JOURNAL OF VIROLOGY, Aug. 1995, p. 5147–5151 Vol. 69, No. 8
0022-538X/95/$04.00+0

Papillomavirus L1 Capsids Agglutinate Mouse Erythrocytes through a Proteinaceous Receptor

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Received 13 March 1995/Accepted 19 April 1995

Virus-like particles (VLPs) composed of L1 derived from bovine papillomavirus type 1 (BPV-1), several human papillomavirus types, or cottontail rabbit papillomavirus (CRPV) agglutinated mouse but not human or rat erythrocytes. Treatment of mouse erythrocytes with trypsin prevented hemagglutination (HA) by BPV-1. Sera from rabbits immunized with native CRPV VLPs, which protect against experimental CRPV infection, exhibited high titers of antibodies that inhibited CRPV VLP HA activity, while sera from rabbits immunized with denatured CRPV VLPs or native BPV VLPs, which do not protect against CRPV infection, were not inhibitory. Testing for HA inhibition is a rapid and simple method for examining the serological relatedness of papillomaviruses and measuring protective antibody titers after VLP vaccination.

A wide range of viruses have been shown to agglutinate erythrocytes in vitro (21). This property has been exploited for the characterization and identification of cell surface receptors for a number of viruses, such as polyomavirus, influenza virus, and B19 parvovirus (3, 9, 22). Serologic variants of viruses can be characterized by the ability of neutralizing antibodies to interfere with virus-mediated hemagglutination (HA).

The papillomavirus capsid is composed of two proteins, L1 and L2 (17). The function of the L2 protein is unclear, but evidence suggests that although it is not required for receptor binding (19), L2 is required for infectivity (25). The major capsid protein, L1, is arranged in 72 pentamers with T=7 icosahedral structure (1, 11) and has the capacity to self-assemble into virus-like particles (VLPs) when expressed in eukaryotic cells (14). The VLPs have a structure very similar to that of virions at a resolution of 3.5 nm (11) and are able both to bind cell surfaces and to compete for bovine papillomavirus type 1 (BPV-1) infection of mouse C127 cells (7, 18, 19). In addition, the VLPs display the same type-specific, conformationally dependent neutralizing epitopes as native virions (5, 10, 14). This property makes VLPs a promising candidate for a prophylactic vaccine to protect against papillomavirus infection (2, 6). VLPs have also been used in an enzyme-linked immunosorbent assay (ELISA) to measure levels of serum antibodies to papillomavirus capsids and thereby detect patients who have been exposed to papillomaviruses (15).

It has been reported that BPV-1 but not cottontail rabbit papillomavirus (CRPV) virions can agglutinate mouse erythrocytes (8, 23). Human papillomavirus type 1 (HPV-1) is unable to agglutinate erythrocytes from a number of different species, but interaction with mouse erythrocytes was not tested (4). Since other data suggest that papillomaviruses share a common receptor (18, 19), we have reexamined their hemagglutinating properties by using VLPs derived from a number of different papillomaviruses.

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BPV-1 VLPs composed of L1 are sufficient for HA of mouse erythrocytes. Favre et al. demonstrated that BPV-1 virions and empty capsids purified from bovine papillomas were able to agglutinate mouse erythrocytes but not erythrocytes of any other species tested (8). We confirmed these results (Fig. 1) and examined whether BPV-1-derived VLPs were hemagglutinating.

VLPs were purified from Sf9 insect cells infected with recombinant baculovirus expressing either L1 or L1-L2 papillomavirus capsid proteins. The purification of VLPs was performed as described previously (16), except that the sucrose cushion pellet was resuspended in 10 ml of ice-cold phosphate-buffered saline (PBS) by sonication, layered over two step gradients (20 ml of 40% [wt/vol] sucrose in PBS over 10 ml of 70% [wt/vol] sucrose in PBS), and subjected to centrifugation for 150 min at 25,000 rpm in an SW28 rotor at 4°C. Material at the 40 to 70% (wt/vol) sucrose interface was harvested, resuspended in 27% (wt/wt) CsCl in PBS, and subjected to centrifugation for 20 h at 28,000 rpm in an SW28 rotor at 10°C. The band of VLPs forming at a density of approximately 1.28 g/cm^3 was harvested, repurified by isopycnic CsCl gradient centrifugation, dialyzed exhaustively in PBS containing 0.5 M NaCl, and stored at −80°C. The protein concentration was measured by the bicinchoninic acid assay (Pierce), and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Erythrocytes from Swiss Webster mouse (or rat or human) fresh citrated blood (Rockland) were harvested by centrifugation for 5 min at 1,000 × *g* and 4°C. The erythrocytes were washed and resuspended at 1% (vol/vol) in PBS containing 1 mg of bovine serum albumin (BSA) per ml. Purified VLPs were diluted in PBS containing 1 mg of BSA per ml and mixed with an equal volume of a 1% (vol/vol) suspension of erythrocytes in PBS, and 100 μl was transferred to a well of a 96-well plate (Falcon). The plates were incubated for 3 h at 4°C and photographed.

