Human papillomavirus (HPV) type 11 recombinant virus-like particles induce the formation of neutralizing antibodies and detect HPV-specific antibodies in human sera

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Recombinant human papillomavirus type 11 (HPV-11) virus-like particles (VLPs) were tested for their ability to induce the formation of neutralizing antibodies, and were also tested for serodiagnostic capabilities in an ELISA in comparison with HPV-11 whole virions. VLPs, purified by CsCl density gradient centrifugation from the cell-free supernatant of Ac11L1-infected Sf9 suspension cell cultures, were used to immunize rabbits and anti-VLP antibodies were tested in the athymic mouse model of HPV-11 infection. Pretreatment of infectious HPV-11 virions with the immune serum of VLP-treated animals caused a marked reduction of graft growth \((P < 10^{-4})\) and viral gene expression \((P < 10^{-4})\), similar to the effects obtained using whole virion post-immune serum, and consistent with immune neutralization. To assess the serodiagnostic capabilities of VLPs, a VLP ELISA was developed and used to analyse sera that were tested previously in an HPV-11 whole virion ELISA. Specific antibodies were detected in 49% of patients’ sera \((P = 2 \times 10^{-4})\), and individual VLP seroreactivities correlated with those previously obtained using whole virions as the antigen \((r = 0.87; P < 10^{-4})\). These results indicate that recombinant VLPs can be used to elicit a neutralizing antibody response, and can substitute faithfully for native virions in the development of HPV-serodiagnostic immunoassays.

Human papillomaviruses (HPVs) are small DNA viruses that infect cutaneous and mucosal stratified epithelia (Reichman & Bonnez, 1990). Genital HPVs are of particular medical importance due to their close association with the development of anogenital warts, intraepithelial neoplasias and invasive cancers (zur Hausen, 1976; Gross, 1983). Although intensive investigations of the aetiology and epidemiology of genital HPV infections have been made, studies of the pathogenesis of and immune responses to infection have been hindered by the inability to propagate these highly tissue-specific viruses \(in vitro\). This difficulty has been partially overcome by the development of the athymic mouse–human xenograft system, in which HPV-11 virions can be produced and recovered (Kreider et al., 1985, 1987). Using these virus particles in an ELISA, we have demonstrated the presence of specific antibodies in the sera of patients with condyloma acuminatum (Bonnez et al., 1991) or with recurrent respiratory papillomatosis (Bonnez et al., 1992a), two diseases associated with HPV-11 or the closely related HPV-6. These observations, along with other evidence (Kienzler et al., 1983; Steele & Gallimore, 1990), indicate the importance of conformational epitopes in the development of an antibody response to HPV capsid proteins. This antibody response may be biologically relevant, as we and others have shown that HPV-11 virions can induce neutralizing antibodies in the rabbit (Christensen & Kreider, 1990; Bonnez et al., 1992b). There is also preliminary evidence that neutralizing antibodies are present in the sera of patients with condyloma acuminatum or recurrent respiratory papillomatosis (Christensen et al., 1992).

The athymic mouse human xenograft model is costly and currently limited to the production of HPV-1 or HPV-11 virions (Kreider et al., 1987, 1990). As an alternative, we and other investigators have produced virus-like particles (VLPs) of papillomaviruses in recombinant expression systems (Zhou et al., 1991; Kirnbauer et al., 1992; Hagensec et al., 1993; Rose et al., 1993). In addition, we have shown that HPV-11 VLPs constructed from L1 protein possess conformational epitopes that are immunologically similar to those present on native HPV-11 virions (Rose et al., 1993). In this study, we sought to characterize further the immunological properties of recombinant VLPs by testing their ability to induce a serum neutralizing antibody response and by investigating their suitability for use as antigens in the serodiagnosis of HPV infection.
Using a modification of our published method (Rose et al., 1993), VLPs were recovered and purified directly from the cell-free supernatants of AcHI1L1-infected Sf9 suspension cultures. AcHI1L1-infected Sf9 cells (0.21 suspension culture) were incubated at 27 °C for 120 h and then centrifuged at 1000 g for 10 min. The cell-free supernatant was recovered, centrifuged at 100,000 g for 90 min at 4 °C, and the pellet was resuspended in a total volume of 5 ml of buffer A (PBS, 1 mM-MgCl2, 1 mM-CaCl2, 1 mM-PMSF, 10 μg/ml leupeptin). After adding 5.2 g of solid CsCl, the volume was adjusted to 13 ml with buffer A (0.4 g/ml final concentration of CsCl), and the sample was centrifuged at 100,000 g for 22 h at 10 °C. The single visible band obtained was removed with an 18-gauge needle, diluted in buffer A to 12 ml, and centrifuged at 100,000 g for 90 min at 4 °C. The pellet was resuspended in buffer A (50% glycerol), the A~280 of this solution was measured and it was stored at −20 °C.

