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Cross-neutralization of cutaneous and mucosal Papillomavirus types with anti-sera to the amino terminus of L2

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Abstract

Vaccination with papillomavirus L2 has been shown to induce neutralizing antibodies that protect against homologous type infection and cross-neutralize a limited number of genital HPVs. Surprisingly, we found that antibodies to bovine papillomavirus (BPV1) L2 amino acids 1–88 induced similar titers of neutralizing antibodies against Human papillomavirus (HPV)16 and 18 and BPV1 pseudoviruses and also neutralized HPV11 native virions. These antibodies also neutralized each of the other pseudovirus types tested, HPV31, HPV6 and Cottontail rabbit papillomavirus (CRPV) pseudoviruses, albeit with lower titers. HPV16, HPV18, HPV31, HPV6 and CRPV L2 anti-sera also displayed some cross-neutralization, but the titers were lower and did not encompass all pseudoviruses tested. This study demonstrates the presence of broadly cross-neutralizing epitopes at the N-terminus of L2 that are shared by cutaneous and mucosal types and by types that infect divergent species. BPV1 L2 was exceptionally effective at inducing cross-neutralizing antibodies to these shared epitopes.
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Introduction

HPVs can be classified into those that infect non-genital cutaneous sites and those that infect ano-genital and oral mucosal sites (reviewed in (Lowy and Howley, 2001)). Non-genital skin warts are a prevalent disease of childhood, while most ano-genital and oral infections affect adult populations. Among the genital–mucosal genotypes, persistent infection with "high-risk" or "oncogenic" HPV genotypes is a necessary but not sufficient cause of cervical cancer (Bosch et al., 1995; Clifford et al., 2003; Walboomers et al., 1999). Infection with "low-risk" HPV genotypes (especially HPV6 and HPV11) accounts for most cases of genital warts. Elimination of cervical cancer will require effective vaccination against more than 15 known genital HPV types (Bosch and de Sanjose, 2003; Munoz et al., 2004). Protection from infection by immunization with virus-like particles (VLPs) and by passive transfer of antibodies in animal models (Breitburd et al., 1995; Christensen et al., 1996b; Kirnbauer et al., 1996; Suzich et al., 1995) have provided the rationale for initiating vaccine trials in humans in an effort to curb the morbidity and mortality associated with these viruses. Immunization with VLPs composed of the Papillomavirus (PV) major capsid protein, L1, generates neutralizing antibodies that are primarily type specific (Christensen and Kreider, 1990, 1991; Christensen et al., 1990; Ghim

et al., 1991; Kirnbauer et al., 1994; Koutsky et al., 2002; Roden et al., 1995). Limited cross-reactivity has been observed among closely related types such as HPV18 and 45, and HPV6 and 11 (Christensen et al., 1994; Combita et al., 2002; Giroglou et al., 2001; Roden et al., 1996; White et al., 1998) when L1 or L1/L2 VLPs were the immunogen. Furthermore, both animal challenge (Breitburd et al., 1995; Jarrett et al., 1990) and clinical studies (Koutsky et al., 2002) have suggested that protection will be largely type-specific. The plethora of oncogenic types (notably HPV16, HPV18, HPV45, HPV31, HPV33, HPV52 and HPV58, which together are responsible for 87% of cervical cancer cases (Munoz et al., 2004)), indicate that broad protection will require a multi-valent vaccine directed against as many of the >15 oncogenic HPV types as possible. Alternative vaccination protocols yielding cross-protective antibodies against a single antigen would greatly reduce the complexity and expense of generating and analyzing multi-type vaccines if they achieved similar protection levels.

Vaccination with L2 can provide immunity from homologous PV challenge in animal model systems. This protection appears to be mediated by a relatively low-titer neutralizing antibody response in vaccinated animals (Chandrachud et al., 1995; Christensen et al., 1991; Embers et al., 2002). In contrast to the predominantly type-specific antibodies directed against L1 neutralization epitopes, cross-neutralization has been observed among divergent genital HPV types, that is, HPV6, 16 and 18 (Roden et al., 2000), or HPV6 and 16 (Kawana et al., 1999) with antibodies generated against HPV L2 proteins or peptides. Although they provide protection, neutralizing antibody titers elicited by L2 vaccination are typically 2–3 orders of magnitude lower than those found in L1 VLP vaccinated animals. Furthermore, it is unclear how effective anti-L2 antibodies would be at neutralizing even more distantly related papillomaviruses. Here, we study the cross-neutralizing activity of antibodies generated against recombinant L2 proteins and peptides from different animal and human papillomavirus types that represent both genital and cutaneous groups.

Results and discussion

Cross-neutralization of BPV1, HPV16, and HPV18 pseudoviruses with anti-full-length L2 serum

Neutralization of different papilloma pseudoviruses with sera from rabbits immunized with L2 (full-length or peptides) was performed. As positive and type-specificity controls, and for inter-assay normalization of the different papillomavirus neutralization assays, L1 VLP antiserum or monoclonal antibodies were used; H16.V5 monoclonal for HPV16 (Christensen et al., 1996a), 5B6 monoclonal for BPV1 (Roden et al., 1994), or sera from rabbits immunized with VLPs from the homologous type for the remaining pseudoviruses. As described previously (Roden et al., 2000), none of the sera raised against L1 or L1/L2 VLP immunogens derived from HPV6, HPV16, HPV18, BPV1 or CRPV induced cross-neutralizing antibodies in the pseudovirus infectivity assays tested (data not shown). This is consistent with the presence of immuno-dominant neutralizing epitopes in L1 and the sub-dominance of L2 in the context of the capsid (Roden et al., 2000).

