INTERACTION OF HUMAN IMMUNODEFICIENCY AND PAPILLOMA VIRUSES:
ASSOCIATION WITH ANAL EPITHELIAL ABNORMALITY IN HOMOSEXUAL MEN

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During the 7th annual follow-up of our cohort of homosexual men in 1989, we tested the hypotheses that infection with human immunodeficiency virus (HIV) may enhance the expression of human papilloma virus (HPV) and that the development of anal epithelial abnormality is related to a biologic interaction between these two viruses. Overall, 41 (39%) of the 108 men had anal swabs positive for one or more genotypes of HPV 6/11, 16/18 or 31/33/35. Twenty-three (53%) of the 43 HIV-positive subjects harbored HPV compared to 18 (29%) of the 64 HIV-negative subjects (p = 0.012), including higher prevalence rates for HPV genotypes 16/18 (p = 0.01), 6/11 (p = 0.007), and 31/33/35 (p = 0.07). Multivariate logistic regression analysis of the HIV-positive subjects showed low CD4+ cell counts to be an independent risk factor for detection of HPV (p = 0.04) and in particular for HPV genotypes 31/33/35 (p = 0.02) and 6/11 (p = 0.07). In contrast, similar analysis of the HIV-negative subset showed that a positive antibody test for syphilis was associated with HPV (p = 0.03). Anal epithelial abnormalities were found in 13 (14%) of 92 technically adequate cytologic smears and were strongly associated with detection of any HPV genotypes by the dot-blot method (p = 0.01), and in particular with HPV genotypes 6/11 (p = 0.001). None of 15 subjects with HPV detected only by PCR had anal epithelial abnormality. We propose a viral interaction model, in which HIV-related immune deficiency allows reactivation of HPV, with a subsequent or concomitant appearance of epithelial abnormality.

The human papillomaviruses (HPV) include at least 60 genotypes and represent an important group of human pathogens (Koutsky et al., 1988). Although some genotypes of HPV may be considered benign, as they cause common dermal warts (Jablonska et al., 1985), genotypes 16, 18, 31, 33 and others have been implicated in the pathogenesis of squamous epithelial neoplasms (Ostrow et al., 1987), including cervical dysplasia and invasive cervical cancer (Koutsky et al., 1988), anal carcinoma (zur Hausen, 1985b; Beckman et al., 1989), and perhaps bronchial carcinoma (Byrde et al., 1987; Syrjanen et al., 1987). Furthermore, HPV infection of the ano-genital tract is frequent in both males and females, and some HPV are sexually transmitted (Koutsky et al., 1988). In males, HPV genotypes 6 and 11 have been detected in urethral specimens (Gruessendorf-Conen et al., 1987), and HPV genotypes 2 and 5 have been detected in semen samples (Ostrow et al., 1986). A high prevalence of HPV genotypes 16 and 33 has also been detected in colposcopically directed biopsies from lesions of female intra-epithelial neoplasia (Barascho et al., 1989).

The expression and detection of HPV are modulated by the immune status of the host. For example, the mild and transient immunodeficiency of pregnancy is associated with a concomitant increase in HPV prevalence (Schneider et al., 1987). Similar trends have been seen with iatrogenic immune suppression in renal allograft patients (Alloub et al., 1989) and in homosexual men with human immunodeficiency virus (HIV) infection (Frazer et al., 1986). Taken together, these observations imply that HIV-associated immunosuppression may result in an enhanced expression of HPV. Consequently, we evaluated anal HPV infection in a cohort of homosexual men to test the hypothesis that co-infection with HIV may enhance the expression of HPV. In addition, we evaluated whether the development of anal epithelial abnormality is related to a biologic interaction between these two viruses.

