Serological relationship between cutaneous human papillomavirus types 5, 8 and 92

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Evidence of a possible association of cutaneous human papillomavirus (HPV) types, especially members of the genus Betapapillomavirus, and the development of non-melanoma skin cancer (NMSC) is accumulating. Vaccination with virus-like particles (VLPs) consisting of self-assembled L1, the major capsid protein, has been introduced to control anogenital HPV infection. This study examined the serological relationship between betapapillomavirus (β-PV) types 5 and 8 and the new type HPV-92, which has recently been isolated from a basal cell carcinoma containing a high number of viral genomes. Following expression by recombinant baculoviruses, the L1 protein of HPV-92 self-assembled into VLPs that elicited high-titre antibodies after immunization, similar to VLPs from HPV-5 and -8. Haemagglutination inhibition (HAI) assays were used as a surrogate method for the detection of virion-neutralizing antibodies, which correlates with protection from infection. Antisera raised against HPV-5 and -8 VLPs displayed HAI activity not only against the homologous type, but also against heterologous HPV types 5, 8 and 92, whereas HAI activity of antisera against HPV-92 VLP was restricted to the homologous type. The results of neutralization assays using HPV-5 pseudovirions were consistent with those from HAI assays. Cross-neutralizing immune responses by VLP vaccination against heterologous HPV types may provide broader protection against the multiplicity of HPV types detected in NMSC. If a close link to HPV infection can be conclusively established, these results may provide a basis for further evaluation of VLPs of β-PVs as candidates for a prophylactic skin-type HPV vaccine, aimed at reducing the incidence of NMSC.

INTRODUCTION

Papillomaviruses (PVs) comprise a large group of small, non-enveloped DNA tumour viruses that infect epithelial cells of the skin and mucosa, causing papillomas and warts in many different vertebrate species including humans. More than 100 human papillomavirus (HPV) types have currently been completely characterized. The aetiological role of high-risk mucosal HPV types in the development of anogenital – especially cervical – neoplasias and a subset of oropharyngeal carcinomas is firmly established (Howley & Lowy, 2007; Kirnbauer et al., 2008). In addition, the possible involvement of cutaneous HPVs in the pathogenesis of non-melanoma skin cancer (NMSC) is under active investigation.

NMSCs, in particular basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs), are the most prevalent malignancies in the Caucasian population and their incidence is increasing (Diepgen & Mahler, 2002). The main risk factor for development of these keratinocyte skin cancers is UV radiation, with immunosuppression and genetic factors appearing to play an adjunct predisposing role. Due to behavioural changes and ageing of the world’s population, these NMSCs represent increasing health and socioeconomic problems. It is estimated that at least half of white-skinned transplant recipients will develop NMSC (Diepgen & Mahler, 2002). Preventative strategies have focused on raising public awareness to use precautions against UV exposure. In recent years, evidence of a possible association of HPV infection and development of NMSC has accumulated. In contrast to cervical cancer, a defined adjunct causal role for HPV infection, in addition to UV radiation-induced DNA damage, has yet to be established.
The oncogenic potential of certain HPV types in human skin cancer was originally recognized in studies of patients with the rare genodermatosis epidermodysplasia verruciformis (EV) (Orth, 2006). EV patients display increased susceptibility to cutaneous HPV infection, and mutations in the EVER1 or EVER2 genes have been linked to the disease (Ramozy et al., 2002). There exist 24 fully characterized HPV types belonging to the genus Betapapillomavirus (which historically have been designated EV types), which have been isolated from the characteristic macular and verrucous EV skin lesions. Approximately 50 % of EV patients develop SCC on sun-exposed areas. HPV-5 and HPV-8 are the types most often present in EV skin cancers, and viral genes are always transcribed (Orth, 2006).

