cervical cancers (Bosch *et al.,* 1995), and it would be important to protect against as many of these types as possible. Although there are no animal models for HPV infection, animals have been vaccinated with VLPs of various HPV types to assess the degree of capsid antibody cross-reactivity between HPV types. Results from ELISAs (Kirnbauer *et al.,* 1994), inhibition of mouse erythrocyte agglutination (HAI) (Roden *et al.,* 1995), *in vitro* neutralization of authentic virus (Christensen and Kreider, 1990; White *et al.,* 1998), and *in vitro* neutralization of pseudovirions (Roden *et al.,* 1996a) support the conclusion that sera raised against VLPs can neutralize the corresponding HPV type, but generally do not neutralize other types. Exceptions to this are the limited cross-neutralization seen between some very closely related types such as HPV16 and 33; HPV18 and 45; and also HPV6 and 11 (Christensen *et al.,* 1994; Roden *et al.,* 1996b; White *et al.,* 1998). Because of the lack of cross-reactivity between types, a VLP-based vaccine will probably have to include several HPV types. HPV16 was chosen as an initial model for some VLP-based vaccines, since it is the HPV type found most frequently in cervical cancer, present in about 50% of the tumors (Bosch *et al.,* 1995; Kirnbauer *et al.,* 1993).

Numerous HPV16 variants have been isolated from different geographic regions, and evidence suggests that the evolution of these variants coincides with the migratory patterns of early humans (Ho *et al.,* 1991). The variants have been grouped according to nucleotide ho-

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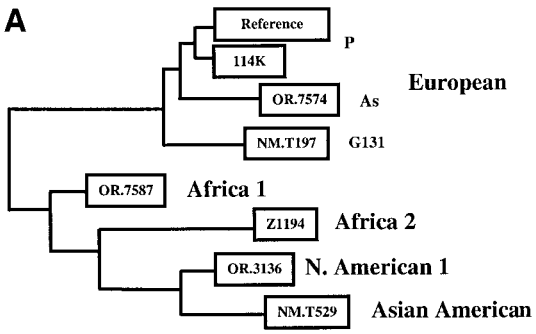
mology and have been placed along five major phylogenetic branches (or classes) (Chan *et al.,* 1992; Yamada *et al.,* 1995). Nucleotide differences that result in amino acid changes for L1 in 85 HPV16 variants map closely to the principal neutralization epitopes of monoclonals V5 and E70 (Chen *et al.,* 2000), which might suggest that the variants were established to escape neutralization. In support of this model, monoclonal E70 was able to neutralize European (114K) but not Africa 2 (Z-1194) pseudotype virions (Roden *et al.,* 1997), and a single amino acid change can result in the loss of V5 reactivity (White *et al.,* 1999). However, other studies suggest that at least some cross-reactivity between variants is possible: sera from women with naturally occurring HPV16 infections recognized VLPs of HPV16 variants 114K and Z1194 (Cheng *et al.,* 1995), and another study with human sera also confirmed cross-reactivity between divergent variants (Touze *et al.,* 1998). Regardless of what mechanism induced the divergence between HPV16 variants, it is important to determine whether the mutations affect antibody-mediated neutralization. It is difficult to predict the effect of a given mutation on neutralization, since the immunodominant neutralizing antibodies recognize conformational epitopes (Christensen *et al.,* 1994; Christensen and Kreider, 1990; Roden *et al.,* 1994).

In addition to the possibility of providing insight into the selective pressures that may contribute to the phylogenetic diversity between HPV16 variants, determining the degree to which immunization with L1 VLPs of one variant may induce antibodies that cross-neutralize other HPV16 variants has important implications for the design of vaccine efficacy trials and for vaccine valency. If an HPV16 L1 VLP vaccine were deficient in neutralizing HPV16 variants outside its own phylogenetic branch, it would be necessary in an efficacy trial to power the trial according to the expected number of HPV16 infections by variants within that single branch, or to use a multivalent vaccine that contained HPV16 VLPs from several branches. The issue of poor cross-neutralization would be of particular concern for a monovalent HPV16 VLP vaccine that was being tested in a population where the variant in the vaccine was not from the predominant branch found in that population (Ho *et al.,* 1993). On the other hand, the demonstration of efficient cross-neutralizing activity across the various phylogenetic branches of HPV16 would imply that a monovalent vaccine would display a similar degree of protection against the entire spectrum of HPV16 variants.

