Antigenicity of bovine papillomavirus type 1 (BPV-1) L1 virus-like particles compared with that of intact BPV-1 virions

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Virus-like-particles (VLPs) of various papillomavirus (PV) types have been produced by expressing recombinant L1 proteins in eukaryotic cells. Although VLPs have the same ultrastructural appearance as native virions and their immunogenicity appears to be similar, their antigenicity has not been carefully evaluated. For this reason, the antigenicity of intact bovine PV type 1 (BPV-1) virions was compared with that of BPV-1 recombinant L1 VLPs by ELISA using a well-characterized panel of polyclonal and monoclonal antibodies generated against intact and denatured BPV-1 particles. The structural integrity of the authentic virions and recombinant VLPs was verified by electron microscopy. The specificity of antibodies raised against intact BPV-1 virions and their reactivity with VLPs revealed that the immunodominant, type-specific, conformational epitopes of intact virions were reproduced on VLPs. However, many monoclonal antibodies that define cross-reactive, non-conformational (linear) epitopes cryptic to the authentic BPV-1 virion tested positively when reacted with intact VLPs. One monoclonal antibody, which recognizes a BPV-1 and deer PV surface conformational epitope, did not react with VLPs. Therefore, although VLPs can be used to immunize animals against infection, the external exposure of broadly cross-reactive epitopes of intact L1 VLPs suggests that the use of L1 VLPs in antigenicity studies such as serological screening should be done with caution.

Introduction

Papillomaviruses (PVs) are species-specific and anatomic site-restricted tumorigenic viruses (Baker, 1987; de Villiers, 1989; Pfeister, 1984). In their natural host, they produce fibropapillomas or papillomas with productive infections occurring only in differentiating keratinocytes of low grade tumours. Virions have a non-enveloped capsid, 55 nm in diameter (Baker et al., 1991; Crawford & Crawford, 1963; Pfeister, 1987). PV capsids are icosahedral and are composed of 12 five-coordinated and 60 six-coordinated capsomeres arranged on a $T=7$ surface lattice (Baker et al., 1991; Finch & Klug, 1965). The genome consists of double-stranded circular DNA, approximately 8 kb in size (Baker, 1987; Pfeister, 1984, 1987). PVs have two capsid proteins. The major capsid (L1) protein has a molecular mass of 55 kDa, representing about 80% of the total viral protein (Pfeister, 1984). The minor capsid (L2) protein migrates aberrantly at a molecular mass of 77 kDa in SDS-PAGE gels (Jin et al., 1989). The density of complete PV virions in CsCl has been shown to be 1.34 g/ml (Crawford & Crawford, 1963).

Studies of the immunology and cell biology of papillomaviruses have been hampered by the lack of an in vitro system for replicating the virus. However, productive infections induced by PVs such as bovine PV type 1 (BPV-1) and human PV type 1 (HPV-1) have yielded quantities of virions sufficient to be characterized immunologically (Baker et al., 1991; Lancaster & Olson, 1978; Lim et al., 1990; Yaegashi et al., 1991). Although intact BPV-1 virions induce a type-specific, protective immune response against infection in the natural bovine host (Ghim et al., 1991; Jarrett et al., 1990), the topographical locations of the neutralizing epitopes have not been defined. In anticipation of the development of subviral PV vaccines, it was suggested that the neutralizing immune response would target either type-specific conformational or nonconformational (linear) epitopes on the surface of virus particles (Cowsert et al., 1987). Subsequently, it was demonstrated that conformational epitopes were targeted by high-titred, type-specific, neutralizing antibodies to BPV-1 (Ghim et al., 1991). Recombinant L1 proteins expressed in either the vaccinia
virus expression system (Hagensee et al., 1993; Zhou et al., 1993) or the baculovirus expression system (Christensen et al., 1994; Kirnbauer et al., 1992; Rose et al., 1994) have been shown to assemble into VLPs, composed of conformationally correct L1 proteins that reproduce neutralizing epitopes of authentic PV virions by mimicking the capsid ultrastructure (Ghim et al., 1994; Hagensee et al., 1993, 1994; Kirnbauer et al., 1992; Rose et al., 1994; Zhou et al., 1993). BPV-1 VLPs induce high-titred neutralizing antibodies that can be measured by prevention of BPV-1-induced focus formation (FF) in immortalized murine cell lines (Kirnbauer et al., 1992).

