Detection of Multiple Human Papillomavirus Types in Condylomata Acuminata from Immunosuppressed Patients

Darron R. Brown, Janine T. Bryan, Harvey Cramer, Barry P. Katz, Victoria Handy, and Kenneth H. Fife

Divisions of Infectious Diseases and of Biostatistics, Departments of Medicine, of Microbiology and Immunology, and of Pathology, Indiana University School of Medicine; Richard L. Roudebush VA Medical Center; and Regenstrief Institute for Health Care, Indianapolis, Indiana

Using the hybrid capture method, condylomata acuminata from healthy patients (controls) and patients with altered cell-mediated immunity were analyzed for multiple human papillomavirus (HPV) DNA: 82.9% and 38.0% of lesions from 41 controls and 21 patients, respectively, were HPV DNA–positive only with probes for low-risk HPV types ($P = .00035$). Using probes for both low- and high-risk HPV types, 16.3% and 52.3% of lesions from 43 controls and 21 patients, respectively, were positive for both probes ($P = .0038$). Evidence of multiple HPV types was also found by Southern blot and in situ hybridization studies. The mean HPV copy number detected by either probe did not differ significantly among patient groups. Using sensitive techniques, such as hybrid capture, multiple HPV types, including those associated with genital malignancy, can be detected in condylomata acuminata. Serial biopsies demonstrate the dynamic nature of genital HPV infection and that changes in the predominant HPV types may be reflected in tissue pathology.

Condylomata acuminata (genital warts) are caused by infection of genital epithelial surfaces with human papillomaviruses (HPVs) [1]. About one-third of the 75 known HPV types regularly infect the genital tract, with results ranging from asymptomatic, latent infection to the typical exophytic cauliflower-like growths known as condyloma acuminata or to dysplasia and invasive carcinoma. Condylomata acuminatum is a benign condition, and most lesions contain low-risk HPV types (HPV-6 or -11), but high-risk types (HPV-16 and -18) have occasionally been detected [2–4].

Previous studies using a variety of detection methods, including in situ hybridization, polymerase chain reaction, and Southern blot, have shown that >1 HPV type can be found in up to 32% of condylomata acuminata [5–10]. In a recent study evaluating the hybrid capture method, we detected >1 HPV type in 14 of 40 biopsy samples of condylomata acuminata [3]. Most of the lesions containing multiple HPV types were removed from patients with conditions known to depress cell-mediated immunity (CMI), including infection with the human immunodeficiency virus (HIV) and use of immunosuppressive medications after organ transplantation. However, >1 HPV type was detected in some lesions from patients with no known immune defects.

In this study, we analyzed biopsy samples of exophytic condylomata acuminata removed from men and women, some of whom had conditions known to depress CMI.

Materials and Methods

Patient populations. Patients were evaluated for sexually transmitted diseases, including HPV infection, in a hospital-based gynecology outpatient clinic, Indiana University Transplantation Service, or a sexually transmitted disease clinic. Biopsies were done if patients had clinical evidence on routine examination of genital HPV infection, such as typical condylomata acuminata of the external genitalia, perianal area, or vagina. Several organ transplant recipients were evaluated on more than one occasion. Patients with visible recurrent lesions underwent second biopsies in the same anatomic area as the first.

Excision biopsies. Biopsies of typical exophytic condylomata acuminata were done as previously described [11]. Lesions were cleansed with an iodine solution before biopsy. Samples were held in normal saline until processed, generally within 2 h. Biopsies of exophytic lesions were ≥ 2 mm^3. Lesions were split into two equal fragments: One, used to extract DNA, was frozen in liquid nitrogen, and the other was placed in zinc formalin to prepare paraffin-embedded sections. One section from each sample was deparaffinized and stained with hematoxylin-eosin for confirmation of histology consistent with HPV infection.