BPV virions disrupted at high pH (50 mM sodium bicarbonate [pH 10.8], 1 mM dithiothreitol) did not hemagglutinate mouse erythrocytes (data not shown). VLPs composed of L1 and L2 (L1-L2) derived from BPV-1 (14) were able to agglutinate Swiss Webster mouse (Fig. 1) but not human or rat

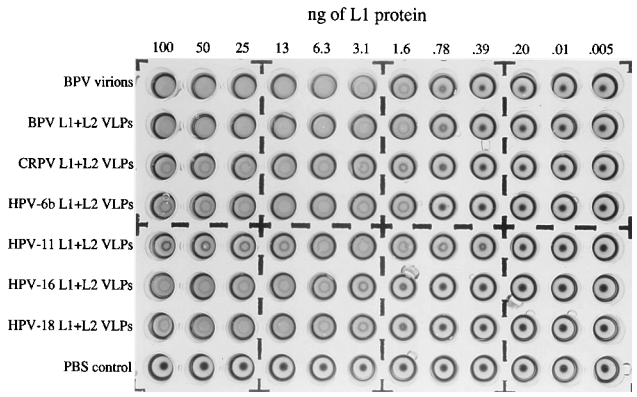

FIG. 1. Papillomavirus virions and VLPs agglutinate mouse erythrocytes. Purified papillomavirus virions and VLPs (1 μg each) were serially diluted twofold in PBS–0.1% BSA and mixed with mouse erythrocytes at a final concentration of 0.5% (vol/vol) in 0.1 ml of PBS–0.1% BSA per well of a 96-well plate. The samples were incubated for 3 h at 4°C and photographed.

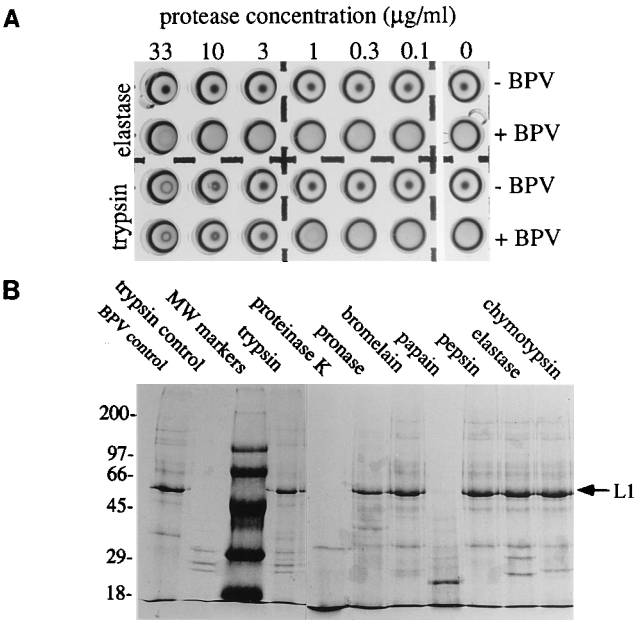

FIG. 2. Effect of protease treatment on BPV-1 virions and BPV-1-mediated HA of mouse erythrocytes. (A) A 1% (vol/vol) suspension of mouse erythrocytes washed in PBS was incubated with various concentrations of trypsin, elastase, and PBS for an hour at 37°C. The erythrocytes were washed once and resuspended at 1% (vol/vol) in PBS containing 1% (wt/vol) BSA, 20 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. Pretreated erythrocytes (50 μl) were mixed with an equal volume of PBS containing 1% (wt/vol) BSA, 20 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, and 15 ng of BPV-1 virions, incubated for 3 h at 4°C, and photographed. (B) Purified BPV-1 virions at 1 mg/ml were incubated in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2), 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$ for 1 h at 37°C with 0.1 mg of the proteases indicated per ml. The samples were separated under reducing conditions on an SDS–10% PAGE gel and Coomassie stained. MW, molecular weight. The numbers at the left are in kilodaltons.