Detection of betapapillomavirus (β-PV) DNA in skin cancer is not restricted to EV patients, as NMSCs of immunocompetent patients frequently harbour β-PV DNA, although generally at very low copy numbers of less than one in 100 cells. Nested PCR-based epidemiological studies have reported β-PV DNA in about 30–50 % of NMSCs of immunocompetent individuals (Harwood et al., 2004; Pfister, 2003), whereas in organ transplant recipients, a group with a 50–100-fold increased risk of developing NMSC, the detection rate increases up to 90 % (de Jong-Tieben et al., 1995). Furthermore, β-PV DNA has been found in pre-malignant skin lesions such as actinic keratoses and extragenital Bowen’s disease, but also in normal or psoriatic skin and plucked hairs (Antonsson et al., 2000; Boxman et al., 1997; Favre et al., 1998; Forslund et al., 2003c). Independently, several epidemiological studies have found an association between the detection of β-PV DNA in normal skin or hair follicles, preferentially on sun-exposed sites, and increased risk of developing pre-malignant or invasive SCC (Harwood et al., 2004; Termorshuizen et al., 2004).

Gene transcription of β-PV types has been demonstrated in a proportion of SCCs from both immunocompetent and immunocompromised individuals. Although the level of transcriptional activity has been low, these results imply a possible biological role in tumour development (Pfister, 2003; Purdie et al., 2005). The oncogenes E6 and E7 of β-PVs have weak transforming potential in vitro compared with E6 and E7 of high-risk mucosal types such as HPV-16. However, β-PV E6 targets the pro-apoptotic protein Bak for degradation (Jackson et al., 2000) and thus may protect cells from UV-induced apoptosis. By favouring accumulation of UV-induced DNA damage, β-PVs may contribute at an early stage of skin carcinogenesis. In addition, E6 of some β-PVs (HPV-8 and -38) can activate telomerase, and a fraction of HPV-8 E6/E7 transgenic mice spontaneously develop SCCs (Bedard et al., 2008; Schaper et al., 2005).

Seroepidemiological data have also suggested an association between NMSC and skin-type HPV infection. Increased seroprevalence for antibodies (Abs) to β-PV types, including HPV-5 and -8, has been observed in patients with SCC or actinic keratosis, a precursor lesion of SCC (Feltkamp et al., 2003).

A relatively new HPV type, designated (candidate) HPV-92, was isolated from a BCC of an 89-year-old immunocompetent Australian male (Forslund et al., 2003a). Intriguingly, a high viral load comprising approximately 100 DNA copies per tumour cell was observed, compared with the low amount of HPV DNA usually found in keratinocyte skin cancers. Additionally, HPV-92 has been detected in a fraction of BCCs, raising the possibility of a causal association (Forslund et al., 2007). HPV-92 DNA has also been detected in a fraction of SCCs, solar keratoses and keratoacanthomas of immunocompetent as well as immunocompromised patients (Forslund et al., 2003a, b, c). HPV-92 belongs to species 4 β-PVs (de Villiers et al., 2004) and is phylogenetically distantly related to the oncogenic types HPV-5 and -8, with an amino acid identity of 71 % in the L1 protein (Fig. 1). HPV-5 and -8, both species 1 β-PVs, share 87 % amino acid sequence identity in L1.

The major capsid protein L1 of PVs self-assembles into virus-like particles (VLPs) that are highly immunogenic (Kirnbauer et al., 1992, 1993). Generally, systemic immunization with VLPs induces mainly type-restricted neutralizing antiserum, so a polyvalent vaccine would be required to protect against the multiplicity of β-PV types that may play a role in the development of SCCs of the skin. However, low levels of cross-neutralization have been detected after VLP immunizations, but only between the closely related genital types HPV-6 and -11, HPV-16 and -33, HPV-31 and -33, and HPV-18 and -45 (Giroglou et al., 2003b).
2001; Harper et al., 2006; Roden et al., 1996; White et al., 1998), showing amino acid sequence identity in the L1 protein of 92.4, 80.9, 78.4 and 87.4 %, respectively. If a preventative β-PV VLP vaccine were developed, information on cross-neutralization would be clinically relevant, as vaccine formulations are likely to include a limited number of HPV VLP types.

The aim of this study was to determine the serological relationship between the cutaneous types HPV-5, -8 and -92, which have been associated with the pathogenesis of NMSC.

