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High Prevalence of Human Papillomavirus Infections in Urine Samples from Human Immunodeficiency Virus-Infected Men

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Infection with human immunodeficiency virus (HIV) and the resulting immunosuppression are associated with an increased risk for human papillomavirus (HPV) persistence and related malignancies. In the present study we investigated the prevalence of HPV in urine samples from 104 HIV-infected men with low CD4$^+$ cell counts (<100 per mm^3) and 115 urine samples from HIV-negative men. A high prevalence of HPV DNA (39.4%) was found in the HIV patients. Most of the HPV types were high risk (81.4%), with HPV 52 as the most prevalent type (12.5%), followed by HPV 18 (6.7%), HPV 35 (5.8%), and HPV 70 (4.8%). Multiple HPV genotypes were observed in 17 (41%) of the 41 HPV- and HIV-positive men. In contrast, only 11 (9.6%) HPV DNA-positive cases were observed among the 115 HIV-uninfected men, and 3 (27.3%) contained multiple genotypes. Quantitative analyses indicated that the HPV viral load, as measured in urine samples, is significantly higher in HIV-positive men compared to HIV-negative men. In the present study we show that urine samples are useful for detecting HPV DNA, there is a high prevalence of HPV in HIV-positive men, and the HPV viral load is substantially higher in HIV-positive than in HIV-negative men. More studies are needed to evaluate the risk and natural development of HPV-related malignancies in HIV-positive men.

Human papillomaviruses (HPVs) are the causative agents of benign and malignant lesions of cutaneous and epithelial tissues (6, 23). So far, approximately 100 HPV types have been identified. About 40 types have been detected in the (ano)genital area, and these are classified as low risk, intermediate, or high risk on the basis of their ability to cause cellular transformation in vitro and their association with cervical cancer (18, 22). The high-risk types include HPV 16, 18, 31, 33, and 45, while HPV 6 and 11 are considered low-risk types because these are predominantly detected in benign lesions (e.g., genital warts) (8).

Clinical and epidemiological evidence strongly suggests that the (ano)genital HPVs are sexually transmitted (26). About two-thirds of individuals who have sexual relations with an infected partner will become infected. However, only a minority of HPV-infected individuals will eventually develop HPV-related malignancies.

Molecular biological methods to detect DNA in urine are routinely used to diagnose common sexually transmitted diseases (STDs) such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections (10). Also, several studies showed that HPV DNA could be detected with high sensitivity in urine samples (7, 19). An advantage to detecting STDs in urine specimens is that it is a noninvasive sampling method and permits simultaneous detection of various infectious agents (7).

Data from studies on human immunodeficiency virus (HIV)-positive women show increased HPV incidence, more persistence of high-risk HPV infections, and increased frequencies of multiple infections (17), dysplasia, and cervical cancer. Apparently, HIV alters the natural history of HPV infection through an HIV-impaired immune system, resulting in an increased risk for the development of cervical cancer (9). Although many data are available from HPV studies in women (7, 12, 14, 21), data on infections in men are very limited (30). In most cases HPV infection in men is subclinical, resulting in a reservoir of asymptomatic carriers who can serve as vectors for the virus. However, in HIV-positive men, a high prevalence and incidence of anal intraepithelial neoplasia has been reported (13). HPV has also been closely linked with anal cancer and certain penile cancers (32).

The aim of the present study was to determine the prevalence of HPV infection in HIV-infected and HIV-uninfected men, as measured in urine samples, and to investigate the presence of specific HPV genotypes in the two groups. Finally, we wanted to investigate possible quantitative viral differences (HPV viral load) between these two groups.

MATERIALS AND METHODS

Study population. Urine samples were obtained from HIV-infected patients attending a special HIV clinic at the Slotervaart Hospital, Amsterdam, The Netherlands, during a routine visit to the physicians between 1993 and 1997. Only materials from patients (n = 104) with a CD4$^+$ cell count below 100 per mm^3 were used in this study. All patients provided informed consent for the use of these materials for HIV and HIV-related research purposes. As a control, urine samples from randomly selected non-HIV-infected males (n = 115) were provided anonymously from the Microbiology Department at the Slotervaart Hospital, Amsterdam, The Netherlands. All urine samples were stored at −20°C until further processing.