Extraction of DNA. DNA was extracted from biopsy samples as previously described [3]. Briefly, biopsy samples were frozen with liquid nitrogen, then processed with a mikro-dismembrator II (B. Braun Instruments, Melsungen, Germany). The resulting material was solubilized, treated with proteinase K, and extracted with phenol–chloroform–isoamyl alcohol. DNA was pre-

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Reprints or correspondence: Dr. Darron R. Brown, Division of Infectious Diseases, Indiana University School of Medicine, Emerson Hall, Rm. 435, 545 Barnhill Dr., Indianapolis, IN 46202-5124.

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cipitated and quantitated by spectrophotometry. The presence of high-molecular-weight DNA was established by agarose gel electrophoresis followed by staining with ethidium bromide. The yield from tissue samples was ~10–50 μg of DNA.

Hybrid capture assay. HPV DNA was detected using a hybrid capture assay (ViraType Plus; Digene Diagnostics, Beltsville, MD) as previously described [3]. Briefly, under high-stringency conditions, RNA probes for 14 HPV types were allowed to hybridize to alkali-denatured specimen DNA. Positive specimens were detected by binding the hybridization reaction to tubes coated with a monoclonal antibody to RNA-DNA hybrids. Bound hybrids were detected by the addition of an alkaline phosphatase–conjugated antibody to RNA-DNA hybrids followed by addition of LumiPhos 530 (Digene) and reading in a luminometer (Optocomp I; MGM Instruments, Hamden, CT).

Probes were divided into two pools on the basis of the association of each type with genital tract malignancy. Probe group A contained the "low-risk" HPV types 6, 11, 42, 43, and 44; probe group B contained the "high-risk" HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56. Positive controls consisted of 1 pg each of HPV-11 DNA for probe A or HPV-16 DNA for probe B diluted in 5 μg of HPV-negative DNA. Controls were run in triplicate with each assay. Patient samples were considered positive if the number of relative light units read from the luminometer was greater than the mean of the positive control values. The test was considered valid only if the value of the positive control was ≥1.5 times that of the negative control.

Southern blots. Southern blots were done, under stringent conditions (t_m −15°C) as previously described [3], if at least 5 μg of DNA was available in addition to the 10 μg required for the hybrid capture assay. To summarize, 5-10 μg of sample DNA was digested with *Pst*I for 2 h at 37°C. Following electrophoresis, DNA was transferred to nylon membranes (Hybond-N+; Amersham, Arlington Heights, IL) by the method of Southern [12]. After transfer, membranes were prehybridized for 16 h, then hybridized in a solution containing 5×10^6 cpm/mL heat-denatured ^{32}P-labeled genomic HPV DNA (a mixture of HPV-6b, -11, -16, -18, and -31) purified away from the pBR 322 vector. The nylon membranes were then washed in decreasing salt concentrations and autoradiographed.

To determine HPV types in samples that were positive in the hybrid capture for both probes, replicate Southern blots were done on DNA from 7 samples in which sufficient DNA was available after the initial Southern blot and hybrid capture assay. For the replicate Southern blots, agarose gels were loaded with equal amounts of DNA digested with *Pst*I. After transfer, membranes were hybridized as above with either a mixture of HPV-6b and -11 probes or a mixture of HPV-16, -18, and -31 probes labeled with ^{32}P. The remaining conditions of the Southern blot assay were as described above.

In situ DNA hybridization. To detect HPV DNA in tissue sections, in situ hybridization was done using a tissue hybridization kit (Digene) to assay sections from 10 genital lesions. These lesions were chosen because the hybrid capture assay was positive for both probes. Adjacent sections from each tissue were deparaffinized with xylene and ethanol, heated to 100°C, then hybridized for 18 h at 37°C using either biotinylated probes for HPV-6 and -11 or probes for HPV-16, -18, -31, -33, and -35. Both probe mixtures were used for 1 large biopsy sample. Two tissues were used as controls for cross-hybridization of the two probe mixtures. The first was an HPV-11–infected human foreskin implant grown in an athymic mouse [13]. The second was a vulvar carcinoma sample containing 12.43 copies/cell HPV-16 by hybrid capture. These tissues were treated in the same manner as the biopsy samples.

