Pseudovirion-binding and neutralizing antibodies to cutaneous human papillomaviruses (HPV) correlated with the presence of HPV DNA in skin

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Whereas the antibody response to the anogenital human papillomaviruses (HPVs) is known to be mainly type-specific, correlated with the presence of viral DNA and mainly directed to conformational epitopes of the virion, it is not known if this applies also to the antibody response to cutaneous HPVs. For 434 non-immunosuppressed patients with skin lesions (squamous cell carcinoma and basal cell carcinoma of the skin, actinic keratosis and benign skin lesions), we compared HPV DNA status with seroreactivity to HPV pseudovirions (PsV) and to GST-L1 fusion proteins from HPV types -5, -6, -15, -16, -32 and -38. Biopsies from the skin lesions were tested for the presence of HPV DNA using three different PCR methods, with typing by sequencing. Serum samples from subjects with HPV DNA-positive biopsies and randomly selected serum samples from subjects with HPV DNA-negative biopsies were also tested with neutralization assays with HPV5, -38 and -76 PsV. Agreement of the three serological methods varied from poor to moderate. Type-specific seroprevalences among patients positive for the same type of HPV DNA (sensitivity of serology) was improved with the PsV-based method (mean of 40%, maximum 63%) compared with the GST-L1 method (mean of 20%, maximum of 25%). Neutralization was the most sensitive assay for HPV38 (50%). In summary, cutaneous HPVs also appear to induce a type-specific antibody response that correlates with the presence of HPV DNA and that can be detected with improved sensitivity using PsV-based serology.

INTRODUCTION

Human papillomaviruses (HPV) are the main causative factors for cervical cancer and several other human cancers (de Villiers et al., 2004; zur Hausen, 2002). About 90% of the characterized HPV types belong to the alpha, beta and gamma genera. While HPV types in the genus alpha mainly have a mucosal tropism, the viruses in the beta and gamma genera mostly have cutaneous tropism (de Villiers et al., 2004). Cutaneous papillomaviruses are found both in healthy skin and in skin lesions such as actinic keratosis (AK) and squamous cell carcinoma of the skin (SCC) (reviewed in Feltkamp et al., 2008).

For anogenital HPVs, HPV serology is an important epidemiological tool to observe past and present HPV infections, as a substantial proportion of infected individuals develop type-specific serum antibodies to the major capsid protein L1 (Dillner, 1999). Seropositivity correlates past or current presence of the same type of viral DNA at the cervix (Dillner, 1999). Most of the antibody response is directed to conformational epitopes, with type-specificity being lost for denatured antigens (Dillner, 1999). Serology is also a tool to test for immunogenicity in HPV vaccination trials (Schiller & Lowy, 2009). The most widely used method in HPV serology is ELISA measuring IgG antibodies to virus-like particles (VLPs) (Kirnbauer et al., 1994). VLPs spontaneously assemble after overexpression of L1 or L1 and L2 in vitro (Hagensee et al., 1993; Kirnbauer et al., 1992). HPV pseudovirions (PsV) mimic natural viruses in containing both the major capsid protein L1 and the minor capsid protein L2. PsV can also carry a genome and be infectious (Buck et al., 2004). Neutralizing antibodies as detected in an HPV PsV neutralization assay are protective against virus challenge, at least in the case of mucosal HPV types (Pastrana et al., 2004). For HPV16 and -18, the neutralization assay is known to be as sensitive as ELISA, and also more specific, making it attractive for vaccine studies (Dessy et al., 2008; Pastrana et al., 2004).

