Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies

Neil D. Christensen, Reinhard Höpf, Susan L. DiAngelo, Nancy M. Cladel, Susan D. Patrick, Patricia A. Welsh, Lynn R. Budgeon, Cynthia A. Reed and John W. Kreider

Departments of Pathology, Microbiology and Immunology, The Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033, U.S.A. and Department of Dermatology, University of Innsbruck, Innsbruck, Austria

Baculovirus-expressed human papillomavirus type 11 (HPV-11) major capsid protein (L1) virus-like particles (VLPs) were produced in insect cells and purified on CsCl density gradients. The VLPs retained conformational neutralizing epitopes that were detected by a series of HPV-11-neutralizing monoclonal antibodies. Electron microscopy determined that the HPV-11 L1 VLPs were variable in size with a surface topography similar to that of infectious HPV-11. The VLPs were very antigenic, and induced high titres of neutralizing antibodies in rabbits and mice when used as an immunogen without commercial preparations of adjuvant. These VLP reagents may be effective vaccines for protection against HPV infections.

Introduction

Conformational, strongly neutralizing epitopes on papillomaviruses (PVs) have been identified by a series of monoclonal antibodies (MAbs) (Christensen et al., 1990b; Christensen & Kreider, 1991, 1993). Although several weakly neutralizing linear epitopes have also been identified on both the major (L1) and minor (L2) capsid proteins of PVs, immunization against PV infection has been successful in some cases (Jarrett et al., 1991; Campo et al., 1993), but weak or ineffectual in others (Jin et al., 1990; Christensen et al., 1991; Ghim et al., 1991; Lin et al., 1992). In contrast the conformational epitopes located on intact virions induce high titres of neutralizing antibodies with subsequent strong protection against viral challenge (Olson, 1963; Christensen & Kreider, 1990; Jarrett et al., 1990a,b). Strategies to protect against human PV (HPV) infection therefore require the development of antigenic reagents that mimic the conformational neutralizing epitopes located on intact virions.

One such strategy to generate the correct surface topography of viruses synthetically is to produce artificial virus-like particles (VLPs) by overexpression of the viral coat proteins in various expression systems (Urakawa et al., 1989; Saliki et al., 1992; Brown et al., 1991; Kajigaya et al., 1991; Montross et al., 1991; French et al., 1990; Spehner et al., 1991; Zhou et al., 1991, 1992; Haffar et al., 1990; Rumenapf et al., 1991; Jones et al., 1990; Martinez et al., 1992; Kirnbauer et al., 1993). The production of VLPs for some viruses such as polyomavirus and parvovirus has even been accomplished successfully by expression of the major coat protein alone (Montross et al., 1991; Brown et al., 1991; Martinez et al., 1992). Recently, VLPs of various PVs have been developed using recombinant vaccinia viruses (Zhou et al., 1991, 1993; Hagensee et al., 1993), or recombinant baculoviruses (Kirnbauer et al., 1992, 1993; Rose et al., 1993). As found for polyomavirus and parovirus, successful PV VLP formation was possible by overexpression of the major coat protein L1 in the absence of the minor coat protein L2 (Kirnbauer et al., 1992, 1993; Zhou et al., 1993; Rose et al., 1993; Hagensee et al., 1993). For the formation of some PV VLPs however, dual expression of both L1 and L2 was necessary (Zhou et al., 1991).

In this report we developed a recombinant baculovirus producing human PV-11 L1 that self-assembled into VLPs in insect cells. These VLPs were screened by a set of neutralizing MAbs to determine whether they retained conformational neutralizing epitopes, and were also used as an antigen in the immunization of mice and rabbits. The results demonstrated that HPV-11 L1 VLPs were recognized by the neutralizing MAbs and induced high titres of neutralizing antibodies when used as immunogen without adjuvant.
Methods

