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Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18

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Abstract

Sensitive high-throughput neutralization assays, based upon pseudoviruses carrying a secreted alkaline phosphatase (SEAP) reporter gene, were developed and validated for human papillomavirus (HPV)16, HPV18, and bovine papillomavirus 1 (BPV1). SEAP pseudoviruses were produced by transient transfection of codon-modified papillomavirus structural genes into an SV40 T antigen expressing line derived from 293 cells, yielding sufficient pseudovirus from one flask for thousands of titrations. In a 96-well plate format, in this initial characterization, the assay was reproducible and appears to be as sensitive as, but more specific than, a standard papillomavirus-like particle (VLP)-based enzyme-linked immunosorbent assay (ELISA). The neutralization assay detected type-specific HPV16 or HPV18 neutralizing antibodies (titers of 160–10240) in sera of the majority of a group of women infected with the corresponding HPV type, but not in virgin women. Sera from HPV16 VLP vaccinees had high anti-HPV16 neutralizing titers (mean: 45000; range: 5120–163840), but no anti-HPV18 neutralizing activity. The SEAP pseudovirus-based neutralization assay should be a practical method for quantifying potentially protective antibody responses in HPV natural history and prophylactic vaccine studies.

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Introduction

Human papillomaviruses (HPVs), which compose a group of more than 100 genotypes, cause genital and cutaneous warts (reviewed in (Fazel et al., 1999; Handsfield, 1997; Lowy and Howley, 2001)). A subset of HPV genotypes, especially HPV16 and HPV18, has been implicated in the etiology of cervical cancer (Bosch and de Sanjose, 2003; Bosch et al., 1995). Even for those HPV genotypes associated with cancer, most HPV infections are self-limit-

ing, with immune responses to earlier HPV exposure perhaps contributing to the relative resistance of older individuals to the acquisition of HPV (Koutsky et al., 1992; Schiffman, 1994).

In other viral infections, resistance to infection may correlate with the development of neutralizing antibodies. Such data have been difficult to obtain with HPV infection because HPV neutralization assays are cumbersome, and most lack sufficient sensitivity to reliably detect serum-neutralizing antibodies following natural infection. Currently available papillomavirus neutralization assays rely on neutralization of either authentic virions (Christensen et al., 1992; Smith et al., 1995; White et al., 1998), pseudotyped virions (Roden et al., 1996a), pseudovirions that have encapsidated reporter genes (Kawana et al., 1998; Rossi et al., 2000; Stauffer

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et al., 1998; Touze and Coursaget, 1998; Unckell et al., 1997), or capsids carrying a reporter gene on their surface (Bousarghin et al., 2002). Most serological analyses of responses to natural HPV infection have relied on assays that are surrogates for neutralization, especially virus-like particle (VLP)-based enzyme-linked immunosorbent assays (ELISAs) (Dillner, 1999) or antibody displacement assays (Opalka et al., 2003; Yeager et al., 2000). These surrogate assays are more sensitive than most current neutralization assays and are simple enough that they can be used to analyze hundreds of samples. However, one problem with VLP-based ELISAs is that while the immunodominant neutralization epitopes of HPVs have been shown to be genotype-specific, ELISAs may also detect non-neutralizing and cross-genotype reactive antibodies, thus complicating the interpretation of some results.

The development of prophylactic HPV vaccines represents another area of active investigation in which the measurement of serologic responses plays a key role. The leading candidate prophylactic vaccine is composed of the L1 major capsid protein assembled into VLPs that possess the type-specific immunodominant neutralization epitopes present in authentic virions. In preclinical vaccine studies involving animal papillomavirus challenge, L1 VLP immunization conferred high-level protection attributable to type-specific neutralizing antibodies (Breitburd et al., 1995; Christensen et al., 1996b; Kirnbauer et al., 1996; Suzich et al., 1995). Early phase trials in humans have confirmed that L1 VLP vaccines are well tolerated and immunogenic, and that an HPV16 L1 VLP vaccine may protect against HPV16 infection (Koutsky et al., 2002) (also reviewed in (Galloway, 2003; Schiller and Lowy, 2001; Stanley, 2002)). As with natural history studies, most vaccine responses have been determined by VLP ELISA or other surrogate assays. Because it is expected that commercialized L1 VLP vaccines will be composed of VLPs from two or more HPV types, the partial cross-genotype reactivity of VLP-based ELISAs might compromise their utility.

These considerations suggest that a scalable papillomavirus neutralization assay with sensitivity comparable to VLP-based ELISAs would have utility for natural history studies of HPV infection and for the analysis of vaccine responses. We have recently described (Buck et al., 2004) the development of a new procedure for the intracellular generation of papillomavirus pseudoviruses (also known as papillomaviral vectors) whose production efficiency was at least 10 million-fold greater than that of our previous method of intracellular pseudovirus production (Rodén et al., 1996a). In this communication, we report the application of this technology to the development and validation of a quantitative high-throughput papillomavirus neutralization assay by analyzing the serum neutralization titers of VLP-vaccinated women and women naturally infected with HPV.

Results

Neutralization of GFP pseudovirus in C127, HaCaT, and 293TT cells

A method for generating high-titer pseudovirus stocks composed of a reporter plasmid encapsidated by major and minor structural proteins (L1 and L2) of bovine papillomavirus 1 (BPV1) and HPV16 has recently become available (C.B. Buck et al., 2004). The method depends on the high-level coexpression of L1 and L2 and overreplication of a reporter plasmid carrying the SV40 origin of replication (SV40 ori) in a cell line, 293TT, engineered to overexpress the SV40 T antigen. To determine if such pseudoviruses could be adapted to an in vitro neutralization assay, we generated an HPV16 pseudovirus containing a plasmid encoding a green fluorescent protein (GFP) reporter gene and the SV40 ori. A crude pseudovirus stock was generated by cotransfecting this GFP reporter plasmid, pYafW, into 293TT cells together with plasmids expressing codon-modified HPV16L1 (p16L1h) and HPV16L2 (p16L2h) genes (Leder et al., 2001).

We used the HPV16-based GFP pseudovirus at a dilution of 1:2500 to infect several lines, namely mouse C127, human HaCaT keratinocytes, and 293TT cells (Table 1). FACS analysis of the cells showed that each line could be infected with the pseudovirus and that infection could be neutralized by preincubating the pseudovirus inoculum with an HPV16-neutralizing monoclonal antibody (V5). The neutralization titer for the monoclonal antibody was about two million in each line. Neutralization was HPV16 antibody specific, in that an anti-BPV1 monoclonal antibody (5B6) failed to prevent infection by the pseudovirus. Compared with infection of C127 cells, the mean fluorescence in infected HaCaT cells was about 2-fold higher, while in 293TT cells there were 4–5-fold more GFP-expressing cells and a 14–35-fold higher mean fluorescence. Both increases in the 293TT cells probably reflect the ability of the GFP plasmid to be overreplicated by the high levels of SV40 T antigen in these cells. This higher signal strength in 293TT cells suggested that this line would be the preferred indicator cell for a neutralization assay.

