Detection of L1, infectious virions and anti-L1 antibody in domestic rabbits infected with cottontail rabbit papillomavirus

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INTRODUCTION

Papillomavirus L1, the major capsid protein, is essential for the assembly of infectious virions during infection. L1 can also form virus-like particles (VLPs) in vitro. VLPs are highly immunogenic and induce high titres of serum-neutralizing antibodies in both preclinical and clinical settings (Stanley, 2003; Ling et al., 2000). Human papillomavirus (HPV) infections fail to occur in laboratory animal tissues because of the strong species-restrictive barriers of papillomaviruses. However, several animal papillomavirus models have contributed significantly to our understanding of pathogenesis and immunogenicity of papillomavirus infection and tumour development (Fausch et al., 2003; Campo, 2002). The cottontail rabbit papillomavirus (CRPV)/rabbit model is one of the more extensively studied animal papillomavirus models (Christensen et al., 1999). One unique property of the CRPV/rabbit model is that the infection mimics high-risk type HPV-induced malignancy in patients, and therefore provides an ideal preclinical model to study the pathogenesis of papillomavirus infection in vivo (Brandsma, 2005).

The natural host, the cottontail rabbit, typically produces papillomas with copious quantities of virus, but is a difficult model to work with because there are no commercial cottontail rabbit sources. In earlier studies, investigators achieved very limited success in passaging virus in domestic rabbits (Shope, 1935; Friedewald & Kidd, 1944). Further study suggested that CRPV infection produced masking viruses which was interpreted as immature particles located in the infected cell nuclei of domestic rabbit papillomas (Selbie & Robinson, 1947; Ito & Evans, 1961; Evans et al., 1964; Evans & Rashad, 1967). However, because of limited reagents for the detection of virus proteins, especially late protein L1 and L2, it was not clear whether CRPV infection in domestic rabbit led to expression of L1 and infectious virions. Our recent studies suggested that our CRPV strain consistently induced high levels of anti-L1 antibody in infected domestic rabbits (Hu et al., 2006b). Other investigators observed a higher incidence of anti-L1 antibody in animals with malignancy (Selvakumar et al., 1994). Based on these findings, we revisited the question as to whether domestic rabbit tissues...
could express CRPV L1 and generate infectious virus. We used recently developed monoclonal anti-CRPVL1 antibodies, targeting both linear and conformational epitopes, for the detection of L1 protein in CRPV-induced papillomas (Hu et al., 2006b). In addition, we used a sensitive in vitro infectivity assay to quantitatively detect infectious virions extracted from papillomas from these domestic rabbits (Culp & Christensen, 2003). Interestingly, most domestic rabbits showed modest to high levels of L1 protein in CRPV virion-induced papillomas and in about half of viral DNA-induced papillomas. Similar results were found when anti-L1 antibody was assayed in sera from these infected rabbits. We conclude that the CRPV/rabbit model can be used additionally as an in vivo model to study all aspects of the virus life cycle.

METHODS

Virus and DNA for challenge. Hershey CRPV infectious virus stock (50 μl of a 10⁻² dilution is a standardized dose per site used for most of our previous infection studies) was used for viral infection in the experiments. DNA cloned from this virus stock was identified as wild-type CRPV (or progressive CRPV) and used for infection of rabbit skin (Hu et al., 2006a).

Induction of skin papillomas. The DNA construct described above was purified by caesium chloride ultracentrifugation and adjusted to 200 μg ml⁻¹ in 1 × TE buffer (Hu et al., 2002) for challenge on animals. Outbred New Zealand White (NZW) rabbits and EIII/JC inbred rabbits were maintained in the animal facilities of the Pennsylvania State University College of Medicine. The studies were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University. For application of virus and viral DNA, rabbits were sedated using ketamine/xylazine anaesthesia. Back skin tissue from the same site was challenged with 10⁶–10⁷ virus particles. Back skin papillomas (Hu et al., 2006b). In addition, we used a quantitative RT-PCR (QRT-PCR) was also conducted to detect CRPV L1 and anti-L1 Ab in domestic rabbits

ELISA. Serum samples were collected from rabbits infected with virions and viral DNA. Standard ELISA was used to measure plasma titres of anti-CRPVL1 antibodies as previously described (Christensen et al., 1990). 96-well Maxisorp ELISA plates (Nunc) were coated with CRPV L1 VLP (generated in our laboratory) at room temperature for 30 min in PBS, pH 7.0 (native antigen ELISA) or overnight in 0.1 M NaHCO₃ buffer, pH 9.0 (denatured antigen ELISA). After several washes with PBS, the wells were blocked with 5 % non-fat milk protein in PBS for 1 h. Rabbit serum was diluted 1:50 in blocking buffer and added to the wells. After 1 h incubation at room temperature, the plates were washed three times and then incubated with a 1:1000 dilution of an alkaline phosphatase-conjugated swine anti-rabbit IgG secondary antibody (Dako) for 1 h. The plates were then washed three times, developed with 1 mg ml⁻¹ of p-nitrophenylphosphate substrate in alkaline phosphatase buffer, and analysed at 450 nm with an Opsys MR microplate reader (Dynex Technologies).