Analysis of anti-full-length L2 sera was initially performed with BPV1, HPV16 and HPV18 pseudoviruses. The sera showed neutralization of the homologous types with reciprocal titers that were 600 for BPV1; 2100 for HPV16; and 1350 for HPV18 (Table 1A). Unlike L1 VLP antiserum, anti-HPV18 and HPV16 full-length L2 sera showed reciprocal cross-neutralization. These two types had been shown to share neutralizing L2 epitope(s) (Roden et al., 2000), and their L2 proteins share 53.4% amino acid identity (Fig. 1). Anti-sera raised against recombinant full-length HPV16, and HPV18 L2 protein were also able to neutralize BPV1 pseudoviruses in this assay, albeit with lower titers (Table 1A). In a previous study, neutralizing activity against BPV1 pseudoviruses was not detected in the sera of sheep immunized with these proteins (Roden et al., 2000); however, the lack of neutralization might be attributable to the less sensitive neutralization method used in that study. Unexpectedly, a serum raised against full-length BPV1 L2 neutralized HPV16 and HPV18 pseudovi-

Table 1A

Cross-neutralization of divergent human and animal papillomaviral pseudoviruses by polyclonal antisera to full-length L2 from diverse papillomaviruses

Anti-serum	Neutralization titer*					
	BPV1	HPV16	HPV18	HPV31	HPV6	CRPV
Preimmune full length BPVL2	<50 (2)	<50 (2)	<50 (2)	<50 (2)	<50 (2)	<50 (2)
Full-length BPV L2-His	**600 (4)**	780 (4)	1780 (4)	<50 (2)	<50 (2)	<50 (2)
Full-length HPV16 L2-GST	<50 (2)	**12,150 (4)**	1350 (2)	2340 (2)	260 (2)	450 (3)
Full-length HPV16 L2-His	90 (8)	**2100 (10)**	650 (6)	260 (2)	110 (4)	90 (2)
Full-length HPV18 L2-His	150 (2)	4050 (2)	**1350 (2)**	450 (2)	70 (3)	70 (3)
Full-length HPV31 L2-His	<50 (2)	110 (4)	<50 (2)	**450 (2)**	<50 (3)	<50 (3)
Full-length HPV6 L2-His	<50 (2)	90 (2)	150 (2)	50 (2)	**4050 (3)**	50 (3)
Full-length CRPV L2-His	<50 (2)	1350 (4)	<50 (2)	780 (2)	260 (2)	**10,120(6)**

* The bolded and shaded titers in this table represent the geometric mean neutralization titer against the homologous virus. The number in parenthesis indicates the number of times the assay was performed.

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BPV      ---MSARKRVKRASAYDLYRTCKQAGTCPPDVIPKVEGDTIADKILKFGG
HPV16    MRHKRSAKRTKRASATQLYKTCKQAGTCPPDIIPKVEGKTIADQILQYGS
HPV18    -MVSHRAARRKRASVTDLYKTCKQSGTCPPDVVPKVEGTTLADKILQWSS
HPV31    MRSKRSTKRTKRASATQLYQTCKAAGTCPSDVIPKIEHTTIADQILRYGS
HPV6b    -MAHSRARRRKRASATQLYQTCKLTGTCPPDVIPKVEHNTIADQILKWGS
CRPV     ---MVARSRKRRAAPQDIYPTCKIAGNCPADIQNKFENKTIADKILQYGS
         *  :**:   ::* *** :*.**.*:   *.*   *:**:**::..
BPV      LAIYLGGLGIGTWSTGRVAAGGSPRYTPLRTAGSTSSLASI
HPV16    MGVFFGGLGIGTGS----GTGGRTGYIPLGTRPPTATDTLA
HPV18    LGIFLGGLGIGTGS----GTGGRTGYIPLGGRSNTVVDVGP
HPV31    MGVFFGGLGIGSGS----GTGGRTGYVPLSTRPSTVSEASI
HPV6b    LGVFFGGLGIGTGS----GTGGRTGYVPLQTSAKPSITSGP
CRPV     LGVFFGGLGISSAG----GSGGRLGYTPLSGGGGRVIAAAP
         :.:::*****.:  .    .:**    * **
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"*" denotes identical residues in that column in all sequences in the alignment.
":" denotes conserved substitutions
"." denotes semi-conserved substitutions.

Fig. 1. Homology of Amino Termini of Papillomavirus L2 protein.

rions as effectively as it neutralized BPV1 pseudovirions. HPV16 and HPV18 L2 proteins only share 38.4% or 35.7% amino acid identity, respectively, with BPV1 L2 (Fig. 1). Another unexpected result was the relative lack of cross-neutralization of HPV types that are more closely related. For example, serum against HPV31 L2, which shares 69% identity with HPV16 and 54.3% with HPV18 (Fig. 1), only neutralized HPV16 at a titer of 110 and did not neutralize HPV18 at the highest concentration tested (1:50). The anti-HPV6 L2 serum cross-neutralized HPV16 and HPV18, but not BPV1. CRPV L2 shares 34–39% identity with BPV1, HPV16 and HPV18 L2, and although it also unexpectedly cross-neutralized HPV16 with relatively high titers (1350) (Table 1A), it failed to neutralize BPV1 and HPV18.

These data together suggested that BPV1 L2 might have an epitope(s) that is either more immunogenic or more efficiently displayed/folded and allows for generation of reactive, broadly cross-neutralizing anti-L2 sera. The apparent broad cross-neutralizing activity obtained with the full-length BPV1 L2 serum, although promising, also generated some concerns because it was obtained from a single animal. Immunization of another animal with the same recombinant preparation showed similar homologous and heterologous (HPV16) neutralization results (Table 2). Furthermore, similar results were obtained with GST and 6-His tagged HPV16 L2 antiserum (Table 1A), suggesting that the tag plays no role in generating neutralizing antibodies.

