

# A Human Papillomavirus (HPV) *In Vitro* Neutralization Assay That Recapitulates the *In Vitro* Process of Infection Provides a Sensitive Measure of HPV L2 Infection-Inhibiting Antibodies

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Papillomavirus L2-based vaccines have generally induced low-level or undetectable neutralizing antibodies in standard *in vitro* assays yet typically protect well against *in vivo* experimental challenge in animal models. Herein we document that mice vaccinated with an L2 vaccine comprising a fusion protein of the L2 amino acids 11 to 88 of human papillomavirus type 16 (HPV16), HPV18, HPV1, HPV5, and HPV6 were uniformly protected from cervicovaginal challenge with HPV16 pseudovirus, but neutralizing antibodies against HPV16, -31, -33, -45, or -58 were rarely detected in their sera using a standard *in vitro* neutralization assay. To address this discrepancy, we developed a neutralization assay based on an *in vitro* infectivity mechanism that more closely mimics the *in vivo* infectious process, specifically by spatiotemporally separating primary and secondary receptor engagement and correspondingly by altering the timing of exposure of the dominant L2 cross-neutralizing epitopes to the antibodies. With the new assay, titers in the 100 to 10,000 range were measured for most sera, whereas undetectable neutralizing activities were observed with the standard assay. *In vitro* neutralizing titers measured in the serum of mice after passive transfer of rabbit L2 immune serum correlated with protection from cervicovaginal challenge of the mice. This “L2-based” *in vitro* neutralization assay should prove useful in critically evaluating the immunogenicity of L2 vaccine candidates in preclinical studies and future clinical trials.

Clinical trials of human papillomavirus (HPV) L1 virus-like particle (VLP) prophylactic vaccines have demonstrated a high degree of safety, immunogenicity, and effectiveness at preventing infection and neoplastic disease caused by the vaccine-targeted types (reviewed in reference 24). Despite this success, vaccines based on the L2 minor capsid protein are attractive candidates for second-generation HPV prophylactic vaccines because, in contrast to L1 VLP vaccines, they induce broad cross-type protection as measured both with *in vitro* neutralization assays and with *in vivo* protection assays based on challenge with animal papillomavirus types or HPV pseudovirions (5, 14, 15, 16). For instance, immunization of rabbits with an HPV16-derived L2 peptide induced cross-protection against both cutaneous infection with cottontail rabbit papillomavirus (CRPV) and mucosal infection with rabbit oral papillomavirus (ROPV) (15). However, *in vitro* neutralization titers against homologous types induced by L2 immunogens have been much lower than the titers induced by L1 VLP-based vaccines, regardless of the adjuvant employed (34).

One possible explanation for the differences in neutralization titers is that an ordered multivalent display of epitopes on the VLP surface induces B cell activation and survival signals that cannot be matched by a monomeric antigen in conjunction with current adjuvants. However, virus-like display of L2 peptides has thus far not resulted in the induction of high titers of *in vitro* neutralizing antibodies (4, 35). A second possibility is that only a small proportion of the L2 antibodies generated by the vaccines can actually inhibit HPV infection. In this study, we have evaluated a third possibility, namely, that the current *in vitro* neutralization assays are insensitive measures of infection-inhibiting L2 antibodies and therefore underestimate the protective potential of L2 vaccines (29).

There are several reasons to believe that the latter explanation is at least partially responsible for this phenomenon. We have

repeatedly observed that mice injected with L2-based polypeptide vaccines are fully protected from cervicovaginal challenge with HPV pseudovirions, although at the time of challenge, their sera do not contain *in vitro* neutralizing antibodies against the corresponding virus that are detectable in the standard neutralization assay. We believe that *in vivo* protection following L2 immunization is antibody mediated because passive transfer of L2 antibodies can fully protect against challenge (11). Additionally, L2 is not encoded by the pseudovirions used in the challenge studies (2). Finally, the main L2 cross-neutralization epitopes are poorly exposed on mature virus, and current neutralization assays, if they predominantly detect this subset of L2 epitopes, could substantially underestimate the potential neutralizing activity of a given L2 immune serum.

Our recent delineation of the *in vivo* infectious process, utilizing our murine cervicovaginal model, is relevant to the possible insensitivity of current *in vitro* neutralization assays for the measurement of L2 neutralizing antibodies (21, 32). *In vivo*, HPV pseudovirions bind initially to heparan sulfate proteoglycan (HSPG) moieties, located on the basement membrane (BM), that are exposed following disruption of epithelial integrity. This binding induces a conformational change in the capsid that

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causes exposure of a furin/proprotein convertase cleavage site located at the amino terminus of L2. Subsequent furin/PC cleavage leads to a second conformational change, which further exposes the L2 amino terminus, including the major cross-neutralization epitopes (21). According to our model, these events expose a previously occluded surface of L1 that can now engage an unidentified (or putative) cell surface receptor on the keratinocytes as they migrate over the exposed BM to repair the disrupted epithelium (10, 21). L2 neutralizing antibodies bind to capsids following furin cleavage, preventing infection because the bound antibodies prevent capsids from transferring to, or stably associating with, the keratinocyte cell surface receptor (11, 12). Importantly, the L2 cross-neutralizing epitopes are exposed for an exceptionally long period, several hours, prior to internalization by the cell.