ELISA was also used to detect virus particles from papilloma extracts or fractions from Optiprep ultracentrifugation. Crude papilloma extracts (50 μl) or 2 μl aliquots from fractions diluted in 50 μl PBS were used as antigen to coat wells of 96-well plates overnight at 4 °C. Supernatant (1:100) of a mouse monoclonal antibody (CRPV1A) detecting a conformational epitope of CRPV L1 was used as primary antibody. A phosphatase-conjugated goat-anti-mouse IgG (1:5000, Pierce) was used as secondary antibody.

Immunohistochemistry. Biopsies from papillomas initiated with CRPV virions or viral DNA were stored in liquid nitrogen (for native protein analyses) and fixed in cold methanol after dissection or fixed in 10 % neutral-buffered formalin (for denatured protein analyses). Three antibodies were used for L1 detection in rabbits: GSA (group-specific antigen, a commercially available polyclonal antibody), CRPV4B (an in-house prepared monoclonal antibody targeting conformational epitopes in frozen tissues) and G4B (a second in-house prepared monoclonal antibody targeting a linear epitope). Mouse anti-CRPV L1 mAbs were diluted 1:500 and used to probe tissue sections (Hu et al., 2006b). Proteins were detected using Histostain (Invitrogen) containing goat anti-mouse streptavidin peroxidase followed by aminoethylcarbazol substrate. Positive-staining cells appeared red.

Papilloma extract preparation. Papillomas induced by infectious CRPV virions and viral DNA were harvested after the rabbits were euthanized. The tissues were then stored at −70 °C. Papilloma tissue (0.5 g ml⁻¹ in PBS) was homogenized at high speed with a Polytron PT 10-35 (Brinkman) for 3 min. The mixture was then centrifuged at 15 000 r.p.m. for 30 min (rotor diameter 27 cm) to remove cellular debris. The supernatant was collected as crude papilloma extracts for in vitro and in vivo infectivity assays.

In vitro infectivity assay. Extracts from papillomas (0.5–200 μl) were incubated with rabbit fibroblast cells or RK-13 (rabbit kidney) cells for 2 days. Total RNA was extracted from infected cells. A two-step nested RT-PCR was conducted to detect EIII/JC spliced viral transcripts using the GeneAmp RNA PCR kit (Applied Biosystems) in accordance with the manufacturer’s instruction. The primers for nested RT-PCR pairs are outsider primers (Upstream 5’-CCAGAAGCCATAAAGAATTCTTGAAT-3’ and Downstream 5’-GTGCCCCCTTCTCAAGCAATT-3’ and primer upstream (Upstream 5’-CCCCGGAGTTGTGTAAGCTTAAA-3’ and Downstream 5’-AACCTCGGGAAACGTCTTAT-3’). A quantitative RT-PCR (QRT-PCR) was also conducted to detect relative infectivity of two samples. The probe for CRPV EIII/E4 transcripts was 5’-6-FAM-d(TGAAAATGGCTGAAGCCTCCC-3’). The upstream primer was 5’-GTGCCGGGATGGTTGTA-3’ and downstream primer is 5’-GGTGCTCTTCAAGGGGACT-3’. QRT-PCR (QuantiTect RT-PCR kit, QIAGEN) was conducted as described previously (Culp & Christensen, 2003).

In vivo infectivity assay. Papilloma extracts were purified using an Optiprep gradient ultracentrifugation as reported previously (Pyeon et al., 2005). Different fractions were collected and checked for L1 protein by ELISA. The fractions with highest OD readings were used for in vivo infectivity. Seven EIII/JC inbred rabbits were sacrificed at day 3 and challenged with 100 μl crude extracts or 50 μl purified fractions per site. An infectious CRPV stock (50 μl) with a dilution of 10⁻⁴ was used as a positive control. The rabbits were monitored for papilloma outgrowth weekly after the infection. To eliminate possible viral DNA contamination, we incubated papilloma extracts with DNase (5 μg of CRPV DNA incubated with DNase for the digestion
control). The fractions were also incubated with a specific monoclonal antibody against CRPV L1 for specificity control.