Therefore, we sought in this study to determine whether sera from a recent trial of human volunteers immunized with VLPs of the European variant 114K (Clayton Harro, Johns Hopkins University, manuscript submitted) were able to neutralize representative variants from each of the five phylogenetic branches of HPV16. To make this determination, we first produced a series of infectious pseudotype virions that contained BPV1 genomes, which had been packaged by HPV16 L2 and the HPV16 L1 capsid protein from a specific variant (HPV/BPV pseudovirions). We then used a previously described neutralization assay (Roden *et al.,* 1996a) to assess human sera from normal volunteers who had been immunized with L1 VLPs from a single HPV16 variant for their ability to neutralize the infectivity of this series of HPV/BPV pseudovirions.



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Amino Acid Position			76	176	181	191	282	353	389	474
Variant Class		Variant name								
EUROPEAN	Subclass P	114K	H	T	N	I	S	T	T	L
	As	OR.7574			T					
	G131	NM.T197				L				
AFRICA 1		OR.7587	Y	N						F
AFRICA 2		Z1194	Y	N	T		P	P		F
N. AMERICAN 1		OR.3136	Y	N				P		F
ASIAN AMERICAN		NM.T529	Y	N	T			P	S	F

FIG. 1. (A) HPV16 phylogenetic tree. Modified from Yamada *et al.* (1995). Constructed from E6, L2, L1, and LCR sequences. Only those variants used in this study are shown. The horizontal length of each branch is proportional to the number of steps (reconstructed point mutations) along that branch. The tree shows variant classes in larger font, and European lineage subclasses P, As, and G131 in smaller font. (B) Divergence in L1 amino acid sequence for HPV16 variants. Positions are shown only for those amino acids that differ from variant 114K. Amino acids at those positions are shown for 114K using standard single-letter amino acid designations. For other variants alternate amino acids are shown, and empty spaces indicate the same amino acid as 114K.

RESULTS

Assembly into VLPs and generation of pseudovirions from seven HPV16 variants

To assess the potential for cross-variant protection, we selected a total of seven HPV16 variants: one variant from each of three European lineage subclasses and one from each of the four other phylogenetic classes. These variants are depicted in a phylogenetic tree (Fig. 1A) modified from Yamada and coworkers (1995). The tree was originally constructed from the sequences of E6, L2, and L1 of 30 isolates. For the current study, only changes in the L1 sequence are relevant. When com-

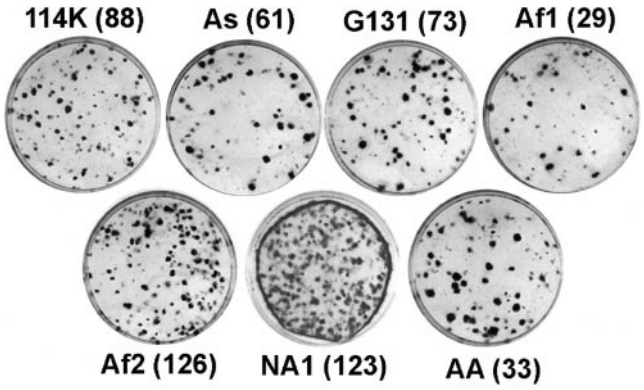

FIG. 2. Focus formation induced by HPV16 pseudovirion variants in C127 mouse cells. Pseudovirions (HPV16 capsid/BPV genome) carrying variant L1 capsids were used to infect C127 monolayers, which were grown 14–17 days postinfection and stained for visualization of transformed foci. The variant name is shown above or below its corresponding dish; numbers in parenthesis indicate the average number of foci per dish from four dishes for each variant.

pared to 114K L1, the most divergent variants were Af2 and AA, each having six amino acid substitutions (Fig. 1B). The least divergent were As and G131, which belong to subclasses of the same branch as 114K. The sequence of 114K L1, the European variant used in the vaccine, was originally reported as having an A at nucleotide position 6432. Our laboratory, and others (White *et al.,* 1999), have since found that position to be a G. This change results in an alanine instead of a threonine at amino acid 266.