Hybridized probe was detected by incubation of slides with a streptavidin–alkaline phosphatase conjugate and reaction with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium substrate according to the manufacturer's instructions. Cells positive for HPV DNA were identified by purple nuclear staining.

Statistical analysis. Statistical analysis was done only on the initial biopsy from each patient. χ^2 analysis or Fisher's exact test (two-tailed) was used to determine if an association was present between the 2 patient groups and HPV types corresponding to probe A or B (or both) in the hybrid capture assay. The Mann-Whitney *U* test was done on copy number data for each probe group. In addition, copy number data were log-transformed to meet the assumption of equal variances. The separate variance t test was used on transformed data. These tests addressed whether there was a significant difference between the mean HPV copy numbers of the 2 groups.

Results

Characterization of patients. Sixty-two subjects underwent biopsy of condylomata acuminata, including 41 with no underlying illness (27 men, 14 nonpregnant women), 12 who were HIV-infected (10 men, 2 women), and 9 women who were organ transplant recipients (7 kidney, 2 liver). The HIV-infected patients were at various stages of disease, with CD4 cell counts ranging from 60 to 650/μL. Three of the transplant recipients were evaluated on more than one occasion, and biopsies of recurrent lesions were done in the same anatomic area as the first biopsy (patients 55, 56, and 58).

Hybrid capture assay. The relative light unit values were converted to estimate the HPV DNA copies per cell, using the conversion 1 pg of HPV DNA/5 μg of cellular DNA = 0.05 HPV copies/cell. All 62 initial biopsy samples produced a positive signal for at least one of the two probes in the hybrid capture assay (figure 1). Overall, 42 (67.7%) of 62 lesions produced positive signals for probe A only, 2 (3.2%) for probe B only, and 18 (29%) for both probes.

There were positive signals with probe A only in 34 (82.9%) of 41 lesion samples from control patients and in 8 (38.1%) of 21 from study patients ($P = .00035$, χ^2 test) (table 1). There were no positive signals with only probe B in 41 lesion samples from control patients, but there were 2 (9.5%) in 21 samples from study patients ($P = .11$, Fisher's exact test). Positive signals were detected with both probes in 7 (17%) of 41 samples from controls and 11 (52.4%) of 21 from study patients ($P = .0038$, χ^2 test). The mean HPV copy number per cell corresponding to the probe A HPV type was