erythrocytes (data not shown). The mouse erythrocytes were agglutinated by BPV-1 L1-L2 VLPs at the same concentrations as BPV-1 virions purified from a bovine wart; approximately 5 to 20 ng of particles per 100-μl reaction mixture containing 0.5% (vol/vol) erythrocytes was required for HA (Fig. 1). Furthermore, VLPs composed of BPV-1 L1 alone were also able to agglutinate mouse erythrocytes, but reproducibly at only fourfold higher concentrations of L1 protein than those of L1-L2 (data not shown). This difference is consistent with the ability of L2 to enhance the efficiency of VLP assembly in insect cells by approximately fourfold (16).

HA is a common activity of papillomavirus VLPs. Attachment of a virus to its host cell surface is the obligatory first step of infection and frequently is the major determinant of viral host range. Different virus types within the same genus, e.g., the major and minor serotypes of human rhinovirus and the retroviruses, may use different cell surface receptors (12). Therefore, we tested whether L1-L2 VLPs derived from CRPV and from HPV types 6b, 11, 16, 18, and 31 (13a, 14, 16) could agglutinate mouse erythrocytes. VLPs derived from all papillomavirus types tested were able to do so at concentrations similar to those required for BPV-1 L1-L2 VLPs and BPV-1 virion-mediated HA (Fig. 1). This is consistent with the ability of VLPs composed of BPV-1 L1 or HPV-16 L1-L2 to compete with BPV-1 virion infection of mouse C127 cells (18, 20).

HPV-16 L1-L2 VLPs did not agglutinate rat or human erythrocytes (data not shown). The inability of papillomavirus VLPs to agglutinate erythrocytes of species other than the mouse is in accord with previous results (4, 8) but is nonetheless unexpected since BPV-1 binds to nucleated cells derived from all species tested (18, 20). Although it has not been established whether binding is mediated by the same receptor on each cell type, the absence of receptors on rat and human erythrocytes, the ease of the HA assay, and convenient purification of large quantities of erythrocyte membranes make this an attractive model for study of papillomavirus receptor molecules.

In addition, the hemagglutinating activity per unit of virion protein provides a rapid method for assessing the quality of preparations of papillomavirus VLPs for use in vaccination, since improperly folded viral L1 protein does not contribute to the HA assay.

BPV-1 binds a proteinaceous receptor on the mouse erythrocyte membrane. A diverse range of molecules, including sialic acid for polyomavirus, class I major histocompatibility complex for simian virus 40, acetyl choline receptor for rabies virus, blood group P antigen for B19 parvovirus, and CD4 for human immunodeficiency virus, have been shown to be virus receptors (3, 9, 12, 22, 24). The cell surface receptors for papillomaviruses have not been identified. However, neuraminidase treatment of cell surfaces has been reported to have no effect upon agglutination of mouse erythrocytes by BPV-1 (8) or upon BPV-1 infection of mouse C127 cells (data not shown), suggesting that unlike polyomavirus, BPV-1 does not bind to sialic acid.

In an attempt to determine if the papillomavirus receptor on mouse erythrocytes is proteinaceous, we pretreated the erythrocytes with a number of proteases. The erythrocytes were then washed twice in PBS containing 1 mg of BSA per ml, 20 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride, and the HA assay was performed with BPV-1. Prior treatment of the erythrocytes with trypsin or proteinase K, but not elastase or chymotrypsin A4, abolished the ability of BPV-1 to hemagglutinate (Fig. 2A; data not shown for proteinase K and chymotrypsin A4). It should be noted that at high concentrations, all of the proteases caused the erythrocytes to clump, presumably by completely stripping the membranes of proteins. BPV-1 is resistant to high concentrations (0.1 mg/ml) of trypsin, elastase, chymotrypsin A4, pronase, and bromelain but not papain and proteinase K, as assessed by SDS-PAGE analysis (Fig. 2B). Therefore, it is unlikely that pretreatment of the erythrocytes with trypsin prevented BPV-1-mediated HA by degradation of the virions. Furthermore,