Cross-neutralization of BPV1, HPV16, and HPV18 pseudoviruses with anti-L2 a.a. 1–88 serum

To map which polypeptide of the BPV1 L2 was responsible for the cross-neutralization, sera raised against different regions of BPV1 L2 were tested for their anti-BPV1 and HPV16 activity. The only defined cross-neutralizing epitope described so far is for residues 108–120 of HPV16 L2 (Kawana et al., 1999). This peptide is a conserved region in L2 with 46% identity between HPV16 and HPV18. However, there is little homology with BPV1 L2 in this region (15% identity). In contrast, the amino termini of L2

proteins of different PV types exhibit considerably higher percent identities, even for distantly related papillomaviruses. There is 67.4% identity between HPV16 and HPV18 within the first 88 amino acids of these proteins, and both HPV16 and HPV18 share roughly 55% identity with BPV1 L2 (Fig. 1), suggesting a conserved structure and perhaps also function. Of note, BPV1 L2 amino acids 1–88 encompass a region that can bind to the surface of a variety of cell lines, interfere with BPV1 infection, and may be involved in transport of particles across the cytoplasm (Yang et al., 2003). Furthermore, a portion of the region appears to be displayed on BPV1 virion surfaces (a.a. 61–123) (Liu et al., 1997). In addition, Kawana et al. (1999) showed that the epitope of an HPV16 monoclonal antibody that neutralizes HPV16 but not HPV6 also maps to residues 69–81. The only two cysteines of L2 and a region very rich in glycines (Fig. 1), both with unknown function, are in this 1–88 region. Highly conserved lysines and arginines involved in DNA binding (Zhou et al., 1994) and infection (Roden et al., 2001) are also in this region. The high homology in this region and its importance in infectivity raised the possibility that an immunogen based on a peptide from this region might induce cross-neutralizing antibodies against divergent HPV types.

Anti-BPV1 L2 1–88 sera from two rabbits were able to neutralize HPV16 and BPV1 pseudovirions with approximately the same titers (Table 2), attesting to the reproducibility and to the ability of sera raised against the N-terminal portion of BPV1 L2 to have good neutralizing activity against HPV16. Anti-BPV1 L2 1–88 sera had neutralizing titers ranging between 1780 and 4740 (Table 2), confirming what had been seen with full-length BPV1 L2 immunization. Furthermore, serum from a third rabbit, immunized with a different preparation of the BPV1 L2 1–88 recombinant protein, had similar activity (data not shown).

Cross-neutralization of native HPV11 virions by L2 a.a. 1–88 antiserum

Although BPV1 L2 1–88 antisera neutralizes BPV1 virions in the focal transformation assay (Roden et al., 1994),

it was possible that the observed cross-neutralization was an artifact of the pseudovirion system. To address this issue and extend evidence of cross-neutralization to another HPV type, we tested the ability of BPV1 L2 1–88 antisera to neutralize native HPV11 in vitro (Fig. 2). Both neutralizing monoclonal antibody H11.H3 and rabbit antiserum to full-length HPV11 L2 (1:50) completely neutralized HPV11 infection. Furthermore, a 1:50 dilution of BPV1 L2 1–88 antiserum, but not the pre-immune serum, also neutralized native HPV11 virions. In addition to showing that BPV1 L2 1–88 can also neutralize HPV11, this result supports the conclusion that the neutralization data obtained with pseudovirus assay are biologically relevant.

We sought to further define the nature of the cross-neutralizing epitopes. An anti-BPV1 L2 45–172 serum that had previously been shown to neutralize BPV1 (Roden et al., 1994) failed to neutralize HPV16, although it did neutralize BPV1 in our assay at low titers. Sera raised against peptides 130–257, 216–340, 300–425 and 384–469 failed to neutralize either BPV1 or HPV16 pseudovirus detectably (Table 2). Analysis for all experiments in Tables 1A and 1B and 2 shows that rabbits vaccinated with the L2 1–88 peptide (n = 7) do not generate significantly higher titers against homologous type virus than rabbits (n = 8) vaccinated with the full-length L2 protein (P = 0.28). Our data are consistent with the localization of the major cross-neutralizing epitopes at the N terminus of L2. The high-titer cross-neutralizing response obtained when immunizing with the amino terminus of BPV1 L2 suggests that vaccination strategies should focus on this highly conserved region.

To further define the nature of the cross-neutralizing epitopes in HPV16 L2 recognized by BPV1 L2 1–88 antiserum, we tested its reactivity with a peptide array comprising 20 mer peptides of the first hundred amino
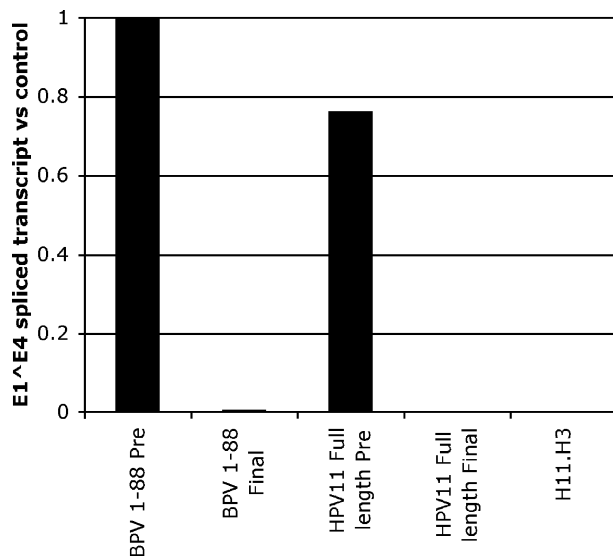
Table 1B

Cross-neutralization of divergent human and animal papillomaviral pseudovirus types by polyclonal antisera to BPV1 or HPV16 amino-terminal L2 peptides