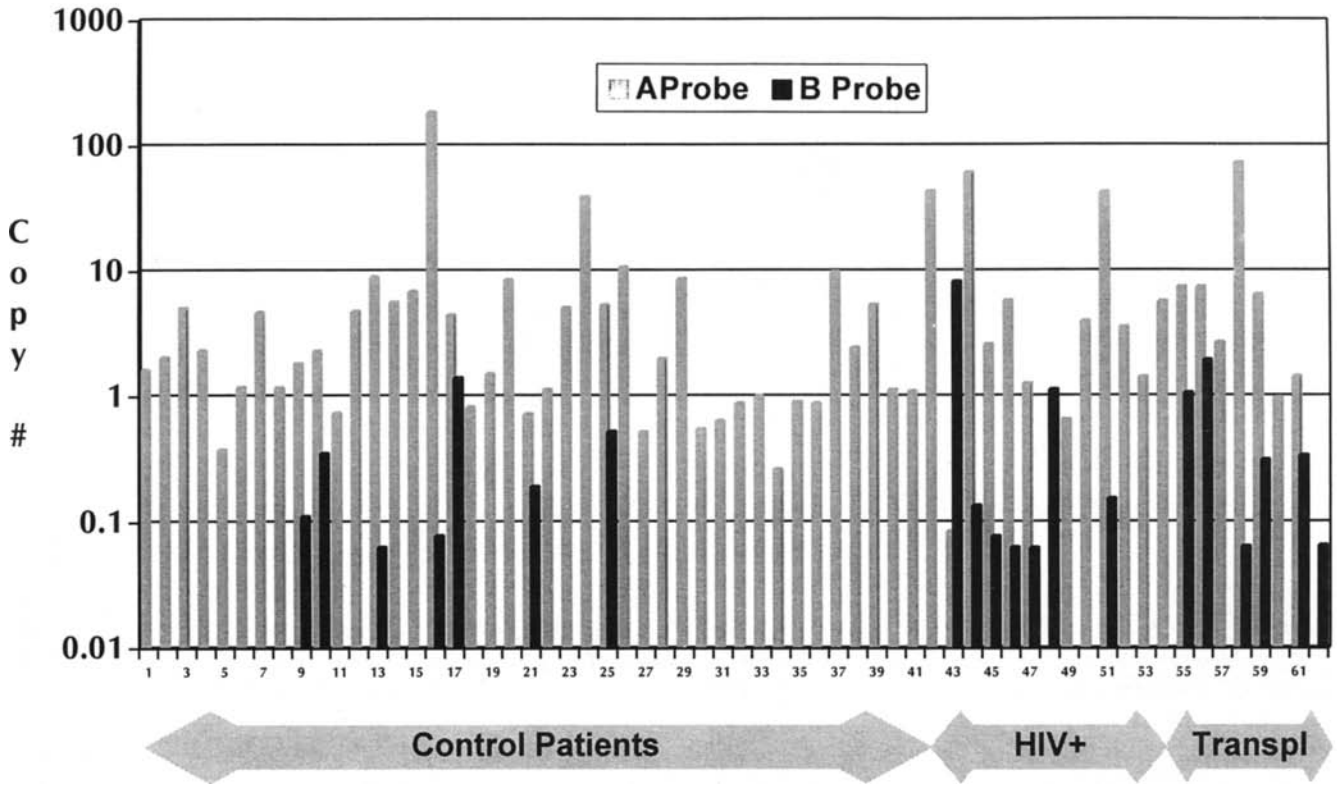

Figure 1. Estimated copy number for A and B probe groups in lesion samples from patients and controls, detected by hybrid capture assay. Samples negative for probe group have no bar. Transpl = transplant recipients.

8.4 in lesion samples from controls and 14.2 in those from study patients ($P = .18$, separate variance t test); the mean copy number per cell corresponding to the probe B HPV type was 0.39 in control samples and 1.0 in study patient samples ($P = .74$, separate variance t test).

Both probes had positive signals in the first biopsy samples from the 3 transplant patients who had >1 biopsy (figure 2). The signal for probe A was initially stronger than the signal from B in all 3 cases. The second biopsy sample from each patient demonstrated a change in the relative amounts of the two signals, with the probe B signal increasing in relationship to the probe A signal. The third biopsy from patients 55 and 56 produced a signal only with probe B. A fourfold increase in the virus copy number corresponding to probe B occurred between the first and second biopsies from patient 58 (0.06–0.27 copies/cell).

Table 1. Controls and patients with positive results for low- and high-risk HPV DNA on the basis of hybrid capture assay.

Probe group	Controls ($n = 41$), no. (%)	Patients ($n = 21$), no. (%)
A only	34 (82.9)	8 (38)*
B only	0	2 (9.5)†
A and B	7 (17)	11 (52.4)‡

NOTE. Probe A = low-risk HPV; B = high-risk HPV.
* $P = .00035$.
† $P = .11$.
‡ $P = .0038$.

Southern blots. After the hybrid capture assay, sufficient DNA was available to do Southern blots, using a mixture of HPV-6b, -11, -16, -18, and -31 probes, on 59 of the 62 initial biopsy samples (40 from controls, 19 from study patients). For the combined patient groups, HPV-6 subtypes were detected in 36 samples, HPV-11 in 15, HPV-16 in 2, and HPV-31 in 1. Three samples contained indeterminate HPV types thought to be HPV-6 variants. Two samples were negative for HPV in the Southern blot assay (patients 2 and 62). Southern blot analysis revealed HPV-6 (including variants) or HPV-11 in 39 (97.5%) of 40 lesion samples from controls and in 13 (68.4%) of 19 from study patients. HPV-16 and -31 were not found in any of 40 samples from controls, but they were found in 3 (15.7%) of 19 lesions from study patients.