DNA isolation. DNA was extracted from 200 μl urine by guanidinium thiocianate lysis, binding to silica particles, and washing and elution with 120 μl of Tris-EDTA buffer as described by Boom et al. (5).

HPV DNA amplification and detection. After DNA isolation, HPV DNA was amplified using the SPF10 PCR primer set (15). Each run was accompanied by several positive and negative quality control samples. During each PCR run one

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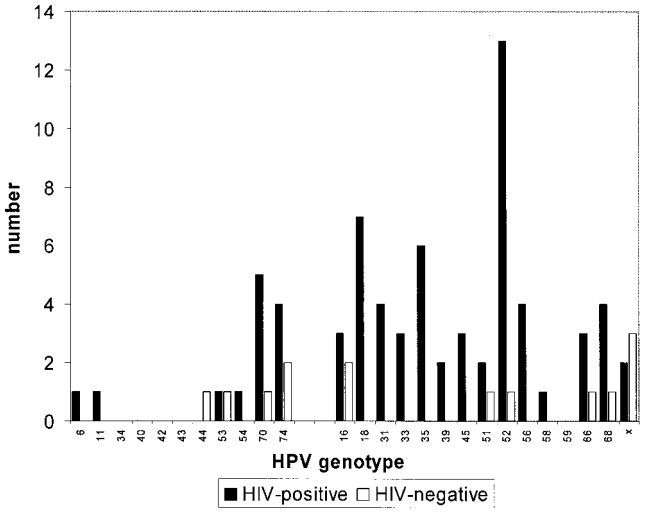

FIG. 1. Bar graph illustrating the distribution of HPV genotypes detected by SPF10 HPV DNA PCR and HPV LiPA in 52 HPV DNA-positive men (104 HIV-positive and 115 HIV-negative men). The HPV types are grouped into low-risk types on the left and high-risk types on the right. Bars indicate the number of samples from control (HIV-uninfected) and immunosuppressed (HIV-positive) patients.

negative control (water) and one positive control sample (HPV18-positive cells) were tested. The SPF10 PCR was performed in a final reaction volume of 50 μl containing 10 μl of the isolated DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 μM of each deoxynucleoside triphosphate, 15 pmol of each of the forward and reverse primers, and 1.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, Calif.). The PCRs were performed as follows: activation of AmpliTaq Gold enzyme for 9 min at 94°C, followed by 40 cycles of 30 s at 94°C, 45 s at 52°C, and 45 s at 72°C, with a final extension of 5 min at 72°C (15). Amplification of a part of the beta-globin gene was used to assess the presence of human cells and the quality of the target DNA. HPV-negative samples with a negative PCR result for the β-globin gene were analyzed again. HPV-negative samples with a positive β-globin gene PCR were regarded as true HPV negatives.

Amplification products were tested in a DNA enzyme immunoassay (DEIA), a probe hybridization assay in a microtiter plate to detect the presence of HPV DNA, as described earlier (16). This assay also included appropriate controls: one high positive, one low positive, and a negative control. SPF10 amplimers from HPV DNA-positive samples were subsequently analyzed using the HPV reverse hybridization line probe assay (LiPA). This assay comprises a membrane strip containing HPV type-specific oligonucleotides to specifically detect 25 HPV genotypes, 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. Negative and positive controls were used to monitor the performance of the assay. The layout of the HPV LiPA and reaction conditions have been described before (15).

Quantitative analysis of HPV DNA. Quantification of HPV DNA in urine samples was performed with the iCycler IQ real-time detection system (Bio-Rad) using SYBR Green as the fluorescent dye. The HPV SPF10 quantitative PCR (qPCR) was performed in a final volume of 50 μl containing 10 μl isolated DNA, 15 pmol of each SPF10 forward and reverse primer, and 1× SYBR Green supermix (Bio-Rad). The conditions for amplification were 9 minutes at 95°C for enzyme activation, followed by 50 cycles of 30 seconds at 95°C for denaturation, 45 seconds at 52°C for primer annealing, and 45 seconds at 72°C for extension, and a final extension of 5 minutes at 72°C. For a melting curve the additional melting program was 10 seconds at 30°C followed by an increase of 0.5°C/s up to 95°C.

HPV DNA copy numbers were determined by using the standard curve generated in the same PCR run with known concentration of HPV 16 plasmid DNA dilution series. To correct for the number of input cell equivalents in a patient sample, the single-copy-number gene beta-actin was quantified in a SYBR Green qPCR test using the primers 5′-TCACCCACACTGTGCCCATCTACGA-3′ and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′.