*In vitro* infection depends on similar sequential changes but differs from the *in vivo* process in several key respects. Although cultured keratinocytes produce an extracellular matrix (ECM) with some similarities to the BM, the role of the ECM in HPV infection *in vitro* is not the same as that of the BM *in vivo*. Pseudovirions can bind to ECM *in vitro*, but this interaction is not critical for subsequent infection, since virus bound directly to cells in suspension can lead to infection (P. M. Day, unpublished observations). It is the HSPGs on the cell surface, rather than those on the ECM, that are critical for capsid binding and infection (13, 17). Furin cleavage and L2 epitope exposure also occur on the cell surface, not generally on the ECM (10). Therefore, all of the described conformational changes that occur prior to infectious entry take place on the cell surface *in vitro*, unlike the *in vivo* situation, where initial attachment and furin cleavage occur on the BM, independent of the cell surface. This topological difference may result in the *in vivo* situation offering a greater opportunity for antibodies directed against L2 to bind to exposed epitopes on the BM prior to any interaction of the capsids with cell surfaces.

Given this new understanding of the differences between the mechanisms of *in vivo* and *in vitro* HPV infection, we sought to devise a more sensitive *in vitro* assay for measuring neutralizing L2 antibodies. This improved assay is based on the construction of an *in vitro* infection process that closely mimics our mechanistic model for *in vivo* infection. In the current study, we present the developmental steps in the formulation of this neutralization assay and demonstrate its sensitivity, relative to a traditional pseudovirion-based neutralization assay, in the detection of potentially protective L2 antibodies.

## MATERIALS AND METHODS

**Pseudovirions.** All pseudovirions were produced as previously described (3), utilizing the “ripcord” variation for *in vitro* assays and the “standard” variation for *in vivo* assays as detailed on the laboratory website (<http://home.ccr.cancer.gov/lco/default.asp>). The capsid-encoding plasmids p16Llw, p31Llw, p45Llw, p52Llw, p18Llw, p58Llw, and p11Lw and p11lw were utilized. For *in vivo* imaging, the plasmid pCLucf (a firefly luciferase expression plasmid) was encapsidated. For neutralization assays, either pYSeAP (a secreted alkaline phosphatase [SeAP] expression plasmid) or pfwB (a green fluorescent protein [GFP] expression plasmid) were encapsidated. All plasmids are described on the laboratory website.

**Cell lines.** The HeLa cell line was obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (P/S). 293TT cells, which were previously described, were cultured in the same medium (1). 804G cells were a kind gift

of Jonathan Jones (31) and were also cultured in DMEM-FBS and P/S. The pgsa-745 cell line was also obtained from ATCC and cultured in DMEM-FBS, P/S, and 10 mM proline. The same media were utilized for propagation of FD11 cells and CHO $\Delta$ furin ( $\Delta$ furin) cells obtained from Steven Leppa (NIAID, NIH) and David Fitzgerald (NCI, NIH), respectively. MCF10A cells, obtained from ATCC were cultured in DMEM/F12 medium supplemented with 5% horse serum, P/S, 500  $\mu$ g/ml hydrocortisone (Sigma H-0888), 10  $\mu$ g/ml insulin (Sigma I-1882), and 20  $\mu$ g/ml epidermal growth factor (100-15; Peptrotech).

**Animal studies.** Six- to eight-week-old female BALB/cAnNCr mice were obtained from the National Cancer Institute and housed and handled in accordance with the NCI-approved guidelines, and all protocols were approved by the NCI’s Animal Care and Use Committee. Animals were immunized subcutaneously with 5  $\mu$ g of HPV16 VLPs or 25  $\mu$ g of His<sub>6</sub>-tagged L2 11-88 $\times$ 5 peptide (19) precipitated onto Inject Alum (77161; Thermo) in a final volume of 100  $\mu$ l. Animals were immunized three times, with each injection spaced 2 weeks apart, and sera were collected 2 weeks after the final immunization in order to create the serum pools. Polyclonal rabbit serum was generated against the L2 11-88 $\times$ 5 polypeptide by Lampire Biological Laboratories. Three immunizations with 300  $\mu$ g peptide at 3-week intervals were performed. The first immunization was in complete Freund’s adjuvant. The following two were in incomplete Freund’s adjuvant. The exsanguination bleed was 2 weeks following the final immunization.

***In vivo* pseudovirus delivery and infection.** The mice received 3 mg of Depo-Provera (NDC 0009-0626-01; Pfizer) 5 days prior to infection. Animals were pretreated with 4% nonoxynol-9 approximately 5 h prior to administration of pseudovirus, as previously described (8, 20, 32). A pseudovirus inoculum of 10  $\mu$ g, based on L1 content, was used regardless of HPV type, since this would equalize the potential number of target epitopes for the neutralizing antibodies. For those mice receiving the passively transferred rabbit sera, the indicated volume of sera was diluted in 1 $\times$  phosphate-buffered saline (PBS) to a final volume of 100  $\mu$ l and was administered intraperitoneally 24 h prior to infection. Infection was measured 48 h after pseudovirus delivery by intravaginally instilling 20  $\mu$ l of D-luciferin-K<sup>+</sup> salt (0.3 mg, 122796; Caliper Life Sciences), followed by imaging with an Ivis 100 system (Caliper Life Sciences) at 4°C, as previously described (8, 20). Raw data were computed using the Living Image software program (Caliper Life Sciences). An identical region of interest (ROI) was drawn around the area from which the luciferase signal was emitted for each mouse, and the average radiance within the ROI was determined. Data represent the mean luminescence for 5 mice per group.

**L1-based neutralization assay.** The L1-based neutralization assay was performed as previously described (28). Briefly, pYSeAP-containing pseudovirions were incubated with immune serum on ice for 60 min prior to addition to previously plated 293TT cells. SeAP production was measured using the Great EscAPE kit (631737; Clontech) 72 h following pseudovirus addition.