The VLP ELISA and PsV neutralization assay can only test for one HPV type at a time, resulting in the fact that studies of HPV serology for multiple genotypes require large amounts of serum and labour. Therefore, immunoassays that measure HPV type-specific antibodies to several HPV genotypes simultaneously are becoming increasingly used. High-throughput multiplex HPV serology has used either VLPs (Dias et al., 2005; Opalka et al., 2003), capsomeres of
L1 proteins expressed as glutathione S-transferase (GST) fusion proteins (Waterboer et al., 2005) or PsV bound to heparin-coated beads (Faust et al., 2010) as antigens. A competitive multiplex HPV serology method using HPV6, -11, -16 and -18 L1 VLPs (Dias et al., 2005; Opalka et al., 2003) has been extensively used to measure antibody levels after vaccination with the quadrivalent HPV vaccine (Villa et al., 2003). Lately, this competitive assay has been replaced with a method for measuring IgG binding directly to nine HPV types as the competitive assay may underestimate the total protective antibody level (Opalka et al., 2010). The use of the readily produced GST-L1 proteins as antigen enables testing for antibodies against a very large number of HPV types in a high-throughput multiplex system making it a widely used seroepidemiology tool (Michael et al., 2008; Waterboer et al., 2005). All conformational epitopes identified by monoclonal L1-VLP antibodies to mucosal HPV types are presented by GST-L1 proteins (Rizk et al., 2008). However, studies evaluating the sensitivity to detect corresponding anti-HPV antibodies in patients positive for the DNA of cutaneous HPV types have reported a relatively low sensitivity (Andersson et al., 2008; Plasmeijer et al., 2010) and a lack of type-specific concordance between GST-L1 protein serology and presence of HPV DNA (Paaso et al., 2011). Since there is no international standardization of the serological methods for cutaneous HPV types, it is difficult to interpret and compare the results of different studies (Antonsson, 2012). Papillomavirus serology has been standardized only for HPV16 so far (Eklund et al., 2012).

We have previously developed a high-throughput HPV serological method to detect IgG antibodies to mainly mucosal HPV types. The method is based on PsV bound to heparin-coated Luminex beads and was validated by comparison with the HPV DNA status of the serum donors (Faust et al., 2010). The aim of the present study was to expand the PsV-based multiplex method to include more HPV types, in particular the cutaneous ones, and validate the method by comparison with the presence of HPV DNA in the corresponding tumour tissue. As a clear correlation with the presence of viral DNA has not been shown before for cutaneous HPVVs, this aim also includes investigating whether cutaneous HPVs do indeed induce type-specific antibody responses. A second aim was to compare different methods by reanalysing serum samples that had been analysed previously by the GST-L1 multiplex serology assays (Andersson et al., 2008) with the newly developed PsV-based Luminex assay. As neutralization assays for cutaneous HPV types had also not been validated in relation to type-specific HPV DNA presence, we also wished to investigate whether a type-specific and neutralizing antibody response that correlates with the presence of the same type of viral DNA could be demonstrated.

RESULTS

The design and production of PsV was successful for HPV3, -15, -32, -33, -68 and -76. These did not differ in size or shape from previously described HPV PsV (Faust et al., 2010) (Fig. 1). For unknown reason, HPV15 PsV were not functional in the neutralization assay (data not shown), but were functional as VLPs.

In the PsV Luminex, the beta types had generally higher seroprevalences than the alpha types (mean 25 vs 5 %, respectively). HPV5 (29 %), -38 (26 %) and -76 (27 %) had the highest seroprevalence and HPV45 (0.7 %) the lowest (Table 1). Among the mucosal types, HPV16 and -6 had the highest seroprevalences (both 6 %). The HPV seroprevalences did not differ between the different patient groups (Table 1). The HPV type with highest seropositivity when tested with GST-L1 Luminex was HPV6 (32 %), while only 6 % of the subjects were seropositive for HPV6 in the PsV Luminex analysis (Table 1). Antibodies against HPV16 had similar prevalences with both methods (8 and 6 %, respectively). Antibodies against HPV5, -15, -38 and -32 were less prevalent with the GST-L1 method than with the PsV Luminex method (15 vs 29 %, 14 vs 21 %, 2 vs 6 % and 18 vs 26 %, respectively) (Table 1).

The HPV seropositivity analysed with all three methods was compared to the presence of HPV DNA in skin biopsies from the same donors (Table 2). Information about HPV DNA status was available for 427 subjects and the most commonly detected HPV were the beta types -5, -15, -38 and -76.