Anti-Serum	Neutralization titer[a]					
	BPV1	HPV16	HPV18	HPV31	HPV6	CRPV
BPV1 L2 a.a. 1–88	3460 (7)	4740 (7)	7020(5)	220 (3)	340 (4)	780 (3)
HPV16 L2 aa 1–88#1	<50 (4)	3080 (4)	150 (2)	260 (2)	<50 (4)	260 (2)
HPV16 L2 aa 1–88#2	50 (4)	1350 (4)	150 (2)	260 (2)	<50 (4)	<50 (2)
HPV16 L2 aa 1–88#3	50 (4)	1780 (4)	150 (2)	90 (2)	90 (4)	260 (2)
HPV16 L2 aa 1–88#4	<50 (4)	780 (4)	90 (2)	150 (2)	60 (4)	90 (2)

[a] Titers are given as geometric means. The number in parenthesis indicates the number of times the assay was performed.

acids of HPV16 L2 that overlapped by 12 residues. While no single epitope was dominant, reactivity mapped predominantly to the central region of this peptide (Fig. 3).

We also examined sera from amino acids 1–88 of HPV16 L2 for cross-neutralizing activity. Sera from all four immunized rabbits had relatively high homologous neutralization titers (Table 1B) that ranged from 780 to 3080 and differed by less than 4-fold. All of the anti-HPV16 L2 1–88 sera were able to cross neutralize HPV18, but with lower titers than those observed with the anti BPV1 L2 1–88 sera. Only two of the four anti-HPV16 L2 1–88 sera tested were able to neutralize BPV1 pseudoviruses at 1:50. Although the mean titer of the anti BPV1 L2 1–88 serum appears to be higher against the heterologous HPV16 and HPV18 pseudovirions than against the homologous type, in each case, the median neutralization titers are 4050, whether it is against BPV1, HPV16 or HPV18 pseudovirus, and they do not differ more than 3-fold from the median for each type. However, subtle effects of differing particle to infectivity ratios between pseudovirions preparations are also possible. The neutraliz-

Fig. 2. Neutralization of native HPV11 virions by BPV1 L2 1–88 antiserum.

Table 2

Neutralization of BPV1 and HPV16 pseudoviruses by polyclonal antisera to BPVL2 polypeptides

Anti-serum	Neutralization titer	
	BPV1	HPV16
Pre-immune full length BPV1 L2	<50 (2)	<50 (2)
Full length BPV1 L2#1	600 (4)	780 (4)
Full length BPV1 L2#2	780 (2)	780 (2)
BPV1 L2 a.a. 1–88#1	2600 (5)	1780 (4)
BPV1 L2 a.a. 1–88#2	3460 (7)	4740 (7)
BPV1 L2 a.a. 45–173	90 (4)	<50 (4)
BPV1 L2 a.a. 130–257	<50 (4)	<50 (4)
BPV1 L2 a.a. 216–340	<50 (2)	<50 (2)
BPV1 L2 a.a. 300–425	<50 (2)	<50 (2)
BPV1 L2 a.a. 384–469	<50 (2)	<50 (2)

Titers are given as geometric means. The number in parenthesis indicates the number of times the assay was performed.
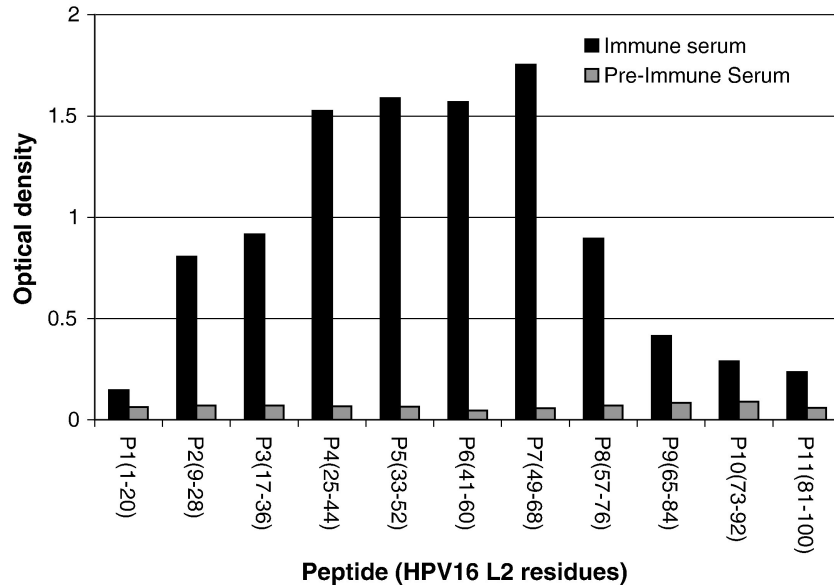
Fig. 3. Reactivity of BPV1 L2 1–88 antiserum with an HPV16 L2 peptide array.

ing titer against homologous type pseudovirus generated by vaccination with residues 1–88 from BPV1 (3 rabbits) and HPV16 (4 rabbits) were not significantly different ($P = 0.67$). By contrast, the neutralizing titer of antiserum to BPV1 peptide 1–88 neutralized HPV16 pseudovirus with a higher titer than antiserum to HPV16 peptide 1–88 against BPV1 pseudovirus ($P = 0.02$). These results again pointed to BPV1 L2 1–88 being a better immunogen than HPV16 L2 for eliciting cross-neutralizing HPVs.

It is possible that the neutralization titers against homologous type virus versus heterologous type virus might vary after repeated immunization. Therefore, we measured the BPV1 and HPV16 pseudovirion neutralizing titers of L2 1–88 antisera of BPV1 and HPV16 from sera obtained 74 days (P1), 88 days (P2) and 102 days (Final) after the initial immunization from animals that had received 3 or 4 boosts (Table 3). Both sera demonstrated a consistent neutralization titer against homologous type and heterologous type virus. Thus, we find no evidence of narrowing of BPV1 L2 1–88 antiserum specificity over this time window and with repeated boosts.