The single visible band obtained was resuspended in buffer A (50% glycerol), the A~280 of this solution was measured and it was stored at −20 °C. Immunoactive L1 protein was detected in the CsCl-purified sample by SDS–PAGE/Western blot immunoassay (data not shown), and the presence of intact VLPs with the morphological characteristics of papillomavirus particles was verified by electron microscopy (data not shown).

To produce HPV-11 L1 VLP-specific antisera, New Zealand white rabbits were immunized intramuscularly at two sites with a 1:1 emulsion of purified VLPs (containing 50 μg protein) in complete Freund’s adjuvant (0.5 ml per site), and boosted after 2 weeks with a VLP emulsion prepared in incomplete Freund’s adjuvant. Immune sera were collected 14 days later. We first sought to confirm our previous observation that HPV-11 whole virion antibodies are immunologically cross-reactive with recombinant VLPs (Rose et al., 1993). VLP preimmune and immune sera were tested for cross-reactivity with HPV-11 whole virions in a dot-blot immunoassay; VLP and control whole virion immune sera were equally reactive with whole virions and with recombinant VLPs, thus confirming the reciprocal nature of this cross-reactivity. Preimmune sera did not react with either of these antigen preparations (data not shown).

VLP antibodies were tested for neutralizing activity using our previously published method (Bonnez et al., 1992b). The preparation of the infecting HPV-11 (Kreider) virus suspension (originally provided by John Kreider) has been described (Bonnez et al., 1992b). In four parallel experiments, 450 μl of the infecting virus suspension (batch 4/90; 1:10 dilution) was incubated at 37 °C for 1 h with 50 μl (1:10 final dilution) of either preimmune anti-HPV-11 serum (group 1), immune anti-HPV-11 serum (group 2), preimmune anti-VLP serum (group 3), or immune anti-VLP serum (group 4). Groups 1 and 2 were neutralization controls that we have described previously (Bonnez et al., 1992b), and groups 3 and 4 were the test groups. The preparation of human foreskins excised for routine circumcision has also been described (Bonnez et al., 1991). Foreskins were cut into 1 mm squares and a small number of fragments from each foreskin were snap-frozen and saved. The remaining fragments were divided equally into four groups, and each group was added to one of the four virus suspension/serum mixtures at the end of the incubation period. Mixtures were incubated for 1 h at 37 °C. For each experimental group, one foreskin fragment was placed under the renal capsule of each kidney of three female, litter-matched, 4 to 6 week old, athymic nu/nu mice on a BALB/c background (Taconic Farms). The experiment was replicated on a different day, with a different foreskin. Thus, for each experimental group a total of 12 grafts were implanted. The animals were sacrificed 12 weeks after grafting, at which time the grafts were removed and processed as previously described (Bonnez et al., 1992b). For graft size comparison, the geometric mean diameter (GMD) was calculated as ³\sqrt[³]{\text{length} \times \text{width} \times \text{height}}.

In our experience (based on unpublished observations), uninfected foreskin grafts do not grow significantly when implanted in athymic mice, and grafts pretreated with other (non-neutralizing) rabbit post-immune sera generally become infected and grow to the same extent observed with grafts infected but not pretreated with serum. Since the total number of samples is limited by logistical considerations, these previous observations were considered in the experimental design of the present study. Therefore non-infected grafts, and grafts pretreated with other (non-neutralizing) rabbit immune sera, were not included as controls for these analyses.