Replicate Southern blots demonstrated the presence of >1 HPV type in 3 of 7 lesion samples (figure 3, table 2). All 7 samples were positive for both probes in the hybrid capture assay. The sample from patient 17 contained HPV-11 and -18, the sample from patient 25 contained HPV-11 and -16, and the sample from patient 55 contained HPV-16 and a

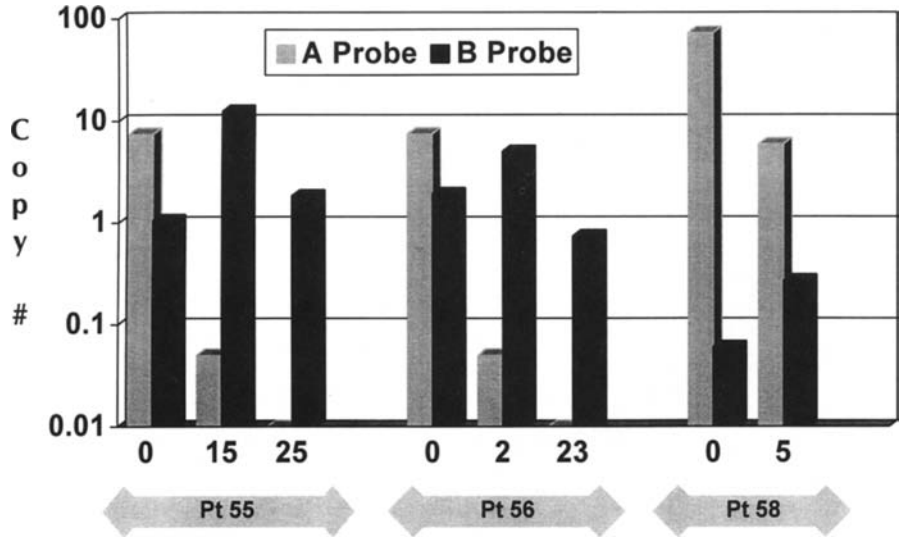

Figure 2. Results of hybrid capture assay of serial biopsy samples from same anatomic sites on 3 organ transplant recipients. Estimated copy number for A and B probe groups are as shown in figure 1. Horizontal axis, time in months between specimens. Time 0 sample results correspond to those of initial biopsies in figure 1.

variant of HPV-6. In the remaining 4 patients (51, 56, 58 [second biopsy], and 59), replicate Southern blots did not clearly demonstrate bands consistent with specific HPV types in both blots (not shown).

Histology. Each of the biopsy samples, which were from the penis, vulva, or perianal areas, were submitted for routine histopathologic evaluation; characteristic condyloma acuminatum features were identified in all 62 initial and all repeat samples. All biopsy samples were entirely condylomata acuminata; no normal tissue was present. While all samples had the characteristic exophytic papillary pattern under low-power examination, the number, size, and contour of these papillary processes were variable. Koilocytosis was focal in some samples and more extensive in others. In cases lacking koilocytosis, other features characteristic of condylomata acuminata, such as acanthosis, hyperkeratosis, parakeratosis, and dyskeratosis, were noted.

Six specimens of condylomata acuminata with koilocytosis (all 3 biopsies from patient 55, the second and third from patient 56, and 1 from patient 62) demonstrated changes characteristic of a high-grade squamous intraepithelial lesion (SIL). The third biopsy from patient 55 contained areas of invasive carcinoma of the vulva. In these cases of high-grade SIL (nuclear crowding and moderate nuclear atypia characterized by nuclear enlargement and pleomorphism), hyperchromasia and coarsening of the nuclear chromatin involved about two-thirds of the epithelium.