RESULTS

Anti-CRPV L1 antibody generation in rabbits challenged with infectious virus

Sera collected from domestic rabbits infected with infectious virus (week 12 after challenge) were screened for anti-L1 antibody by standard ELISA. L1 or L1 + L2 VLP antigens were used in this assay. All the rabbits showed modest to high levels of anti-L1 antibodies. These high levels of anti-L1 antibody could be detected 2 years following initial infection (antibody titre ranged from 1:120 to 1:10,000). Higher levels of anti-L1 antibody were also found in rabbits with the largest tumour mass \((n=24, \text{Fig. 1a})\) or with malignant cancers (Lin et al., 1993).

We also measured serum anti-L1 antibody levels in regressor rabbits \((n=5)\). All rabbits with regressed lesions showed high titres of anti-L1 antibody (500–8,000) and this level was maintained for more than 2 years after papilloma regression. Animals with tumours surgically removed lost their anti-L1 antibody after 200 days (Christensen et al., 1999).

To demonstrate that the anti-L1 antibody was not generated from challenge virus, we conducted a time-course study of anti-L1 antibody levels (Fig. 1b) during the early stages of infection on two animals. A small increase in serum anti-L1 antibody was observed in the first 2 weeks (day 11–18) after viral infection prior to clinical appearance of the papillomas, most probably generated from challenge virus, but after day 28 (papillomas became visible), anti-L1 levels dramatically increased and reached peak levels by day 46.

Anti-CRPV L1 antibody production in rabbits challenged with viral DNA challenge

A CRPV genome (which was cloned at SalI site in pUC19) was used for viral DNA infections. When papilloma growth rates were compared between sites challenged with DNA versus virus, significantly slower growth of papillomas was found in sites challenged with DNA in the first 9 weeks of challenge, but no significant difference was found after week 10 (Hu et al., 2006b). Anti-L1 antibody was also detected in 50% of the animals with papillomas initiated by viral DNA, whereas no anti-L1 antibody was detected in the animals by a CRPV L1ATGko mutant.

CRPV L1 expression in virus- and DNA-induced papillomas

CRPV L1 protein was detected in rabbit papilloma tissues using routine immunohistochemistry. We measured CRPV L1 protein with an in-house prepared anti-L1 monoclonal antibody (CRPV4B) that detects a surface conformational epitope, and high to low levels of L1 were detected (Table 1, Fig. 2a). Our initial studies utilized a commercially available polyclonal antibody to GSA, but no signals could be found in most CRPV DNA-induced rabbit papillomas biopsies. We then tested a more effective monoclonal antibody prepared in-house (G4B) to CRPV L1, which detects a denatured epitope for comparison. Low to high levels of L1 protein were now detected in all papilloma biopsies (Fig. 2b). CRPV L1 protein was also detected in papillomas induced by wild-type CRPV but not with L1ATGko DNA (Fig. 3a). Additional immunohistological staining of papillomas in this study demonstrated that the levels of L1 protein in DNA-induced papillomas are not as high as those in virus-induced papillomas.
CRPV DNA without plasmid insert produced papillomas containing more L1 protein

We next determined whether papillomas induced by CRPV DNA retained in the plasmid contributed to reduced L1 protein production due to the plasmid sequence insertion site (E5 gene) in the CRPV viral genome. The plasmid sequence at this site may contribute to a delay or disruption of late gene expression. CRPV DNA was digested with Sall overnight and the DNA challenged onto rabbit back sites on left side sites only. CRPV retained in pUC19 was challenged onto right side sites as control papillomas for the presence of inserted plasmid/CRPV DNA. Papilloma biopsies were harvested after week 13 and examined for L1 protein. Higher levels of L1 were observed in papillomas initiated with viral DNA uninterrupted with the bacterial plasmid sequence when compared to DNA retaining the pUC19 fragment (Fig. 3b).

Table 1. CRPVL1 protein detection in frozen and formalin-fixed sections of CRPV virions- and DNA-induced papillomas

Three antibodies were used for L1 detection in rabbits: GSA (a commercially available polyclonal antibody), CRPV4B (an in-house prepared monoclonal antibody targeting conformational epitope in frozen tissues) and G4B (a second in-house prepared monoclonal antibody targeting a linearized epitope).