Thirty-eight per cent of the patients who were positive for HPV5 DNA also had antibodies against HPV5 when using the PsV Luminex method, but only 15 % when using the GST-L1 Luminex method and 30 % when using the neutralization assay (Table 2). Thirty-nine per cent of the HPV38 DNA-positive patients were also seropositive for HPV38 when using the PsV Luminex method, 25 % when using the GST-L1 Luminex method and 50 % when using the neutralization assay. For HPV38, the neutralization assay was significantly associated with detection of HPV38 DNA (P=0.0002; Fisher’s exact test). HPV76 antibodies were detected by PsV Luminex in 63 %, but by neutralization assay only in 13 % of the HPV76 DNA-positive subjects. For HPV76, the PsV Luminex assay was significantly associated with detection of HPV76 DNA (P=0.03; Fisher’s exact test). GST-L1 serology data were not available for HPV76. The number of HPV15 seropositive patients out of the HPV15 DNA-positive patients was low for both high-throughput serology methods (7 and 14 %, respectively) and the HPV15 neutralization assay was not functional (Table 2). Seroprevalences among subjects positive for some other HPV DNA type did not differ from the seroprevalences seen among HPV DNA-negative subjects (Table 2).

Comparison of the anti-HPV antibody response measured by the two high-throughput serology methods was possible for the HPV5, -6, -15, -16, -32 and -38 (Table 3). Both continuous and categorical data obtained with the two different Luminex methods were compared by calculating R² and Kappa agreements. The continuous data agreement
was best for HPV16 ($R^2=0.44$) and worst for HPV6 ($R^2=0.07$), with agreement for the other studied types having a correlation coefficient around $R^2=0.2$. The categorical data had an agreement that ranged from 'poor' (HPV6, $\kappa=-0.06$), 'slight' (HPV32, $\kappa=0.17$), 'fair' (HPV15, $\kappa=0.38$) to at best 'moderate' (HPV38 $\kappa=0.55$, HPV16 $\kappa=0.45$ and HPV5 $\kappa=0.45$) (Landis & Koch, 1977). The PsV Luminex detected more positive samples compared with the GST-L1 method for HPV5 (74 vs 12), HPV15 (56 vs 23), HPV32 (22 vs 4) and HPV38 (51 vs 17) but fewer positives for HPV6 (23 vs 135) and HPV16 (11 vs 20) (Table 3).

Patients DNA positive for HPV5, -38 and -76 ($n=40$, 36 and 8, respectively) as well as 40 DNA-negative patients for each type, were used to test the neutralization assay. Categorical data obtained with the neutralization assay was compared with data from the PsV and GST-L1 Luminex assays (Table 3). The agreement between the high-throughput Luminex methods and the neutralization assay

**Fig. 1.** Transmission electron microscopy images of HPV PsV. (a) HPV3, (b) HPV15, (c) HPV32, (d) HPV33, (e) HPV68 and (f) HPV76.
<table>
<thead>
<tr>
<th>HPV type (genus)</th>
<th>Antigen</th>
<th>SCC n total=72</th>
<th>positive (%)</th>
<th>BCC n total=160</th>
<th>positive (%)</th>
<th>AK n total=81</th>
<th>positive (%)</th>
<th>Benign n total=121</th>
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<td>Any HPV</td>
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<td>98 (61)</td>
<td>41 (51)</td>
<td>81 (67)</td>
<td>265 (61)</td>
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varied from ‘fair’ to ‘moderate’. For the PsV Luminex and the neutralization assay the kappa value for HPV5 was 0.36 (fair) and for HPV38 the kappa value was 0.49 and for HPV76 it was 0.41 (both ‘moderate’) (Table 3). The GST-L1 Luminex and the neutralization assay agreed at a ‘moderate’ level for HPV5, $\kappa = 0.44$ and ‘fair’ for HPV38, $\kappa = 0.24$ (Table 3).

**DISCUSSION**

In the present study, we have characterized the antibody response to cutaneous HPV types by expanding an already existing high-throughput method for HPV-specific antibody detection (Faust et al., 2010) by adding PsV of three $\beta$ and three $\alpha$ HPV types to the assay and by developing PsV-based neutralization assays. Our validation revealed that the PsV-based antibody detection methods did correlate with the presence of the same type of $\beta$ papillomavirus DNA, at least for HPV38 and -76. This suggests that the cutaneous HPVs induce a type-specific antibody response to conformational epitopes present on PsV. Both the PsV Luminex and GST-L1 assays were performed using multiplexing with fluorescent beads. The multiplexing enables simultaneous testing for a large number of viruses is of interest to enable more comprehensive serological studies. Theoretically, the fact that all the different antigens are incubated at the same time with the serum should also provide an increased ability to detect type-specific antibodies, as cross-reacting antibodies would absorb to the other antigens. However, the fact that the three serological methods evaluated in this study were not in good agreement, implies that cutaneous HPV serology is in need of further development and international standardization.