**L2-based neutralization assay.** MCF10A cells were plated at a concentration of  $2 \times 10^4$ /well in a 96-well assay plate in a volume of 100  $\mu$ l of growth medium and propagated for 24 h. Following this incubation, which allows for deposition of the ECM, the cells were washed twice with PBS and lysed by incubation for 5 min at 37°C with 50  $\mu$ l of prewarmed lysis buffer (PBS containing 0.5% [vol/vol] Triton X-100–20 mM NH<sub>4</sub>OH). Following lysis, 100  $\mu$ l of PBS was added to each well, and a 100- $\mu$ l volume was removed. This gentle washing was repeated two more times. Then the entire remaining volume was gently removed. The ECM-containing wells were then washed twice further with 100  $\mu$ l of PBS. A pseudovirion (with the pfwB plasmid packaged) solution, prepared in conditioned medium from CHO $\Delta$ furin cells in a total volume of 120  $\mu$ l/well, was added to the prepared ECM-containing wells following removal of the final PBS wash. This pseudovirus solution also contained 5  $\mu$ g/ml of heparin (Sigma H-4784). Addition of this small amount of heparin results in an improved consistency of antibody titers (data not shown). This virus-furin-heparin mixture was incubated overnight at

37°C. The next day, the medium was removed and the wells were washed twice with PBS. The final wash was replaced with an antibody dilution series made in pgsa-745 growth medium at a volume of 100  $\mu$ l/well. The plate was then incubated at 37°C for 6 h to allow efficient antibody binding to target epitopes. This incubation can be shortened if desired. No change in antibody titers was observed with incubation times of 1 h, 2 h, 4 h, or 6 h (data not shown). Following this incubation, pgsa-745 cells were added directly to the antibody-containing wells at a concentration of  $8 \times 10^3$ /well in a volume of 50  $\mu$ l. Infection was allowed to continue for 2 days at 37°C, and individual cells were scored by flow cytometric analysis for GFP expression as a measure of infection. Although pseudovirus with a packaged GFP expression plasmid was used for the development of the assay to facilitate enumeration of individual infectious events, the method should be readily transferable to other described pseudovirion-based expression assay systems (e.g., secreted alkaline phosphatase and Gaussia luciferase).

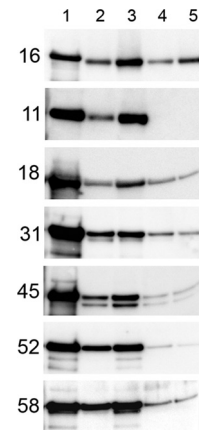
All pseudovirions were titrated in the assay to determine the amount of virus needed to yield 20 to 30% GFP-positive cells. This amount was used for the neutralization assays. Conditioned medium from CHO $\Delta$ furin cells was prepared by plating  $1 \times 10^6$  cells in 17 ml of growth medium 4 days prior to harvesting. For all experiments, 3-fold dilution series of antisera were made starting with an initial dilution of 1/50. Data were analyzed, and 50% inhibitory concentrations (IC<sub>50</sub>s) were determined using the GraphPad Prism software program. Where indicated, furin inhibitor (calbiochem, 344930) was added at a final concentration of 5  $\mu$ M.

**Western blot.** Fifty nanograms of capsids were added to ECM extracted from cultured cells as described above or 1  $\mu$ g of Cultrex EHS-derived ECM (Trevigen 3432-005-01) and allowed to attach at 37°C overnight. Following this incubation, wells were washed three times with PBS. Bound capsids were liberated directly into Laemmli SDS-PAGE dye, run on a 4 to 12% Tris-glycine gel, and transferred to a polyvinylidene difluoride (PVDF) membrane for Western analysis. HPV16 and HPV18 were detected with Camvir-1 antibody (Abcam ab69). All other HPV types were detected with rabbit polyclonal sera generated against the respective virus-like particles (sera were generated as previously described) (33).

## RESULTS

**Examination of binding to various ECMs.** The first step in the development of an *in vitro* infection assay that more closely mimicked *in vivo* events was to identify an ECM source that could support strong capsid association for multiple HPV types. We examined the binding of HPV16, HPV11, HPV18, HPV31, HPV45, HPV52, and HPV58 capsids to ECM derived from HaCaT cells, MDF10A cells, 804G cells, and Cultrex, a commercial ECM preparation derived from Engelbreth-Holm-Swarm murine sarcoma cells. These cell lines were examined because MCF10A and 804G were reported to secrete an ECM that is rich in laminin 332 (22, 26), a proposed ECM receptor for HPV11 (9), and we have previously observed a strong association of HPV16 capsids with HaCaT-derived ECM (10). All HPV types bound well to the ECM produced by HaCaT and MCF10A cells. The binding to 804G-derived ECM was weaker for all HPV types examined, and binding to the Cultrex ECM preparation was weak for all types except HPV16 (Fig. 1). The factors responsible for these observed differences are unclear and are under investigation.