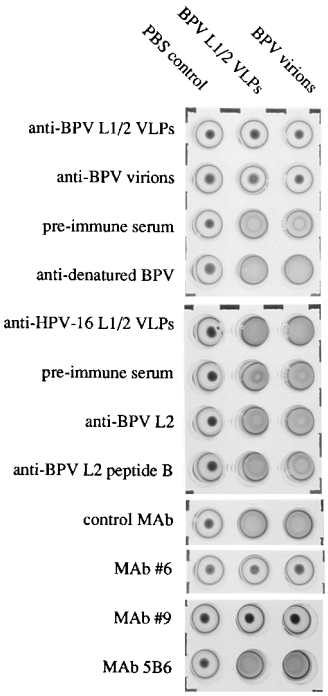

FIG. 3. Effect of antibodies to capsid proteins on BPV-1-mediated HA. BPV-1 virions and VLPs (15 ng each) were incubated for 1 h at ambient temperature with 1 μl of polyclonal serum or 1 μg of purified monoclonal antibody (MAb) or PBS. The samples were mixed with mouse erythrocytes at a final concentration of 0.5% (vol/vol) in 100 μl of PBS–0.1% BSA per well of a 96-well plate, incubated for 3 h at 4°C, and photographed.

addition of the protease inhibitors aprotinin and phenylmethylsulfonyl fluoride (to 20 μg/ml and 1 mM, respectively) during the HA assay (which is performed at 4°C) did not restore the ability of BPV-1 to agglutinate erythrocytes that had been pretreated with trypsin (Fig. 2A) or proteinase K (data not shown). The data suggest that BPV-1 binds to a protein on the surface of mouse erythrocytes, although it is not clear if this occurs via a protein-protein or protein-carbohydrate interaction.

For some viruses, such as encephalomyocarditis virus, the receptors in the erythrocyte and host cell membranes are different (13). Indeed, it is possible that receptors on the murine erythrocytes differ from those used by papillomaviruses in their natural life cycle. However, the binding characteristics of HA by the papillomavirus VLPs are very similar to those of VLP binding in cell lines, suggesting that they may be identical. In both systems, only properly folded VLPs are able to bind (data not shown). Antibodies that inhibit BPV binding to C127 cells also prevent HA (see below and references 8 and 20). Furthermore, the papillomavirus receptors on mouse erythrocytes, as in fibroblasts (18), are proteinaceous, as they may be cleaved by trypsin.

HA is inhibited by type-specific, neutralizing antisera to conformational papillomavirus epitopes. In an effort to confirm the specificity of VLP-mediated HA, a number of antibodies specific for the papillomavirus capsid proteins were tested for their ability to inhibit HA (Fig. 3). Prior to use, all antisera were cleared of nonspecific HA and complement activity by incubation with 4 volumes of a 25% (vol/vol) suspension of mouse erythrocytes overnight at 4°C. The erythrocytes were removed by centrifugation for 5 min at 1,000 × *g* and 4°C, and the supernatant was heated to 56°C for 30 min and cleared by centrifugation for 5 min at 16,000 × *g* and 4°C. Purified VLPs were diluted in PBS containing 1 mg of BSA per ml and incubated with pretreated antisera for 1 h at room temperature in a final volume of 50 μl. The samples were mixed with an equal volume of a 1% (vol/vol) suspension of erythrocytes and transferred to a well of a 96-well plate (Falcon). The plates were incubated for 3 h at 4°C and photographed.

Antiserum generated by denatured BPV-1, which is nonneutralizing, did not inhibit HA by BPV-1. In contrast, neutralizing antiserum generated by intact BPV-1 virions completely inhibited HA, demonstrating that HA-inhibiting antibodies, like neutralizing antibodies, recognize conformational virion epitopes. Comparison of the HA inhibition (HAI) titer and neutralizing titer of this antiserum suggests that the HAI assay is approximately 10- to 30-fold less sensitive than the neutralization assay (data not shown). Antiserum to VLPs composed of BPV-1 L1 alone (14) also inhibited HA (data not shown), while antiserum to bacterially derived full-length BPV-1 L2 did not (20), consistent with the finding that L1 is sufficient for receptor binding (Fig. 3).

