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HERPES SIMPLEX VIRUS TYPE 2 AND HUMAN PAPILLOMAVIRUS TYPE 16 IN CERVICITIS, DYSPLASIA AND INVASIVE CERVICAL CARCINOMA

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Sera and biopsies of cervical lesions from 55 women with benign or malignant disease were analyzed for evidence of infection with herpes simplex virus type 2 (HSV-2) or human papillomavirus (HPV). In addition, information regarding known risk factors for cervical cancer was obtained by interview. The sera were tested for HSV-2 antibodies and the biopsies were tested for HPV or HSV DNA sequences by Southern blot hybridization. HSV-2 sequences were detected in 2/13 (15%) invasive neoplasms and in 1/12 (7%) benign lesions. Under non-stringent conditions of hybridization, reactions with HPV DNA were detected in biopsies of 2/17 (12%) inflammatory lesions, 6/12 (50%) intraepithelial neoplasms and 13/20 (65%) invasive neoplasms. All but one of the positive biopsies of invasive cancer, but only 4/11 biopsies of non-invasive lesions, contained HPV-16 DNA as determined by stringent hybridization conditions. Women with cervical cancer possessed the risk factors associated with the disease. Cigarette smoking and the presence of HPV-16 DNA were the most prominent risk factors. No evidence of an interaction between HSV-2 and HPV-16 was found among the cases of invasive cervical cancer.

Epidemiological studies have demonstrated that women whose sexual behavior is conducive to acquisition of venereal infections are at increased risk of developing cervical cancer (reviewed by Rotkin, 1973; Kessler, 1976). Herpes simplex virus type 2 (HSV-2), a venereally transmitted agent, has been incriminated as a possible cause of cervical cancer on the basis of a greater occurrence of antibodies to HSV-2 among women with cervical cancer than among control women (reviewed by Rawls and Campione-Piccardo, 1981). In addition, HSV-2 is capable of transforming cells *in vitro* (reviewed by Hayward and Reyes, 1983) and there is indirect evidence suggesting that HSV-2 genetic information may be expressed in cancer cells in some cases of cervical cancer (Gupta *et al.*, 1981).

Recently, human papilloma virus (HPV) has been suggested as a risk factor for invasive cervical cancer (zur Hausen, 1977). This hypothesis derives from several lines of evidence. Several animal papillomaviruses are oncogenic and HPV may have oncogenic potential (reviewed by Pfister, 1984). Certain types of HPV infect the cervix, producing flat condylomata or koilocytotic atypia which resemble mild or moderate dysplasia (CIN I and II) (Meisels, 1981). In addition, HPV structural antigens have been demonstrated in all grades of cervical dysplasia including carcinoma *in situ* and HPV DNA has been found in preneoplastic cervical lesions (Guillet *et al.*, 1983; Crum *et al.*, 1983; Fu *et al.*, 1983; Kurman *et al.*, 1983; Walker *et al.*, 1983; Zachow *et al.*, 1982; Green *et al.*, 1982; Gissmann *et al.*, 1983; McCance *et al.*, 1983; Durst *et al.*, 1983; Lancaster *et al.*, 1983; Boshart *et al.*, 1984). Several important questions remain in this hypothesis. A wide variety of closely related types of HPV exist and present serologic methods cannot distinguish antibodies directed against genital HPV from those directed against wart viruses infecting other parts of the body; thus, case-control seroepidemiologic studies have not yet been possible. HPV antigens are found frequently in mild and moderate dysplasia but much less frequently in severe dysplasia or carcinoma *in situ* (Guillet *et al.*, 1983; Crum *et al.*, 1983; Fu *et al.*, 1983; Kurman *et al.*, 1983; Walker *et al.*, 1983). The reagents used to assay these antigens are broadly group-reactive, and tests screening for viral antigens are insensitive indicators of infection. Most recently, 4 HPV types have been identified as specifically infecting the genital tract (HPV types 6, 11, 16 and 18) (Boshart *et al.*, 1984). Durst and co-workers (1983) have cloned HPV-16 DNA from a cervical carcinoma and detected sequences related to HPV-16 DNA in a large proportion of cervical cancer cases.

Cervical cancer appears to cluster in Latin America. In the Republic of Panama, invasive cervical cancer accounts for one-third of all diagnosed female malignancies and the average annual incidence in women between 30–55 years of age exceeds 1/1000 (Reeves *et al.*, 1982). Because cervical cancer is so common in Panama, the National Oncology Institute routinely evaluates large numbers of patients with all degrees of cervical disease. The intent of this study was to compare epidemiologic factors elucidated by interview and anti-HSV-2 antibody, HSV-2 sequences and HPV-16 DNA sequences in samples obtained from women with preinvasive and invasive cervical neoplasia and with non-neoplastic cervical lesions.