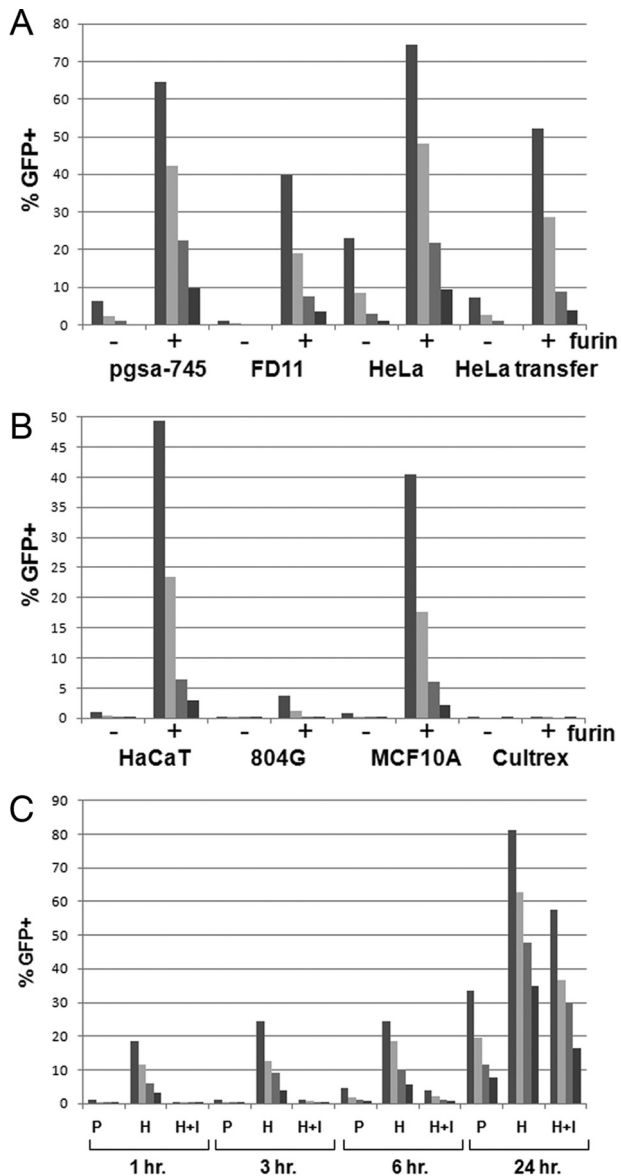
**Examination of ECM-based infection.** As noted earlier, furin cleavage is required for papillomavirus infection *in vivo* and in cultured cells, although the location of the furin cleavage occurs on the basement membrane *in vivo* and on the cell surface in standard cell culture-based infectivity assays (11, 21, 30). To confirm that infection resulting from virus adhered to ECM prior to addition of cells was also furin dependent, we bound GFP-expressing HPV16 pseudovirus to ECM produced by MCF10A cells,



**FIG 1** Pseudovirion binding to extracellular matrices. Pseudovirions were bound overnight to the indicated matrices at 37°C. Unbound capsids were removed by washing. Bound capsids were solubilized in SDS-PAGE loading dye, separated on a 4 to 12% acrylamide gel, and processed for Western analysis. The HPV type is indicated on the left of each row. Input virus was loaded in lane 1. Virus bound to ECM preparations is shown as follows: lane 2, HaCaT; lane 3, MCF10A; lane 4, 804G; lane 5, Cultrex, a commercial ECM preparation derived from EHS cells.

one of the two ECM substrates that strongly bound the various HPV types, in the presence of conditioned medium from a furin-deficient CHO cell line, FD11, or a furin-secreting cell line, CHO $\Delta$ furin, which is also derived from CHO (6, 18). Therefore, these cell lines presumably differ only in furin production. Following overnight virus adsorption, media and unbound virus were removed, and wells were washed thoroughly. Susceptibility to infection was evaluated for three different target cells, HeLa, FD11, and the HSPG-deficient cell line pgsa-745, which differ with respect to their expression of furin and of cell surface HSPG. The cells were added to the ECM-virus-containing wells, and infection was allowed to proceed for 48 h. Additionally, for one experimental set, the saturation of capsid binding to the ECM substrate was estimated by placing the unbound virus and media from the wells into a new plate, to which HeLa cells were added.

As shown in Fig. 2A, the addition of  $\Delta$ furin supernatant greatly increased infection in all cases. Furthermore, it was essential for infection of the FD11 cells, as expected, since these cells are deficient for endogenous furin production, although they express HSPGs. The results confirm the requirement for furin for HPV infection. The pgsa-745 cell line expresses furin but lacks HSPG, due to a deficiency of xylosyltransferase (25). Therefore, infection of this line is also highly dependent on the  $\Delta$ furin supernatant, since it is robustly infected only after the requirement for HSPG has been bypassed by furin precleavage of the capsids (12). Since HeLa cells express both HSPG and furin, they can be infected without addition of exogenous furin. However, the finding that exogenous furin increases infection of HeLa cells indicates that furin is a limiting factor for *in vitro* infection of this commonly employed cell line. The examination of virus transferred to new wells and tested with HeLa cells indicates that infection by the transferred virus was consistently about one-half that of the direct HeLa infection, regardless of the amount of input virus. Therefore, the ECM binding was not saturable under the conditions employed, although the lower titer of the transferred virus may imply a relatively unstable interaction with the ECM.



**FIG 2** Furin-dependent infection following ECM attachment. (A) A titration series of HPV16 pseudovirus containing a packaged GFP expression plasmid was applied to MCF10A-derived ECM in conditioned medium from FD11 cells or CHO $\Delta$ furin cells (indicated as  $-/+$  furin, respectively). Unbound virus and medium were removed by washing, the indicated target cells were added, and infection was allowed to proceed for 2 days. Pseudovirus infection was determined by flow cytometric analysis of GFP $^{+}$  cells. The HeLa transfer condition shows the infection derived from unbound pseudovirus that was transferred to empty wells. HeLa cells were then added, and infection was allowed to proceed as described above. (B) A titration series of HPV16 pseudovirus containing a packaged GFP expression plasmid was applied to the matrices indicated in conditioned medium from either FD11 cells or CHO $\Delta$ furin cells (indicated as  $-/+$  furin). Unbound virus and medium were removed by washing, pgsa-745 target cells were added, and infection was allowed to proceed for 2 days. Pseudovirus infection was determined by flow cytometric analysis of GFP $^{+}$  cells. (C) A titration series of HPV16 pseudovirus containing a packaged GFP expression plasmid was applied to MCF10A-derived ECM in conditioned medium from CHO $\Delta$ furin cells for the indicated time. Unbound virus and medium were removed by washing, the target cells were added, and infection was allowed to proceed for 2 to 3 days, depending upon the time of virus removal. The target cells were either pgsa-745 (P), HeLa (H), or HeLa and 5  $\mu$ M furin inhibitor (H+I). Pseudovirus infection was determined by flow cytometric analysis of GFP $^{+}$  cells.