Although we tested 434 serum samples for antibodies against 16 different HPV types, four of them belonging to genus $\beta$ (5, 15, 38 and 76) and 12 in the genus $\alpha$ (3, 6, 11, 16, 18, 31, 32, 33, 45, 52, 58 and 68), a limitation of the study is that only for six HPV types did we have data from both high-throughput methods and that the neutralization assay was only performed for a subset of the study population (type-specific HPV DNA-positive cases and 40 controls per HPV type) as neutralization is very laborious. The comparison of neutralization assay with the two high-throughput Luminex serology assays is therefore limited.

In the present study population, antibodies to $\beta$ HPV types were more prevalent than antibodies to $\alpha$ types. This is expected, as the prevalence of cutaneous HPV types on the skin is usually greater than the prevalence of mucosal HPVs in the genital tract. Also, the age of the study group was high (mean age 72) and seroprevalences for cutaneous HPV types tend to continuously increase during a lifetime, whereas antibodies against mucosal anogenital types are less common in elderly people (Michael et al., 2008; Newall et al., 2008).
We previously reported that when the GST-L1 Luminex results were compared to the presence of HPV DNA in tissue samples (Andersson et al., 2008), the sensitivity for HPV beta type serology was low. In this study, we found better agreement between HPV DNA in tissue samples and type-specific antibodies with PsV Luminex than for GST-L1 for HPV5 and 38 (38 vs 15% and 39 vs 25%, respectively). Neutralization should be a more specific method than measurement of binding IgG antibodies, and for HPV38 the neutralization assay was the most sensitive method as well. The difference in sensitivity to detect HPV76 between PsV Luminex and neutralization assay may indicate the existence of a non-neutralizing antibody response against this virus. For the PsV Luminex assay, a lack of association with the presence of the same type of HPV DNA in the patient was only found for HPV type 15. This problem could be technical, but it is also possible that it may reflect a difference in biology. While different serotypes for the same genotype have not been described for anogenital HPV types, there is clear evidence that HPV5 (a cutaneous HPV types) does contain at least three different serotypes corresponding to genetic subtypes of HPV5 (Favre et al., 2000). If other cutaneous HPV genotypes also have several different genotypes, a lower concordance with the presence of viral DNA would be expected.

When comparing agreements between methods using kappa values, the highest agreement was ‘moderate’. The largest discordance was for HPV6, where the antibody prevalence by PsV Luminex was almost five times lower than with the GST-L1 Luminex method and the agreement was non-existent. We have previously demonstrated validity of our PsV Luminex HPV6 test using serum samples from condylomata acuminata patients (Faust et al., 2010) and found the method to detect HPV6-specific antibodies with high specificity, suggesting that the high HPV6 seroprevalences seen with GST-L1 Luminex may be due to low specificity.

The PsV Luminex serology method was found to, in general, have an improved sensitivity and specificity. We hope to expand the method to cover more HPV types by multiplexing and thus make it possible to perform high quality seroepidemiological studies for the cutaneous HPVs. The present study demonstrated that, in particular for the cutaneous HPVs, there are large differences in the results obtained with three HPV serology methods. International collaborative studies for validating and standardizing HPV serology also for cutaneous HPVs would thus be important to enable informative seroepidemiological studies for elucidating the natural history and possible role in human disease for the cutaneous HPV types.

## METHODS

### Patients and collection of data and samples.

The enrolment of cases and controls is described in detail elsewhere (Andersson et al., 2008). Briefly, the study included 434 immunocompetent patients attending dermatology clinics in Sweden (400 patients) or Austria (four patients) (Forslund et al., 2007). Cases were defined as having a histologically confirmed diagnosis of SCC (n=72, mean age 80 years, range 50–94) or basal cell carcinoma (BCC) (n=160, mean age 73 years, range 34–93), whereas for the pre-malignant actinic keratosis, AK (n=81, mean age 75 years, range 53–95) clinical diagnosis was sufficient. The control patients had a variety of benign skin lesions (n=121, mean age 71 years, range 29–97), the most common being seborrhoeic keratosis (SK, n=62).