Recent studies have focused on the immunogenicity of virus-like particles (VLPs) and their possible use as subunit recombinant PV vaccines in the natural host (Breitbart et al., 1994; Ghim et al., 1995). It has been assumed that intact BPV-1 VLPs are antigenically similar to intact BPV-1 virions (Kirnbauer et al., 1992), which react only with antibodies that recognize surface epitopes (Cowsert et al., 1987). In this study, using well-characterized polyclonal and monoclonal antibodies produced against intact and disrupted BPV-1 virions, we compared, by ELISA, the antigenicity of conformational and linear epitopes on BPV-1 VLPs with that of BPV-1 virions to determine the feasibility of using VLPs as antigenic substrates for tests such as serotyping.

**Methods**

**Purification of BPV-1 virions.** BPV-1 virions were purified from bovine fibropapillomas as described previously (Cowsert et al., 1987; Ghim et al., 1991). Intact virions were stored as aliquots of stock solution at −80 °C until used.

**Antibodies.** A panel of monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs) was used to compare the antigenicities of BPV-1 virions and VLPs. The antibodies have been previously characterized as recognizing either linear or conformational epitopes on the surface of intact BPV-1 virions or linear epitopes internal (cryptic) to the intact authentic capsid (Table 1). MAbs AU-1 to AU-6 (Lim et al., 1990; Nakai et al., 1986) and 1H8 (Cowsert et al., 1987) recognize linear epitopes internal to the BPV-1 virion. AU-1, AU-2, AU-5 and 1H8 have been defined to the level of one amino acid by epitope mapping (Lim et al., 1990). MAb 13D6 is cross-reactive with BPV-1 and deer PV (DPV), and recognizes external, non-neutralizing conformational epitopes (Cowsert et al., 1987). MAb B1A1, kindly provided by N. D. Christensen, is type-specific and conformationally dependent, and neutralizes BPV-1 virions (Christensen & Kreider, 1990). Rabbit PAbs raised against disrupted BPV-1 virions (Rdis, DAKO) recognize surface and cryptic linear epitopes. Rabbit sera raised against intact BPV-1 virions (Rint) recognize surface conformational epitopes, some of which are neutralizing. The monoclonal antibody MAB45, which defines a type-specific linear epitope on the HPV-1 capsid (Yagasaki et al., 1991), was used as a negative control.

**Construction of BPV-1 L1 recombinant baculovirus.** The BPV-1 L1 open reading frame (from nt 5609 to 7161) was amplified by PCR from p142-6, which contains the full-length wild-type BPV-1 genome (Lambert & Howley, 1988). The primers used were 5' GAC ATG GGA TCC GCC ACC ATG GCG TTG TGG CAA 3' (BamHI) and 5' GAC CTC CTG TCA ATT AGG TGC AGT TGA 3' (PstI). This fragment was cloned into the BamHI and PstI sites of the baculovirus transfer vector pHueBacIII (Invitrogen), creating pBIL1 (Fig. 1). To produce recombinant baculovirus, pBIL1 was co-transfected with linearized wild-type AcMNPV DNA by cationic liposome-mediated transfection as instructed by the manufacturer (Transfection module; Invitrogen). The resulting recombinant baculovirus, rBacB1L1, was plaque purified using Grace’s complete medium (Gibco/BRL) containing 1.25% SeaPlaque agarose (FMC Bioproducts) and X-Gal (for colour screening). High-titred recombinant baculovirus stocks were prepared as described by Invitrogen.

**Immunofluorescence.** BPV-1 L1 protein expression in Si9 insect cells was verified using immunofluorescence (IF). For IF, cells were grown on coverslips and infected with rBacB1L1 or with wild-type baculovirus. At 72 h post-infection, the coverslips were fixed in cold acetone for 5 min and air dried. After rehydration in PBS, the coverslips were then incubated with Rint (diluted 1:100 in PBS) for 1 h at room temperature (RT), washed with PBS, and incubated with fluorescein-conjugated goat anti-rabbit IgG (H+L) (diluted 1:100 in PBS) under the same conditions. After the final washing, the slips were mounted in Elvanol and viewed with a fluorescent microscope (Olympus).