In situ hybridization. Analysis of 10 exophytic condylomata acuminata with positive signals for both probes in the hybrid capture assay revealed nuclear staining in 9 lesions with both probe groups in the in situ hybridization assay (table 2). One sample (patient 21) was negative for both probes. In positive tissue samples, nuclear staining was often seen in the same regions in serial sections, but in some cases, different regions of tissue were positive for the two probe groups. The intensity of nuclear staining correlated well with the strongest probe group signal determined in the hybrid capture assay (data not shown). For the biopsy from patient 25, the probe mixtures were used individually on adjacent sections, then together on a third adjacent section, revealing more positive nuclei than either probe mixture alone and, thus, suggesting that the pooled probe mixture was detecting the sum of the separate populations. Sections of the control samples containing high copy numbers of either HPV-11 or -16 showed no cross-hybridization with the two probe groups in the in situ hybridization assay (not shown).

Discussion

This study shows that condylomata acuminata removed from immunosuppressed persons but not from otherwise healthy persons frequently contain >1 HPV type. In addition, the predominant HPV type in genital lesions may not remain constant but rather can change dramatically over a period of a few months in immunosuppressed patients. The changes in HPV type may coincide with changes in histopathology. There are several important questions regarding the natural history of genital HPV infection. First, why is the frequency of detection of HPVs that correspond to probe B higher in immunosuppressed patients than in control groups? Second, why do HIV-infected patients and organ transplant recipients have condylomata acuminata that contain HPV types from both the low- (e.g., HPV-6) and high-risk groups (HPV-16) more often than healthy persons who are not receiving immunosuppressive treatment?

Possibly, immunosuppressed patients had more sex partners and, therefore, were exposed to multiple HPV types. It would then be expected that multiple HPV types would be identified frequently in these patients. We cannot directly address this issue because sex histories were not taken. It is

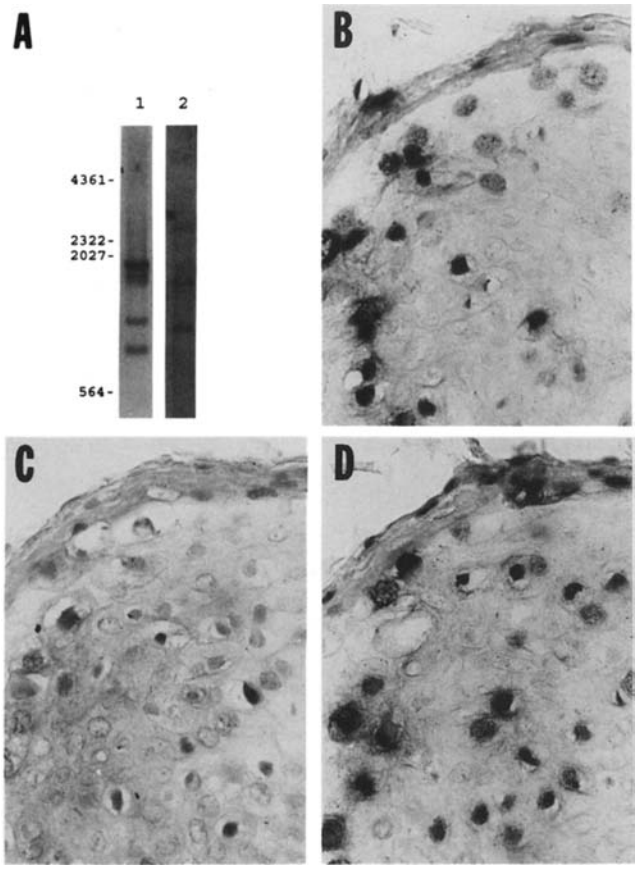

Figure 3. Analysis of condylomata acuminata from patient 25. **A**, Southern blot of DNA extracted from biopsy sample and probed with mixture of HPV-6b and -11 (lane 1) or HPV-16, -18, and -31 (lane 2). Fragment size (bp) on left. Autoradiograph developed at 24 h (lane 1) and 7 days (lane 2). **B-D**, in situ hybridization assay of 3 adjacent tissue sections from same genital lesion. HPV DNA detected by probing with mixture of HPV-6b and -11 (**B**), HPV-16, -18, and -31 (**C**), or both mixtures (**D**). Original magnification, ×100.