A serum sample was obtained from each subject and stored at −20 °C. At the same visit as the serum sampling, two biopsies were collected from each patient, one 2 mm punch biopsy from the lesion and one from healthy skin 10–15 cm from the lesion. Before taking
the biopsy, the skin was anesthetized and stripped with tape to avoid surface contaminations of virus (Forslund et al., 2004). Seven individuals were excluded from HPV DNA testing (but were included in serology testing) because the skin had inadvertently not been stripped before a biopsy was taken (SCC, n = 1; BCC, n = 2; AK, n = 1; benign, n = 3).

**HPV DNA analysis.** HPV DNA was analysed as described previously (Andersson et al., 2008). Briefly, the DNA from each punch biopsy was extracted using a phenol-free method (Forslund et al., 1999). Sample adequacy was tested by PCR of the human β-globin gene (de Roda Husman et al., 1995; Forslund et al., 2002). All samples were tested with four different HPV PCR methods in three different laboratories. All primers were located in the L1 ORF and typing was done by sequencing of ampilizers. If a sample was positive in at least one HPV test in at least one laboratory, the sample was scored as HPV DNA positive.

**PsV.** Expression vectors to produce HPV PsV types -5, -6, -11, -16, -18, -31, -45, -52 and -58 were kindly provided by Drs John Schiller and Christopher Buck, National Cancer Institute, Bethesda, MD, USA. Nucleotide sequences of these plasmids can be found at http://home.ccr.cancer.gov/lco/default.asp. Expression vectors to produce HPV PsV types -3, -15, -32, -68 and -76 (GenBank accession numbers in NCBI database X74462, X74468, NC_001586, M12732, DQ080079 and Y15174, respectively) were cloned using the same strategy as described for HPV38 (U31787) (Faust et al., 2010). Animal PsV were not produced and used as negative control.

PsV carrying secreted alkaline phosphatase reporter gene were generated by transfection of 293T cells as described elsewhere (Buck et al., 2004). Virus particles were adsorbed to carbon-coated grids, stained with uranyl formate and examined with transmission electron microscopy.

**PsV Luminex.** The method was performed as described previously (Faust et al., 2010) in extended format that added PsV of HPV types -3, -15, -32, -33, -68 and -76 to the previously established multiplex mix of PsV of HPV3, -5, -6, -11, -15, -16, -18, -31, -32, -33, -38, -45, -52, -58, -68 and -76. A mean fluorescence intensity (MFI) of 250 was used as cut-off for seropositivity for all studied HPV types. All sera were tested in two serum dilutions (1 : 50 and 1 : 150) and were scored as positive if they had >250 MFI in at least one dilution. The bead coupled with heparin only acted as a background control.

**GST-L1 Luminex.** The serum samples had been tested previously (Andersson et al., 2008) for antibodies to the major capsid protein L1 of the HPV1, -5, -6, -8, -9, -10, -15, -16, -20, -24, -32, -36, -38 and -52, and to the early proteins E6 and E7 of the HPV types -8 and -38. Briefly, the GST-L1 Luminex serology used a GST capture ELISA in combination with fluorescent technology (Sehr et al., 2001; Waterboer et al., 2005, 2006). For the six of these HPV types (HPV5, -6, -15, -16, -32 and -38) comparison with present PsV Luminex data were possible.

**Neutralization assay.** To detect HPV5, -38 and -78-specific neutralizing antibodies in human serum, protocol by Pastrana et al. (2004) was followed using a serum dilution of 1 : 50. Chemiluminescence was read for 0.25 per well using a Wallac Victor 1420 Multilabel counter. The serum was counted as neutralizing, if the secreted alkaline phosphatase signal was reduced by more than 50 %.

**Statistics.** Agreement of categorical values between assays was quantified calculating kappa values using GraphPad QuickCalcs online calculator. \( R^2 \) was calculated using Microsoft Excel to compare continues data of GST-L1 and PsV Luminex assays. Seroprevalences among subjects positive for the same HPV DNA type and HPV-negative subjects were compared by using the Fisher’s exact test (two-sided).

The study adheres to the Declaration of Helsinki Guidelines and was approved by the Ethics Committees of the Karolinska Institutet and of Lund University, Sweden, and of the Medical University of Vienna, Austria.

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**REFERENCES**