Production of HPV-11 L1 recombinant baculovirus and purification of VLPs. A recombinant baculovirus expressing the HPV-11 L1 protein was produced by recombination of wild-type baculovirus DNA with the transfer vector pVL1392 (Invitrogen) containing an HPV-11 L1 insert. HPV-11 DNA cloned from the Hershey isolate (Kreider et al., 1985) was digested with restriction enzymes EcoRI and Bsu36I to obtain the fragment spanning 5678 bp to 7718 bp containing the entire L1 open reading frame. The fragment was blunt-ended using Klenow enzyme, then ligated into the transfer vector pVL1392 and recombinant baculoviruses were then produced by standard methods (Summers & Smith, 1988). Isolated recombinants were screened for L1 protein production by Western blotting using anti-PV group-specific antigen (GSA) antibody (Dako). The method for VLP purification was identical to that used to purify infectious PV from cysts produced in the athymic mouse xenograft system (Kreider et al., 1985). HPV-11 L1 VLPs were produced by infection of Spodoptera frugiperda-derived insect cells (Sf9) at an m.o.i. of 10, and the infected cells were lysed 72 h later by one freeze-thaw cycle. The lysed cells were homogenized in a Virtis homogenizer for 5 min at 30000 r.p.m., cellular debris was removed by low speed centrifugation and then the supernatant was placed onto a CsCl gradient for banding of VLPs. Aliquots of the gradient were removed and their density was determined from the measured refractive indices. Fractions of the gradients were analysed for VLPs and L1 protein by Western blotting and by ELISA using MAb (Christensen et al., 1990b).

Electron microscopy (EM) of VLPs. HPV-11 L1 VLPs were examined by EM to determine whether their surface topography was similar to that of infectious PVs (Kirnbauer et al., 1992). CsCl gradient-purified infectious HPV-11 and VLPs were dialysed against PBS then 5 μl of this suspension was applied to formvar-coated EM grids and the excess liquid was drawn off with a paper tissue. The sample was then stained with 1% phosphotungstic acid and viewed under a Philips EM400 electron microscope.

ELISA and Western blot. Intact and disrupted HPV-11 L1 VLPs were tested in ELISA for reactivity to HPV-11-neutralizing MAb by previously published methods utilizing infectious PV (Cowart et al., 1987; Christensen et al., 1990b). Intact VLPs were attached to ELISA plate wells at neutral pH, and disrupted VLPs were attached in alkaline and reducing conditions (0.2 M Na2CO3, 0.01 M DTT pH 10.5) (Favre et al., 1975; Christensen et al., 1990b). Western blot analysis of gradient fractions of CsCl-banded VLPs were also examined using anti-PV GSA antibody as previously described (Christensen et al., 1991).

Immunizations. Rabbits and mice were immunized with HPV-11 L1 VLPs diluted in PBS. Rabbits received 50 μg protein per injection intramuscularly and mice received 1 μg protein per injection subcutaneously at 2 week intervals. The L1 protein content of VLP preparations was estimated by densitometric scanning of Coomassie blue-stained gels using BSA as a standard. Approximately 23% of total VLP protein was calculated to be L1 protein. Serum samples were collected 3 to 4 days after each booster immunization, heat-inactivated at 56 °C for 30 min, then tested in ELISA for reactivity to intact infectious HPV-11 (Christensen et al., 1990b).

Neutralization of HPV-11 by anti-VLP sera. Neutralization of HPV-11 was assayed using the athymic mouse xenograft system (Kreider et al., 1985) as previously described (Christensen et al., 1990b). Dilutions of rabbit sera were added to aliquots of infectious HPV-11 and incubated for 1 h at 37 °C, then foreskin chips were added and incubated for a further 1 h at 37 °C. The foreskin chips were transplanted under the renal capsule of athymic mice and were examined 70 days later for morphological transformation and HPV-11 DNA by in situ hybridization (Christensen et al., 1990b). Morphologically normal epithelial cysts that were negative in situ were considered experimental evidence of complete virus neutralization, and cysts morphologically transformed (HPV-11 DNA-positive) as non-neutralized. Positive controls for neutralization included previously tested rabbit anti-HPV-11 sera, and neutralizing MAb (Christensen et al., 1990b). Negative control sera included preimmune rabbit sera and non-immune mouse sera.

Results

Production of HPV-11 L1 VLPs in insect cells

A recombinant baculovirus that expressed HPV-11 L1 in insect cells was developed. CsCl gradient fractions of lysates of infected Sf9 cells were tested in ELISA using a neutralizing MAb to test the hypothesis that the VLPs retained conformational neutralizing epitopes (Fig. 1). Strong reactivity with the intact (but not disrupted) VLPs by the neutralizing MAb H11.B2 was obtained