L1 amino acid sequence can influence the efficiency of VLP assembly (Sasagawa *et al.,* 1995; Kirnbauer *et al.,* 1993; Touze *et al.,* 1998), and can presumably also affect the production of infectious pseudovirions. 114K and Z1194, from the European and Africa 2 variant classes, respectively, have been used previously in *in vitro* neutralization assays (Roden *et al.,* 1997) and were shown to yield similar numbers of focal transformation events. However, for variants representing Af1, NA1, AA, As, and G131, it was not known whether they would self-assemble into capsids and would therefore be predicted to provide the L1 functions required for generating infectious pseudovirions. We thus first expressed each L1 in BHK-21 cells, using a Semliki Forest Virus system (Roden *et al.,* 1996a). The resulting L1 proteins were purified by sequential sucrose cushion and cesium chloride isopycnic density gradient centrifugation (Kirnbauer *et al.,* 1993) and the assembly into VLPs verified by transmission electron microscopy (data not shown).

We then determined whether L1 from the variants could participate in the formation of infectious pseudovirions. BPHE-1 cells contain multiple episomal copies of the BPV1 genome. The formation of infectious pseudovirions in BPHE-1 cells requires L1 and L2 coassembly for packaging the BPV1 genome (Roden *et al.,* 1996a). We used the L2 gene from 114K for the production of all pseudovirions, rather than create an SFV for L2 from each variant and coexpressing it with its cognate L1 gene.

Thus, BPHE-1 cells were coinfected with an SFV that expressed an L1 variant and an SFV that expressed the 114K L2, and pseudovirion production was measured by testing the ability of lysates from these cells to induce (BPV1 genome-dependent) focal transformation in C127 mouse fibroblasts (Dvoretzky *et al.,* 1980). All L1 variants were found to be able to use 114K L2 and to give rise to infectious pseudovirions, as reflected by this bioassay (Fig. 2). The number of foci induced from a given amount of lysate was quite similar for each variant, of which the highest number (Af2) was about four times greater than the lowest (Af1). Analysis of additional independently derived preparations of pseudovirions indicated that these differences in focus forming activity were not variant-specific; coinfection of BPHE-1 cells with the same recombinant L1 and L2 SFV stocks in separate assays could lead to substantially differing numbers of infectious particles (data not shown).

It was previously reported that the level of L1 expression can affect VLP yields (Touze *et al.,* 1998). To examine whether the relative levels of L1 or L2 might correlate with the infectivity of a given preparation, extracts of the pseudovirion lysates used in Fig. 2 were analyzed by immunoblot for the presence of L1 and L2 (Fig. 3). The highest levels of L1 expression were found with G131 and Af1; however, these two variants produced intermediate and low numbers of foci, respectively. Conversely, the two preparations with the lowest levels of L1 expression (K and As) did not induce the lowest numbers of foci. Thus, L1 did not seem to be the limiting factor for infectious pseudovirion formation. The L2 used in these experiments was from a single variant (114K), and its expression levels also did not correlate with pseudovirion formation. L2 was expressed at low levels in the Af2 coinfections, and at high levels in NA1 coinfections, but they yielded similar numbers of foci (126 and 123, respectively).

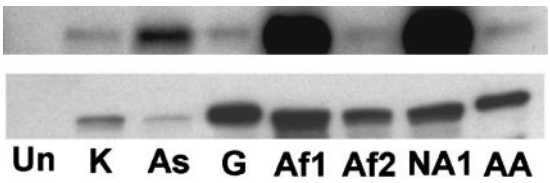

FIG. 3. SFV-infected BPHE-1 cells express L1 from each variant and 114K L2. After coinfection with recombinant L1 and L2 SFV, BPHE-1 cells were lysed by sonication. Protein extracts were subjected to 10% SDS–polyacrylamide gel electrophoresis, transferred onto PVDF membranes, and immunoblotted for the presence of L2 (top panel) or L1 (bottom panel). For L2, rabbit anti-114K L2-GST polyclonal serum was used. For L1, monoclonal antibody CAMVIR-1 (McLean *et al.,* 1990) was used. This monoclonal antibody recognizes a linear epitope which is identical in all variants.