Analogous results were obtained in studies of HPV-16 VLP-mediated HA. Rabbit antiserum raised against L1 VLPs derived from wild-type HPV-16 prevented HPV-16 VLP- but not BPV-1 VLP-mediated HA (data not shown). Therefore, the epitopes on VLPs recognized by HA-inhibiting antibodies are type specific. Furthermore, the type specificity of antibody inhibition argues strongly that HA is not caused by a contaminant in the VLP preparations (Fig. 3). Neither antiserum to the HPV-16 L1 of the reference strain, which contains a point mutation that reduces the efficiency of VLP assembly 1,000-fold (16), nor antiserum to bacterially derived HPV-16 L2 fused with glutathione *S*-transferase blocked HA (data not shown).

We have previously generated four monoclonal antibodies which neutralize BPV-1 and recognize type-specific conformational epitopes on L1 (20). Three of these monoclonal antibodies (MAbs 3, 6, and 9) were able to prevent binding of iodinated BPV-1 to C127 cells. However, monoclonal antibody 5B6 did not inhibit binding, demonstrating that neutralization can occur by two mechanisms. MAbs 3 (data not shown), 6, and 9 but not 5B6 were also able to prevent HA of mouse erythrocytes by BPV-1 VLPs and virions (Fig. 3).

HAI activity correlates with protection from experimental CRPV infection. A recent study has tested the ability of immunization with VLPs to protect rabbits against subsequent experimental challenge with infectious CRPV virions (2). No protection against papilloma formation or against progression to cancer was observed in rabbits immunized with native or denatured BPV-1 VLPs or with denatured CRPV L1-L2 VLPs. By contrast, substantial protection was induced in rabbits immunized with native CRPV VLPs composed of L1 alone or L1-L2, although a few animals developed a small number of lesions, none of which progressed to carcinomas (2).

Therefore, we wished to determine the relationship between protection from CRPV infection and antibody titers as measured by HAI and ELISA by using CRPV L1-L2 VLPs (Table 1). Although the ELISA was approximately 100-fold more sensitive than the HAI assay, protection from infection was found to correlate better with HAI titers than with ELISA titers. For example, the ELISA shows relatively high titers of antibodies to CRPV VLPs which were not protective in several rabbits immunized with denatured CRPV VLPs. By contrast, the HAI titer is less than 1:200 (the lowest dilution tested) in all eight rabbits from this group. The high ELISA titers in rabbits immunized with denatured CRPV VLPs are probably

TABLE 1. Comparison of HAI titers, ELISA titers, and susceptibility to CRPV infection in rabbits immunized with papillomavirus capsid proteins

Antigen for vaccination	HAI titer[a]	Serum ELISA titer ($\log_{10}$)[b]				Papilloma growth[c]
		CRPV L1-L2 VLPs	BPV-1 L1-L2 VLPs	Denatured CRPV L1-L2	Denatured BPV-1 L1-L2	
CRPV L1 VLPs	800	4		1		Abortive
	1,600	4		1		Negative
	200	3		1		Mild
	800	4		1		Moderate
	12,800	4		1		Negative
	800	4		1		Negative
	2,400	5		1		Abortive
	800	4		3		Negative
CRPV L1-L2 VLPs	800	4		2		Negative
	1,600	4		2		Negative
	1,600	4		2		Abortive
	800	4		<2		Mild
	1,600	4		<2		Negative
	800	4		2		Negative
	1,600	4		<2		Negative
	800	4		<2		Mild
Disrupted CRPV L1-L2	<200	3	1	2	1	Severe
	<200	3	1	2	<1	Severe
	<200	1	<1	<2	<1	Severe
	<200	3	<1	2	<1	Severe
	<200	3	1	2	<1	Severe
	<200	4	<1	2	<1	Severe
	<200	2	<1	2	<1	Moderate
	<200	4	<1	4	<1	Severe
Disrupted BPV L1-L2	<200	<3	4		3	Severe
	<200	<3	4		3	Severe
	<200	<3	4		2	Severe
	<200	<3	4		2	Mild
	<200	<3	4		3	Severe
	<200	<3	4		4	Severe
	<200	<3	4		2	Severe
	<200	<3	4		2	Severe
Adjuvant	<200	<1	<1	<1	<1	Severe
	<200	1	1	<1	<1	Severe
	<200	<1	<1	<1	<1	Severe

[a] Sera from immunized rabbits were analyzed for CRPV L1-L2 VLP-mediated HAI titers.