Results of the VLP antiserum neutralization assay are summarized in Table 1. At the time of euthanasia, one graft was missing from each of the neutralization control preimmune and immune anti-HPV-11 virion antibody-treated groups. Thus, the number of grafts available for analysis in each of these groups was 11. The median [range] GMDs (mm) of the grafts in the preimmune and immune control groups were respectively 2.9 [1.0, 4.9] and 1.3 [1.0, 2.6]. The difference, 1.6 mm, was statistically significant (P = 0.004, Mann-Whitney U test). All 12 implanted grafts were available for analysis in both the preimmune and immune anti-VLP antibody-treated groups. The median [range] GMDs (mm) of the grafts were respectively 2.3 [1.3, 4.2] and 1.0 [1.0, 1.8]. The difference in size, 1.3 mm, was statistically significant (P < 10-4). Although the difference in graft sizes between the first and second replicate experiments was not statistically significant (P = 0.62) in the preimmune group, it was significant (P = 0.007) in the immune group. Therefore, we compared the differences in graft
sizes between the preimmune and immune anti-VLP antibody-treated groups within each replicate experiment. Both were statistically significant (P = 0.002 and P = 0.04, respectively for the first and second replicate).

Neutralization was confirmed by analysing all grafts for the presence of the HPV-11 El^E4 spliced mRNA transcript, as previously described (Bonnez et al., 1992b). Primers complementary to sequences in the second and fourth exons of the β-actin gene were designed to produce a 582 bp spliced cDNA PCR amplification product using reverse transcriptase (RT) and used for all samples as a positive control for mRNA integrity. Samples negative for β-actin mRNA were excluded from further analysis. El^E4 mRNA was detected in 10/11 (91%) and 0/10 (0%) of the grafts from groups pretreated with preimmune or immune VLP sera, respectively (P < 10^-4). Similarly, for the control groups pretreated with preimmune or immune anti-whole virion sera, El^E4 mRNA was detected in 8/11 (73%) and 0/10 (0%) grafts, respectively (P = 10^-3). Thus, treatment of the viral inoculum with the immune VLP serum was associated with marked inhibition of both graft growth and viral gene expression, effects which are consistent with immune neutralization.

VLPs were tested in an ELISA to assess their usefulness for the detection of specific antibodies in patients' sera, and the results were compared with results previously obtained using the same sera in an HPV-11 whole virion ELISA (Bonnez et al., 1993). The antigen was diluted in PBS to give an amount equivalent to that of the amount used in the previous whole virion ELISA (Bonnez et al., 1993), and either the antigen solution or PBS without any antigen was applied in aliquots into alternate rows of wells in a 96-well plate. After coating for 16 h at 4 °C, these solutions were aspirated and wells were blocked with diluent/blocking solution (Kirkegaard and Perry Laboratories) at room temperature for 16 h at 4 °C. These solutions were aspirated and wells were blocked with diluent/blocking solution (Kirkegaard and Perry Laboratories) at room temperature for 16 h at 4 °C. Then 100 μl aliquots were added to wells treated either with PBS alone or with antigen solution (two replicates per serum sample). Plates were incubated at room temperature for 90 min and then washed four times with wash solution (Kirkegaard and Perry Laboratories). Anti-human IgG–alkaline phosphatase conjugate (100 μl aliquots, diluted 1:5000; Tago) was added to each well and plates were incubated at room temperature for 90 min. Plates were washed four times and developed with alkaline phosphatase substrate (p-nitrophenyl phosphate in diethanolamine buffer). Specific absorbance at 405 nm for each sample serum was calculated by subtracting the value obtained from the PBS-treated well from the value obtained from the antigen-containing well for each replicate and mean replicate differences were calculated. In our previous whole virion ELISA, we analysed 42 patients' sera (and 20 control sera) for changes in capsid antibody levels during the course of treatment (Bonnez et al., 1993). All sera tested in the present study were collected at entry into the previous study. One of the patients' sera analysed in the previous study had not been reported for reasons related to treatment outcome and not to the results of the immunoassay. However, because the absorbance value of this serum was available, the serum was included in the present assay, which increased the number of patients' sera analysed in the present study to 43. The number of control sera analysed was reduced from 20 to 16 for logistical considerations pertaining to the assay.

The median [range] seroreactivity of the 16 control sera, expressed as an A405 value, was 0.005 [−0.029, 0.025], compared to 0.024 [−0.063, 0.512] for the 43 patients' sera (Fig. 1), a statistically significant difference (P = 0.01; Mann-Whitney U test). Using the highest A405 value in the control group as a cut-off, the sensitivity of the assay was 49% (P = 2 × 10^-4; Fisher's exact test). Therefore, the HPV-11 VLP ELISA was able to discriminate between patients with condyloma acuminatum and controls. In addition, there was excellent correlation (Pearson's product-moment r = 0.87; P < 10^-4) between sample seroreactivities with the HPV-11 VLP ELISA and the HPV-11 virion ELISA.