TABLE 1

Neutralizing Titer of 114K L1 VLP Vaccine Volunteer Sera against Homologous and Heterologous Variants

Volunteer	114K	As	G131	Af1	Af2	NA1	AA
25	640	2560	640	2560	640	640	640
26	640	2560	640	2560	640	640	640
27	640	2560	640	2560	640	640	640
28	2560	2560	2560	2560	2560	2560	2560
29	<40	<40	<40	<40	<40	<40	<40
30	640	2560	2560	2560	640	640	2560
31	10,240	10,240	10,240	10,240	2560	10,240	10,240
32	160	640	160	640	160	160	640
33	<40	<40	<40	ND	<40	<40	<40
34	2560	2560	2560	2560	640	2560	2560
35	2560	2560	2560	2560	640	2560	2560
36	640	2560	640	640	160	160	640
V5[a]	$\geq 1 \times 10^6$	$\geq 1 \times 10^6$	1×10^5	$\geq 1 \times 10^6$	1×10^5	1×10^5	$\geq 1 \times 10^6$
No. of foci[b]	88	61	73	29	126	123	33

Note. Neutralizing titers for each vaccine volunteer are shown for variants from each of the five phylogenetic branches of HPV16 (E, Af1, Af2, NA1, and AA) and for two lineage subclasses of the European branch (As and G131). Volunteers 25–28, 30–32, and 34–36 were immunized with 114K L1 VLPs, while Volunteers 29 and 33 were placebo-vaccinated. ND, not determined.

[a] Neutralizing titer of monoclonal V5 (Christensen *et al.,* 1996) for the various variants.

[b] Average number of foci seen for each variant from four positive control plates.

Neutralization of HPV16 variants with sera from volunteers of a 114K L1 HPV16 VLP vaccine trial

Our laboratory is currently evaluating a prophylactic HPV16 L1 VLP vaccine. Sera from volunteers immunized with the 114K L1 vaccine have been shown to neutralize 114K L1 HPV16 pseudovirions in *in vitro* assays (Clayton Harro, Johns Hopkins University, manuscript submitted). To assess the degree to which the antibodies generated by the vaccine would neutralize other HPV16 isolates, the sera were used to carry out titrations of *in vitro* neutralization of the infectious pseudovirion variants described above. The sera tested were from 10 volunteers who had been immunized with three 50-μg doses of VLPs without adjuvant, and from two control subjects from the same study who had received the placebo (saline solution). The serum samples were those that had been taken 5 months after initial vaccination, 1 month after the third vaccination.

To assess the neutralizing activity, the sera were diluted 1:40 and the ability of fourfold serial dilutions to neutralize a standard virus inoculum for each was determined (Table 1). Figure 4 shows the neutralization assay results for a serum which gave a neutralization titer of 640. As expected, sera from the placebo-vaccinated volunteers (29 and 33) was unable to neutralize pseudovirions from any variant, even at the highest concentration tested (1:40). By contrast, all of the sera from the volunteers who had been immunized with vaccine were able to neutralize all variants tested, although the sera included those with a strong neutralizing response (i.e., 31) and those with a relatively weaker one (i.e., 32). Furthermore, the calculated titers appeared to depend primarily on the serum being tested, rather than on the pseudovirion variant. Compared with the titer obtained with the 114K variant (the variant used as the vaccine trial), the titer of any individual serum was similar regardless of the variant examined, differing by no more than a single fourfold dilution from what was seen with 114K. The preimmune sera from all of the volunteers lacked neutralizing activity at 1:40, the highest concentration tested.

Another way of evaluating similarities between variants is to measure the ability of neutralizing monoclonal antibodies to bind the variants and to neutralize the infectivity of the pseudovirions. In HPV16 ELISA assays, neutralizing monoclonal V5 (Christensen *et al.,* 1996a)

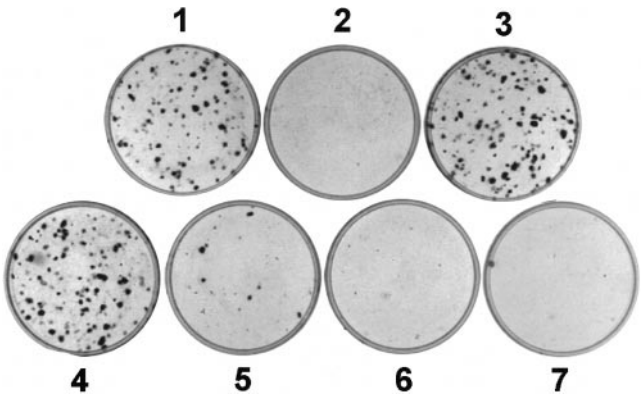

FIG. 4. Neutralization of pseudovirion infection by human immune serum. BPHE-1 lysates (250 μl) containing 114KL1/L2 infectious pseudovirions were combined with PBS (1), 1:40 dilution of placebo vaccinated serum (3), or 1:2560 (4), 1:640 (5), 1:160 (6), 1:40 (7) serum dilution of a 114K L1 VLP vaccinated volunteer and used to infect C127 cells, which were grown and processed as described in Fig. 2. Negative control (PBS without pseudovirions) (2).