MATERIAL AND METHODS

Subjects and study design

During May and June of 1982, we initiated a prospective cohort study, enrolling 245 homosexual and bisexual men who were consecutive patients of one primary care physician in Manhattan, NY, and 2 primary care physicians in Washington, DC. Enrolled subjects were re-evaluated at approximately yearly intervals. Previous reports have provided details of enrollment and follow-up procedures; demographic, drug use, and sexual practice data; and rates and risk factors for HIV and AIDS (Goedert et al., 1984, 1985a, 1987; Wikt er et al., 1990). As sexual practices had markedly changed between 1982 and 1989, we analyzed associations with levels of sexual activity and smoking (at least 1 cigarette per day) at time of enrollment (1982) questionnaire because we expected that the pre-1982 behavior would be more indicative of outcome. The current analysis included the 105 subjects from whom adequate samples for HPV testing were obtained during the 1989 evaluation and included current (1997) CD4+ and CD8+ lymphocyte counts determined by flow cytometry (Goedert et al., 1985a).

Collection of specimens

HIV, hepatitis B and syphilis antibodies were measured by standard techniques (Goedert et al., 1987). Epithelial cells from the transformation zone of the ano-rectal junction were collected by dacron swabs that were wetted in saline, directly inserted to the ano-rectal junction and gently rotated before being withdrawn. The first swab was smeared on a clean glass slide, sprayed immediately with a water-soluble fixative for exfoliative cytology (Clay Adams, Becton Dickinson, Parsippany, NJ), and read later for cytologic abnormalities. Second and third swabs were collected similarly, transferred to transport media (Virapap, Life Technologies, Gaithersburg, MD), and kept at 4°C for 2 to 18 hr, when they were frozen at −20°C until testing.

Detection and typing of HPV DNA

The presence of HPV was detected by dot blot hybridization and the polymerase-chain reaction (PCR). Commercially available dot blot hybridization kits (Virapap and Viatype, Life Technologies) were used to screen for HPV and for detection

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of specific HPV genotypes 6/11, 16, 18, 31, 33 and 35, following the manufacturer’s instructions.

PCR was performed directly on the same virus transport medium. Following the protease digestion step of the dot blot, 100 μl of the transport medium was precipitated with 100% ethyl alcohol and 1.2 M sodium acetate (pH 5.2) overnight at −20°C. The precipitated DNA was dried under vacuum and redissolved in 100 μl of sterile distilled water. Prior to use for PCR, the target DNA was denatured by boiling at 100°C for 5 min, followed by cooling for 15 min on ice. Specific primer pairs, corresponding to the E6 region of the HPV genomes, were designed to detect the presence of HPV 6, 11, 16, 18, 31 and 33 sequences. Amplification was carried out in a DNA Thermal Cycler (Perkin-Elmer/Cetus, Emeryville, CA) for 30 cycles. The reaction was carried out in 50 μl final volume consisting of 20 μl of denatured target DNA, 2.5 units of the enzyme Taq polymerase and 50 picomole of HPV primer pairs in a PCR buffer. The composition of the PCR buffer was 10 mM each of dATP, dGTP, dCTP, 2.5 mM magnesium chloride, 10 mM Tris-hydrochloride (pH 8.3), 50 mM KCl, 0.1 mg/ml of gelatin (Sigma, St Louis, MO, Catalog No 6507), 0.45% Nonidet P40 (Sigma Catalog No N6507) and 0.45% Tween 20. The reaction cycle consisted of 3 steps—primer annealing for 30 seconds at 50°C, primer extension for 2 min at 37°C and denaturation for 1 min at 90°C. Based on our experience, we amplified HPV 16 and 18 sequences in one reaction mixture and HPV 6/11, 31 and 33 in a different reaction. The integrity of the amplification reaction was monitored by the incorporation of a primer pair for the hemoglobin gene in the tube containing HPV 16 and 18 primers. The amplified product was detected by hybridization with specific HPV probes at Tm=20 (Caussey et al., 1988).

**Scoring criteria for HPV positivity**

No HPV-35 probe was available for PCR. Otherwise, dot blot and PCR were done on every sample for HPV genotypes 6/11, 16, 18, 31, 33 and 35. A specimen was scored positive for a given genotype of HPV if it gave a positive hybridization signal with either the dot blot kit or the PCR reaction. For the purpose of analysis and in keeping with the known biologic parameters we studied. Statistical significance was assessed by considering a p value of 0.05 or less in a 1-tail test.