Table 3
Stability of neutralization titers with repeated immunization

Serum	Bleed	Boosts	Day	Anti HPV16 titer	Anti BPV1 titer
Rabbit S844-2	Prebleed	0	0	<50	<50
anti HPV16	P1	3	74	4050	50
L2 1–88	P2	4	88	4050	150
	Final	4	102	4050	50
Rabbit 5315#2	Prebleed	0	0	<50	<50
anti-BPV1	P1	3	74	12,150	12,150
L2 1–88	P2	4	88	12,150	12,150
	Final	4	102	12,150	12,150

Cross-neutralization of HPV31, HPV6, and CRPV pseudoviruses with anti-full-length L2 serum

Sera raised against full-length L2s were also tested against additional pseudoviruses. The BPV1 full-length L2 sera, which had cross-neutralized HPV16 and 18 pseudoviruses, was unexpectedly negative against HPV31, CRPV and HPV6 (Table 1A). HPV16 L2 shares about 69.1% identity with HPV31, but only 53.6% and 39.4% with HPV6 and CRPV, respectively. However, anti-HPV16 L2 sera cross-neutralized HPV31, HPV6 and CRPV, and the titers differed by no more than 3-fold (Table 1A). HPV31 L2 shares 38.7%% identity with CRPV, an identity similar to that between HPV16 and CRPV, but anti-HPV31 L2 was unable to neutralize CRPV pseudoviruses. It is tempting to attribute the lack of cross-reactivity to the relatively low homologous titer of that serum (450); however, the homologous BPV1 L2 titer was comparable (600), and as noted above this serum was able to cross-neutralize HPV16 and HPV18 with similar titers (Table 1A). Anti-HPV31 full-length L2 serum also failed to neutralize CRPV and HPV6 pseudovirions. In addition to its HPV16 cross-neutralizing capabilities, the anti-CRPV L2 serum was also cross-neutralizing for HPV31 and HPV6, but not for HPV18 or BPV1, despite a similar degree of homology with these proteins.

Cross-neutralization of HPV31, HPV6, and CRPV pseudoviruses with anti-L2 a.a. 1–88 serum

We tested anti-BPV1 L2 a.a. 1–88 serum. Despite the failure of anti-full-length BPV1 L2 serum to neutralize HPV31, we found that the anti-BPV1 L2 1–88 peptide serum was able to cross-neutralize HPV31, with titers similar to those obtained with anti-HPV16 L2 peptide 1–88

(Table 1B). Identities in this region of L2 between HPV16 and HPV31 are 77% (Fig. 1), and 58% between BPV1 and HPV31. The anti-BPV1 L2 serum was also able to neutralize HPV6 with a titer of 340 and CRPV with a titer of 780. Of note is the fact that the 1–88 a.a. region of BPV1 L2 has about the same amount of homology (56–58%) with HPV16, HPV18, HPV31 and HPV6; and only slightly lower (47.6%) with CRPV (Fig. 1).

Serum IgG is responsible for the cross-neutralizing activity

To determine whether immune IgG was responsible for the cross-neutralization, the following two experiments were conducted. In the first experiment, sera depleted of IgG using protein G Sepharose was tested in HPV16 and BPV1 neutralization assays. Neither pre- nor post-immune IgG depleted sera were able to neutralize either HPV16 or BPV1 pseudovirus (Table 4). In the second experiment, IgG from pre- and post-immune samples were purified using protein G columns (Pierce). Purified IgG from pre-immune sera was unable to neutralize either pseudovirus (Table 4) at the highest concentration tested, 1:30 (28 ng/μl), while purified IgG from anti-BPV1 L2 1–88 serum neutralized both BPV1 and HPV16 at a dilution of 6400 (0.2 ng/μl). Purified IgG from rabbits immunized with HPV16 L2 1–88 neutralized both HPV16 and BPV1, but the titers were higher against HPV16 compared to BPV1, as had been observed with whole serum (Table 4).

Immune responses to L2 are suboptimal, since the anti-L2 neutralizing titers are significantly lower than anti-L1 neutralizing titers raised against L1 only VLPs (data not shown); yet studies in rabbits and cattle have consistently shown that L2 immunization can be sufficient to provide protection, probably via neutralizing antibodies, from experimental challenge with homologous virus (Campo et al., 1997; Embers et al., 2002). Furthermore, vaccination of both humans and mice with full-length HPV16 L2 peptides elicits cross-neutralizing antibodies, suggesting that this phenomenon is not restricted by species (Kawana et al., 2001, 2003).

Taken together, the data might suggest that BPV1 L2 a.a. 1–88 constitutes an immunogen capable of generating broad cross-neutralizing responses against both cutaneous and genital types, including all pseudoviruses tested to date. Since the corresponding peptide from HPV16 failed to yield similar results, it suggests that the BPV1 L2 immunogen in particular may have sequences that foster re-folding of the peptide such that a relevant cross-neutralizing epitope is efficiently displayed. Alternatively, BPV1 L2 1–88 may less efficiently display non-neutralizing epitopes, which inhibit induction of antibodies to the cross-neutralizing epitope(s). Further mapping of this region using monoclonal antibodies would be informative as to which residues comprise the neutralizing epitope(s), which might lead to a broadly protective HPV L2 peptide vaccine. Indeed vaccination of patients with the HPV16 L2 108–120 peptide generate antibody that neutralized both HPV16 and HPV52 (Kawana et al., 2003).