Secreted alkaline phosphatase pseudovirus neutralization assay

The GFP pseudovirus assay represented a substantial improvement over our previous focus assay in terms of ease of pseudovirus production and shortening of assay time. To develop an assay with even higher throughput, we sought an alternative readout that might be easier to monitor than FACS-based GFP detection. For this purpose, we constructed a pseudovirus encoding secreted alkaline phosphatase (SEAP) which can be detected using a highly sensitive chemiluminescent reporter system. SEAP is secreted into cell-culture supernatants, thus reducing handling

Table 1
Antibody-dependent neutralization of infection with GFP HPV16 pseudovirus

Cell type	Treatment	Mean fluorescence	Percentage of FL1-positive cells	Percentage of neutralization ^a	
C127	No pseudovirus	9	0.1	NA	
	Pseudovirus + No Ab	20	10.2	0	
	V5 at $1:5 \times 10^4$	10	0.2	100	
	V5 at $1:2.5 \times 10^6$	18	4.6	66	
	V5 at $1:1.25 \times 10^8$	20	9.6	7	
	5B6 at $1:5 \times 10^4$	22	11.8	0	
	HaCaT	No pseudovirus	21	0.3	NA
		Pseudovirus + No Ab	49	7.8	0
V5 at $1:5 \times 10^4$		18	0.4	100	
V5 at $1:2.5 \times 10^6$		40	3.0	65	
V5 at $1:1.25 \times 10^8$		51	9.0	0	
5B6 at $1:5 \times 10^4$		50	9.7	0	
293TT		No pseudovirus	21	0.3	NA
		Pseudovirus + No Ab	710	44.2	0
	V5 at $1:5 \times 10^4$	19	0.7	99	
	V5 at $1:2.5 \times 10^6$	512	32.8	26	
	V5 at $1:1.25 \times 10^8$	647	44.6	0	
	5B6 at $1:5 \times 10^4$	690	45.0	0	

^a Percent neutralization was defined as +100 minus the ratio of the specific percentage of cells that were FL1-positive with V5 (obtained by subtracting background percentage of FL1-positive cells with no pseudovirus) divided by the specific percentage of cells that were positive without antibody (obtained by subtracting background percentage of FL1-positive cells with no pseudovirus) were FL1 positive with V5 divided by the percent of cells that were positive without antibody. were FL1 positive with V5 divided by the percent of cells that were positive without antibody.

of cells and allowing repeated testing of the same cells for more than several days.

In preliminary experiments, we compared SEAP expression from an HPV16 pseudovirus containing a commercially available plasmid, pSEAP2Control (SEAP expression is under the control of SV40 early promoter), to pseudoviruses carrying two newly constructed SEAP reporter plasmids; p2CMVSEAP (SEAP expression is under the control of CMV immediate early promoter) and pYSEAP (uses human elongation factor-1 α (EF1- α) promoter). Each of the three plasmids also contains the SV40 ori. We found that 293TT target cells infected with the pseudovirus containing pYSEAP expressed approximately 10 or 100 times more SEAP (measured as relatively light units, RLU) than cells infected with p2CMVSEAP or pSEAP2Control, respectively (data not shown).

To determine the degree to which replication of pYSEAP by SV40 T antigen enhanced SEAP production, we transduced 293H (T antigen-negative) or 293TT (T antigen-positive) cells with a pYSEAP pseudovirus preparation. In preliminary studies, we found that SEAP was not detectable at 24 h posttransduction in either cell line, consistent with previous observations that papillomavirus infection, as well as pseudovirus transduction, is a relatively slow process (Day et al., 2003) (C.B. Buck et al., 2004). SEAP was readily detected in both lines by 48 h posttransduction, and its concentration increased about 8-fold at 72 h, with a further 3-fold increase at 96 h, where it plateaued. In view of this time course, we chose to assay SEAP production at 72 h postinfection in subsequent assays. When the 293H and 293TT cells were infected with 2-fold serial dilutions of pYSEAP HPV16 pseudovirus, there was an approximately linear response for RLU readouts between 20 and 500 (Fig. 1 and data not shown). Analysis of six replicates showed that intra-assay variation in SEAP production had a standard error of the mean of approximately 3–4%. At each dilution, 293TT cells produced approximately 30–40 times more SEAP than 293H cells. Therefore, 293TT cells were used as target cells for all subsequent experiments.

In our previous HPV16 neutralization assay (Roden et al., 1996a), which is based on focal transformation of C127 cells induced by HPV16 pseudovirions that have encapsidated the BPV1 genome, we had observed that the apparent neutralization titer varied inversely with the size of the pseudovirus inoculum (Pastrana et al., 2001). Thus, the smaller inocula increased the sensitivity of the assay, but with a lowering of the signal to background ratio and an eventual loss of the linear response. To examine the inoculum–sensitivity relationship in the SEAP neutralization assay, we performed neutralization with two different concentrations of pYSEAP HPV16 pseudovirus and serial dilutions of monoclonal antibody V5 (Fig. 2). Although the 8-fold larger pseudovirion inoculum (the 1:100 dilution) resulted in a 7.5-fold rise in

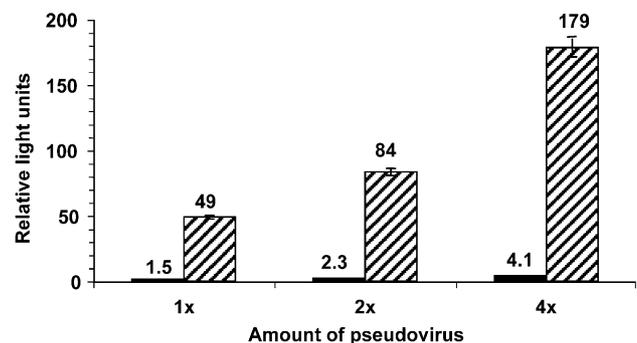


Fig. 1. Effect of SV40 large T antigen on SEAP production after pseudovirion infection. Infection of 293H (solid bars) or 293TT (striped bars) cells with pYSEAP pseudovirions. Two-fold increasing concentrations of diluted pseudovirions were used to infect cells. After 72 h, SEAP in the supernatant of infected cells was assayed by luminometry. Numbers on top of the bars represent the mean of 6 wells, and error bars are the standard error of the mean.