was previously shown to block approximately 75% of the reactivity in sera from naturally infected patients (Wang *et al.,* 1997), suggesting that it may recognize an immunodominant epitope. To determine the presence or absence of known and/or dominant conformational epitopes in the variants used in this study, neutralizing monoclonals V5 and E70 were tested for their ability to recognize the variants in VLP-based ELISAs (Kirnbauer *et al.,* 1994). Except for the lack of recognition of Af2 by E70, as previously noted (Roden *et al.,* 1997), all other variants were recognized by both antibodies (data not shown). The ability of V5 to neutralize all seven variants was also tested. V5 efficiently neutralized all variants (Table 1). Thus, with rare exception, the variants behaved similarly in terms of binding the monoclonal antibodies and their ability to be neutralized by the sera from the vaccinees.

Influence of pseudovirion inoculum size on neutralization titer

By convention, the neutralization titers for HPV16 are the reciprocal of the highest dilution of serum able to block infection from a constant amount of virus (Roden *et al.,* 1996a; White *et al.,* 1998). However, the amount of virus has not been standardized as a defined unit (e.g., 50 infectious particles), which might be used to compare results between laboratories, in part because the particle-to-infectivity ratio may vary (Roden *et al.,* 1996a). Preliminary *in vitro* neutralization assays using monoclonal V5 and 114K pseudovirions suggested that neutralization titers differed if the number of particles used in an assay was varied. In addition, when the number of foci produced by each variant, which varied from 30 to 130 (Fig. 2 and Table 1), was considered, a trend was also observed when the neutralization titers of all vaccine volunteers were compared to the 114K neutralization titers. Neutralization titers were the same as or higher than those seen with 114K if fewer foci were produced by a particular variant, while the titers were the same as or lower than those seen with 114K if more foci were produced by a particular variant.

To experimentally examine the relationship between the size of the virus inoculum and neutralization titers, we used a single stock of 114K pseudovirions and varied the size of the inoculum used in the neutralization assay. Fourfold serial dilutions of serum from a single HPV16 VLP vaccine volunteer were combined with either high or low numbers of the pseudovirion focus-forming units before adding the mixtures to C127 monolayers. By this assay, an eightfold increase in the number of pseudovirions used resulted in an apparent 16-fold reduction of the neutralizing titer (Table 2). To confirm that this effect was neither variant-specific nor serum-specific, we performed similar neutralization experiments with the AA variant (one of 114K's more distantly related L1 variants) and another serum. Results similar to those obtained with 114K were also seen with AA, although the titers against the two variants cannot be compared directly, since limited serum availability dictated that the sera used for each variant be different. A 10-fold increase in the inoculum of the AA variant resulted in a 16-fold reduction in the neutralizing titer for a vaccine volunteer's serum. In both variants an increase in the virus inoculum resulted in an increased number of focal transformation events and a decrease in apparent neutralizing titer, although the relationships were not strictly linear. These results demonstrate that the calculated neutralization titer depends on the virus inoculum. They also suggest that at least some of the differences in neutralization titer seen between variants (Table 1) may be more apparent than real, since the neutralization titer tended to correlate inversely with the relative number of focus-forming units in the inoculum used for a particular variant.

TABLE 2

Effect of Pseudovirion Level on Neutralizing Titers

114K			AA		
Volume of virus (ml)	Number of foci	Neutralizing titer	Volume of virus (ml)	Number of foci	Neutralizing titer
0.2	50	2560	0.025	52	640
1.6	150	160	0.250	>200	40

Note. Stocks of BPHE-1 lysates containing infectious 114K or AA pseudovirions were made. Different volumes of the indicated pseudovirion stock were combined with fourfold serial dilutions of serum from 114K L1 VLP vaccine volunteers and used to infect C127 monolayers. For each variant, the first column shows the volume of pseudovirions used for infection; the second column shows the number of foci observed when no serum was added; and the third column shows the determined neutralizing titer for that volume of the pseudovirion preparation.