[b] ELISA titers to CRPV L1-L2 and BPV-1 L1-L2 native VLPs and denatured protein antigens are presented as the logarithms of the highest dilutions of serum that generate an optical density greater than 1.0 in this assay. Data are from reference 2.

[c] The maximum number and size of lesions which developed within 28 weeks after infection with 5×10^{10} particles of CRPV were graded as follows: severe, 10 to 100 papillomas; moderate, 3 to 10 papillomas; mild, 1 to 3 papillomas; abortive, 1 to 3 minute nongrowing papillomas; and negative, no papillomas. Data are from reference 2.

caused by partial denaturation of the VLPs when they bind to the assay plate.

Human sera with antibodies to HPV-16 VLPs do not have significant HAI activity. Sera from six patients attending a sexually transmitted disease clinic in Greenland were tested for antibodies to HPV-16 VLPs by ELISA (15, 18a) and for their ability to inhibit HPV-16 and BPV-1 L1-L2 VLP-mediated HA. Three of the sera had significant levels of antibodies that recognize HPV-16 L1-L2 VLPs in an ELISA (optical density values of 1.426, 1.355, and 1.373 at a 1:5 dilution), and three had reactivity to the antigen below the cutoff point (optical density values of 0.216, 0.277, and 0.295 at a 1:5 dilution) (15). None of the human sera showed significantly different HAI activity against HPV-16 or BPV-1 VLPs, while antisera from rabbits experimentally inoculated with either HPV-16 or BPV-1 VLPs exhibited high titers of type-specific inhibition of HA (data not shown). The negative results may be due to the absence of antibodies that prevent HPV-16 VLPs from binding to mouse erythrocytes or to the insensitivity of the assay relative to the ELISA. We favor the latter explanation on the basis of its analogy with the comparison between ELISA and HAI titers in rabbits immunized with CRPV VLPs.

Potential utility of the HAI assay. Compared with other viral antibody detection assays, the HAI assay was about 10-fold less sensitive than in vitro neutralization (using BPV), while the HAI assays for CRPV and HPV-16 were approximately 100-fold less sensitive than the ELISA (using CRPV or HPV-16). Binding of radiolabeled BPV to C127 cells and HA show similar sensitivities to inhibition by antibodies to the capsid (20). The low titers of anti-VLP antibodies present in patients infected with papillomaviruses make the HAI assay a poor candidate for assessing natural exposure to the virus.

However, in rabbits immunized with CRPV VLPs, high titers of antibodies that inhibited CRPV-mediated HA in a type-specific and conformationally dependent manner correlated strongly with protection from CRPV-induced papillomas and carcinomas (2). The HAI assay eliminated the false positives generated in the ELISA by denatured protein in the VLP preparations, because native capsids are required to bind to the erythrocytes. Therefore, the HAI assay may provide a rapid

and simple measurement of protective antibody titers after VLP vaccination.

It should be recognized, however, that the HAI assay represents a stringent surrogate assay for viral neutralization because it only detects those antibodies that prevent papillomavirus binding. Neither the neutralizing monoclonal antibody 5B6 nor neutralizing polyclonal antiserum to BPV-1 L2 inhibited BPV-1-mediated HA. This fact and previous data which measured the binding of iodinated BPV-1 to C127 cells suggest that the neutralization of papillomaviruses can occur at a step after the virus has bound to the cell (20).

Since antisera generated against papillomavirus capsid proteins are able to inhibit VLP-mediated HA in a type-specific and conformationally dependent manner, the HAI assay should be able to determine the serological relatedness of different papillomavirus isolates. Relatively little is known about this parameter, which represents a common method for classifying viruses within the same group and has potential relevance for the ecology of papillomaviruses. Furthermore, determining whether or not antisera to various HPV types cross-react with each other in an HAI assay should have a predictive value for cross-protection following vaccination. Such results would therefore have implications for the formulation of a polyvalent vaccine designed to immunize individuals against several HPV types.

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