**METHODS**

**Construction of recombinant baculovirus stocks and generation of HPV VLPs.** To construct recombinant baculoviruses expressing the major capsid protein L1 of the new HPV type 92, the L1 open reading frame was amplified from HPV-92 DNA by inverse-touchdown PCR (Slupetzky et al., 2001) using the primers 5’-GGCGCAGATCTCAATATGTCCTATGGGTCCAG-3’ and 5’-GGCGCATGCTCCATACAAGTCCGATTCGTTCC-3’, which correspond to HPV-92 sequences between nt 5638–5656 and 7212–7230, respectively, and contain recognition sites for BglII (underlined), GC clamps (italic) and a polyhedrin leader sequence (AAT) preceding the ATG start codon. The amplified L1 gene was cloned into the baculovirus transfer vector pSynwtVI (italic) and a polyhedrin leader sequence (AAT) preceding the primers were verified by restriction enzyme digest and nucleotide sequencing. By co-transfection of Spodoptera frugiperda (S9) insect cells with transfer vector and linearized baculovirus DNA (BaculoGold; BD Biosciences Pharmingen), recombinant baculoviruses were generated using standard methods (Handisurya et al., 2007). Recombinant baculoviruses expressing L1 of HPV-5 and -8 have been described previously (Favre et al., 2000; Stark et al., 1998). S9 insect cells were subsequently infected at a high m.o.i. and high-molecular-mass structures were separated by density gradient ultracentrifugation. VLP-containing bands were collected and dialysed against PBS containing 0.5 M NaCl and 0.05 % NaN3.

Purified VLPs were analysed by Western blotting or Coomassie Brilliant Blue staining. Briefly, samples were denatured in SDS sample buffer containing 2 % β-mercaptoethanol and analysed by 10 % SDS-PAGE. Following electrophoresis, proteins were stained with Coomassie Brilliant Blue or, for Western blotting, transferred to PVDF membranes. The VLP preparations were analysed by Western blotting using an enhanced chemiluminescence system (Amersham (diluted 1 : 20 000; Kirkegaard & Perry Laboratories) and developed using an ImmunoPure IgG Purification kit A Plus (Pierce Biotechnologies).

**Haemagglutination inhibition (HAI) assay.** Total IgG purified from rabbit pre-immune or immune serum, a murine monoclonal Ab (mAb) specifically recognizing HPV-5 (M. Favre, unpublished data) and an antisera raised against bovine papillomavirus 1 (BPV-1) VLPs were cleared of non-specific haemagglutination (HA) and complement activity by incubation at 56 °C for 30 min followed by centrifugation for 5 min at 10 000 g and 4 °C. VLPs of HPV-5, -8 or -92 were serially diluted in PBS containing 0.1 % BSA in 96-well plates. BPV-1 and BK virus VLPs (a kind gift of J. Schiller, National Cancer Institute, MD, USA) were used as positive controls. A mock VLP preparation derived from S9 cells infected with mutant (prototype) HPV-16 L1 that fails to assemble into intact particles was used as a negative control for HA.

VLPs were incubated with rabbit IgG (final dilution 1:100) for 1 h at room temperature in a final volume of 75 μl, to allow proper adsorption of the Abs to the VLPs. Twenty-five microlitres of a 1 % (v/v) suspension of erythrocytes from fresh citrate-treated blood of BALB/c mice was added to the samples and the plates were incubated for 3 h at 4 °C and photographed.

To determine the HAI titre, thresholds were determined at which specific HA by addition of VLPs was observed. HPV VLPs were dispersed at identical concentrations and serial dilutions (ranging from 1:100 to 1:819200) of rabbit IgG were added to the wells. HAI assays were performed as described above.