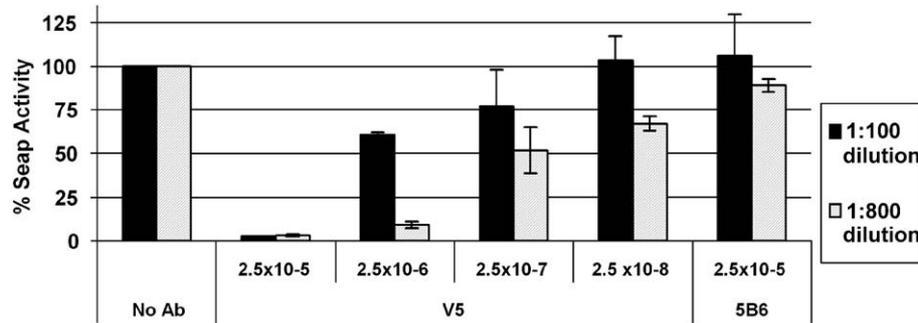


Fig. 2. Effect of inoculum size on neutralization titers. Pseudovirus stock was diluted 1:100 (black bars) or 1:800 (gray bars), preincubated with 10-fold serial dilutions of V5 Monoclonal Antibody starting at 1:250,000 or with 5B6 at the highest concentration, and used to infect 293TT cells. SEAP activity was calculated from an average of 3 wells for each condition and 100% activity was set for wells with pseudovirus but without antibody incubation.

average RLU (418 vs. 55.5 RLU), the increase in the inoculum resulted in more than a 10-fold decrease in neutralizing titer compared with the 1:800 inoculum (the neutralizing titer for the 1:100 dilution was $\geq 2.5 \times 10^5$, and $\geq 2.5 \times 10^6$ for the 1:800 dilution of pseudovirus). Given this trend, we decided to use pseudovirions at concentrations that would yield 20–80 RLU (for this stock 1:800), which represented a level whose positive signal was reproducibly 30–40 times above background and in the linear range.

Analysis of sera from HPV16 VLP vaccine trials

We used the SEAP HPV16 pseudovirus assay to determine the endpoint neutralization titers for sera from 12 volunteers enrolled in a Phase I clinical trial of an HPV16 L1 VLP vaccine (Harro et al., 2001). The serum samples were obtained 1 month after a series of three intramuscular inoculations with 50- μ g doses of VLPs without adjuvant. To examine inter-assay variation, three separate SEAP HPV16 neutralization assays were carried out on different days

(Table 2, columns labeled “crude”) using the same pseudovirus stock. The mean neutralization titers for the VLP vaccinees ranged from 3400 to 68000, and no neutralizing activity was detected in sera from the two placebo controls. There was good inter-assay reproducibility using the crude preparation of pseudovirions, as the same titer for each volunteer was obtained in at least two out of the three experiments and most discordant results differed by only 2-fold. The reproducibility of the assay was statistically significant, with a rho (ρ) exceeding 0.93 and a linear weighted kappa (κ) of greater than 0.63.

The sera from the volunteers had previously been tested by HPV16 ELISA (Harro et al., 2001) and by our previous HPV16 neutralization assay (Pastrana et al., 2001; Roden et al., 1996a), which enabled us to compare these results with the titers obtained in the SEAP assay (Table 2). There was a good correlation between the titers in the two HPV16 neutralization assays ($\rho = 0.96$), but the titers for the SEAP assay were an order of magnitude higher (mean = 16-fold, range = 7–27-fold) than those observed with the focus assay

Table 2
Sensitivity and reproducibility of the SEAP pseudovirus neutralization assay using serum from the HPV16 VLP phase I clinical trial

Patient	16 ELISA ^a	18 ELISA	16 focus assay ^a	SEAP pseudovirus neutralization					
				Crude				Optiprep purified	
				1	2	3	Average	HPV16	HPV18
25	10,240	2560	640	10,240	10,240	10,240	10,240	40,960	<40
26	10,240	2560	640	20,480	10,240	10,240	13,653	30,720	<40
27	10,240	2560	640	20,480	10,240	10,240	13,653	20,480	<40
28	40,960	2560	2560	40,960	20,480	20,480	27,307	61,440	<40
29 ^b	<10	<40	<40	<40	<40	<40	<40	<40	<40
30	10,240	640	640	20,480	10,240	20,480	17,067	20,480	<40
31	40,960	2560	10,240	81,920	81,920	40,960	68,267	163,840	<40
32	2560	640	160	5120	2560	2560	3413	5120	<40
33 ^b	<10	<40	<40	<40	<40	<40	<40	<40	<40
34	40,960	640	2560	81,920	20,480	20,480	40,960	61,440	<40
35	40,960	2560	2560	40,960	20,480	20,480	27,307	40,960	<40
36	10,240	160	640	10,240	10,240	5120	8533	10,240	<40
GMT	15,500	1300	1100				16,970	30,500	

Note. Values in the columns reflect the inverse of serum dilution for the endpoint-neutralizing titer, which was defined as 50% inhibition of the amount of SEAP compared to the virus added without antibody. GMT represents the geometric mean titers.

^a Values for the HPV16 VLP ELISA and the focus neutralization assay were previously published (Harro et al., 2001; Pastrana et al., 2001).

^b Placebo-immunized volunteers.

($P = 0.003$). The SEAP neutralization titers also showed a good correlation with the ELISAs ($\rho = 0.95$, linear weighted $\kappa = 0.74$ {95% CI = 0.39–1.0}), and the increased sensitivity of the SEAP assays resulted in the neutralization titers obtained by this assay being comparable to the ELISA titers.