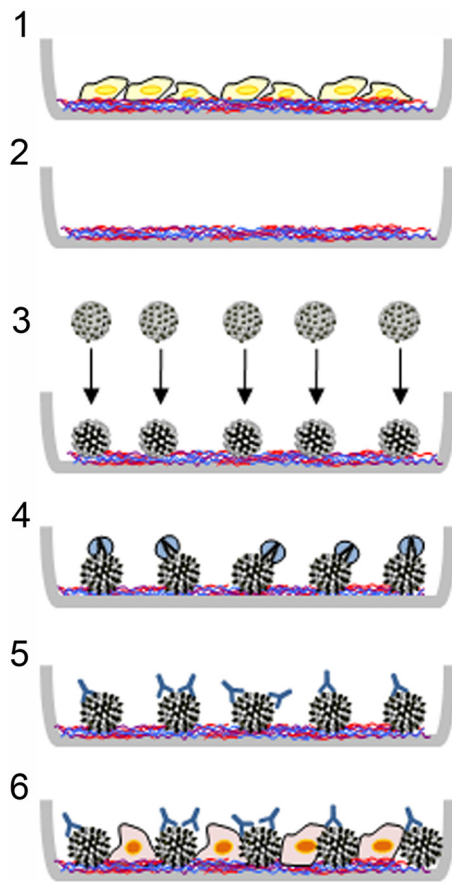
We next determined how the panel of ECM preparations utilized initially (Fig. 1) would perform in this infectivity assay, using pgsa-745 cells as the target cell line and HPV16 as a model pseudovirus. As shown in Fig. 2B, we found that high infectivity was obtained after binding HPV16 capsids to the ECM produced by HaCaT and MCF10A. In all cases, infectivity was greatly increased in the presence of  $\Delta$ furin supernatant compared to results with FD11 supernatant. Surprisingly, neither the 804G-derived ECM nor the Cultrex ECM preparation supported subsequent infection, although, as shown in Fig. 1, HPV16 capsids bound reasonably well to these matrices. We speculate that binding to these matrices does not induce, or inhibits, the conformational changes required for furin cleavage and/or the exposure of the secondary receptor binding site.

For subsequent experiments, we chose to use ECM produced by MCF10A cells as the binding substrate, since all the HPV types examined bound best to this ECM and HPV16 binding resulted in high infectivity of target cells. To maximize secondary receptor-dependent infection, the assay was performed in the presence of  $\Delta$ furin supernatant and utilized pgsa-745 as the target cell (12). Because our *in vivo* studies have demonstrated that the furin-dependent exposure of the major L2 neutralization epitopes is a slow process (21), we decided to examine this parameter within the constraints of this *in vitro* assay. To address this, we adsorbed HPV16 pseudovirus to MCF10A-derived ECM in the presence of  $\Delta$ furin supernatant for 1 h, 3 h, 6 h, or 24 h. Following the incubation, unbound pseudovirus and furin were removed, and infection of pgsa-745 cells, HeLa cells, or HeLa cells in the presence of furin inhibitor was assessed. As seen in Fig. 2C, the infection of both pgsa-745 and HeLa cells with furin inhibitor was poor until the 24-h time point. This indicates that, similar to the situation *in vivo*, *in vitro* exposure of the L2 furin cleavage site is a slow process. We also observe an increase in untreated HeLa cells at 24 h, following furin cleavage, similar to that shown in Fig. 2A. Therefore, the long initial incubation of pseudovirus and ECM in the presence of furin is essential for subsequent infection of HSPG- or furin-deficient cell lines.

A simplified schematic of the finalized assay is shown in Fig. 3. We refer to this newly developed assay as the “L2-based neutralization assay,” as opposed to the original “L1-based neutralization assay.”

**Examination of neutralization titers.** To initiate a comparison of the performance of the L2-based and L1-based neutralization assays, we analyzed sera from animals immunized with an L2 immunogen comprising a fusion polypeptide composed of amino acids 11 to 88 from HPV16, HPV18, HPV1, HPV5, and HPV6, designated L2 (11-88 $\times$ 5) (19). This immunogen is a candidate for clinical development because it induces relatively high titers of cross-neutralizing antibodies in the L1-based neutralization assay and protects against challenge by homologous and heterologous HPV types in a mouse model (11). We determined neutralizing activity against HPV types 16, 31, 33, 45, and 58. These types were selected for the following reasons: HPV16 is a component of the L2 (11-88 $\times$ 5) candidate vaccine, as well as the commercial HPV VLP vaccines; HPV31, -33, and -58 are phylogenetically related to HPV-16 but are progressively more divergent from it and are not included in either the L2- or VLP-based vaccines; the L2 (11-88 $\times$ 5) vaccine protects against HPV58, in contrast to the VLP vaccine (27); and HPV45 is closely related to HPV18.