MATERIAL AND METHODS

Patient samples

Biopsy specimens were collected from women referred to the Gynecologic Oncology Clinic of the Panama National Oncology Institute for evaluation of possible cervical cancer. Sixty-four volunteer patients were studied with a view to sampling approximately equal numbers in 3 categories of histologically confirmed disease: non-neoplastic lesions (22 women with cervicitis, hyperplasia or metaplasia), preinvasive neoplasia (15 patients with CIN I, II or III) and invasive cervical carcinoma (21 subjects with stage Ib or more advanced disease). In addition, 6 women thought to have cervicitis or CIN were found to have condylo-

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matous dysplasia, flat condyloma or koilocytotic atypia when their biopsy specimens were examined histologically, and these women were considered separately. Fifty-five biopsy specimens were satisfactorily processed; the specimens which were not satisfactorily processed were from 5 non-neoplastic, 3 preinvasive and 1 invasive cervical lesions.

After giving informed consent, patients underwent a standard gynecologic examination in which colposcopically directed biopsies were taken for histopathology and virology studies. A standard interview using a questionnaire designed to quantify sexual and other risk factors was also administered. Biopsies intended for virologic studies were snap-frozen in liquid nitrogen for transportation to the Gorgas Memorial Laboratory where they were subsequently stored at −70°C until shipment on dry ice to McMaster University. Biopsies for histopathologic examination were treated by routine methods and evaluated by the pathology staff of the Panama National Oncology Institute. Ten ml of venous blood were obtained from each patient and serum was stored at −20°C until assayed for antibodies at McMaster University where all material was sent under code, testing being done without knowledge of the patients' diagnoses.

Processing of DNA from biopsies

The frozen samples were thawed, washed with phosphate-buffered saline (PBS) and resuspended in a small volume of 50mM Tris-HCl pH 7.5, 150mM NaCl, 2mM Na_2 EDTA. The specimens were minced thoroughly with scissors and digested for 24 hr at 37°C with Pronase or Proteinase K at a concentration of 500 μg/ml, in the presence of 1% (w/v) sodium dodecyl sulphate (SDS). Following 2 extractions with re-distilled phenol saturated with 100mM Tris-HCl pH 8.0, 10mM Na_2 EDTA, the aqueous phases were pooled and extracted twice with anhydrous ethyl ether. Nucleic acids were then precipitated from solution at −20°C by addition of two volumes of 95% ethanol in the presence of 0.3M sodium acetate, pelleted (15,000 *g* for 15 min at 4°C), rinsed briefly with 95% ethanol, drained thoroughly and re-dissolved in 50mM Tris-HCl pH 7.5, 100mM NaCl and 2mM Na_2 EDTA. This solution was incubated first for 2hr at 37°C in the presence of 50 μg/ml RNase A (from bovine pancreas) and then for 24 hr with 100 μg/ml pronase or proteinase K. Phenol extraction, ether extraction and ethanol precipitation were repeated and the DNA pellet was dissolved in 10mM Tris-HCl pH 7.5, 1mM Na_2 EDTA (TE buffer).

The concentration of DNA was determined by optical density (OD) at 260 nm. Complete digestion of each sample was obtained with 1 unit of restriction endonuclease Eco RI per μg of DNA in a reaction mixture containing 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 1mM dithiothreitol (Cleland's Reagent; DTT), 1mM spermidine and 100 μg/ml nuclease-free bovine serum albumin (BSA), for at least 5hr at 37°C. Phenol extraction, ether extraction and ethanol precipitation were again repeated and the resulting DNA pellet was dissolved in TE buffer at a concentration of 0.5 mg/ml. Gel electrophoresis on 1% (w/v) agarose gels was performed using 40-μl (20 μg) aliquots of the digested DNA sample. Included in each gel was a positive control consisting of 0.3 genome equivalents of HSV-2 DNA which had been digested with EcoRI and BglII. Following electrophoresis, the gels were stained with 1 μg/ml ethidium bromide for ultraviolet visualization. The transfer of DNA from the gels to nitrocellulose membranes was accomplished by the Southern blotting method (Southern, 1975) as modified by Wahl *et al.* (1979). After transfer was completed, the nitrocellulose membranes were rinsed briefly in 0.3M sodium chloride, 0.03M sodium citrate pH 7.0 (double strength SSC), baked for 2 hr at 80°C and individually sealed in plastic bags for storage at 4°C.