<table>
<thead>
<tr>
<th>Rabbit ID</th>
<th>Challenge</th>
<th>L1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type CRPV DNA</td>
<td>CRPV L1ATGko DNA</td>
</tr>
<tr>
<td>283</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>288</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>294</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>296</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>858</td>
<td>ND</td>
<td>Positive</td>
</tr>
<tr>
<td>859</td>
<td>ND</td>
<td>Positive</td>
</tr>
<tr>
<td>860</td>
<td>Yes</td>
<td>ND Negative</td>
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<tr>
<td>864</td>
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<tr>
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</tr>
<tr>
<td>654</td>
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<td>Positive*</td>
</tr>
<tr>
<td>339</td>
<td>ND</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*Significantly more positive signals were found in these papilloma tissues.

Extracts from virus-induced papillomas were infectious in vitro

As shown above, virus- and DNA-challenged rabbits generated anti-L1 antibody and, most importantly, L1 expression was detected in most virus-induced papillomas and a portion of DNA-induced papillomas. We next wanted to test if infectious viruses were generated in these papillomas. Papillomas collected from both virus- and viral DNA-infected animals and their extracts were assayed for infectivity using an in vitro infection culture system. Most papilloma extracts were able to generate E1^E4 transcripts by nested RT-PCR (data not shown). To compare the infectivity titres of two papilloma exacts (rabbit 838 and 1035) with wild-type CRPV virus (10^2), we used QRT-PCR analysis. Much lower levels of E1^E4 transcripts were found in these extracts when compared with our standard challenge virus stock (Fig. 4).

Extracts from NZW rabbit papillomas were infectious in vivo

Both virus- and viral DNA-induced papilloma extracts were tested for their infectivity in vivo. Two papilloma
extracts from virus infection and two from viral DNA infection were prepared by homogenization. Supernatant of each papilloma extract (100 μl) was incubated with DNase at room temperature for 1 h. Positive controls were CRPV DNA (5 μg) and CRPV virus stock at 10⁻⁴ dilution with or without DNase treatment. Papillomas grew on sites challenged with viral DNA but not on the sites treated with DNase (Table 2). Papillomas also grew on sites challenged with CRPV virus stock with or without treatment of DNase. However, no sites challenged with either extracts from virus-induced or viral DNA-induced papillomas grew papillomas after week 6 (Table 2).

Suspecting that the purity of papilloma crude extracts and possible ‘masking effects’ interfered with the infectivity of these extracts, we conducted an Optiprep gradient ultracentrifugation of these papilloma extracts. Different fractions were collected and tested for L1 protein by ELISA. Fractions from papillomas induced by infectious viruses (Fig. 5a) showed higher levels of L1 compared with those induced by viral DNA (Fig. 5b). These fractions were then used to challenge EIII/JC inbred rabbits with or without incubation with a neutralizing monoclonal antibody against CRPV L1 (CRPV1A). An infectious virus stock with a dilution of 10⁻³ with and without incubation with CRPV1A was used for positive and negative controls (Table 2). One small papilloma grew on one site of one virus-induced papilloma extract and one viral DNA-induced papilloma extract, respectively, around week 2–3 and regressed soon after (Table 2). Therefore, purified fractions from both virus- and viral DNA-induced papilloma extracts were capable of inducing papillomas on domestic rabbits.

DISCUSSION

The CRPV/rabbit model has been used extensively for studying papillomavirus pathogenesis because this animal model mimics features of high-risk HPV virus-induced malignancy in humans (Christensen, 2005). Early studies reported that domestic rabbit papillomas produced few, if any, infectious viruses (Shope, 1935; Friedewald, 1940; Friedewald & Kidd, 1944; Greene, 1954; Ito & Evans, 1961). However, our studies have detected anti-L1 antibody in the serum of infected domestic rabbits, suggesting the presence of assembled L1 in these papillomas. Our most recent studies also demonstrated that L1 could be a target for cell-mediated immune responses in domestic rabbits (Hu et al., 2006b). These findings led us to revisit the question of L1 expression in domestic rabbits, using newly developed monoclonal anti-CRPV L1 antibodies and sensitive in vitro infection assays. Our data clearly showed that L1 was present in most CRPV-induced papillomas and in a portion of CRPV DNA-induced papillomas in domestic rabbits.
rabbits. The extracts from papillomas with high levels of L1 also contained infectious virions.