The crude HPV16 pseudovirion preparations also contain noninfectious empty VLPs (Buck et al., 2004). Several batches of crude preparations were tested to determine if the pseudovirions performed similarly. When crude preparations were diluted to obtain 30–50 RLUs, the endpoint neutralization titer with V5 was consistently found to be about 2.5×10^6 (data not shown). Because VLPs may contain neutralization epitopes, their presence would be expected to reduce the apparent neutralizing titer of sera by competing with pseudovirions for adsorption of neutralizing antibodies. Given that the use of Optiprep density fractionation can partially separate empty VLPs from DNA-containing pseudovirions (C.B. Buck et al., 2004), we sought to determine whether Optiprep fractionation might increase the sensitivity of the SEAP neutralization assay. We found that neutralization titers were an average of 1.9-fold higher for the 12 volunteers tested with Optiprep purified pseudovirus (Table 2) ($P = 0.003$). However, Optiprep purification resulted in a 97% loss of HPV16 pseudovirus titer. Similar losses in titer were seen after Optiprep purification of HPV18 (development of HPV18 pseudovirions is described below), but not of BPV1 pseudovirus stocks. Despite the reduction in titer, the high initial yield of the production system made it possible to harvest enough Optiprep purified material for approximately 4000 individual tests from a single 162-cm² flask of transfected 293TT cells. To ensure that different batches of pseudovirions had similar ratios of VLP/pseudovirions, the preparations were normalized for RLUs yielded, analyzed by SDS-PAGE, silver stained to determine L1 content, and endpoint titers determined using monoclonal V5 for HPV16, monoclonal 5B6 for BPV1, and a rabbit polyclonal serum for HPV18. Endpoint titers with V5 for HPV16 were 2.5×10^7 , with 5B6 for BPV1 were 1×10^5 , and with rabbit polyclonal serum for HPV18 were 1×10^6 . For the experiments described in this paper using Optiprep purified stocks, a single batch of pseudovirions was used for HPV16, BPV1, and HPV18; 3, 1, and 0.2 ng, respectively, of L1 were added per well to yield about 40 RLUs. To further examine the specificity of the neutralization assay, we tested sera from a panel of vaccinees in a SEAP BPV1 pseudovirus assay as well as the SEAP HPV16 assay with crude- and Optiprep-purified pseudovirus preparations. Because BPV1 is divergent from HPV and human exposure to BPV1 probably occurs only rarely, human sera would not be expected to have significant anti-BPV1 neutralizing capacity. The limited amount of the sera used for generating the data in Table 2 made it necessary to use sera from 17 participants in our Phase II HPV16 L1 VLP vaccine trial (Harro et al., unpublished). As with sera from vaccinees in the Phase I trial, the HPV16 ELISA and SEAP HPV16 neutralization titers of sera from vaccinees in

the Phase II trial were similar to each other (data not shown). A few of the sera from the Phase II vaccinees were able to neutralize a crude preparation of SEAP BPV1 pseudovirus at a dilution of 1:20, but not at higher dilutions (data not shown). This sporadic nonspecific low-titer “neutralizing” activity was also observed with Optiprep-purified preparations of SEAP BPV1 pseudovirus. In addition, low neutralizing activity was detected in some preimmune sera from other sources (rabbits, mice, sheep, and human sera from other studies, see results below and data not shown). From these results, we conclude that the observed inhibition of SEAP BPV1 pseudovirion infection does not result from cross-neutralizing antibodies induced by HPV16 L1 VLP vaccination, but rather from a nonspecific factor(s) present in some sera. Heat inactivation, repeated cycles of freeze thawing, centrifugation at $16000 \times g$, and preabsorption of the sera with heterologous VLPs failed to eliminate the nonspecific inhibition of infectivity (data not shown). Except for one serum (see section on Detection of neutralizing antibodies in sera from unvaccinated women), we have not observed cross-species inhibition of pseudovirus infection at dilutions above 1:40.

The specificity of the HPV16 neutralization titers was also examined by developing an analogous SEAP HPV18 pseudovirus neutralization assay. Despite the presence of high HPV16 neutralization titers (5100–163000 using HPV16 SEAP Optiprep-purified pseudovirions), none of the sera from Phase I HPV16 VLP vaccinees, when diluted to 1:40 or higher, neutralized SEAP HPV18 pseudovirus, confirming the type-specific nature of the neutralizing antibodies induced by the HPV16 VLP vaccine (Table 2). The sera from the vaccinees were also studied with a standard HPV18 ELISA, which detected positive titers that were 4–64-fold lower than their HPV16 ELISA titers (Table 2). This positive result with the HPV18 ELISA is not unexpected, as VLPs contain cross-reactive linear epitopes that are non-neutralizing. The contrast between the positive HPV18 ELISA data and the negative HPV18 neutralization results demonstrates the greater type specificity of the neutralization assay.

Detection of neutralizing antibodies in sera from unvaccinated women

To investigate whether the greater sensitivity of the SEAP pseudovirus neutralization assay might make it useful for detecting type-specific neutralizing antibody responses following genital tract HPV infection, we tested sera from a cohort of Danish women whose cervical HPV DNA status (Kjaer et al., 1996) and HPV16 ELISA reactivity (Kjaer et al., 2001) were known. The cohort was divided into six groups (Table 3). The first group consisted of women who, when they entered the study, were virgins and initially tested negative both for genital HPV DNA and HPV16 ELISA reactivity (Group 1A), but who subsequently became positive for genital HPV DNA following initiation of sexual

Table 3
Neutralization of HPV16 and BPV1 and HPV18 with serum from a natural history study