Fig. 1. ELISA reactivity of CsCl gradient fractions of lysed Sf9 cells infected with recombinant baculovirus expressing HPV-11 L1. One microlitre of individual fractions of the CsCl gradient were added to duplicate wells of an ELISA plate and screened with MAb H11. B2 (□) and polyclonal anti-6L1 peptide antisera (A) for reactivity to intact (a) or disrupted (b) VLPs. A410 values are expressed as the mean ± s.e.m. of duplicate wells. Gradient densities (▽) from left to right: 1.30, 1.32, 1.34 and 1.36 g/ml. A visible band was detected in fractions 11 to 13.
Table 1. ELISA reactivity of MAbs to CsCl-banded HPV-11 L1 VLPs obtained from Sf9 cell lysates infected with recombinant baculovirus

<table>
<thead>
<tr>
<th>MAb/antiserum*</th>
<th>Antigen</th>
<th>Neutralizing</th>
<th>Intact VLPs</th>
<th>Disrupted VLPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>H11.A3</td>
<td>HPV-11</td>
<td>No</td>
<td>0.263 (0.017)</td>
<td>0.001 (0.001)</td>
</tr>
<tr>
<td>H11.B2</td>
<td>HPV-11</td>
<td>Yes</td>
<td>0.528 (0.011)</td>
<td>0.002 (0.001)</td>
</tr>
<tr>
<td>H11.F1</td>
<td>HPV-11</td>
<td>Yes</td>
<td>0.404 (0.049)</td>
<td>0.002 (0.000)</td>
</tr>
<tr>
<td>H11.G5</td>
<td>HPV-11</td>
<td>Yes</td>
<td>0.797 (0.065)</td>
<td>0.001 (0.000)</td>
</tr>
<tr>
<td>H11.H3</td>
<td>HPV-11</td>
<td>Yes</td>
<td>0.784 (0.024)</td>
<td>0.002 (0.000)</td>
</tr>
<tr>
<td>CRPV-1A</td>
<td>CRPV</td>
<td>Yes</td>
<td>0.001 (0.001)</td>
<td>0.001 (0.000)</td>
</tr>
<tr>
<td>2795C-4</td>
<td>HPV-6 L1</td>
<td>No</td>
<td>0.007 (0.000)</td>
<td>0.971 (0.002)</td>
</tr>
<tr>
<td>2975A-4</td>
<td>HPV-16 L1</td>
<td>No</td>
<td>0.006 (0.001)</td>
<td>0.470 (0.042)</td>
</tr>
<tr>
<td>NRS*</td>
<td></td>
<td></td>
<td>0.000 (0.001)</td>
<td>0.006 (0.001)</td>
</tr>
</tbody>
</table>

* MAbs and polyclonal rabbit antisera. Antisera 2795C-4 and 2975A-4 were generated against HPV-6b and HPV-16 L1 peptides and fusion proteins (Firzlaff et al., 1988; Christensen et al., 1990a).
† Results are shown as A410 values, the s.e.m. is shown in parenthesis. Values shown underlined are considered reactive.
‡ Normal rabbit serum.

Fig. 2. ELISA titration of rabbit (B0567) anti-VLP sera against intact infectious HPV-11 particles. Dilutions of rabbit sera from pre-immune sera (●) and after 1 (■), 2 (□), 3 (△) and 4 (△) immunizations at 2 week intervals with 50 μg/VLP protein in PBS. Antibody titres (50% binding) reach 1:20000 to 1:50000 by the second immunization. A410 values represent mean ± s.e.m. of duplicate readings and error bars are given unless smaller than the symbols. One of two similar rabbit responses to VLP immunizations is shown.

VLP disruption was confirmed by positive reaction with polyclonal anti-6L1 peptide sera (Firzlaff et al., 1988; Christensen et al., 1990a), and by loss of binding by MAb H11.B2 (Fig. 1b). All five MAbs (Christensen et al., 1990b) that recognized surface conformational epitopes (four neutralizing and one non-neutralizing) showed strong reactivity to intact but not disrupted HPV-11 L1 VLPs by ELISA (Table 1).

EM of the VLPs was conducted to determine whether their gross surface morphology was similar to that of infectious virus as has been observed by others (Kirnbauer et al., 1992, 1993; Rose et al., 1993; Hagensee et al., 1993). The results showed that VLPs of variable size were produced that had features similar to those of infectious virions and the HPV VLPs produced by other researchers (Kirnbauer et al., 1993) (data not shown).