DISCUSSION

In this study we have determined that immunization with L1 VLPs of a European HPV16 variant induces antibodies that are able to neutralize representative variants from all of the five phylogenetic branches of HPV16. This result suggests that from a vaccine perspective, the spectrum of HPV16 variants form a single serotype and that vaccination with 114K L1 VLPs is likely to confer a similar degree of protection against most HPV16 variants worldwide.

We found that clinical isolates from the five branches (Yamada *et al.,* 1995) were able to self-assemble efficiently into infectious pseudovirions and could utilize a common L2, that of a European variant, to encapsidate the BPV1 genome. This observation implies that, although amino acid substitutions in L1 for a given HPV16

variant are associated with specific substitutions in L2 (Yamada *et al.,* 1995), the L1 substitutions do not prevent the L1 protein from interacting productively with L2 from a different variant. We did note some differences in pseudovirion yields, but the differences were not variant-specific. There was sufficient variation in the yields obtained with different preparations of the same variant that we attribute most of the variation in our system to the complex interaction between the two separate SFV L1 and L2 viruses and the BPV genomes in the BPHE-1 cells, rather than to sequence-specific differences in L1 expression (Touze *et al.,* 1998) or to putative differences in the interactions between the 114K L2 and L1 from other variants. The production of infectious pseudovirions requires that a given BPHE-1 cell be coinfected with both SFV viruses, and infection by one SFV virus can interfere with superinfection by another SFV virus (Singh *et al.,* 1997). In other studies, we have noted that such dual infection occurs only in a minority of the BPHE-1 cells (Day *et al.,* 1998).

The cross-neutralization assays indicated that all sera from volunteers immunized with L1 VLPs from the 114K European variant had neutralizing activity against all the HPV16 variants. There was a correlation between the relative neutralization titer of a given serum against the 114K pseudovirions and its relative titer against the other variants, with the neutralizing activity against the 114K variant being no more than one fourfold dilution higher than that against the other variants.

It is likely that even the fourfold differences observed in neutralization titer against variants other than 114K may be largely a consequence of variables in the neutralization assay, rather than an indication of lower neutralizing activity against particular variants. In principle, neutralization by the sera from volunteers immunized with 114K L1 VLPs should be a function of the amount of properly assembled L1 protein in a pseudovirion preparation. However, it is difficult to determine precisely what proportion of L1 in a given preparation is sufficiently self-assembled to bind the neutralizing antibodies. In addition, different preparations, even of the same variant, may have different particle-to-infectivity ratios, which would also affect the apparent titer, although this ratio is difficult to determine (Roden *et al.,* 1996a). This variable could explain, at least in part, why we did not find a strict correlation between the amount of L1 in a preparation and its infectivity as measured by the focal transformation assay. We also determined experimentally that the apparent neutralization titer varied inversely with the size of the pseudovirion inoculum, and the variants with lower titers in Table 1, compared with those against 114K, tended to be those with higher numbers of focus-forming units. We therefore believe that although a fourfold difference in titer against the same variant in the same assay may be significant, the variables inherent in the cross-neutralization comparisons are such that fourfold differences in comparisons between variants may not be biologically significant.

Several molecular epidemiology studies were previously performed on HPV16 variants, but little is known about the selective pressures that led to the formation of these variants (Chan *et al.,* 1992; Ho *et al.,* 1993; Stewart *et al.,* 1996). The concentration of amino acid mutations around known neutralizing epitopes led some investigators (Chen *et al.,* 2000) to speculate that fixation of HPV16 variants in the human population may be driven by escape from neutralization. It is true that the Africa 2 variant is unable to bind neutralizing monoclonal antibody E70 (Roden *et al.,* 1997). However, to the extent that our studies of the variants from each branch are representative of all members of that branch, our results strongly suggest that the HPV16 genotype is comprised of variants that share at least one important neutralizing epitope (V5), and that hyperimmune sera obtained by vaccination with the 114K isolate recognizes an immunodominant epitope(s) which is shared by all variants.

The failure of most of the amino acid changes in the HPV16 L1 variants to be associated with loss of cross-neutralization against the immunodominant 114K epitope(s) may be accounted for by the relative stringency of changes in L1 that are compatible with efficient virion production. L1 is the most highly conserved of the viral proteins (Yamada *et al.,* 1995), and single amino acid substitutions in a structurally important area of L1 can severely compromise the efficiency of L1 self-assembly into properly folded capsid protein (Kirnbauer *et al.,* 1993). Mutation "hot spots," which generally lie on the surface exposed residues of L1 pentamers (Chen *et al.,* 2000), are likely to be the areas where neutralizing epitopes lie, but even these regions are structurally constrained. Only two of the variants chosen for our studies (Z1194 and NM.T529) contain amino acid changes that localize to the major surface-exposed loops of L1 as reported by Chen *et al.* (2000); however, changes in internal amino acids, such as amino acid 50 (Chen *et al.,* 2000), could presumably also alter neutralization epitopes.