	ID #	DNA	ELISA		Neutralization		
			HPV16	HPV18	HPV16	BPV1	HPV18
Group 1A virgins 1st visit	1A-1	Neg	Neg	Neg	20	<40	<40
	1A-2	Neg	Neg	Neg	40	<40	<40
	1A-3	Neg	Neg	Neg	20	<40	<40
	1A-4	Neg	Neg	Neg	40	<40	<40
	1A-5	Neg	Neg	Neg	40	<40	<40
	1A-6	Neg	Neg	Neg	<20	<40	<40
	1A-7	Neg	Neg	Neg	<40	<40	<40
	1A-8	Neg	Neg	Neg	40	<40	40
	1A-9	Neg	Neg	Neg	40	<40	40
Group 1B initiated sexual activity, 2nd visit	1B-1	X*	640	Neg	2560	<40	<40
	1B-2	16	Neg	160	160	40	160
	1B-3	16	Neg	Neg	400	<40	40
	1B-4	16	Neg	160	<40	<40	<40
	1B-5	16	640	Neg	10,240	<40	160
	1B-6	56	Neg	Neg	640	<40	160
	1B-7	16,18	Neg	160	40	<40	40
	1B-8	16	Neg	Neg	40	<40	<40
	1B-9	16,18,35	Neg	160	40	<40	40
Group 2 16DNA–/ 16ELISA–	2-1	Neg	Neg	NT	40	<40	NT
	2-2	33	Neg	NT	40	<40	NT
	2-3	Neg	Neg	NT	<20	<40	NT
	2-5	Neg	Neg	NT	40	<40	NT
	2-6	Neg	Neg	NT	40	<40	NT
	2-7	Neg	Neg	NT	40	<40	NT
	2-8	Neg	Neg	NT	40	<40	NT
	2-9	Neg	Neg	NT	160	<40	NT
	2-10	Neg	Neg	NT	<40	<40	NT
	2-11	Neg	Neg	NT	40	40	NT
	Group 3 16DNA+/ 16ELISA+	3-1	16	160	NT	640	<40
3-3		16	40	NT	640	40	NT
3-4		16	160	NT	640	<40	NT
3-5		16,31	160	NT	40	<40	NT
3-6		16	160	NT	640	40	NT
3-7		16,33,45	160	NT	160	40	NT
3-8		16	160	Neg	2560	40	<40
Group 4	3-10	16	2560	NT	400	160	NT
	4-1	16,52	Neg	NT	160	<40	NT
	4-2	16	Neg	NT	40	<40	NT
	4-3	16	Neg	NT	160	<40	NT
	4-4	16,18	Neg	NT	160	<40	NT
	4-5	16	Neg	NT	160	<40	NT
	4-7	16	Neg	NT	160	<40	NT
	4-8	16,56	Neg	NT	160	<40	NT
	4-9	16,18	Neg	NT	40	<40	NT
	4-10	16	Neg	NT	160	<40	NT
Group 5 16DNA–/ 16ELISA+	5-1	Neg	40	NT	640	<40	NT
	5-2	56	10,240	40	10,240	<40	40
	5-3	Neg	160	NT	640	40	NT
	5-4	Neg	160	NT	40	<40	NT
	5-6	Neg	640	NT	40	<40	NT
	5-7	35,58	160	NT	160	40	NT
Group 6	5-8	Neg	160	40	2560	<40	40
	5-9	31	640	NT	640	<40	NT
	6-1	18,33	Neg	40	<40	<40	40
	6-2	18	Neg	640	40	<40	640
	6-3	18	Neg	Neg	40	40	40
	6-4	18	Neg	160	160	<40	160
	6-5	18,45	Neg	640	40	<40	2560
	6-6	18	Neg	40	40	<40	160
	6-7	18	Neg	Neg	40	<40	40
6-8	18	Neg	40	40	<40	40	

Table 3 (continued)

	ID #	DNA	ELISA		Neutralization		
			HPV16	HPV18	HPV16	BPV1	HPV18
Group 6	6-9	11,18	Neg	40	160	40	160
	18 DNA+/ 16DNA–/ 16 ELISA–	6-10 18	Neg	40	40	<40	160

Note. For the neutralization assays, titers at or below 40 (depicted in italics) were considered negative. NT denotes not tested.

X* denotes an untyped HPV DNA.

activity (Group 1B). The remaining five groups consisted of sexually active women who were negative both for HPV16 DNA and HPV16 ELISA (Group 2), positive for both assays (Group 3), positive for one assay but not the other (Groups 4 and 5), or positive for HPV18 DNA but negative for HPV16 DNA and HPV16 ELISA (Group 6).

Approximately 10 women from each group were chosen at random for evaluation in the SEAP HPV16 and BPV1 neutralization assays. We also performed HPV16 ELISAs on the samples. Of the sera we analyzed by quantitative ELISA, most (54/60) gave results that were concordant with previously published ELISA results, which used a qualitative assay (Kjaer et al., 2001). Sera from the six volunteers whose endpoint titer and qualitative ELISAs were discordant were not included in the comparisons with the SEAP neutralization assay.

To maximize sensitivity, the sera were tested using Optiprep-purified pseudovirus. The BPV1 pseudovirus was included as specificity control. Of the 60 volunteers tested, 59 had anti-BPV1 neutralization titers of 40 or less. One individual (3-10) had an anti BPV-1 titer of 160 (Table 3). It is not known whether this volunteer might have developed an authentic anti-BPV1 response to BPV1 exposure or had an unusually high serum concentration of a nonspecific inhibitor(s). Based on these results, sera were considered positive for HPV neutralization if their SEAP HPV neutralization titer was greater than 40 and was also at least 4-fold higher than in the BPV1 assay.

Using these criteria, 27 of the 63 (43%) sera from the natural history study were considered HPV16 seropositive by the neutralization assay compared to the 10 of 63 (16%) by ELISA ($P = 0.0002$). The κ between these two tests was 0.26 (95% CI = 0.06–0.46) and the percent agreement was 67%. When the analysis was restricted to those subjects who were HPV16 DNA negative at the time the sera were collected, 14 of 46 (30%) sera from the natural history study were considered HPV16 seropositive by the neutralization assay compared to the 2 of 46 (4%) by ELISA ($P = 0.0005$). The κ between these two tests was 0.19 (95% CI = 0.02–0.36) and the percent agreement was 74%. These results suggest that the neutralization assay is more sensitive, but leaves open the possibility that it might be less specific than the ELISA. This issue was therefore explored by studying the individual groups.

In group 1A (Table 3), all of the nine sera from the virgin women were negative in the SEAP HPV16 neutralization assay. Sera from these same women had also been collected at a second visit, after they had become sexually active and had acquired cervical HPV DNA (Group 1B). HPV16 DNA was detected in Pap specimens from seven of the women, and a different HPV genotype was detected in specimens from the other two women. In the HPV16 ELISA, only two women seroconverted, one who was HPV16 DNA positive and one infected with an unknown type. By comparison, five of nine (56%) sera were considered positive in the SEAP HPV16 neutralization assay. The positive results included both of the sera that were also positive by ELISA, two additional cases that were HPV16 DNA positive, and one case that was positive for HPV56 DNA.

Although the women in Group 2 had been sexually active, they were expected to be at low risk for having anti-HPV16 neutralizing antibodies because their sera were HPV16 VLP ELISA negative and their cervical samples were HPV DNA negative (Table 3). Consistent with this prediction, only 1 of the 10 women in this group was positive in the HPV16 neutralization assay. This woman gave a history of having six sexual partners.

The women in Group 3 were expected to be positive in the neutralization assay because they were positive both for HPV16 DNA and HPV16 ELISA. Indeed, seven of the eight women (87.5%) in Group 3 were positive for neutralizing antibodies. Most of the HPV16-neutralizing titers were higher than the corresponding HPV16 ELISA titers. A notable exception was subject 3–10, who had a 6-fold higher ELISA titer; interestingly, she was also the only subject with a detectable anti-BPV1 neutralizing titer. The trend towards higher sensitivity of the HPV16 neutralization assay compared to the HPV16 ELISA was also borne out in Group 4, with seven of its nine subjects (77.8%) being positive for HPV16 neutralization, even though all were negative by HPV16 ELISA. The women in Group 5 (HPV16 ELISA positive/HPV16 DNA negative) presumably had successfully cleared their HPV infections before enrollment in the study. Sera from six of the eight subjects in this group were HPV16 neutralization positive. The two subjects whose neutralization assays were negative (5-4 and 5-6) had low to intermediate ELISA titers (160 and 640, respectively).