**HPV-5 pseudovirion (PsV) neutralization assay.** HPV-5 PsVs were generated by co-transfection of 293 TT cells and neutralization assays were performed as described previously (Buck et al., 2005). Briefly, 293 TT cells [30 000 cells in 10 μl neutralization buffer (10 % FCS in Dulbecco’s modified Eagle’s medium without phenol red) per well] were plated in non-treated sterile 96-well plates (Nalge Nunc) 3–4 h in advance to allow proper adherence. HPV-5 PsVs were incubated for 1 h at 37 °C in 5 % CO2 with serial dilutions of total IgG purified from rabbit immune serum raised against HPV-5, -8 or -92. Incubation with IgG from pre-immune serum of the same animals served as appropriate controls. After the incubation period, the PsV/IgG mixtures were placed on 293 TT cells and further cultivated for 72 h. Finally, supernatants were harvested and clarified by centrifugation at 1500 g for 5 min. The secreted alkaline phosphatase (SEAP) content in the clarified supernatants was measured at 405 nm, corresponding to the amount of SEAP in the wells. IgG dilutions showing at least 50 % reduction in SEAP respectively homologous VLP preparation as the antigen, as described previously (Kirnbauer et al., 1994; Zamora et al., 2006). Briefly, intact VLPs were diluted in PBS to a final concentration of 0.01 mg ml-1 and 0.1 ml aliquots were coated overnight at 4 °C onto 96-well microtitre plates (Maxisorp; Nunc). After three washes with PBS, wells were blocked with 1 % non-fat dried milk in PBS. Rabbit immune serum raised against HPV-5, -8 or -92 VLPs was used as the primary Ab. Serial dilutions of sera ranging from 10−2 to 10−7 were added to triplicate wells and incubated. Detection was performed using horseradish peroxidase-labelled goat anti-rabbit Ab (Kirkegaard & Perry Laboratories) and the peroxidase substrate diaminobenzene 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonate) (Roche Diagnostics). Absorbance values were determined at 405 nm. Specific A405 values were calculated by subtracting mean values obtained in wells without antigen (PBS only) from values obtained in antigen-coated wells. Replication variation in the assays was less than 5 %.

Total IgG was purified from rabbit pre-immune and immune sera using an ImmunoPure IgG Purification kit A Plus (Pierce Biotechnologies).
activity compared with pre-immune IgG were considered to be neutralizing. Data shown are the means ± SD of triplicate wells of a representative experiment.

RESULTS

The major capsid protein L1 of HPV-92 self-assembles into highly immunogenic VLPs

To determine whether the major capsid protein L1 of the new HPV type 92 had the intrinsic capacity to self-assemble into VLPs, similar to other HPV types, HPV-92 L1 was expressed by recombinant baculoviruses in Sf9 insect cells and particles were purified by density-gradient centrifugation. Denatured HPV-92 L1 VLPs migrated on 10 % SDS-PAGE as an approximately 55–60 kDa protein (Fig. 2a), corresponding to the expected molecular mass for PV L1 proteins. L1 of HPV-5 and -8, although similar in predicted size and molecular mass, migrated slightly differently, possibly due to different post-translational modifications.

Analysis by transmission electron microscopy revealed spherical structures of approximately 50–55 nm in diameter composed of capsomers (the pentamer subunit of VLP, consisting of five L1 molecules) (Fig. 2b). The spherical particles were morphologically similar to the VLPs of other HPV types (Kirnbauer et al., 1992). In addition, assembly of capsomers into long rod-like structures, as well as incomplete particles, was observed. The major capsid proteins of HPV-5 and -8 also self-assembled into VLPs, as described previously (data not shown) (Favre et al., 2000; Stark et al., 1998).

To determine the immunogenicity of HPV-92 VLPs, a New Zealand White rabbit was immunized. Serum samples were taken prior to and after immunizations and tested in an ELISA using intact HPV-92 VLPs as the antigen. HPV-92 VLPs readily induced specific Abs with high titres of >600,000, even after administration of the first dose (data not shown). Similarly, HPV-5 and -8 VLPs were also highly immunogenic when administered by a similar protocol. The rabbit immune sera showed high Ab titres (>600,000) in the ELISA assay against HPV-5 and -8 VLPs. All pre-immune sera lacked immunoreactivity.

Detection of linear cross-reactive epitopes present in HPV-5, -8 and -92 VLPs

To determine whether the VLPs of HPV-5, -8 and -92 shared linear cross-reactive epitopes, purified VLPs were analysed by Western blotting. Polyclonal rabbit immune sera obtained by immunization with HPV VLPs were used as the primary Ab.