**Purification of VLPs.** Si9 cells were cultured in supplemented Grace’s medium containing 10% fetal bovine serum and 0.1% Pluronic F-68 (Gibco/BRL) in spinner flasks. Cells (1.8 × 10^8) were infected with

![Fig. 1. Construction of recombinant baculovirus transfer vector pBIL1.](image)
rBacBL1 at an m.o.i. of 10 to 50. At 72 h post-infection, the infected cells were collected by centrifugation at 160 g for 10 min at 4 °C. The pellet was resuspended in hypertonic lysis buffer (10 mM-Tris-HCl, pH 8, 10 mM-NaCl, 1.5 mM-MgCl₂) and homogenized with 50 strokes in a tissue grinder and centrifuged at 9000 g for 20 min at 4 °C. The nuclei containing the VLPs were resuspended in Dulbecco's PBS containing MgCl₂ and CaCl₂ (D-PBS; Gibco/BRL) and sonicated for 1.5 min at 9 W (Ultrasonic processor VC100; Sonics & Materials Inc.). The supernatant was loaded on top of a 40% (w/v) sucrose-D-PBS cushion and centrifuged at 14000 g using an SW 55.1 Ti rotor for 2 h at 4 °C. The pellet was resuspended in D-PBS, sonicated for 10 s, and separated by centrifugation at 246000 g for 20 h in a CsCl gradient (1.30–1.33 g/ml). The bands were collected separately and re-centrifuged for 2 h at 140000 g. The pellets were resuspended in D-PBS and the presence of VLPs was verified by electron microscopy.

Electron microscopy. BPV-1 virions and VLPs were immobilized on a 300 mesh Formvar/carbon-coated grid and negatively stained with 2% phosphotungstic acid (pH 6.8). VLPs and virions were observed with a transmission electron microscope (JEOL 100S) at a magnification of 30000 x and counted as previously described (Gorra et al., 1985).

Immunoblot of BPV-1 virions and VLPs. Protein samples were denatured in Laemmli sample buffer, heated at 95 °C for 5 min and separated on a 10% SDS-PAGE gel. The separated proteins in the gel were electrically transferred to a nitrocellulose membrane. The VLPs in the heaviest band had the same structural appearance as the BPV-1 virions (Fig. 2 a, b), and, therefore, were used in the antigenicity studies. The VLPs from each band displayed the same antigenicity (data not shown) by ELISA using Rint antibodies. As viewed by electron microscopy, the surface topography of VLPs in all three bands had a similar ultrastructural appearance. The VLPs in the heaviest band had the same ultrastructural appearance as the BPV-1 virions (Fig. 2a, b), and, therefore, were used in the antigenicity studies. The L1 protein of the VLPs had a molecular mass of 55 kDa as shown by immunoblots with Rdis (Fig. 3).

Comparison of reactivities of BPV-1 virions and VLPs against PAbs and MAbs

The L1 protein concentrations of virions and VLPs were quantified by immunoblot. As shown in Fig. 3, comparison of the quantity of L1 protein in 0.5 μg protein of virion solution with that in 0.1 μg protein of VLP solution led to the conclusion that the L1 protein in the VLP solution was approximately two to three times more concentrated than in the virion solution. However, the numbers of authentic virus particles were higher than those of VLPs. The difference in the particle count of virions and VLPs with respect to the L1 protein concentrations was most likely due to increased L1 protein in the single and aggregated capsomeres (which were not counted) in the VLP preparations; capsomeres were not seen in virion preparations.