DNA probes

Recombinant plasmids containing each of the HSV-2 DNA fragments were labelled *in vitro* with ^{32}P-dCTP by nick translation to specific activities of approximately 1×10^8 cpm/μg DNA (Maniatis *et al.*, 1975). The presence of HSV-2 sequences in the biopsies was assessed by using the following cloned BglII fragments of HSV-2 DNA as hybridization probes: J (0.32-0.38 mu [map units]; L (0.88-0.94 mu); N (0.58-0.62 mu); O (0.38-0.41 mu) and P (0.17-0.19 mu). The choice of these fragments was based on preliminary results obtained when DNA from 8 biopsies of invasive cervical cancer were each probed with cloned HSV DNA fragments representing the entire genome except for regions between 0.13-0.18 and 0.72-0.87 mu. Only two biopsies were found clearly positive for sequences hybridizing to the BglII J and O (1 specimen) or BglII L and P (1 specimen) fragments of HSV-2. Two additional biopsies appeared positive for sequences homologous to the BglII N fragment; however, due to background problems the results were not convincing. Nevertheless, the BglII N fragment was included among the probes used in the present screening.

A recombinant plasmid containing human papillomavirus type 16 (HPV-16) DNA was kindly provided by Dr. L. Gissmann (Deutsches Krebsforschung Zentrum, Heidelberg). The HPV DNA was isolated from the plasmid and labelled by digestion with exonuclease III (10 units/μg DNA) for 60 min at 37°C and then incubated with *E. coli* DNA polymerase I in the presence of ^{32}P-dGTP alone or in combination with ^{32}P-dTTP. The specific activity of the HPV-16 probes was between 5×10^7 and 1×10^8 cpm/μg DNA.

Hybridization reactions

Hybridizations with the HSV-2 probes were performed essentially as described by Wahl *et al.* (1979). The nitrocellulose filters were first incubated in prehybridization buffer [50% (v/v) deionized formamide, 5-fold SSC, 5-fold Denhardt's reagent, (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidinone (PVP), 0.1% (w/v) BSA), 200 μg/ml sonicated and denatured DNA from salmon testes (type III), 50mM Tris-HCl pH 7.5, 10% (w/v) dextran sulphate] for 4 hr at 42°C, then in hybridization buffer [50% deionized formamide, 5-fold SSC, 2-fold Denhardt's reagent, 200 μg/ml sonicated and denatured DNA from salmon testes, 20mM Tris-HCl pH 7.5, 10% (w/v) dextran sulphate], containing heat-denatured HSV-2 probe (2×10^6 cpm/ml) for 18-24 hr. The membrane was then washed gently 3 times with double strength SSC, 0.1% (w/v) SDS (5 min/wash) at room temperature. Another 6-8 washes were done in the same buffer at 65°C (1 hr/wash) after which 3 final washes were done in 0.1-fold SSC, 0.1% (w/v) SDS, at room temperature (5-10 min/wash). The membrane was then dried at 37°C and

exposed to XRP-1 X-ray film in an intensifier screen for 10-14 days at −70°C.

For HPV-16, the nitrocellulose filters were prehybridized at 42°C for at least 4 hr in a solution containing 50 mM Tris HCl pH 7.5, 5-fold Denhardt's solution, 5-fold SSC, 20% formamide and 0.5 mg/ml of yeast tRNA. Hybridization was carried out at 42°C for 2-3 days with ^{32}P-labelled HPV-16 DNA (2×10^5 cpm/cm^2 nitrocellulose) in 20 mM Tris HCl pH 7.5, 2-fold Denhardt's solution, 5-fold SSC, 20% formamide and 0.1 mg/ml yeast tRNA. The filters were then washed 5 times with 2-fold SSC containing 0.1 SDS at 42°C (non-stringent), exposed moist at −70°C to X-ray film using intensifier screen for 1 to 5 days, rewashed 5 times at 65°C with 2-fold SSC containing 0.1% SDS (stringent) and exposed again for 2-10 days. Autoradiographs were assessed independently by 5 observers and those samples considered positive by at least 4 observers were taken as indicative of the presence of viral DNA sequences. The specimens were analyzed in batches of 9 and the test for the presence of HSV-2 DNA was considered satisfactory if the HSV DNA control included in the test of that batch was positive. Satisfactory hybridizations (as judged by positive reaction between probe and viral DNA) were performed on 53%, 47%, 32% and 16% of specimens probed with HSV-2 BglII fragments J, L, N, O and P, respectively. The group of specimens probed with BglII J comprised all of the samples screened with the other probes.

Antibodies to HSV-2

Antibodies to HSV-1 and HSV-2 were assayed by a microneutralization test using KOS strain of HSV-1 and 333 strain of HSV-2 (Rawls *et al.*, 1980). For each serum the titres of antibodies to the 2 virus types were determined and the antibody activity was expressed as a II/I index. II/I indices of 85 or more were considered positive for antibodies to HSV-2 (Rawls *et al.*, 1970).