Our conclusion that, from a vaccine standpoint, HPV16 represents a single serotype needs to also be confirmed for natural infections. However at least another HPV type, HPV5, which is comprised of several variants, has been shown to form a single serotype (Favre *et al.,* 1997). HPV5 and HPV16 are members of different HPV supergroups (Chan *et al.,* 1995), with HPV5 being an EV-specific type that typically infects nongenital skin, while HPV16 represents an anogenital type that typically infects the genital skin and mucosa. Since HPV5 and HPV16 represent evolutionarily different HPV types, it is tempting to speculate that other HPV types from which multiple variants have been isolated, such as HPV6, HPV11, and HPV18 (Heinzel *et al.,* 1995; Icenogle *et al.,* 1991; Kitasato *et al.,* 1994; Ong *et al.,* 1993), may also each represent a single serotype. Consistent with this hypothesis, both HPV16

and HPV18, which are found in different groups of PV Supergroup A, seem to have undergone similar patterns of evolution (Ho *et al.,* 1993). However, it remains possible that some HPV types might be comprised of more than one serotype. It is clear from the large number of HPV types that serologically distinct genotypes do eventually evolve. But our results support the idea that this does not usually result from only one or a small number of amino acid changes in L1.

VLPs are proving to be potent immunogens in humans, as well as in animals (Breitburd *et al.,* 1995; Kirnbauer *et al.,* 1996; Suzich *et al.,* 1995; Zhang *et al.,* 2000; Clayton Harro, Johns Hopkins University, manuscript submitted). It should be noted that the preimmune sera of the vaccinated volunteers whose immune sera were tested in the current study were negative for serum antibodies against HPV16 114KL1 VLPs, and the serum antibody titers induced in the volunteers by the HPV16 L1 VLPs were more than one order of magnitude higher than those seen following natural infection with HPV16 (Cheng *et al.,* 1995; Wang *et al.,* 1997; Clayton Harro, Johns Hopkins University, manuscript submitted). Also, although L2 does contain neutralization epitopes (Campo *et al.,* 1997; Kawana *et al.,* 1999; Roden *et al.,* 2000), these epitopes usually induce only low titers of neutralizing antibodies and were not included in the L1 VLPs used to immunize the volunteers. It may therefore be concluded that the efficient neutralization observed for all the HPV16 variants was the result of immunization with the vaccine and did not result from environmental exposure to virions of the variants.

Studies in animals indicate that protection by VLPs against animal papillomavirus infections is mediated largely by neutralizing antibodies (Breitburd *et al.,* 1995; Suzich *et al.,* 1995). The results obtained here with the HPV16 variants indicate that there are sufficient cross-reactive epitopes among the variants to permit efficient cross-neutralization, although some variants may lack a subset of the neutralizing epitopes (Roden *et al.,* 1997; White *et al.,* 1999). These observations imply that whatever protection may be conferred by immunization with HPV16 L1 VLPs from the 114K variant will extend to the vast majority of HPV16 variants, although field trials will be necessary to verify this prediction. The greater risk of persistence and progression associated with some non-114K HPV16 variants to develop CIN 2-3 (Xi *et al.,* 1997) underscores the need for a vaccine that can protect against as many variants as possible.

MATERIALS AND METHODS

Construction of SFV expressing L1 from HPV16 variants

A variant from each of the three lineage subclasses of the HPV16 European branch was selected, as well as one variant from each of the four other phylogenetic branches (Fig. 1). The variants were: European (E) subclass P 114/K, European subclass Asian variant (As) OR.7574, European subclass (G131) NM.T197, Africa 1 (Af1) OR.7587, Africa 2 (Af2) Z-1194, North American (NA1) OR.3136, and Asian American (AA) NM.T529. The L1 genes of 114K and Africa 2 had been previously cloned into SFV expression vectors in our laboratory (Roden *et al.,* 1996a). The As, Af1, and AA variants were provided by C. Wheeler (University of New Mexico) (Yamada *et al.,* 1995). The European and Africa 2 variants were cloned into the *Bam*HI site of pSFV 1 (Life Technologies, Gaithersburg, MD). As, Af1 and AA variants were directionally subcloned from the pCR2 vector into the *Bgl*II and *Hin*dIII sites of pBluescript, and further subcloned into the *Apa*I and *Not*I sites of the pSFV 4.2 vector (Life Technologies). Confirmation of the DNA sequence of each variant was performed by double-pass sequencing through regions of sequence divergence.