We also carried out a smaller pilot characterization of the SEAP HPV18 neutralization assay. As with the HPV16 neutralization assay, all nine sera from the virgins (Table 3, group 1A) were negative in the HPV18 neutralization assay. At their second visit, after the women had become positive for HPV DNA, sera from three of the women were HPV18 neutralization positive, although they were not the two women in whom HPV18 DNA had been detected (1B-7 and 1B-9). To further characterize the HPV18 neutralization assay, women in Group 6 were examined because they were positive for HPV18 DNA but were negative both by HPV16 DNA and by HPV16 ELISA (Table 3). In this group, which

had not previously been tested for HPV18 serologic responses, 8 of 10 were HPV18 ELISA positive, and 6 of 10 were HPV18 neutralization positive (all 6 were HPV18 ELISA positive) (Table 3). For the six sera where both the HPV18 ELISA and HPV18 neutralization results were positive, the neutralization titer was either the same (2/6) or 4-fold higher (4/6). The two subjects whose serum was positive in the HPV18 ELISA but negative in the HPV18 neutralization assay had low ELISA titers (40).

To further explore the type specificity of the two neutralization assays, we tested the HPV18 assay with sera from the three women from the remaining groups who had HPV16 neutralization titers of 2560 or higher (Table 3; subjects 3-8, 5-2, and 5-8). None had detectable HPV18 neutralizing activity, although two were HPV18 ELISA positive at a titer of 40 (subjects 5-2 and 5-8). Taken together, the results argue against the possibility that the HPV16 and HPV18 neutralization assays are measuring cross-neutralizing antibodies in women who were positive in both neutralization assays. It seems more likely that women whose sera were positive in both neutralization assays had been exposed at some point to both HPV types.

Discussion

Here we report the generation and characterization of papillomavirus pseudovirus-based neutralization assays for HPV16, HPV18, and BPV1. In this initial characterization, the neutralization assays appear to be at least as sensitive as, and possibly more HPV genotype-specific than, a standard VLP-based ELISA. In addition, SEAP-based HPV neutralization assays directly measure the activity of antibodies that may be the most relevant to vaccine and natural history studies. The neutralization assay is run in 96-well plates, where it can be performed with a few microliters of sample, which is often available in limited quantities. The assay is amenable to large-scale production, and possibly to automation.

The assay represents an application of our recently described method for generating high-titer papillomavirus pseudoviruses in mammalian cells (Buck et al., 2004). The pseudovirus production method depends in part on the high-level expression of the two viral structural proteins, L1 and L2, whose authentic genes have been found to be poorly expressed in monolayer cultures of mammalian cells. This poor expression may be attributable to the presence of multiple negative regulatory elements (see discussion and references in Buck et al., 2004). One successful approach to overcoming this problem depends on the silent conversion of papillomavirus codons to those most frequently found in highly expressed mammalian proteins (Leder et al., 2001; Zhou et al., 1999) (Buck et al., 2004) (Liu et al., 2002). In this report, we have codon-modified the L1 and L2 genes of HPV18 using the codon modification strategy described by Buck et al. The successful application of codon modification

to the L1 and L2 genes of BPV1 (Zhou et al., 1999) (Buck et al., 2004), HPV16 (Leder et al., 2001), and HPV18 (this report) suggests that it should be an effective strategy for allowing high-level expression of the structural genes of other papillomaviruses.

The SEAP-expressing pseudoviruses used in the neutralization assay can be used in the form of crude cell lysates or following partial purification in an Optiprep gradient. The sensitivity of the crude preparations appears to be similar to that of the standard VLP ELISA, while the Optiprep-purified material has 2-fold greater sensitivity, without apparent loss of specificity. The crude preparations can be used where high titer responses are expected, as in sera from VLP vaccine trials. The Optiprep purification preferentially removes some of the noninfectious particles that can compete with the infectious pseudovirions for binding neutralizing antibodies. Its modestly improved sensitivity may be desirable for samples expected to have low neutralizing activity, such as serum or mucosal samples in natural infection studies or mucosal samples from vaccine trials. For the Optiprep-purified material, we estimate that we have used about 1000–2500 virion equivalents of L1 per cell. By comparison, 25000 viral particles/cell and 150000 particles/cell were previously reported for two HPV11 neutralization assays (Leiserowitz et al., 1997; Yeager et al., 2000). In contrast to previous neutralization assays, the production of infectious particles should not be limiting because crude extracts from a single 162-cm² flask can produce enough SEAP pseudoviruses for 150000 wells. Optiprep purification of HPV16 and HPV18 pseudovirions is associated with a 1.5 log reduction in yield. However, the purification procedure we have used in this report was optimized for use with BPV1 pseudoviruses (C.B. Buck et al., in press, *Journal of Virology*). Further optimization of the procedure might reduce the loss of titer.

One limitation of the SEAP neutralization assay is the nonspecific inhibition of pseudovirus activity that was observed with low dilutions of serum (1:10–1:40). Nonspecific inhibition of pseudovirus infection has been reported for at least one other cell-based neutralization assay (Yeager et al., 2000). It would be useful to identify and eliminate the factor(s) responsible for this inhibition, with the goal of increasing the sensitivity of the assay by detecting specific neutralization in lower dilutions of serum.

Immunodominant neutralizing antibodies are generally directed against L1 conformational epitopes that are type-specific (Christensen and Kreider, 1990, 1991; Christensen et al., 1990; Ghim et al., 1991; Kirnbauer et al., 1994; Roden et al., 1995), although limited cross-reactivity has been observed among closely related types such as HPV16 and 33, HPV18 and 45, and HPV6 and 11 (Christensen et al., 1994; Combata et al., 2002; Giroglou et al., 2001; Roden et al., 1996b; White et al., 1998). Because the VLP-based ELISA is more likely to recognize non-neutralizing linear epitopes that are more cross-reactive between HPV genotypes, the neutralization assay may be substantially more

genotype-specific than the standard ELISA. The greater type specificity of the HPV18 neutralization assay compared with the HPV18 ELISA was seen in the HPV16 VLP vaccinees. Sera from vaccinated individuals with very high anti-HPV16 neutralization titers were unable to neutralize HPV18 pseudovirions, while the same sera were positive in the HPV18 ELISA. We do not have sera from individuals vaccinated only with HPV18 VLPs. However, one woman, who was naturally infected with HPV18 and had anti-HPV18 neutralizing titers of 2560, was negative for HPV16 neutralization. This result, plus a lack of relationship between HPV16 neutralization titer and HPV18 neutralization titer in the sera from the HPV natural history cohort, suggests that the HPV16 neutralization assay is also type-specific.