A polyclonal rabbit serum was tested first, because rabbits gen-



**FIG 3** Schematic of L2-based neutralization assay. The simplified diagram illustrates the main steps in this assay. 1, MCF10A cells are plated in a 96-well plate and grown for 24 h to allow for ECM deposition; 2, the ECM remains on the plastic following lysis of the MCF10A cells; 3, a pseudovirus preparation is plated onto the ECM; this interaction results in a conformational change in the capsid that exposes the N terminus of L2; 4, exogenous furin is applied with the pseudovirions to allow cleavage of the conserved N-terminal furin cleavage site in L2; this results in exposure of the major cross neutralization epitopes in L2; 5, following removal of unbound pseudovirus, an antibody titration series is applied to the plate and incubated at 37°C for 6 h; 6, HSPG-deficient pgsa-745 cells are plated into the virus/antibody-containing wells. Infection is assessed following a 48-h incubation.

erally generate higher titers of *in vitro* neutralizing antibodies against L2 immunogens than mice. As shown in [Table 1](#), the mean titer against HPV16 was 120-fold higher in the L2-based assay, and similar increases in titers were observed against the other tested

**TABLE 1** Comparison of neutralization titers obtained with the L2-based assay versus those obtained with the L1-based assay<sup>a</sup>

Parameter	Value for virus				
	HPV16	HPV31	HPV33	HPV45	HPV58
Neutralization titer					
Nonimmune	<50	<50	<50	<50	<50
Rabbit anti-L2 L1 assay	12,150	1,350	4,050	12,150	4,050
Rabbit anti-L2 L2 assay	$1.5 \times 10^6$	$1.2 \times 10^5$	$4.6 \times 10^5$	$9.0 \times 10^5$	$2.7 \times 10^5$
Fold increase	120	85	114	74	66

<sup>a</sup> The L1-based neutralization assay was compared to the new L2-based neutralization assay using a rabbit polyclonal serum raised against L2 (11-88×5) as a sample immune serum. Serum from a nonimmune rabbit was used as a control. The fold increase in sensitivity between the two assays is indicated.

**TABLE 2** *In vitro* neutralization titers for sera from immune animals<sup>a</sup>

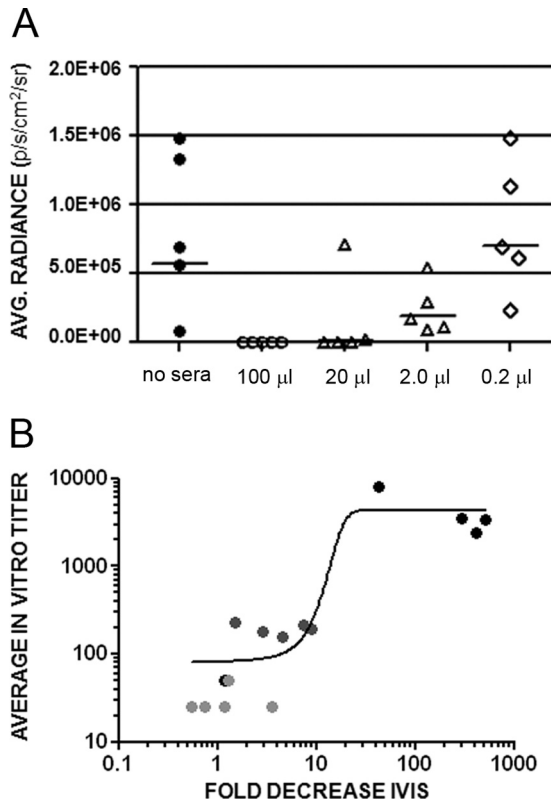
Assay and serum pool	Neutralization titer for virus				
	HPV16	HPV31	HPV33	HPV45	HPV58
<b>L2-based assay</b>					
PBS, pool 1	<50	<50	<50	<50	<50
PBS, pool 2	<50	<50	<50	<50	<50
HPV16 VLP, pool 1	172,080	<50	<50	<50	<50
HPV16 VLP, pool 2	306,550	<50	<50	<50	<50
L2 11-88×5, pool 1	7,500	50	4,315	2,780	5,370
L2 11-88×5, pool 2	13,760	765	4,130	2,640	4,025
L2 11-88×5, pool 3	4,095	<50	<50	365	700
L2 11-88×5, pool 4	10,730	7,865	27,145	7,805	13,820
L2 11-88×5, pool 5	20,900	2,600	1,605	5,890	3,395
L2 11-88×5, pool 6	27,630	480	210	5,005	10,940
L2 11-88×5, pool 7	3,420	190	22,385	1,375	1,140
<b>L1-based assay</b>					
PBS, pool 1	<50	<50	<50	<50	<50
PBS, pool 2	<50	<50	<50	<50	<50
HPV16 VLP, pool 1	7,600	<50	<50	<50	<50
HPV16 VLP, pool 2	60,605	<50	<50	<50	<50
L2 11-88×5, pool 1	<50	<50	<50	<50	<50
L2 11-88×5, pool 2	<50	<50	<50	<50	<50
L2 11-88×5, pool 3	<50	<50	<50	<50	<50
L2 11-88×5, pool 4	<50	<50	50	50	<50
L2 11-88×5, pool 5	<50	<50	<50	50	<50
L2 11-88×5, pool 6	<50	<50	50	<50	<50
L2 11-88×5, pool 7	<50	<50	150	50	<50
<i>P</i> value, 1-7 (L2-based vs L1-based assay)	0.001	0.011	0.012	0.002	0.001

<sup>a</sup> Cross-neutralization titers obtained with “L1-based” and “L2-based” assays. Sera were obtained from mice that had been immunized with L2 (11-88×5) and shown to be immune to *in vivo* challenge with HPV16, -31, -45, or -58. Sera from 5 animals that underwent identical treatment were pooled and assessed for *in vitro* neutralization with the newly described L2-based neutralization assay or the “L1-based” assay. As specificity controls, pooled sera from HPV16 VLP-immunized animals were also assayed. The assay was performed in duplicate, and the average titer is shown. Triplicate experiments were not possible due to limited sample volume. The bottom row indicates the *P* value of the difference between the two groups of 7 pools for each virus type. Titers that were found to be <50 were assigned a value of 25 for statistical analysis. The *P* value is based on the Mann-Whitney test with Gaussian approximation.

types, ranging from 66- to 114-fold. These results encouraged us to examine the sera from L2-immunized mice.