Materials and methods

For generation of N-terminally 6-histidine tagged L2 fusion proteins, the open reading frames for L2 genes were cloned into the pQE-12 (Qiagen) or pPro-EXHt (Invitrogen) vectors. Full-length L2 genes included those for BPV1, HPV16, HPV18, HPV31, HPV6 and CRPV. BPV1 His-fusion peptides spanning the length of BPV1 L2: peptide A (a.a. 1–88), peptide B (a.a. 45–173), peptide C (a.a. 130–257), peptide D (a.a. 216–340), peptide E (a.a. 300–425) and peptide F (a.a. 384–469) and full-length BPV1 L2 (a.a. 1–469) were those described previously (Roden et al., 1994). A similar histidine fusion peptide spanning amino acids 1–88 of HPV16 was also constructed, expressed and purified as for the BPV1 peptides. The proteins were expressed in *E. coli* by induction with IPTG, purified after being solubilized in 6 M guanidine HCl on a Nickel agarose column (NTA from Qiagen) and eluted at low pH (4.5) in 8 M urea. The preparations were dialyzed overnight against PBS and stored at −80 °C. The HPV16 L2-GST fusion protein that was described by Roden et al. (2000), was gel-purified after induction in *E. coli*. Immunization of New Zealand white rabbits with these fusion proteins was also previously described (Roden et al., 1994). Briefly, rabbits were primed with aggregates of L2 protein (300 μg) resuspended in

Table 4
Cross-neutralization of BPV1 and HPV16 pseudoviruses is IgG-dependent

Anti-serum	Neutralization titer					
	BPV1			HPV16		
	Untreated	IgG depleted	Purified IgG	Untreated	IgG depleted	Purified IgG
Pre-immune serum#1	<50	<50	<30 (28 ng/μl)	<50	<50	<30 (28 ng/μl)
Pre-immune serum#3	<50	<50	<30 (28 ng/μl)	<50	<50	<30 (28 ng/μl)
BPV1 L2 a.a. 1–88	4050	<50	6400 (0.2 ng/μl)	4050	<50	6400 (0.2 ng/μl)
HPV16 L2 aa 1–88#1	<50	<50	80 (10.4 ng/μl)	4050	<50	6400 (0.1 ng/μl)
HPV16 L2 aa 1–88#2	50	<50	240 (4.3 ng/μl)	1350	<50	2130 (0.5 ng/μl)
HPV16 L2 aa 1–88#3	50	<50	80 (11.2 ng/μl)	1350	<50	2130 (0.4 ng/μl)
HPV16 L2 aa 1–88#4	<50	<50	80 (14.1 ng/μμl)	1350	<50	710 (1.6 ng/μl)

Titers are given as geometric means.

complete Freunds adjuvant on day 1, and boosted on days 28, 42, 60 and 76 with incomplete Freunds adjuvant. A test bleed is taken on 56 and additional bleeds taken on 74 and 88 before exsanguination on day 102 (Proteintech).

Maps of plasmids used for generation of high-titer pseudoviruses are available at the website http://www.ccr.cancer.gov/Staff/links.asp?profileid=5637. Generation of pseudoviruses using the codon-modified L1 and L2 genes of BPV1 (plasmid pSheLL) (Buck et al., 2004; Zhou et al., 1999), HPV16 (plasmids p16L1h and p16L2h) (Leder et al., 2001), and HPV18 (plasmids peL1fB and peL2bhb) (Pastrana et al., 2004) has been described previously. HPV6 and CRPV pseudoviruses were produced using expression plasmids carrying L1 and L2 genes that were entirely codon-modified using a previously described strategy (Buck et al., 2004). For HPV31, it was possible to express limited amounts of L1 and L2 using minimally codon modified genes in the context of an expression plasmid carrying the woodchuck hepatitis virus post-transcriptional regulatory element (WRPE) (Donello et al., 1998).

Pseudovirions were produced as previously described (Buck et al., 2004; Pastrana et al., 2004) with minor modifications. Briefly, plasmids encoding L1 and L2 genes were cotransfected into 293TT cells along with a reporter plasmid encoding secreted alkaline phosphatase (pYSEAP) (Pastrana et al., 2004). After 48 h, cells were lysed with 0.2% Brij-58, 9.5 mM $MgCl_2$, 0.1–0.2% Benzonase (Sigma) and 0.1% plasmid safe (Epicentre) and incubated at 37 °C for 15 min. The resulting pseudovirions were then matured by overnight incubation of the lysates at 25 °C (BPV1, HPV16 and HPV18) or 37 °C (HPV31, CRPV, and HPV6) overnight (Buck et al., 2005). The mature pseudovirions were solubilized by addition of 0.17 volumes of 5 M NaCl, then clarified by low speed ($1500 \times g$) centrifugation. Pseudoviruses were purified on a pre-formed 27, 33, 39% Optiprep (Sigma) step gradient. Optiprep fractions containing SEAP-transducing activity were pooled and frozen.

Serum from individual rabbits was diluted 3-fold, with 1:50 being the highest concentration of serum tested. Diluted sera were incubated with pseudoviruses at 4 °C for 1 h and the combination was then used to infect 293TT cells. The supernatants were analyzed for SEAP activity after 72 h with the GreatEscape Chemiluminescent substrate (BD Clontech Biosciences) on a luminometer (Dynex Technologies). The geometric mean of the inverse neutralizing titer of 2–10 experiments is reported.

Neutralization assays using native HPV11 virions were performed using Q-PCR as described in (Culp and Christensen, 2003, 2004). Briefly, sera were maintained at 1:50 during a 1-h pre-incubation with virions and during the entire first 48 h of infection of HaCaT cells. At 48 h, the spent medium was removed, monolayers were rinsed with DMEM-10 and cells were fed fresh DMEM-10 containing H11.H3 to neutralize any virions possibly remaining at the cell surface. RNA was harvested at 72 h p.i. The relative expression of spliced E1^4 message was determined by reverse transcription and Q-PCR (Culp and Christensen, 2003).