The limited cross-reactivity of L1 neutralizing antibodies has led vaccine researchers to conclude that protection from VLPs will be relatively type-specific. This characteristic is expected to lead to the development of multivalent VLP vaccines, as multiple HPV types are implicated in benign and malignant HPV infections (Bosch and de Sanjose, 2003; Bosch et al., 1995). The tendency of the standard ELISA for a heterologous HPV genotype to yield potentially false-positive results is likely to become progressively more important as the number of HPV genotypes in the vaccine is increased. Probable false-positive VLP ELISA results can be overcome with the SEAP neutralization assay, or with a VLP-based biochemical assay where a neutralizing monoclonal antibody competes with serum antibodies for binding to VLPs (Opalka et al., 2003; Yeager et al., 2000). While both assays are more genotype-specific than the standard ELISA, the biochemical assay may not detect classes of neutralizing antibodies whose binding to VLPs is not inhibited by the neutralizing monoclonal antibody (Wang et al., 1997). It is also possible that a subset of non-neutralizing L1 antibodies could sterically hinder VLP binding of the labeled neutralizing monoclonal antibody, thereby producing false-positive results.

The SEAP neutralization assays may also find application in the development of L2-dependent vaccines, which have the potential of inducing cross-reactive neutralizing antibodies (Kawana et al., 1999; Roden et al., 2000). The production of pseudoviruses depends upon the presence of L2 (C.B. Buck et al., in press, *Journal of Virology*), and preliminary analysis indicates that they can be neutralized by anti-L2 sera (unpublished data).

Seroconversion, as measured by VLP ELISA, does not occur in all natural infections, may be delayed by several months relative to the initial detection of genital tract HPV DNA, and is more likely to occur in persistent HPV infection (reviewed in (Konya and Dillner, 2001)). It was therefore of interest to evaluate the relationship between the presence of cervical HPV DNA and seropositivity in the HPV16 and HPV18 neutralization assays.

Among women with prevalent (mostly asymptomatic) HPV16 or 18 infections (Table 3, Groups 3, 4, and 6), 19 of 27 (73%) scored positive in the corresponding neutralization

assay. These results are consistent with those of Kawana et al. (2002), who detected HPV16 pseudovirus neutralizing antibodies in 6/7 women with asymptomatic HPV16 infection. While most neutralizing titers in our natural infection study were in the 160–640 range, several of the women had titers of 2560–10250 (Table 3), overlapping the lower range of titers in VLP-vaccinated women (Table 2). In previous studies attempting to detect neutralizing antibodies in HPV-infected women, the sera were tested at only a single low dilution of 1:10 (Wang et al., 1997), 1:20 (Bousarghin et al., 2002; Leiserowitz et al., 1997), 1:100 (Kawana et al., 2002), or 1:160 (Smith et al., 1995).

Our results suggest that the HPV16 neutralization assay, using Optiprep-purified pseudovirions, is possibly more sensitive than the HPV16 ELISA. The greater sensitivity was seen most strikingly in the groups of subjects who were selected for study because they were HPV16 DNA positive but HPV16 ELISA negative. Among this group, seven of nine were positive in the HPV16 neutralization assay. However, we note that the SEAP-based HPV neutralization titers among these discordant results were low (160), and thus we cannot rule out false positivity as the consequence of an arbitrary positive cutpoint. Moreover, this same trend was not observed among the smaller set of women with prevalent HPV18 infections, where 6/8 HPV18 ELISA-positive women were neutralization positive compared with 0/2 women who were ELISA negative.

Analysis of larger and more varied study populations will be needed to more precisely establish the relationship among neutralization titers, genital tract HPV DNA, and VLP ELISA reactivity in various types of human subjects. Nevertheless, this initial report establishes the SEAP HPV pseudovirus neutralization assay as a specific, sensitive, and practical method for evaluating potentially protective antibody responses in natural history and prophylactic vaccine studies.

Materials and methods

Cell lines

C127 clone C (Dvoretzky et al., 1980) and HaCaT cells (Boukamp et al., 1988) were grown in complete Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% heat-inactivated fetal bovine serum (HyClone). 293H and 293TT (Buck et al., 2004) cells were grown in the same medium supplemented with 1% Glutamax and 1% nonessential amino acids (Invitrogen).

Plasmids

Nucleotide sequences of plasmids constructed for the manuscript can be found at <http://ccr.cancer.gov/Staff/Links.asp?StaffID=443>. pSEAP2Basic and pSEAP2Control were purchased from BD Biosciences Clontech. Plasmid con-

structs 16L1h and 16L2h expressing codon-modified HPV16 L1 and L2 genes were kindly provided by Martin Müller (Leder et al., 2001). For bovine papillomavirus type 1 (BPV1) codon modified genes, we used a single plasmid, pSheLL, which codes for both BPV1 L1 and BPV1 L2 (Buck et al., 2004). We used the As Different As Possible (ADAP) strategy (C.B. Buck et al., in press, *Journal of Virology*) to codon-modify HPV18 L1 and L2 open reading frames (GenBank accession numbers AY383628 and AY383629). Based on the successful modification of HPV16 (Leder et al., 2001) and of HPV18 and BPV1 by ADAP, we have created a set of rules that can be applied to modify the late gene codons of other HPV types. A computer program that calculates modifications according to these rules is available at http://genome.nci.nih.gov/publications/papilloma_ADAP.html. The genes were chemically synthesized by Blue Heron Biotechnologies, confirmed by sequencing, and provided in their pBHBCMV mammalian expression vector. The resulting codon-modified 18L1 and 18L2 constructs were named peL1bhb and peL2bhb, respectively. The codon-modified 18L1 ORF was subcloned into vector puL1fB, which contains an EF1 α promoter. The puL1fB construct was made as follows: the Toll-like receptor gene of pUNO-hTLR09 (Invitrogen) was replaced with the ORF of codon-modified HPV 16 L1 from p16L1h to make puL1B. Enhanced green fluorescent protein (GFP) (Clontech) was then inserted into this plasmid to generate puL1fB. To build peL1fB, 16L1 was removed from puL1fB and replaced with codon-modified 18L1 from peL1bhb. GFP reporter plasmid pYafW was generated by inserting a fragment containing GFP into a modified version of pYafB. pYafB was generated by replacing the BPV1 URR and HSV TK polyA signal of pSU-5697 (Buck et al., 2004) with a BstBI–DraI fragment from the BPV1 genome encoding a putative packaging signal (Zhao et al., 1999). For reporter plasmids pYSEAP and p2CMVSEAP, secreted alkaline phosphatase was taken out of pSEAP2Basic and placed into pYafB or pCINeo (Promega) backbones, respectively.