**Cytology**

Cytological smears were stained with the standard Papanicolaou technique and scored by a pathologist (J.G.) who had no prior knowledge of clinical or laboratory findings. Cells were examined for abnormal nuclear shape, size, chromatin pattern and overall nuclear staining (hyperchromasia). Smears were also examined for perinuclear cytoplasmic cavities characteristic of papillomavirus cytopathic effect. Smear cells showing at least 2 of the above nuclear abnormalities were considered to exhibit dysplasia, the grade of which was determined by the amount of squamous differentiation in the involved cells, with less differentiation corresponding to higher-grade dysplasia. Smear cells showing just one nuclear abnormality as well as perinuclear cytoplasmic cavities (koliocytosis) were considered to exhibit condylomatous changes. Smear cells showing just one nuclear abnormality and no other changes were classified as atypical. Smears with dysplasia, condylomatous changes or atypia were considered abnormal. All other smears that were adequately collected and fixed, including those with only anuclear squamous plaques, were considered normal.

**Statistical methods**

Data handling and statistical analyses were done by means of SAS software (SAS Institute, Cary, NC). Associations between HPV and risk factors were determined by contingency table analysis, and significance testing was performed by the Chi square test, except that Fisher’s exact test was used if any cell frequency was below 5. Student’s t-test was used for continuous variables, or the Wilcoxon unpaired rank sum test was used for variables that were not normally distributed. Agreement between the 2 detection methods was computed by Kappa statistics (Fleiss, 1981). Multiple logistic regression was used to determine statistical independence of the effects of the parameters we studied. Statistical significance was assessed by considering a p value of 0.05 or less in a 1-tail test.

**RESULTS**

The anal swabs of 41 (39%) of the 105 homosexual men were positive for one or more genotypes of HPV, including 22 that were positive by both PCR and dot blot methods, 15 that were positive only by the PCR method, and 4 that were positive only by the dot blot method. There was moderate overall agreement between the two methods (k = 0.58, p = 0.008).

**Risk factors and markers for HPV**

Detection of anal HPV was strongly associated with HIV infection, with one or more HPV genotypes detected in 23 (53%) of 43 HIV-positive men compared to 18 (29%) of 62 HIV-negative men (p = 0.01, Table I). As shown in Figure 1, each of the HPV genotypes was more frequent in HIV-positive than in HIV-negative men, including HPV 6/11 (33% vs. 11%, p = 0.007), HPV 16/18 (20% vs. 5%, p = 0.01), and HPV 31/33/35 (39% vs. 16%, p = 0.07). With the dot blot method, 2 men were co-infected with HPV 16/18 and 31/33/35, 3 others were co-infected with HPV 6/11 and 31/33/35, and 3 others were infected with all 3 HPV genotype groups. These 8 men were all HIV-positive. With the PCR method, 13 men were co-infected, including 4 who were HPV-negative. Using both tests, 22 men were infected with at least 2 genotypes of HPV.

The prevalence of anal HPV was moderately associated with hepatitis B virus (p = 0.04) and weakly associated with syphilis (p = 0.19, Table I). HPV-positive men had had significantly more homosexual partners before 1982 (medians 25 vs. 13.5 per year, p = 0.04) and tended toward more frequent unprotected receptive anal intercourse frequency (10–19 vs. 5–9 per year, p = 0.12). In addition, HPV prevalence tended to be higher in those who had not completed college compared to those who had completed college (p = 0.07, Table I), but no significant associations were detected between HPV prevalence and city of enrolment, ethnic origin, age, or cigarette smoking. Subjects co-infected with HIV and HPV had slightly but not significantly lower CD4+ lymphocyte counts than those with HIV only (p = 0.19, Table I); however, co-infection did not appear to affect CD8+ lymphocyte counts (p = 0.65). Among HIV-negative subjects, CD4+ and CD8+ lymphocyte counts were similar in HPV-positive and HPV-negative subjects (p = 0.78).