For IgG depletion, 68 μl of packed Protein G sepharose beads (Pierce) were used to absorb 60 μl of serum, and 180 μl of PBS were added to ensure proper mixing of the sample. The samples were rocked at 4 °C for 5 h. After a brief centrifugation, the supernatant was transferred to a fresh tube with an additional 68 μl of beads and rocked at 4 °C overnight. The samples were centrifuged and the supernatants used in neutralization assays.

For IgG purification, samples were purified with the Pierce's Nab Protein G spin purification kit, according to the manufacturer's recommendation.

Multiple sequence alignments were performed using the T-Coffee computer program (Notredame et al., 2000).

References

Bosch, F.X., de Sanjose, S., 2003. Chapter 1: human papillomavirus and cervical cancer-burden and assessment of causality. J. Natl. Cancer Inst. Monogr. (31), 3–13.

Bosch, F.X., Manos, M.M., Munoz, N., Sherman, M., Jansen, A.M., Peto, J., Schiffman, M.H., Moreno, V., Kurman, R., Shah, K.V., 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide prospective. J. Nat. Cancer Inst. 87, 796–802.

Breitburd, F., Kirnbauer, R., Hubbert, N.L., Nonnenmacher, B., Trin-Dinh-Desmarquet, C., Orth, G., Schiller, J.T., Lowy, D.R., 1995. Immunization with virus-like particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J. Virol. 69 (6), 3959–3963.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. 78 (2), 751–757.

Buck, C.B., Thompson, C.D., Pang, Y.Y., Lowy, D.R., Schiller, J.T., 2005. Maturation of papillomavirus capsids. J. Virol. 79 (5), 2839–2846.

Campo, M.S., O'Neil, B.W., Grindlay, G.J., Curtis, F., Knowles, G., Chandrachud, L., 1997. A peptide encoding a B-cell epitope from the N-terminus of the capsid protein L2 of bovine papillomavirus-4 prevents disease. Virology 234 (2), 261–266.

Chandrachud, L.M., Grindlay, G.J., McGarvie, G.M., O'Neil, B.W., Wagner, E.R., Jarrett, W.F., Campo, M.S., 1995. Vaccination of cattle with the N-terminus of L2 is necessary and sufficient for preventing infection by bovine papillomavirus-4. Virology 211 (1), 204–208.

Christensen, N.D., Kreider, J.W., 1990. Antibody-mediated neutralization in vivo of infectious papillomaviruses. J. Virol. 64, 3151–3156.

Christensen, N.D., Kreider, J.W., 1991. Neutralization of CRPV infectivity by monoclonal antibodies that identify conformational epitopes on intact virions. Virus Res. 21 (3), 169–179.

Christensen, N.D., Kreider, J.W., Cladel, N.M., Patrick, S.D., Welsh, P.A., 1990. Monoclonal antibody mediated neutralization of infectious human papillomavirus type 11. J. Virol. 64, 5678–5681.

Christensen, N.D., Kreider, J.W., Kan, N.C., DiAngelo, S.L., 1991. The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. Virology 181 (2), 572–579.

Christensen, N.D., Kirnbauer, R., Schiller, J.T., Ghim, S.J., Schlegel, R., Jenson, A.B., Kreider, J.W., 1994. Human papillomavirus types 6 and 11 have antigenically distinct strongly immunogenic conformationally dependent neutralizing epitopes. Virology 205, 329–335.

Christensen, N.D., Dillner, J., Eklund, C., Carter, J.J., Wipf, G.C., Reed, C.A., Cladel, N.M., Galloway, D.A., 1996. Surface conformational and linear epitopes on HPV-16 and HPV-18 L1 virus-like particles as defined by monoclonal antibodies. Virology 223, 174–184.

Christensen, N.D., Reed, C.A., Cladel, N.M., Han, R., Kreider, H.W., 1996. Immunization with virus-like particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomaviruses. J. Virol. 70, 960–965.

Clifford, G.M., Smith, J.S., Plummer, M., Munoz, N., Franceschi, S., 2003. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br. J. Cancer 88 (1), 63–73.

Combita, A.L., Touze, A., Bousarghin, L., Christensen, N.D., Coursaget, P., 2002. Identification of two cross-neutralizing linear epitopes within the L1 major capsid protein of human papillomaviruses. J. Virol. 76 (13), 6480–6486.

Culp, T.D., Christensen, N.D., 2003. Quantitative RT-PCR assay for HPV infection in cultured cells. J. Virol. Methods 111 (2), 135–144.

Culp, T.D., Christensen, N.D., 2004. Kinetics of in vitro adsorption and entry of papillomavirus virions. Virology 319 (1), 152–161.

Donello, J.E., Loeb, J.E., Hope, T.J., 1998. Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. J. Virol. 72 (6), 5085–5092.

Embers, M.E., Budgeon, L.R., Pickel, M., Christensen, N.D., 2002. Protective immunity to rabbit oral and cutaneous papillomaviruses by immunization with short peptides of L2, the minor capsid protein. J. Virol. 76 (19), 9798–9805.

Ghim, S., Christensen, N.D., Kreider, J.W., Jenson, A.B., 1991. Comparison of neutralization of BPV-1 infection of C127 cells and bovine fetal skin xenografts. Int. J. Cancer 49 (2), 285–289.

Giroglou, T., Sapp, M., Lane, C., Fligge, C., Christensen, N.D., Streeck, R.E., Rose, R.C., 2001. Immunological analyses of human papillomavirus capsids. Vaccine 19 (13–14), 1783–1793.

Jarrett, W.F.H., O'Neill, B.W., Gaukroger, J.M., Smith, K.T., Laird, H.M., Campo, M.S., 1990. Studies on vaccination against papillomaviruses: the immunity after infection and vaccination with bovine papillomaviruses of different types. Vet. Rec. 126, 473–475.