We have used a CRPV strain (Hershey progressive CRPV strain) for most of our previous studies (Hu et al., 2006a). We also reported a second natural CRPV strain which led to a high incidence of papilloma regression in rabbits. The major difference between these two strains resides in the E6 and E7 genes (Hu et al., 2002). Other investigators have used CRPV strains that are genetically different to the progressive strain reported in our studies (Salmon et al., 1997). When these progressive strains are compared, very high sequence homology is found in the late genes. However, some differences were found in early genes such as E5, which shows the greatest degree of divergence among these strains (Han et al., 1998). Therefore, despite sequence identity of CRPV L1, subtle differences between strains may alter L1 expression levels and hence L1 protein content in papillomas. Nevertheless, our results confirmed that the CRPV/rabbit model can be used to study aspects of the viral life cycle in vivo.

Different viral challenge methods are used by different laboratories studying CRPV DNA infection in rabbits (Ito, 1963; Jensen et al., 1997; Brandsma & Xiao, 1993; Kreider et al., 1995; Xiao & Brandsma, 1996; Salmon et al., 2000; Jeckel et al., 2003; Nonnenmacher et al., 2006; Hu et al., 2006a). Most current challenge methods result in modest rates of infectivity per challenge site or high variation in the size of papillomas at different sites on the same animal (Hu et al., 2006a). In this study, we have applied our recently optimized challenge method (manuscript in preparation) to achieve effective and consistent results from DNA infection. This improved method has helped us to minimize the variations in papilloma size between different challenge sites on the same animal and between animals in the same experiment.

The detection of CRPV L1 protein has been hindered by the lack of sensitive and specific anti-CRPV L1 antibody (Hu et al., 2006b). A commercially available polyclonal antibody to GSA, which detects a conserved region in L1, was commonly used to detect CRPV L1 in our previous studies. This commercial antibody was less sensitive and often resulted in high background levels of staining in our rabbit tissues. We have recently developed several monoclonal antibodies against CRPV L1 that recognize conformational (CRPV4B and CRPV1A) (Christensen & Kreider, 1991) and linear epitopes (G4B) (Hu et al., 2006b). These antibodies helped us to better detect L1 in both virion- and DNA-infected domestic rabbit papillomas. In some cases, the expression level of L1 was as high as that observed in cottontail rabbit papillomas (Hu et al., 2006b).

Lower levels of L1 protein were found in viral DNA-induced papillomas when compared to virus-induced lesions. Our standard method of viral DNA infection includes CRPV genomes contained within the bacterial plasmid sequence inserted within the E5 gene. We have recently observed that linearized CRPV DNA can initiate infection following direct application to scarified rabbit

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**Table 2. In vivo infectivity assay for the papilloma extracts**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Papilloma extract (animal numbers)</th>
<th>Challenge sites</th>
<th>Papilloma sites</th>
<th>Regression sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (crude extract)</td>
<td>Wild-type CRPV ($10^{-4}$) ($n=4$)</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Virus induced papillomas ($n=2$)</td>
<td>8</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>DNA induced papillomas ($n=2$)</td>
<td>8</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>2 (purified fractions from Optiprep)</td>
<td>Wild-type CRPV ($10^{-3}$) ($n=3$)</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Virus induced papillomas ($n=1$)</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DNA induced papillomas ($n=2$)</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Fig. 5.** ELISA readings of different fraction of papilloma extract after purification by Optiprep gradient ultracentrifugation. (a) OD value after 2 h development of the fractions collected from an infectious virus-induced papilloma extract; (b) OD value after 4 h development of the fractions from a CRPV viral DNA-induced papilloma extract.
skin. Rabbits infected with both circular (plasmid) and linearized viral DNA developed serum anti-L1 antibodies. This latter preparation of viral DNA eliminates the plasmid sequence and should improve transcription and translation of the late genes. Our results clearly demonstrated that CRPV DNA without interruption of plasmid (pUC19) induced higher expression of L1 in the papilloma tissues (Fig. 3b). Using the same strategy, we detected L1 expression in papillomas induced by a second progressive CRPV strain (known as the Washington B strain) (Zeltner et al., 1994), DNA used by several other investigators (data not shown).

We purified papilloma extracts induced by both infectious virus and viral DNA by Optiprep gradient ultracentrifugation and tested their infectivity in EIII/JC inbred rabbits. Our in vivo data demonstrated that the papilloma extracts contained infectious virions that were capable of inducing papillomas in rabbits. The small size of these papillomas indicated that the amount of infectious virions in these papillomas was low.

We conclude that domestic rabbit papillomas can generate low to moderate levels of both L1 and infectious virions and thus can be used to study the infectious life cycle in vivo. Some potential differences in L1 protein production and virion yield may occur when different genetic isolates of CRPV are used. Future comparative studies may help explain our results when compared with earlier published reports suggesting that domestic rabbits supported only abortive infection of CRPV.

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REFERENCES