Stepwise logistic regression modelling was used to assess the relationship of each HPV genotype to the 5 following variables: current CD4+ lymphocyte count, serological evidence of syphilis or hepatitis B virus infection, and historical number of male homosexual partners and frequency of receptive anal intercourse. Due to the strong associations between HPV and HIV status, separate models were run for HIV-positive and HIV-negative men. Among the HIV-positive subjects, lower numbers of CD4+ lymphocytes were independently associated...
with the detection of one or more HPV genotypes \(p = 0.04\), after adjusting for the number of sexual partners, anal intercourse and syphilis (Table II). This associated adjustment with CD4 counts was strongest with HPV genotypes 31/33/35 \(p = 0.02\), weaker with HPV genotypes 6/11 \(p = 0.07\), and not detectable with HPV genotypes 16/18 \(p = 0.93\), Table II). The only other independent association in the HIV-positive men was a trend toward higher frequency of anal epithelial abnormality, which was stronger \(i.e., p < 0.01\) after serological evidence of hepatitis B virus was added to this model. Among the HIV-negative men, a multivariate logistic regression model demonstrated a significant association between detection of any HPV and a positive syphilis serology \(p = 0.03\) but not with CD4 counts, anal sex or sexual partners \(p > 0.18\). This multivariate association was particularly strong for syphilis with HPV 31/33/35 \(p = 0.01\).

**Risk factors for anal epithelial abnormality**

Thirteen (12\%) of the anal cytologic smears obtained were technically unsatisfactory and could not be interpreted. Of the 92 smears that could be interpreted, 13 (14\%) demonstrated epithelial abnormality, including 1 with dysplasia, 3 with condylomatous changes, and 9 with atypia. Nine (24\%) of the 37 HIV-positive men had epithelial abnormality, compared to 4 (7\%) of the 55 HIV-negative men \(p = 0.03\). Cigarette smoking had a weaker association, with epithelial abnormality in 6 (21\%) of 28 smokers compared to 7 (11\%) of 65 non-smokers \(p = 0.15\).

The prevalence of anal epithelial abnormality was dependent on both the HPV genotypes and the detection method used. Thus, when the dot blot technique was used, anal epithelial abnormality was strongly associated with HPV \(p = 0.012\), Table II), particularly with genotypes 6/11 \(p = 0.0014\) and to a lesser extent with genotypes 31/33/35 \(p = 0.063\). However, when HPV was detectable by the PCR method only, none of the 15 subjects (4 HPV-positive and 11 HPV-negative) had an epithelial abnormality. Six men with mild abnormalities (atypia) were not positive in the HPV dot blots. Three of these men were cigarette smokers. On further assessment of statistical independence by the logistic regression technique, anal
epithelial abnormality was strongly related to dot-blot detection of HPV 6/11 (p < 0.002), very weakly related to smoking (p = 0.07 to 0.13), and unrelated (after adjustment for HPV status) to CD4 counts (p > 0.3) or HIV status (p > 0.3).

**DISCUSSION**

In our study of homosexual men, 39% had one or more genotypes of HPV detected by molecular analyses of anal swabs and 14% had epithelial abnormality on anal cytologic smears. In the presence of HIV infection, the prevalence rates of all HPV genotypes tested were increased, and in particular the prevalence rates of HPV genotypes 31/33/35 and 6/11 were inversely related to CD4+ lymphocyte counts. Furthermore, anal epithelial abnormality was more frequent with certain HPV genotypes as detected by the dot blot method.

Morphologic features indicative of HPV infection have been previously noted in anal intra-epithelial neoplasia (AIN) of HIV-infected men (Croxton et al., 1984). Scholefield et al. (1989) using DNA hybridization on colposcopically-directed anal biopsies, have reported that HPV genotypes 6/11 were associated with low-grade AIN and HPV genotypes 16/18 with high-grade AIN, similar to the pattern observed in cervical intra-epithelial lesions (Crum et al., 1985). Cytologic evidence of HPV infection in HIV-infected homosexual men has also been noted (Frazier et al., 1986). By a combination of molecular and cytologic methods, we have been able to confirm the presence of, and to further evaluate the relationships between, specific HPV genotypes and anal epithelial abnormality. We found that the prevalence of anal epithelial abnormality was related to genotypes 6/11 and to a lesser extent with HPV genotypes 31/33/35. It is noteworthy that these associations were strongest for the dot-blot detection method, which requires a high copy number of HPV genomes (Caussy et al., 1988), and weakest for the PCR method, which does not. As there were no high-grade AIN lesions in our population, our observation on HPV 6/11 and anal epithelial abnormality is consistent with that of Scholefield et al. (1989).