A genomic DNA dilution series with known concentration was analyzed in the same PCR run to determine the standard curve. The HPV DNA load was defined as the number of HPV DNA copies/ng total DNA.

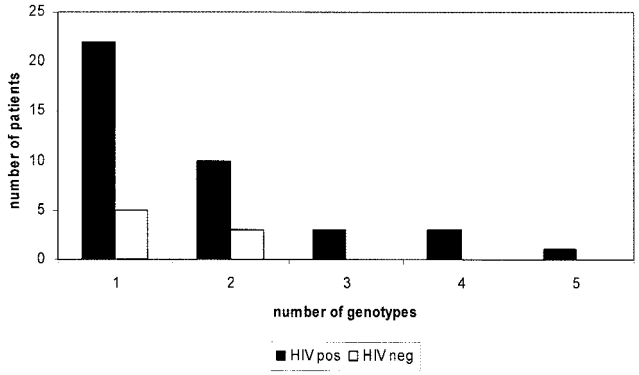

FIG. 2. Frequencies of the number of HPV genotypes observed in HIV-infected and HIV-uninfected men.

Statistical analyses. For statistical analyses of the (quantitative) HPV DNA results, SPSS software (SPSS Inc., Chicago, Illinois, version 11.0) was used. Data presented here were analyzed using the *t* test. A *P* value of 0.05 was considered the threshold level for significance.

RESULTS

Urine samples from 104 HIV-positive and 115 HIV-negative males were tested for the presence of HPV. HPV DNA was detected in 41 (39.4%) of the 104 HIV patients. In the HIV-negative men only 11 (9.6%) were positive for HPV DNA.

All the positive and negative controls gave the appropriate results. HPV amplimers of the HPV DNA-positive samples were further genotyped by using the HPV LiPA. This genotyping assay permits specific detection of 25 HPV genotypes, of which HPV 6, 11, 34, 40, 42, 43, 44, 53, 54, 70, and 74 were considered low-risk types, whereas HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were considered high-risk types (6, 22, 33, 34). The results are shown in Fig. 1.

In the HIV-negative men the overall HPV prevalence (9.6%) was much lower compared to the HIV-positive group (39.4%) ($P < 0.001$). In the HIV-negative group, HPV 16 and 74 were numerically the most important types. In the HIV-positive men HPV 52 was the most prevalent genotype and was observed in 13 men (12.5%), followed by HPV types 18 (6.7%), 35 (5.8%), and 70 (4.8%). HPV 6 and 11 were only observed in two HIV-positive men and were absent in the HIV-negative group. Five of the 52 HPV-positive samples were positive in the HPV DNA screening assay but the HPV-LiPA did not reveal a genotype on the HPV LiPA system. These samples are likely to contain HPV genotypes for which no probes are present on the LiPA.

In both groups, high-risk HPV types were detected. The overall ratio of high-risk HPV to low-risk HPV was different in the HIV-positive population (57 high-risk versus 13 low-risk types) than in the HIV-negative population (9 high-risk versus 5 low-risk types).

The presence of multiple genotypes in both groups is shown in Fig. 2. The number of multiple genotypes was much lower in the HIV-negative group (3/11 multiples; all had two types) versus the HIV-positive men (17/41 multiples, ranging from two to five HPV types). This difference, using the chi-square test, was statistically not significant ($P > 0.05$).

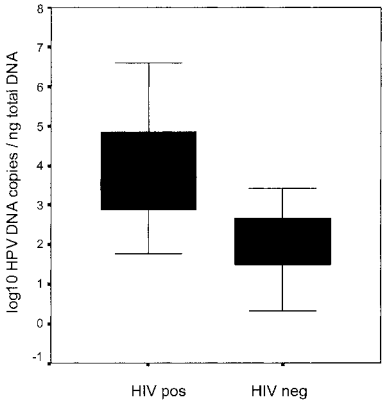

FIG. 3. Comparison of HPV DNA viral load in a subset of urine samples from HIV-positive ($n = 20$) and HIV-negative ($n = 9$) men. The HPV DNA load was quantified by real-time PCR and plotted on a logarithmic scale in a box plot using the statistical program SPSS, version 11.0. The HPV DNA copy number was divided by the total amount of cellular DNA. The correlation between HPV DNA copy number and HIV status was found to be highly significant (t test, $P < 0.0001$).