ELISA values indicating the reactivity of antibodies with saturated BPV-1 virions and VLPs were highly reproducible. Table 2 shows the representative ELISA values for the comparison of the antigenicity of virions and VLPs reactive with a panel of antibodies. It reveals that the most striking antigenic differences between virions and VLPs were detected using antibodies produced against denatured antigens. It also shows that BPV-1 virions and VLPs have the same reactivity by ELISA.
Table 2. Comparison by ELISA of reactivities of BPV-1 virions and VLPs with different antibodies

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<th>Antibody</th>
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<th>Disrupted form</th>
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<td></td>
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<td>VLPs</td>
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Discussion

VLPs are important for studying the immunogenicity and antigenicity of authentic PV virions because virions are not recoverable in large quantities from most PV-induced lesions. In contrast to virions, VLPs can be produced in large quantities by various recombination techniques (Christensen et al., 1994; Hagensee et al., 1993; Kirnbauer et al., 1992; Rose et al., 1994). After genotyping PV-induced lesions, the L1 ORF can be cloned into different eukaryotic expression vectors and expressed as VLPs that have the same ultrastructural appearance as native virions (Christensen et al., 1994; Ghim et al., 1995; Hagensee et al., 1993; Kirnbauer et al., 1992; Rose et al., 1994). These VLPs reproduce type-specific, neutralizing conformational L1 protein epitopes that can be used as immunogens for vaccination against PV infection (Breitburd et al., 1994; Ghim et al., 1995; Kirnbauer et al., 1992). VLPs of BPV-1, HPV-11,
Cottontail rabbit PV and canine oral PV induce neutralizing, conformationally dependent antibodies that can prevent infection and/or transformation of C127 cells (Krnibauer et al., 1992), murine xenografts (Christensen et al., 1994; Rose et al., 1994) or animal hosts (Breitburd et al., 1994; Ghim et al., 1995). Although the immunogenicity of a protein does not necessarily reflect its antigenicity, VLPs are candidates as antigenic substrates for detection of previous infection or for vaccination. For this reason, we compared the antigenicity of BPV-1 virions with that of recombinant L1 VLPs to determine if there were any significant differences between capsids of authentic BPV-1 virions and BPV-1 L1 VLPs.

PV type-specific conformational and linear epitopes are displayed on the surface of virions, whereas broadly cross-reactive linear epitopes are cryptic to the capsid (Cowsett et al., 1987). Cryptic epitopes can be identified in productive PV infections by immunocytocchemical techniques and on synthetic peptide homologues, fusion proteins or denatured L1 proteins by ELISA or by immunoblotting (Viscidi & Shah, 1992). In this study, well-characterized polyclonal and monoclonal antibodies produced against disrupted BPV-1 virions revealed that VLPs are antigenically different from authentic virions (Table 2). Specifically, many cryptic epitopes that are not exposed on intact virions are exposed on intact VLPs. MAbs which recognize cryptic, non-conformational epitopes have previously been used as controls to ensure the fidelity of intact virions on ELISA (Cowsett et al., 1987; Ghim et al., 1991). Of these MAbs, AU-1 displays the strongest reactivity with BPV-1 VLPs. It is unlikely that the reactivity of VLPs with MAbs that recognize cryptic epitopes of virions is an artifact, since the experiment was repeated nine times. VLPs were reactive with MAb B1A1 and PAb Rint which recognize the strongest reactivity with BPV-1 VLPs. It is unlikely that epitopes have previously been used as controls to ensure the apparent flexibility of VLPs will have on immune responses. It might be necessary to treat VLPs with a cross-linking fixative such as formalin to decrease their apparent flexibility or to identify their type-specific neutralizing conformational epitopes in antigen-capture ELISAs for serotyping in order to synthesize them in vitro for the serotyping of HPVs.

Not all conformational epitopes appear to be reproduced by VLPs. Of particular interest is MAb 13D6, which recognizes BPV-1 and DPV but not BPV-2 intact virions (Cowsett et al., 1987), but was not reactive with BPV-1 VLPs. The most likely reason for this difference in reactivity is the flexibility of the BPV-1 L1 VLP when compared with authentic virions. However, it cannot be excluded that some conformational epitopes may be intermolecular, perhaps composed of amino acid residues contributed by both L1 and L2 proteins. Regardless, it demonstrates that VLPs may not have the same antigenicity as homologous virions.

References


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