Immune serum to each of the three HPV VLP types was able to recognize the homologous VLP preparations by Western blotting, showing a prominent band with the expected molecular mass of approximately 55–60 kDa. In addition, each immune serum reacted with the L1 protein of the other two HPV types. Fig. 2(c) shows a representative Western blot using rabbit immune serum (at a dilution of 1:2000) raised against HPV-5 VLPs, which recognized the L1 of purified HPV-5, -8 and -92 VLP preparations at the expected molecular masses.

Abs raised against HPV-5 and -8 display HAI activity against HPV-5, -8 and -92

Incubation of PV virions or VLPs with mouse erythrocytes leads to HA due to cellular receptor binding (Favre et al., 1974; Roden et al., 1996). The HAI assay detects Abs that bind to correctly assembled capsids and appears to correlate with inhibition of virion binding to extracellular matrix (Day et al., 2007). This assay does not measure non-neutralizing Abs to internal or denatured epitopes, but represents a stringent surrogate assay to detect neutralizing Abs and thus has been useful for the examination of serological relatedness of PVs and for measuring protective Ab titres after VLP vaccination (Roden et al., 1996).

IgG purified from rabbit sera raised against the VLPs of HPV-5, -8 or -92 were next tested for their HAI activity to homologous and heterologous HPV VLP types (Table 1). IgG from the respective pre-immune sera served as negative controls.
As expected rabbit immune IgG to HPV-5, -8 or -92 showed HAI of homologous VLPs. Unexpectedly, anti-HPV-5 IgG also revealed HAI of heterologous HPV-8 and -92 VLPs. Similarly, IgG to HPV-8 VLP displayed HAI activity to VLPs of heterologous HPV-5 and -92. In contrast, anti-HPV-92 IgG displayed HAI activity restricted to HPV-92 VLPs, and not to HPV-5 or -8 VLPs. As a control, a non-neutralizing mAb against HPV-5 VLPs did not reveal activity in the three HAI assays (data not shown). These data are the first to demonstrate that β-PV VLPs bind to mouse erythrocytes similarly to the genital HPV types, suggesting a common receptor.

To verify the specificity of the results, IgGs were further tested for HAI using VLPs of BPV-1. No HAI activity was observed for IgG raised against HPV-5, -8 or -92 (Table 1), whereas neutralizing antiserum raised against BPV-1 VLPs inhibited HA induced by BPV-1 VLPs. The haemagglutinating VLPs of BK virus were used to exclude non-specific serum HA of the IgG purified sera (data not shown). Finally, to rule out the possibility that insect cell proteins contaminating VLP preparations non-specifically mediated HA, a mock VLP preparation derived from Sf9 insect cells infected with mutant (prototype) HPV-16 L1, known to fail to assemble into intact particles, was included. As expected, this preparation did not induce HA of mouse erythrocytes, thus ruling out false-positive HA by insect cell proteins (data not shown) (Roden et al., 1996).

To determine HAI titres of immune IgG, VLPs of HPV-5, -8 and -92 were dispersed at identical concentrations, and serially diluted purified IgG was added. IgG to HPV-5 VLPs displayed HAI activity against homologous HPV-5 VLPs with a titre of 3200, but lower titres of 200–400 against VLPs of HPV-8 and -92 (Table 1). Remarkably, anti-HPV-8 IgG showed equal HAI titres of 800 to HPV-8 and heterologous HPV-5 VLPs and HPV-92 VLPs. In contrast, anti-HPV-92 VLP IgG showed HAI restricted to the homologous HPV-92 VLP preparation, with an HAI titre of 1600 (Table 1). The observed differences in HAI titres to homologous types might be attributed, at least in part, to variable efficiency of assembly for VLPs used in the assays.