Immunization with HPV-11 L1 VLPs

Rabbits and mice were immunized intramuscularly and subcutaneously respectively with HPV-11 L1 VLPs...
Table 2. Neutralization of HPV-11 by antisera generated against HPV-11 L1 VLPs

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antigen</th>
<th>Cyst size†</th>
<th>Morphology‡</th>
<th>HPV-11 DNA§</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0567 preimmune</td>
<td>–</td>
<td>3.3 ± 0.4</td>
<td>6/7/8</td>
<td>6/7/8</td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0567 immune</td>
<td>HPV-11 L1 VLP</td>
<td>1.7 ± 0.4</td>
<td>0/6/7</td>
<td>2/6/6</td>
</tr>
<tr>
<td>1:1000</td>
<td>HPV-11 L1 VLP</td>
<td>3.8 ± 0.3</td>
<td>0/5/8</td>
<td>0/5/6</td>
</tr>
<tr>
<td>1:10000</td>
<td>HPV-11 L1 VLP</td>
<td>2.2 ± 0.3</td>
<td>0/6/8</td>
<td>0/6/7</td>
</tr>
<tr>
<td>Preimmune mouse</td>
<td>–</td>
<td>3.5 ± 0.5</td>
<td>5/5/8</td>
<td>5/5/7</td>
</tr>
<tr>
<td>serum 1:100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse immune</td>
<td>HPV-11 L1 VLP</td>
<td>2.6 ± 0.5</td>
<td>0/4/8</td>
<td>0/4/7</td>
</tr>
<tr>
<td>sera 1:100</td>
<td>HPV-11 L1 VLP</td>
<td>1.7 ± 0.2</td>
<td>1/6/8</td>
<td>1/5/6</td>
</tr>
<tr>
<td>1:1000</td>
<td>Infectious HPV-11</td>
<td>3.9 ± 0.3</td>
<td>0/5/7</td>
<td>0/5/6</td>
</tr>
<tr>
<td>639 1:100</td>
<td>Infectious HPV-11</td>
<td>1.4 ± 0.2</td>
<td>0/7/7</td>
<td>0/7/7</td>
</tr>
<tr>
<td>H11. B2</td>
<td>Infectious HPV-11</td>
<td>2.0 ± 0.2</td>
<td>0/5/7</td>
<td>1/5/7</td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Antiseras were as follows: B0567, Dutch belted rabbit B0567; 639, New Zealand white rabbit polyclonal HPV-11-neutralizing antisera (Christensen et al., 1990b); H11. B2, HPV-11-neutralizing MAb supernatant (Christensen et al., 1990b).
† Surviving cysts only, results are given as geometric mean diameter (mm), the S.E.M. is given in parenthesis. Underlined values have P < 0.005 compared to cyst sizes developing in response to preimmune rabbit or mouse serum treatment.
‡ Number of grafts morphologically transformed/number of surviving grafts/number attempted.
§ Number of grafts HPV-11 DNA-positive by in situ hybridization/number surviving/number attempted.

Neutralization of HPV-11 infectivity by anti-VLP sera

Antibody reactions to intact infectious PVs have been shown to be predominantly neutralizing (Christensen et al., 1990b; Jarrett et al., 1990b; Ghim et al., 1991). However, the induction of neutralizing antibodies to an HPV type by HPV VLPs has not been documented. We therefore tested the polyclonal anti-VLP sera for HPV-11 neutralizing activity in the xenograft system. Dilutions of rabbit and mouse anti-VLP sera were mixed with aliquots of infectious HPV-11 then foreskin chips were added and transplanted subrenally. The presence of normal epithelial cysts (Kreider et al., 1985) that were HPV-11 DNA-negative by in situ hybridization was evidence for the presence of neutralizing antibody. The anti-VLP sera were strongly neutralizing, with complete protection against HPV-11 infection at dilutions greater than 1:10000 for the rabbit sera (Table 2). Sera obtained from mice immunized four times with 1 µg VLP protein were also strongly neutralizing with partial protection against HPV-11 infection at dilutions greater than 1:1000. Positive controls for neutralization included a rabbit anti-HPV-11 serum previously shown to be neutralizing and a neutralizing MAb (Christensen et al., 1990b).