Given the higher titers of the L2 (11-88×5)-immune rabbit serum in the L2-based neutralization assay, we examined sera from mice that had been immunized with the L2 (11-88×5) antigen and shown to be protected against experimental cervicovaginal challenge with HPV16, -31, -45, or -58 pseudovirus (11). Using pools of sera obtained from 7 groups of animals (5 mice/group) immunized with L2 (11-88×5), *in vitro* neutralizing titers were measured by the L2-based assay. This assay gave positive results with HPV16, -45, and -58 for all 7 pools and positive results with HPV31 and -33 for 6 of the 7 pools ([Table 2](#), L2-based assay). The titers for the heterologous HPV types ranged from  $2 \times 10^2$  to  $2.7 \times 10^4$ , with titers of  $>10^3$  for 19 of the 26 positives.

In contrast, the L2-based assay gave negative cross-neutralization results with pooled sera of mice immunized with HPV16 VLPs, confirming the specificity of the cross-neutralization observed with the anti-L2 sera in the L2-based assay. Furthermore, the L2-based assay was much more sensitive than the L1-based



**FIG 4** *In vivo* protection and correlation with *in vitro* neutralization titers. (A) *In vivo* infection of animals that were passively transferred with the indicated volume of rabbit anti-L2 (11-88 $\times$ 5) serum was compared to control HPV16 infection (no sera). The volume of serum transferred per animal is indicated. All sera were diluted to a total volume of 100  $\mu$ l/animal. Each symbol represents an individual animal. (B) The correlation of these values with the *in vitro* neutralization titers (Table 3) obtained for the circulating antibodies from these animals obtained immediately prior to challenge. The fold decrease in the *in vivo* infection is indicated on the horizontal axis. The average *in vitro* titer is indicated on the vertical axis. Each circle represents an individual animal. The black circles represent the 20- $\mu$ l dose, dark gray represents the 2- $\mu$ l dose, and light gray represent the 0.2- $\mu$ l dose.

assay for detecting the L2 cross-neutralizing antibodies, since most of the pooled sera from the L2 (11-88 $\times$ 5)-immunized mice were negative for the heterologous HPV types in the L1-based assay, and the titers of those that were positive never exceeded 150 (Table 2, L1-based assay). The L2-based assay produced higher titers for sera from animals immunized with HPV16 VLPs, but the sensitivity was increased by 1 order of magnitude, compared with an increase of at least 2 orders of magnitude for antibodies to L2.

It is not surprising that L1 neutralizing antibodies are also efficiently detected in the new assay. Although previous *in vitro* studies have shown that high levels of neutralizing anti-L1 sera prevent L2 epitope exposure (10), these sera are also able to completely neutralize furin-precleaved pseudovirus (unpublished data). This additional mechanism of neutralization is consistent with the well-described ability of anti-L1 antibodies to prevent *in vitro* infection after cell surface binding (7). Additionally, *in vivo* analysis revealed that neutralization at lower anti-L1 antibody concentrations occurred by a mechanism that permitted BM binding and L2 epitope exposure but prevented stable association with the keratinocytes (11).

**Passive transfer of L2 (11-88 $\times$ 5) antisera: *in vivo* and *in vitro* correlation.** To further validate the L2-based neutralization assay, we assessed the correlation between the *in vitro* neutralizing titers of passively transferred L2 antibodies in the circulation of mice at the time of cervicovaginal challenge and the protection of the mice from cervicovaginal infection. The mice were given an intraperitoneal injection of a dilution series of the rabbit polyclonal serum raised against L2 (11-88 $\times$ 5) and were challenged 24 h later with HPV16 pseudovirions.

*In vivo* protection from infection was observed at the two lowest dilutions of immune rabbit serum (100  $\mu$ l and 20  $\mu$ l) but lost at the two highest dilutions (2  $\mu$ l and 0.2  $\mu$ l) (Fig. 4A). When *in vitro* neutralization titers were determined for the individual mice with sera taken at the time of challenge, all animals that had been protected from *in vivo* challenge possessed detectable neutralization titers in the L2-based assay (Table 3). The average *in vitro* neutralization titers for these animals is shown in Table 3. One striking example of the generally good correlation between the two assays is that the high outlier seen in the *in vivo* experiment at the 20- $\mu$ l dose had an exceptionally low neutralization titer measured *in vitro* (Table 3, mouse 4). The correlation between the *in vitro* titers and the *in vivo* inhibition, which is shown in Fig. 4B, had a Spearman coefficient correlation of 0.8376 with a two-tailed *P* value of <0.0001.

## DISCUSSION

In this study, we have developed an *in vitro* neutralization assay that appears to be at least 100-fold more sensitive for the detection of infection-inhibiting L2 antibodies than the standard neutralization assay, which was developed to measure neutralizing antibodies elicited by L1 VLP vaccines. It is likely that this new assay

**TABLE 3** *In vitro* neutralization titers of sera obtained from animals that had passive transfer of immune serum<sup>a</sup>

Serum (immunization dose [ $\mu$ l])	<i>In vitro</i> neutralization titer	Ivis value	% inh.
Preimmune 1	<50	$1.3 \times 10^6$	
Preimmune 2	<50	$1.5 \times 10^6$	
Mouse 1 (20)	2,419	$2.0 \times 10^3$	100
Mouse 2 (20)	3,497	$2.8 \times 10^3$	100
Mouse 3 (20)	3,350	$1.6 \times 10^3$	100
Mouse 4 (20)	50	$7.1 \times 10^5$	14
Mouse 5 (20)	8,100	$1.9 \times 10^4$	98
Mouse 6 (2.0)	211	$1.1 \times 10^5$	87
Mouse 7 (2.0)	154	$1.8 \times 10^5$	82
Mouse 8 (2.0)	190	$9.3 \times 10^4$	89
Mouse 9 (2.0)	183	$2.9 \times 10^5$	65
Mouse 10 (2.0)	228	$5.4 \times 10^5$	35
Mouse 11 (0.2)	<50	$2.3 \times 10^5$	70
Mouse 12 (0.2)	50	$6.4 \times 10^5$	23
Mouse 13 (0.2)	<50	$1.1 \times 10^6$	-33
Mouse 14 (0.2)	<50	$1.5 \times 10^6$	-80
Mouse 15 (0.2)	<50	$7.0 \times 10^5$	16