unlikely, however, that the group of patients with the highest percentage of lesions containing multiple HPV types (organ transplant recipients) were the most sexually active, because these patients are often hospitalized and are frequently chronically debilitated. In addition, all male control patients and ~25% of female control patients might be expected to have a relatively large number of sexual contacts because they were evaluated at a sexually transmitted disease clinic. It is also possible that the probe B HPV types, such as types 16 and 18, are acquired at the same time as probe A types but are present at very low levels until the degree of immunosuppression is sufficient to allow virus activation. Immunosuppression may allow proliferation of probe B HPV types but may have less effect on proliferation of probe A HPV types.

Another possibility is that probe A types can avoid the immune system of infected patients better than probe B types. This hypothesis could explain the observation that probe A types were found more often in condylomata acuminata from immunocompetent patients, while probe B types were detected more often in immunosuppressed patients.

The percentage of lesions from control patients in our study that contained evidence of >1 HPV type (17%) is consistent with several other reports that used methods other than hybrid capture. For example, Schneider et al. [14] analyzed cervical cells by an in situ filter hybridization method using a probe for HPV-6 and -11 or for HPV-16 and -18. Positive hybridization with both probes occurred in 19% of samples. Nuovo et al. [15] analyzed cervical and vulvar lesions by an in situ hybridization technique using formalin-fixed sections [15]. They found evidence of infection with >1 HPV type in 2.4% of lesions. Using a polymerase chain reaction (PCR) technique with type-specific primers for 8 HPV types, 2 types were detected in 18% of tissues.

Bergeron et al. [6] found evidence of >1 HPV type in 17% of lesions analyzed by Southern blot hybridization in multicentric lesions. Wickenden et al. [16] analyzed condylomata acuminata and cervical cells by a dot hybridization assay for the presence of HPV. Using whole genomic probes, including HPV-6, -11, -16, and -18, they found that 32% of the samples contained DNA for >1 HPV type. Langenberg et al. [10] found evidence of >1 HPV type in 6.3% of condyloma acuminatum samples using the Southern blot method.

A study by Hildesheim et al. [17] showed the highest frequency of mixed infections in histologically normal patients: Mixed HPV infections were identified in 43% of patients with typeable HPV detected by consensus-primer PCR. However, the samples analyzed in that study were cervical lavages and,

Table 2. Evidence of infection with multiple types of HPV in condylomata acuminata biopsy samples analyzed by various methods.

	Hybrid capture copy no. for		Southern blot		In situ hybridization	
Patient no.	Probe A	Probe B	Low-risk HPV	High-risk HPV	Probe A	Probe B
9	1.80	0.11	ND	ND	+	+
10	2.27	0.35	ND	ND	+	+
17	4.43	1.39	11	18	+	+
21	0.72	0.19	ND	ND	–	–
25	5.31	0.53	11	16	+	+
51	42.39	0.15	6a	EQ	+	+
55	7.45	1.05	6*	16	+	+
56	7.45	1.93	EQ	16	+	+
58†	5.92	0.27	6a	–	+	+
59	6.39	0.31	6a	–	+	+

NOTE. Probes A and B = low- and high-risk HPV types, respectively. ND = not done; EQ = equivocal, some hybridization but no clear pattern.
* Related to HPV-6 but pattern slightly different from described subtypes.
† Second biopsy.

thus, were obtained from a large anatomic area, whereas our samples were biopsies of external skin containing only abnormal cells by histology. Hildesheim et al. estimated their assay to be about five times more sensitive than our hybrid capture assay (0.01 vs. 0.05 copies/cell, respectively).

More of our control patients might have demonstrated mixed infections by a more sensitive assay. The quantitative data from the hybrid capture assay suggest that the difference we demonstrated in mixed infections among the control and study patients was not an artifact of the lower sensitivity of the hybrid capture assay. There was no significant difference between the control and study groups in the quantity of HPV DNA detected from either probe group. This suggests either an increase in the true frequency of mixed infections in the immunocompromised patients or at least a selective up-regulation of probe B group viruses in that population. We cannot distinguish between those two possibilities.