Generation of pseudoviruses

Pseudoviruses were generated by cotransfection of 293TT cells as described elsewhere (C.B. Buck et al., in press, *Journal of Virology*). Briefly, 20 million 293TT cells were plated 16 h before transfection in 162-cm² flasks. Lipofectamine 2000 (Invitrogen) was used for transfection, as directed by the manufacturer, using 175 μ l of the lipid reagent and a total of 80 μ g of plasmid DNA per flask. For the production of HPV16 pseudovirus, the cells were transfected with 27 μ g each of pYSEAP, p16L1h, and p16L2h. For HPV18, cells were transfected with 27 μ g each of pYSEAP, peL1fB, and peL2bhb. For BPV1, we used 40 μ g each of pSheLL and pYSEAP. After 4–6 h, the media on the transfected cells were replaced. Cells were split into two 225-cm² flasks at 20–24 h and harvested

40–48 posttransfection. For crude extracts, cells were trypsinized and rinsed with Dulbecco's (D) PBS (Invitrogen). Pellets were resuspended at 50 million cells/ml in DPBS supplemented with an additional 9.5 mM MgCl₂. Brij 58 and Benzonase (Sigma) were added to a final concentration of 0.5% each, and Plasmid-Safe ATP-Dependent DNase was at 0.2% (Epicentre). The mixture was incubated at 37 °C for 45 min. NaCl was adjusted to 850 mM and the mixture placed at 4 °C for 10 min, then clarified by spinning at 1500 × *g* for 10 min at 4 °C. Supernatants were aliquoted into siliconized microcentrifuge tubes (BioPlas) and frozen at –80 °C. For purified preparations, the clarified supernatant was loaded on top of a 27,33,39% Optiprep (Sigma) density gradient that was performed at room temperature for 4 h. The material was centrifuged at 234000 × *g* for 3 h at 16 °C in an SW50.1 rotor (Beckman) and collected by bottom puncture of the tubes. Fractions were inspected for purity on 10% SDS–Tris–glycine gels (Bio-Rad), titrated on 293 TT cells to test for infectivity by SEAP detection, then pooled and frozen at –80 °C until needed.

Neutralization assay

Neutralization buffer was prepared by mixing DMEM without phenol red, 10% heat-inactivated FBS, 1% glutamate, 1% nonessential amino acids, 1% penicillin–streptomycin–fungizone, and 10 mM HEPES (Invitrogen). 293TT cells were preplated 3–4 h in advance in 96-well tissue culture-treated flat bottom plates (Corning-Costar) at 30000 cells/well in 100 µl neutralization buffer. To avoid evaporation, perimeter wells were filled with medium. Crude pseudovirus preparations were diluted 10000-fold, and Optiprep-purified material was diluted 150–400-fold. At these dilutions, target cells typically generated enough secreted alkaline phosphatase (SEAP) for an output reading of 30–70 relative light units (RLUs) (see below). Diluted pseudovirus stocks (80 µl/well) were placed in 96-well nontreated sterile, polystyrene plates (Nalge-Nunc), combined with 20 µl of diluted serum, and placed on ice for 1 h. The 100-µl pseudovirus–antibody mixture was transferred onto the preplated cells and incubated for 68–72 h. At the end of the incubation, 50 µl of supernatant was harvested and clarified at 1500 × *g* for 5 min. The SEAP content in the clarified supernatant was determined using the Great ESCAPE SEAP Chemiluminescence Kit (BD Clontech) as directed by the manufacturer, with 15 µl of the clarified supernatant. Twenty minutes after the substrate was added, samples were read in either white Microlite 1 (Dynex) or Optiplate-96 (Perkin-Elmer) opaque 96-well plates for 0.20 s/well using an MLX Microplate Luminometer (Dynex Technologies) set at Glow-Endpoint. Serum neutralization titers were defined as the reciprocal of the highest dilution that caused at least a 50% reduction in SEAP activity. A serum was considered to be positive for neutralization in the HPV16 or

HPV18 assay if it was neutralizing at a dilution at least 4-fold higher than the titer observed in the BPV1 neutralization assay.

Monoclonal antibodies and sera

Monoclonal antibody V5 was kindly provided by Neil Christensen (Christensen et al., 1996a). Monoclonal Antibody 5B6 was previously generated in our laboratory (Roden et al., 1994). Sera from vaccinated individuals were from a phase I HPV16 L1 VLP vaccine trial (Harro et al., 2001) and an unpublished phase II trial. Sera from naturally infected women were from a prospective cohort study of HPV and cervical neoplasia in Copenhagen (Kjaer et al., 1996). Reactivity for this group had been previously determined in an HPV16 ELISA assay (Kjaer et al., 2001), sera was analyzed at a single dilution of 1:10 and classified as positive or negative compared to a pooled standard. For this study, endpoint ELISA reactivity was performed as described below.

ELISA

IgG-specific HPV16 L1 VLP-based enzyme-linked immunosorbent assays (ELISAs) were performed in a 96-well plate format as described previously (Harro et al., 2001). Four-fold serial dilutions of each serum were assayed, starting at a dilution of 1:10. Sera were designated ELISA positive at a given dilution if the optical density (OD) was greater than or equal to 0.2 and also greater than or equal to the reactivity of a standard pooled serum (Wideroff et al., 1995), which was assayed on the same plate.

Statistical analysis

Spearman correlation coefficients (ρ) and linear weighted kappa values (κ) were calculated to evaluate reproducibility of the titers for the SEAP neutralization assay on crude preparations and to compare the titers among the SEAP neutralization assays on Optiprep-purified pseudovirions, the VLP ELISA, and the focus assay on subjects participating in the HPV16L1 VLP vaccine trials. Wilcoxon matched-pair sign-rank test was used to test differences in median titers. VLP ELISA and the SEAP neutralization assay test results (seropositive or seronegative) on sera from the natural history study were compared using McNemar's χ^2 test, unweighted kappa values and percent agreement were also calculated.

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