<sup>a</sup> Comparison of *in vitro* neutralization titers and *in vivo* protection. The sera from the experiment shown in Fig. 4A (passive transfer of rabbit immune serum) were used to obtain *in vitro* neutralization titers with the L2-based assay. The assay was performed in duplicate, and the average titer is shown. The Ivis value indicates the average radiance value indicating *in vivo* infection from Fig. 4A. Percent inhibition (% inh.) is determined based on the average *in vivo* infection for the five animals shown in Fig. 4A ( $8.3 \times 10^5$ , average radiance). A negative value for percent inhibition indicates a value over the average radiance of the control conditions.

will be critical for immunogenicity analyses of the anticipated early-phase clinical trials of L2-based vaccines. Since antibody responses to subunit vaccines, e.g., L1 VLPs, are often quantitatively similar in mice and humans, it would be difficult to move forward with clinical testing of an L2 vaccine that generated low or undetectable *in vitro* neutralizing activity in preclinical studies, as has generally been found using the *in vitro* L1-based assay. It should also be useful in preclinical studies to reevaluate the potential of some of the many published and unpublished L2 candidates that might have been abandoned because they generated low or no neutralizing antibodies, as measured in the standard L1-based assay.

The rationale for development of the L2-based assay was based on the recognition that passive transfer of sera from animals vaccinated with L2-based immunogens conferred protection against genital HPV challenge, although the sera lacked detectable neutralizing activity in the standard L1-based *in vitro* neutralization assay. The passive transfer results are encouraging for L2 immunogens because they indicate that protective antibodies are induced. However, such *in vivo* assays are too cumbersome for routine use in the immunogenicity analyses of a clinical trial.

Development of the L2-based assay was enabled by the new understanding of the early *in vivo* steps involved in HPV infection, especially those that occur on the basement membrane and lead to exposure of the L2 cross-neutralization epitopes. The assay was based on the hypothesis that generating a process of *in vitro* infection that more closely mimics the model of *in vivo* infection would increase the sensitivity with which L2 neutralizing antibodies can be detected. The fact that this hypothesis was correct provides strong support for the broad outlines of the model, particularly as it relates to the exposure of the L2 cross-neutralization epitopes (21).

The passive transfer experiments indicate a correlation for the rabbit serum between *in vivo* protection and the *in vitro* titer obtained with the L2-based assay. Complete *in vivo* protection (98% to 100%, with the exception of one outlier) was observed following transfer of 20  $\mu$ l of serum. *In vitro* titers in the range of 2,419 to 8,100 were obtained for the protected animals. Transfer of 2  $\mu$ l of serum resulted in intermediate *in vivo* protection (35% to 89%) and *in vitro* titers from 154 to 228. *In vitro* titers were below the limit of detection for 4/5 animals at the 0.2- $\mu$ l dose, whereas 3 animals were partially protected *in vivo* (16, 23, and 70% protection). Therefore, the *in vivo* assay may still be slightly more sensitive when considering serum concentrations that offer a lower level of protection. However, the *in vitro* assay is not subject to the intrinsic variability associated with animal experimentation.

However, it should also be noted that the pools of L2 (11-88 $\times$ 5) immune sera demonstrated rather large variations in titers against specific HPV types, and the titers measured for a specific pool against one type did not predict the titers measured against other types (Table 2, L2-based assay). For instance, pool 7 had the lowest titer against the homologous type HPV16 (3,420) but had an exceptionally high titer against heterologous type HPV33 (22,385). Pool 3 was exceptional in that it had no measurable titer against HPV31 and -33 in the new assay, despite a titer of over 4,000 against HPV16. In contrast, pool 4 had rather consistent titers against all types tested, in the 8,000 to 27,000 range. In future studies, it will be important to compare L2 candidate vaccines for consistency in the induction of neutralizing antibodies among individuals and across types.

A recent mouse study identified substantial differences in the relative sensitivities of the *in vivo* genital HPV challenge assay and the standard L1-based *in vitro* assay as measures of protective L1 antibodies (23). These authors demonstrated that the *in vivo* detection of protection was slightly more sensitive (30-fold) than the *in vitro* assay for a murine L1-specific monoclonal antibody. However, examination of a polyclonal murine anti-Gardasil serum indicated that the *in vivo* assay was 500-fold more sensitive. It is possible that multiple mechanisms of neutralization are at play in the polyclonal response *in vivo* and that these are not equally measurable in the *in vitro* L1 neutralization assay. In support of this conjecture, we have previously shown that passively transferred anti-L1 serum could neutralize by different mechanisms when present at low concentrations versus high concentrations (11). In contrast, passively transferred anti-L2 serum neutralized by the same mechanism at both high and low concentrations, which might provide an explanation for our results showing a closer alignment of *in vivo* and *in vitro* assays.

In future work, it will also be important to assess the degree to which the species source of the serum may affect the *in vivo* response and its correspondence to *in vitro* titers. In the current study, an immune rabbit serum was used for the passive transfer experiments. There may exist some species-specific differences in transduction of transferred serum or Fc receptor-mediated mechanisms of neutralization.

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