Our results contrast with those of other studies, which show that infection of individual lesions with >1 HPV type is unusual. For example, Yang et al. [9] analyzed cervical biopsies by in situ hybridization and found evidence of >1 HPV type in only 1 of 23 lesions. Beckmann et al. [8] analyzed 33 condyloma acuminatum samples by a variety of methods and found none containing >1 HPV type. Using RNA in situ hybridization, Wilbur et al. [7] analyzed 180 condyloma acuminatum samples and found 2 coinfected with HPV-6 and -16. These studies provide little or no information about the immunologic status of patients. To our knowledge, there have been no previous studies that address multiple HPV types in immunosuppressed persons.

Using the hybrid capture assay, we documented the presence of >1 HPV type in a substantial number of condylomata acuminata. To corroborate this finding, highly stringent replicate Southern blots and in situ hybridization assays were done on some of the samples. In most cases, the additional assay supported the result of the hybrid capture assay. We have previously shown that cross-reactivity between probes A and B in the hybrid capture assay is minimal, except when a very high signal for a probe B HPV type occurs [3]. Very high signals for probe A types do not appear to cause a false-positive signal with probe B [3]. Because the mean copy number for the probe A HPV types was not different among the control and study groups, the higher frequency of probe B HPV types detected in the hybrid capture assay among study patients could not be explained by a lack of probe specificity.

There are several possible explanations for a negative result with the replicate Southern blot or in situ hybridization assay on a sample that produced positive signals with both probes in the hybrid capture assay. The hybrid capture assay uses probes encompassing 14 HPV types, compared with 5 in our Southern blot and 7 in the in situ hybridization assay. Therefore, some HPV types in a sample may have been detectable only by hybrid capture. Our prior attempts to do Southern blots at low-stringency conditions have not convincingly documented >1 HPV type, because strong bands from the predominant type generally obscure weak bands from the minor HPV species. A second possibility is that the copy number of a less-abundant HPV type may be below the level of detection in our Southern blot assay, which we have previously reported to be ~0.1–0.5 copies/cell [3]. The hybrid capture assay can detect 0.05 virus copies/cell, so samples with a minor HPV type with a copy number between 0.05 and 0.5 may be missed in our Southern blot assay. Samples from patients 51, 58 (second biopsy), and 59 all had virus copy numbers below 0.5 for probe B HPV types.

Data from the hybrid capture assay suggest that a change in the predominant HPV type occurs over time in lesions from women with organ transplants. In all 3 cases in which >1 biopsy was done at the same anatomic site, probe A signal decreased, accompanied by a relative increase in probe B signal. In 2 of 3 cases (patients 55 and 56), probe B became the predominant signal. In the third case (patient 58), the B signal increased fourfold, while the A signal decreased from 72.61 to 5.92 copies/cell.

There are several possible explanations for the shift in the predominant HPV group detected in the lesions from these 3 patients. The initial biopsies may have sampled an area where probe A HPV type was predominant, and in each case, the second biopsy could have sampled an area with an increased amount of a probe B HPV type, such as HPV-16.

Alternatively, the probe B HPV type, present in low copy number in all 3 initial biopsies, may have caused inhibition of replication of the probe A HPV type. This theory was suggested by Langenberg et al. [10], who did sequential biopsies at 2–52 weeks apart on 5 patients with evidence of dual HPV-infected condylomata acuminata. In 4 of 5 cases, the second biopsy demonstrated an HPV type associated with malignant epithelial change, such as HPV-16.

Another possibility is that HPV-6 or -11 may be easier to eradicate with CO_2 laser therapy, which was used in all 3 of our patients, whereas HPV-16 and -18 have a higher clinical recurrence rate [18–21].

A fourth possibility may relate to the degree of immunosuppression of the patient. Decreasing numbers or function of immune cells may preferentially allow replication of some HPV types. A large, prospective study of genital HPV infection in women with organ transplants will help elucidate the mechanisms involved in this apparent change from a low- to a high-risk HPV type.

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