HPV infections may be latent (Ferencycz et al., 1985) and the factors triggering reactivation are not fully defined although immune competence is clearly involved. For instance, the transient immune deficiency associated with pregnancy (Schneider et al., 1987) and iatrogenic immune deficiency of renal transplantation can lead to HPV reactivation (Alloub et al., 1989; Rudlinger et al., 1989). Similarly, we noted that the prevalence of HPV increased inversely with the number of CD4+ lymphocytes among HIV-infected homosexual men. Since our observations suggest that epithelial abnormality is related to active replication of HPV and an increased number of copies of its genome, we postulate that HIV-related immune deficiency is not directly related to anal epithelial abnormality but rather may permit reactivation of dormant HPV (Fig. 2).

In this model, epithelial abnormality is a consequence of actively replicating HPV, with perhaps some independent contribution from smoking. Our model provides a general framework for understanding the role of immune suppression in the reactivation of dormant HPV infection to induce cytologic abnormality.

Anal carcinoma is a relatively rare disease (Young et al., 1981) and the etiological role of HPV in the genesis of anal cancer (Zur Hausen, 1989a), as in cervical cancer (Caussy et al., 1990), has been difficult to substantiate epidemiologically. If epithelial abnormality is a pre-malignant condition, then one might expect an increase in the number of cases of anal cancer among those at high risk of AIDS. In fact, the frequency of anal cancers among never-married men between 20 and 49 years of age increased 10-fold in Manhattan, New York City from 0.3 cases per year before 1977 to 3 cases per year between 1979 and 1985 (Biggar et al., 1989). In addition, increased risks have been noted for homosexual men (Daling et al., 1987; Holly et al., 1989).

Early studies linking HPV to anal carcinoma have employed cytologic (Holly et al., 1989) or immunohistochemical methods (Gal et al., 1987) that could not implicate the particular genotypes of HPV involved. A population-based case-control study did note that HPV 16/18, 31 and 6/11 were associated with anal cancer (Beckman et al., 1989). HPV genotypes 16/18, 31, 33 and 35 have been reported in cervical dysplasia, cervical carcinoma and anogenital carcinoma and are likely to be involved in the genesis of anal carcinoma (Beaudenon et al., 1986; Lorincz et al., 1986, 1987; zur Hausen, 1989). The role of HPV 6/11 in squamous-cell carcinoma remains controversial, since infection with HPV 6/11 is associated with concomitant infection with other genital HPV
genotypes (zur Hausen, 1989a). Nonetheless, genotypes of HPV 6/11 have been identified in a variety of squamous-cell carcinomas (Bryne et al., 1987; Kasher and Roman, 1988; Rando et al., 1986) and the oncogenic potential of this genotype should not be dismissed. Our findings indicate the need for further study of HPV genotypes in the pathogenesis of anal carcinoma.

Retrospective case-control and cross-sectional studies can not unambiguously distinguish etiologically relevant infection with HPV from opportunistic infection of an established cancer (Caussey et al., 1990; Muñoz et al., 1988). Our finding of a high prevalence of HPV infection in a cohort not selected for the presence of anal carcinoma or epithelial abnormality, in addition to the association of certain genotypes with epithelial abnormality, support the hypothesis that one or more genotypes of HPV are etiologically involved in the pathogenesis of anal carcinoma and are not merely another opportunistic agent in HIV-infected subjects. However, only prospective follow-up of these types of cohorts will unambiguously establish which specific genotypes of HPV are directly or indirectly predictive of anal cancer onset.

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