The hybridization signal from HPV samples from the HIV-positive population was much stronger on the HPV-LiPA assay than the signal of the HPV DNA from the HIV-negative group, suggesting that the HPV DNA load in the latter group is much lower than in the HIV-positive group. Therefore, the HPV viral load was assessed by real-time PCR in 20 HIV-positive and nine HIV-negative men. The results are shown in Fig. 3 and showed that the HPV viral load was significantly higher in HIV-infected men (t test, $P < 0.0001$).

DISCUSSION

In this study we observed a high prevalence of HPV DNA (39.4%) in urine samples from HIV-positive men versus 9.6% in those from HIV-negative men. Previous studies also have shown a higher incidence and prevalence level of HPV in HIV patients compared to HIV-negative men and women (1, 28). Comparison of the exact numbers from the different studies is difficult due to the fact that in these studies a variety of clinical samples were analyzed (e.g. biopsies, urine, and smears) sampled from different sites (e.g., penis and anus) (13). As shown in the present study, urine is suitable for our HPV DNA detection system. Previous studies have already showed that urine is appropriate for screening of important STDs such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (10). The SPF10 primer set amplifies a fragment of only 65 base pairs of the HPV genome. This short PCR fragment makes this assay a highly sensitive HPV detection method, which is suitable not only for pap smears but also for paraffin-imbedded tissue and urine samples.

HPV infection is mostly subclinical in men, and only 1% of HPV-infected men have visible genital warts (3). In men who have sex with men (MSM) with a history of receptive intercourse, the incidence of anal cancer was estimated to be 35 per 100,000 per year (25). In MSM infected with HIV, the incidence of anal cancer is even higher than in HIV-negative men (11). In immunosuppressed men, anal HPV infection and anal histologic or cytologic abnormalities are more prevalent in HIV-positive than in HIV-negative men in the absence of anal intercourse (11, 27, 32).

The immune system is considered a crucial factor in viral infections and may determine the development of HPV persistence after a primary infection. The immune system of the HIV-positive men studied here is greatly impaired since these patients have a $CD4^+$ cell count below 100 cells/mm^3 (4). The high prevalence of high-risk HPV types in the HIV-positive men is consistent with data from other studies in immunodeficient men, e.g., renal allograft recipients (24). Immunosuppression may permit increased replication of what may otherwise have been a low-level, possibly undetectable HPV infection. In this respect we found that the HPV DNA signals were much higher in the samples from HIV-positive than in those from the HIV-negative men, suggesting a higher viral load in HIV-positive men. This was confirmed by the results from the quantitative analysis, where the average viral load in HIV-negative men was approximately 10-fold lower than that in HIV-positive men. Our findings are concordant with an earlier study in which HPV loads were determined in HIV-infected and uninfected women, which showed that higher HPV loads in high-grade lesions were correlated with HIV infection, especially advanced HIV disease (31). This indicates that increased HPV viral load is an independent risk factor for the development of HPV-related malignancies.

Another risk factor found in the HIV-infected group was the high prevalence of multiple infections compared to the uninfected men (17/104 versus 3/115). Also, multiple HPV infections are regarded as an independent risk factor associated with a higher chance of developing high-grade lesions in women (17, 20, 29).

A striking difference with other data (2) is the virtual absence of HPV types 6 and 11, associated with condylomata accuminata, a benign lesion which seldom progresses to malignancy. These benign lesions, however, are frequently observed in HIV-positive males.

We show here a high prevalence of high-risk HPV types in HIV-infected men in contrast to uninfected men, indicating the importance of immunosuppression in men at risk for HPV-related diseases. Another unique result from this study was the high prevalence of HPV type 52, an oncogenic HPV type. Maybe this type is specific for the HIV cohort studied here or specific for highly immunosuppressed HIV patients.

In this group of HIV-positive men, a high incidence of several STDs has been reported during recent years. Moreover, the availability of highly active antiretroviral therapy increases the life expectancy of HIV patients, thereby increasing the risk of developing HPV malignancies and spreading HPV to other men and women. Therefore, more prospective cohort studies are needed to address these issues. Investigating the role of the immune system in HIV-infected and noninfected men will lead to better treatment of HPV-related neoplasias in this population.

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