Discussion

In this study, we have shown that HPV-11 L1 when overexpressed in insect cells self-assembles into VLPs that retain conformational neutralizing epitopes as identified by neutralizing MAbs (Christensen et al., 1990b). These results are in agreement with other published studies showing that the major capsid protein, L1, of PVs can self-assemble in isolation from other PV proteins (Kirnbauer et al., 1992, 1993; Rose et al., 1993; Hagensee et al., 1993) and induce neutralizing antibodies (Kirnbauer et al., 1992). All four neutralizing MAbs that were generated against infectious HPV-11 in a previous study (Christensen et al., 1990b) reacted with the intact VLPs demonstrating that neutralizing antibodies against conformation-dependent epitopes are directed predominantly against L1.
In several animal/PV model systems, intact virus (containing conformational neutralizing epitopes) has induced high titres of neutralizing antibodies and strong protection against experimental virus challenge (Olson, 1963; Jarrett et al., 1990a, b; Christensen et al., 1991). Included in these studies are several epithelial tissue-specific PVs [bovine PVs (BPVs)-4 and -6 and cottontail rabbit PV (CRPV)] PVs inducing fibropapillomas (BPV-1 and -2), and a PV that infects mucosal epithelia (BPV-4). Neutralizing epitopes have also been identified on the capsid proteins L1 and L2 (Pilacincki et al., 1984; Jin et al., 1990; Christensen et al., 1991; Lin et al., 1992). These epitopes, however, induced low titres of neutralizing antibodies and were only weakly protective. Recent studies have demonstrated that fragments of L1 proteins are incapable of triggering neutralizing antibodies, indicating that linear epitopes on CRPV L1 are not neutralizing (Lin et al., 1993). The weakly neutralizing epitopes on L2 have not been characterized in the same manner, but are likely to be linear because denatured L2 has been used for immunizations (Christensen et al., 1991; Lin et al., 1992). It is not yet known whether these weakly neutralizing epitopes are capable of protecting hosts against natural PV infections.

Assembled VLPs have recently attracted attention in PV research because they provide an antigen source which retains surface conformational epitopes that are lost when individual and subunit capsid antigens are used. A major group of antigens that are provided by the VLPs are conformational neutralizing epitopes and evidence has accumulated that these epitopes provide the dominant antibody response to capsid proteins in experimental and natural infections (Jarrett et al., 1990a, b; Ghim et al., 1991; Christensen & Kreider, 1990). An important additional result that is demonstrated in this study is the strong antigenicity of these VLPs for the induction of neutralizing antibodies. The VLPs are potent antigens and trigger high titres of neutralizing antibodies in the absence of commercial preparations of adjuvant. This observation is of significance for the development of effective vaccines for humans because commercial adjuvants and their associated problems can be bypassed.

Neutralization of HPV-11 using rabbit anti-HPV-11 L1 VLP antisera indicated that at low dilutions (1:100) there was some breakthrough of infectivity as determined by in situ hybridization (Table 2). This result is intriguing because higher dilutions of rabbit anti-VLP antisera (1:10000) afforded complete protection. One possible explanation is that very high concentrations of anti-VLP antibodies agglutinated the virus into large aggregates which formed an infectious ‘unit’ with the central virions having minimal bound antibody. Another possibility is that a second, higher affinity protective (non-neutralizing) antibody response was also generated that was of lower titre than the neutralizing antibodies but afforded partial protection for infectious virus at low serum dilutions by steric hindrance of the binding of the neutralizing antibodies. A MAb analysis of the antibody profile to VLP immunizations may help to resolve these possibilities, and the outcome may have important consequences for the development of the VLP strategy for vaccines against HPV infections.

Although serological cross-reactivity of HPV types at the level of conformational neutralizing epitopes is unknown, further studies with the HPV-11-neutralizing MAbs (Christensen et al., 1990b) have indicated that HPV-11 and HPV-6 L1 can be immunoprecipitated by the HPV-11-neutralizing MAbs, but HPV-16 L1 cannot (B. Jenson, personal communication). Future studies with L1 VLPs of the different HPV types may provide data to indicate that HPVs (at the level of neutralizing epitopes) are either antigenically type-specific or are grouped into several major serological subsets. If such subsets are identified, then a limited number of HPV L1 VLPs representing each serological subgroup could provide cross-protection to most HPV types if used together as a composite vaccine.

In summary, we have shown that HPV-11 L1 self-assembles into VLPs which are highly antigenic in the absence of adjuvant and which induce high titres of serum neutralizing antibodies. The conformationally correct neutralizing epitopes on these VLPs were further confirmed by a set of HPV-11-neutralizing MAbs prepared against infectious HPV-11. These VLPs have the potential to be safe (non-infectious) vaccines for humans and to serve as an antigen source for serological studies to identify serum neutralizing antibodies following vaccination and natural HPV infection.

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References