**Table 1. HAI activity (titre)**

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<thead>
<tr>
<th>Purified IgG*</th>
<th>VLP type</th>
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<tbody>
<tr>
<td></td>
<td>HPV-5</td>
</tr>
<tr>
<td>Anti-HPV-5</td>
<td>3200</td>
</tr>
<tr>
<td>Anti-HPV-8</td>
<td>800</td>
</tr>
<tr>
<td>Anti-HPV-92</td>
<td>–</td>
</tr>
<tr>
<td>Anti-BPV-1†</td>
<td>–</td>
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* IgG purified from immune serum raised against HPV-5, -8 or -92 VLPs was tested for its ability to inhibit VLP-induced HA of mouse erythrocytes.
† BPV-1 VLPs and IgG purified from anti-BPV-1 rabbit immune serum served as controls.
ND, Titre not determined; –, negative result.

**DISCUSSION**

This study showed that VLPs of skin HPV types 5, 8 and 92 are highly immunogenic and elicit high-titre antisera similar to the VLPs of genital-mucosal types. Using HAI as stringent surrogate assay, induced antisera scored positively with their respective (homologous) types, suggesting the induction of neutralizing Abs. Unexpectedly, the HAI assay detected activity to the respective heterologous type in sera induced by HPV-5 and -8 VLPs, and also to the more distantly related HPV-92, indicating the presence of cross-reactive epitopes on the surface of all three VLP types tested.

HAI activity represents a well-established surrogate marker for detection of neutralizing antisera that correlate with protection from HPV infection and associated disease *in vivo*. Thus, whether HPV VLPs induce HA cross-inhibiting antisera should have a predictive value for cross-protection following vaccination. This raises the possibility of β-PV VLPs as candidates for a prophylactic skin-type HPV vaccine, aimed at reducing the incidence of NMSC, if a close link to HPV infection can be established conclusively. Importantly, and in contrast to anogenital cancer, in NMSC of immunocompetent patients no prevalent high-risk HPV types have emerged so far. Thus, induction of cross-neutralizing immune responses to several heterologous HPV types might also be achieved.

**HPV-5 PsV neutralization assay**

Propagation of PVs is difficult to achieve in keratinocyte raft cultures and is limited to a few types that do not include HPV-5, -8 and -92. Furthermore, infection with native HPV virions can only be quantified by RT-PCR, making neutralization assays complex. These limitations have been partially overcome by the recent generation of PV-based gene-transfer vectors, also known as PsVs, that can readily be utilized in quantitative neutralization assays (Buck et al., 2005). Currently, few HPV PsV types are available, and include HPV-5 only for the genus *Betapapillomavirus* (kindly provided by Christopher Buck, National Cancer Institute, MD, USA). Thus, we used an HPV-5 PsV-based assay to detect (cross-)neutralizing Abs in sera raised against HPV-5, -8 or -92 VLPs. Serial dilutions of IgG purified from rabbit sera immunized with HPV-5, -8 or -92 VLPs, ranging up to a dilution of 1:819,200, were tested for their ability to neutralize HPV-5 PsVs. IgG purified from rabbit serum immunized with HPV-5 VLPs effectively neutralized HPV-5 PsVs with a titre of 1:51,200 (Fig. 3a). In accordance with the results obtained by HAI assays, anti-HPV-8 IgG also neutralized heterologous HPV-5 PsVs, with a mean neutralization titre of 1:12,800, indicating effective cross-neutralization (Fig. 3b). IgG to HPV-92 did not neutralize HPV-5 PsV (Fig. 3c), corroborating the results of the HAI assays.
ous HPV types may be of outstanding significance for vaccine strategies, to provide broad protection against the multiple types found in skin tumours.

Two recently introduced HPV vaccines, Gardasil and Cervarix, comprising VLPs of low-risk (HPV-6 and -11) and/or high-risk (HPV-16 and -18) genital types have shown predominantly type-restricted efficacy in preventing persistent genital HPV infection and associated disease caused by the targeted types (Garland et al., 2007; Paavonen et al., 2007). Protection correlated with the induction of high-titre neutralizing antisera. In addition, limited cross-neutralization in vitro and cross-protection in vivo have been observed for closely related types, e.g. HPV-6 and -11, HPV-16 and -33, HPV-31 and -33, and HPV-18 and -45 (Giroglou et al., 2001; Harper et al., 2006; Roden et al., 1996; White et al., 1998). These genotypes showed 92.4, 80.9, 78.4 and 87.4% amino acid sequence identity in L1, respectively. However, the degree of sequence identity is not necessarily a good predictor of the efficiency of cross-neutralization, as antiserum to HPV-31 VLPs cross-neutralizes HPV-33 pseudovirions (78.4% L1 identity) more efficiently than those of the more closely related HPV-16 (83.5% L1 identity) (Giroglou et al., 2001).