Table 1. Results of the neutralization assay

<table>
<thead>
<tr>
<th>Antibody group</th>
<th>Anti-HPV-11 virion antibody-treated group</th>
<th>Anti-HPV-11-L1 VLP antibody-treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median GMD [range]; (n)</td>
<td>Number RT/PCR positive/total (%)</td>
</tr>
<tr>
<td>Preimmune</td>
<td>2.9 [1.0, 4.9]; (11)</td>
<td>8/11 (73)</td>
</tr>
<tr>
<td>Immune</td>
<td>1.3 [1.0, 2.6]; (11)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>P values</td>
<td>0.004</td>
<td>10^-3</td>
</tr>
</tbody>
</table>
when all 59 sera were included (Fig. 2), or when only the 21 sera positive by HPV-11 VLP ELISA were considered ($r = 0.87; P < 10^{-6}$).

These results show that, under similar experimental conditions, immune sera from rabbits immunized with HPV-11 L1 VLPs can block HPV-11 infection of human tissue as effectively as sera obtained from rabbits immunized with HPV-11 whole virions. This blockade, which was not observed with the respective preimmune sera, was associated with the absence of early viral gene expression. Therefore, the effect was consistent with classic viral neutralization, i.e. the prevention of virus penetration or decapsidation (Dimmock, 1993) (Table 1).

Although neutralizing antibodies to HPV-11 have been identified in the sera of patients with condyloma acuminatum (Christensen et al., 1992), their biological significance has not yet been defined. If HPV neutralization proves to be a protective immune effector mechanism \textit{in vivo}, then immunization with recombinant VLPs may protect individuals who are at risk of infection. Vaccination with VLPs might also be useful for the therapy of established disease. The neutralizing titre of our rabbit anti-VLP antibody was not assessed by limiting dilution, but others have found neutralizing titres as high as 128 with rabbit anti-HPV-11 virion antibodies (Christensen & Kreider, 1990). Neutralizing titres in humans have not been assessed.

Our study also shows that HPV-11 L1 VLPs are suitable as an antigen for ELISA and that this ELISA can distinguish between sera from patients with condyloma acuminatum and controls. Moreover, the results of the HPV-11 L1 VLP ELISA correlate closely with the results of the HPV-11 virion ELISA when the same sera are compared. These results further establish the structural and antigenic similarities between HPV-11 virions and VLPs derived from HPV-11 L1 protein (Rose et al., 1993).

The reported sensitivity of currently available HPV-11 virion-based or VLP-based immunoassays is approximately 50\% (Bonnez et al., 1991, 1992a, 1993; Carter et al., 1993; and the present study). There are several possible explanations for such a relatively low sensitivity. Condyloma acuminatum and recurrent respiratory papillomatosis are diseases of the skin and mucous membranes and therefore HPV antigens may not interact sufficiently with the immune system to elicit a vigorous antibody response in all infected patients. In addition, neither of these two ELISAs has been optimized and the difficulties associated with producing large quantities of HPV-11 virions has made careful standardization of the virion-based assay very difficult. The availability of VLPs should make optimization of the VLP ELISA possible. Another potential explanation for the low sensitivity of the HPV-11-based assays is that they are too HPV type-specific. However, HPV-11 whole virion immunoassays are able to detect specific, presumably cross-reactive, antibodies in the sera of patients with
condyloma acuminatum or recurrent respiratory papillomatisosis who are infected with HPV-6, the other major causal agent of these diseases (Bonnez et al., 1991, 1992a). Therefore, the poor sensitivity of the present arrays cannot be attributed exclusively to narrow type-specificity of the antigen. It is also important to note that a broader HPV type-specificity does exist, since the sera of patients with condyloma acuminatum or recurrent respiratory papillomatisosis do not react with HPV-1 as readily as the sera of patients with plantar warts (Pfister & zur Hausen, 1978; Pfister et al., 1979; Kienzler et al., 1983; Viaë et al., 1990).

Recombinant VLPs are a unique tool which can be used for the exploration of HPV capsid antigenic cross-reactivity. They may also be useful in studies of pathogenesis and in the development of effective vaccines and reliable immunoassays.

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References


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