Kawana, K., Yoshikawa, H., Taketani, Y., Yoshiike, K., Kanda, T., 1999. Common neutralization epitope in minor capsid protein L2 of human papillomavirus types 16 and 6. J. Virol. 73 (7), 6188–6190.

Kawana, K., Kawana, Y., Yoshikawa, H., Taketani, Y., Yoshiike, K., Kanda, T., 2001. Nasal immunization of mice with peptide having a cross-neutralization epitope on minor capsid protein L2 of human papillomavirus type 16 elicit systemic and mucosal antibodies. Vaccine 19 (11–12), 1496–1502.

Kawana, K., Yasugi, T., Kanda, T., Kino, N., Oda, K., Okada, S., Kawana, Y., Nei, T., Takada, T., Toyoshima, S., Tsuchiya, A., Kondo, K., Yoshikawa, H., Tsutsumi, O., Taketani, Y., 2003. Safety and immunogenicity of a peptide containing the cross-neutralization epitope of HPV16 L2 administered nasally in healthy volunteers. Vaccine 21 (27–28), 4256–4260.

Kirnbauer, R., Hubbert, N.L., Wheeler, C.M., Becker, T.M., Lowy, D.R., Schiller, J.T., 1994. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16 [see comments]. J. Natl. Cancer Inst. 86 (7), 494–499.

Kirnbauer, R., Chandrachud, L., O'Neil, B., Wagner, E., Grindlay, G., Armstrong, A., McGarvie, G., Schiller, J., Lowy, D., Campo, M., 1996. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. Virology 219, 37–44.

Koutsky, L.A., Ault, K.A., Wheeler, C.M., Brown, D.R., Barr, E., Alvarez, F.B., Chiacchierini, L.M., Jansen, K.U., 2002. A controlled trial of a human papillomavirus type 16 vaccine. N. Engl. J. Med. 347 (21), 1645–1651.

Leder, C., Kleinschmidt, J.A., Wiethe, C., Muller, M., 2001. Enhancement of capsid gene expression: preparing the human papillomavirus type 16 major structural gene L1 for DNA vaccination purposes. J. Virol. 75 (19), 9201–9209.

Liu, W.J., Gissmann, L., Sun, X.Y., Kanjanahaluethai, A., Muller, M., Doorbar, J., Zhou, J., 1997. Sequence close to the N-terminus of L2 protein is displayed on the surface of bovine papillomavirus type 1 virions. Virology 227 (2), 474–483.

Lowy, D.R., Howley, P.M., 2001. Papillomaviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, pp. 2231–2264.

Munoz, N., Bosch, F.X., Castellsague, X., Diaz, M., de Sanjose, S., Hammouda, D., Shah, K.V., Meijer, C.J., 2004. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int. J. Cancer 111 (2), 278–285.

Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302 (1), 205–217.

Pastrana, D.V., Buck, C.B., Pang, Y.Y., Thompson, C.D., Castle, P.E., FitzGerald, P.C., Kruger Kjaer, S., Lowy, D.R., Schiller, J.T., 2004. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. Virology 321 (2), 205–216.

Roden, R.B.S., Weissinger, E.M., Henderson, D.W., Booy, F., Kirnbauer, R., Mushinski, J.F., Lowy, D.R., Schiller, J.T., 1994. Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. J. Virol. 68 (11), 7570–7574.

Roden, R.B., Hubbert, N.L., Kirnbauer, R., Breitburd, F., Lowy, D.R., Schiller, J.T., 1995. Papillomavirus L1 capsids agglutinate mouse erythrocytes through a proteinaceous receptor. J. Virol. 69 (8), 5147–5151.

Roden, R.B.S., Hubbert, N.L., Kirnbauer, R., Christensen, N.D., Lowy, D.R., Schiller, J.T., 1996. Assessment of the serological relatedness of genital human papillomaviruses by hemagglutination inhibition. J. Virol. 70, 3298–3301.

Roden, R.B., Yutzy, W.I., Fallon, R., Inglis, S., Lowy, D.R., Schiller, J.T., 2000. Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes. Virology 270 (2), 254–257.

Roden, R.B., Day, P.M., Bronzo, B.K., Yutzy, W.H.t., Yang, Y., Lowy, D.R., Schiller, J.T., 2001. Positively charged termini of the L2 minor capsid protein are necessary for papillomavirus infection. J. Virol. 75 (21), 10493–10497.

Suzich, J.A., Ghim, S., Palmer-Hill, F.J., White, W.I., Tamura, J.K., Bell, J., Newsome, J.A., Jenson, A.B., Schlegel, R., 1995. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc. Natl. Acad. Sci. U.S.A. 92, 11553–11557.

Walboomers, J.M., Jacobs, M.C., Manos, M.M., Bosch, F.X., Kummer, J.A., Shah, K.V., et al., 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol. 189, 12–19.

White, W.I., Wilson, S.D., Bonnez, W., Rose, R.C., Koenig, S., Suzich, J.A., 1998. In vitro infection and type-restricted antibody-mediated neutralization of authentic human papillomavirus type 16. J. Virol. 72 (2), 959–964.

Yang, R., Day, P.M., Yutzy, W.H.t., Lin, K.Y., Hung, C.F., Roden, R.B., 2003. Cell surface-binding motifs of L2 that facilitate papillomavirus infection. J. Virol. 77 (6), 3531–3541.

Zhou, J., Sun, X.Y., Louis, K., Frazer, I.H., 1994. Interaction of human papillomavirus (HPV) type 16 capsid proteins with HPV DNA requires an intact L2 N-terminal sequence. J. Virol. 68 (2), 619–625.

Zhou, J., Liu, W.J., Peng, S.W., Sun, X.Y., Frazer, I., 1999. Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. J. Virol. 73, 4972–4982.