Abs reactive to VLPs of HPV-5 variants have been detected in dermatological proliferative disorders, although their neutralizing activity has not been evaluated due to the lack of appropriate assays (Favre et al., 2000). In addition, divergent HPV-16 variants have been found to be serologically cross-reactive (Cheng et al., 1995). Although immunity induced by VLP vaccination is predominantly type-restricted, recent results from human vaccine trials and the high degree of amino acid homology between L1 proteins of HPV-5 and -8 (87%) have indicated that vaccination with HPV-5 VLPs might also induce cross-neutralization to HPV-8 and vice versa, thus providing biologically relevant cross-protection. In contrast, given the more distant relationship between the L1s of HPV-92 and -5 (71%), and HPV-92 and -8 (71%), our finding that immunization with HPV-5 or -8 VLPs generated Abs that inhibited HA by HPV-92 VLPs was unexpected.

β-PV types reveal 81–90% sequence identity for L1 proteins of types 12, 19, 25, 36 and 47 when compared with L1 of HPV-5 or -8. Furthermore, 71–80% sequence identity is shared between the L1s of HPV-5 and -8 and those of HPV-20, -24, -49, -75, -76, -93 and -96. Another cutaneous HPV type putatively involved in NMSC development, HPV-38, shares 69 and 70% identity to L1 of HPV-5 and -8, respectively. Considering the high degree of similarity in the L1 major capsid proteins, which are the major target for neutralizing Abs, it is tempting to speculate that vaccination with, for example, HPV-5 or -8 VLPs might induce cross-neutralization to additional closely related cutaneous types. This hypothesis can be tested once the VLPs or PsVs of other skin HPV types become available. Further determination of the serological relationship between members of the β-PVs may offer interesting information about possible vaccine candidates targeting the multiplicity of skin HPV types.

HAI titres induced by immunization with HPV-5 VLPs were 8–16-fold lower to heterologous HPV-8 or -92 VLPs compared with homologous HPV-5, suggesting that these VLPs share a fraction of the cross-reactive epitopes. Of note, IgG to HPV-8 VLPs displayed comparable HAI titres to HPV-8 VLPs, and also to HPV-5 and -92 VLPs. In contrast, HAI activity of the antiserum raised against HPV-92 VLPs was type-restricted to HPV-92, and did not cross-inhibit HA by HPV-5 and -8 VLPs. These results may indicate that HPV-92 VLPs display cross-reactive epitope(s) in an immuno-subdominant fashion.

It is noteworthy that the HAI assay is stringent in detecting only a fraction of neutralizing Abs that prevent VLP binding to extracellular matrix, but not those Abs that neutralize by a mechanism after cell attachment, which might lead to an underestimate of the neutralization capacity of the sera (Day et al., 2007; Roden et al., 1996).
Native HPV virions are notoriously difficult to propagate in vitro; thus, neutralization assays using PsV technology have been used to detect neutralizing Abs (Buck et al., 2005). However, only a limited number of HPV PsV types are available, among these HPV-5 but not HPV-8 or -92 PsVs. Neutralization assays using HPV-5 PsVs, which were produced in mammalian cells independently by another laboratory (Buck et al., 2005), corroborated the findings obtained by HAI assays. IgG raised against HPV-5 and -8 VLPs did (cross-)neutralize HPV-5 PsVs at a comparable titre, whereas antiserum to HPV-92 VLPs were non-neutralizing in this assay.

Our study demonstrates that β-PVs HPV-5, -8 and -92 are related serotypes, although representing distinct genotypes. If a close link to HPV infection can be established conclusively, these findings may be relevant for further studies on the development of a broad-spectrum vaccine to prevent β-PV infection of the skin, with the ultimate goal of decreasing the incidence of NMSC in both immunosuppressed and immunocompetent individuals.

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