Site-directed mutagenesis was used to obtain the G131 and NA1 variants. The 114K and Af1 variants cloned in pBluescript (Stratagene, La Jolla, CA) were used as templates to make the G131 and NA1 variants, respectively. Divergent nucleotides in 114K and G131, compared with Af1 and NA1, which did not result in amino acid changes were ignored. For G131, mutagenesis produced the necessary I191L codon change; for NA1, mutagenesis resulted in the desired T353P codon change. The G131 and NA1 variants were also subcloned from pBluescript into the *Apa*I and *Not*I sites of pSFV 4.2. Double-pass sequencing of the amplified region confirmed that the PCR procedure had not generated unintended mutations.

Generation of HPV16 pseudotype virions

BHK-21 cells and BPHE-1 cells were grown in complete Dulbecco's minimal essential medium (DMEM) containing 10% FBS and penicillin/streptomycin. Recombinant L1 SFV stocks from each variant were generated as described previously (Roden *et al.,* 1996a). Briefly, 8 μg of recombinant pSFV-1 and Helper 2 plasmids were linearized with *Spe*I (Life Technologies). Recombinant pSFV 4.2 plasmids were linearized with *Nru*I (New England Biolabs; Beverly, MA). DNA was phenol:chloroform extracted, ethanol-precipitated, and resuspended in TE. DNA (2 μg) served as a template for an *in vitro* transcription reaction, using SP6 RNA polymerase (Life Technologies). Helper 2 RNA (20 μl) and the recombinant pSFV RNA (20 μl) were electroporated into 10 million BHK-21 cells. After electroporation, BHK-21 cells were plated and incubated in a T-175 flask for 24 h in 30 ml medium. Supernatants were collected and cleared by a 10-min centrifugation at 1000 *g,* and stored at $-80°$C until used. The L2 SFV stock used for all coinfections was from the European variant 114K. L1 (1 ml) and L2 (0.25 ml) SFV stocks were mixed together, activated with chymotrypsin (10 mg/ml; Boehringer-Mannheim, Indianapolis, IN), and

used to coinfect 14 million BPHE-1 cells (which contain BPV1 episomes) for 2 h at 37°C. Complete DMEM replaced the infectious material, and cells were incubated for 24 h, scraped, pelleted by centrifugation, and kept at −80°C until used.

Vaccine sera

Sera were obtained from 12 healthy volunteers enrolled in a phase I HPV16 L1 VLP vaccine trial of the 114K variant (Clayton Harro, Johns Hopkins University, manuscript submitted). Ten of the volunteers had received intramuscular injections of 50 μg VLP vaccine without adjuvant at 0, 1, and 4 months. Two of the volunteers had received three placebo injections of saline in the same study. The serum samples used in the current studies were those taken 1 month after the third injection. They were stored at −20°C until used.

Neutralization assay

Neutralization of the infectious pseudotyped virions was assessed as described (Roden *et al.,* 1996a). Briefly, the stored pellets from BPHE-1 cells that had been coinfected with recombinant SFV that express 114K L2 and an L1 variant were sonicated in 1 ml PBS just prior to addition of human sera and infection of C127 cells. Aliquots of this solution were mixed for 1 h at 4°C with PBS, or with fourfold serial dilutions of serum starting with a 1:40 dilution. C127 clone C cells, which had been plated the previous day with 1×10^5 cells/60-mm dish, were used in the infectivity assay. The pseudovirus/PBS or pseudovirus/serum combination was added to the cells for 1 h at 37°C, and then replaced with DMEM and 10% fetal bovine serum. The cells were fed twice weekly for 14–18 days, then stained with 2.5% methylene blue, 0.5% carbol fuchsin (w/v) in methanol for visualization of foci. The neutralization titer was defined as the reciprocal of the highest serum dilution that was able to reduce the number of foci induced by the pseudovirions by at least 50%.

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