Statistical analysis

Differences in proportional distributions were tested for significance using the χ^2 test. Comparison of risk attributes for neoplasia was done by computing relative risk (Lilienfeld *et al.*, 1967).

RESULTS

Herpes simplex virus type 2

Evidence of herpes simplex virus infection was sought by assaying sera for HSV-2 antibodies and by probing DNA extracted from tissues for HSV-2 DNA sequences using Southern blot hybridization. Serological evidence of HSV-2 infections was found in 1/6 (17%) patients with cervical condyloma and 2/17 (12%) patients with non-neoplastic cervical lesions (Table I). An even higher rate of HSV-2 antibodies was found among women with intraepithelial neoplasia (25%) and women with invasive cancer had the highest rate (42%).

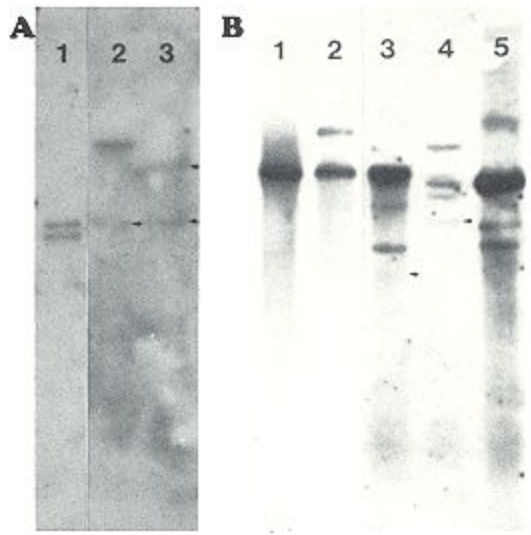

FIGURE 1 – Autoradiographs of Southern blot hybridization reactions of DNA extracted from biopsies, digested with EcoRI and probed with ^{32}P-labelled BglII J fragment of HSV-2 DNA (*a*) or HPV-16 DNA (*b*). The conditions of hybridization with HSV-2 and HPV-16 (non-stringent) are given in "Material and Methods." In gel A, lane 1 contained control HSV-2 DNA at 0.3 genome equivalents while lane 2 contained DNA from an invasive cancer and lane 3 contained DNA from a chronic cervicitis case. In gel B, lanes 1 and 2 contained DNA from metaplastic condylomatous lesions, respectively, while lanes 3, 4 and 5 contained DNA from invasive cancers. Arrows point to minor bands.

HSV-2 sequences were detected by hybridization in DNA extracted from 3 specimens, 2 of which are illustrated in Figure 1*a*. The positive samples all reacted with BglII fragment J only. One positive sample was obtained from a biopsy of chronic cervicitis, and serum from this patient did not contain HSV-2 antibodies. Two biopsies from advanced invasive cancers were also positive for HSV-2 sequences (Table I). Serum from one of these patients contained HSV-2 antibodies while serum from the other patient did not.

TABLE I – DISTRIBUTION OF HSV-2 AND HPV-16 MARKERS IN SERUM AND CERVICAL BIOPSY SPECIMENS

Virus marker	Conditions of hybridization	Histologic diagnosis									
		Condyloma		Non-neoplastic[1]		CIN[2]		Cervical carcinoma[3]			
								I & II		III & IV	
Anti-HSV-2 antibody		1/6	17%	1/7	12%	4/12	25%	4/9	44%	4/10	40%
HSV-2 DNA	Stringent	0/4		1/12	8%	0/9		0/5		2/8	25%
HPV-16 DNA	Stringent	1/6	17%	0/17		3/12	25%	7/9	78%	5/11	45%
	Non-stringent	4/6	67%	2/17	12%	6/12	50%	7/9	78%	6/11	55%
	Ratio str/non-str	1/4	25%	0/2		3/6	50%	7/7	100%	5/6	83%

[1]Includes 1 case of hyperplasia, 11 cases of cervicitis and 5 cases of metaplasia; no differences existed in the distribution of markers between subcategories which were combined.–[2]Includes 1 case of CIN I, 1 case of CIN II, and 10 cases of CIN III; no differences existed in the distribution of markers between subcategories which were combined. [3]Six stage-Ib and 4 stage-IIb patients were combined for presentation. Also combined for presentation were 8 stage-IIIb and 3 stage-IV patients. Results are presented as number positive/number tested and then % positive.

Papillomavirus

The presence of HPV sequences of types other than HPV-16 were detected by hybridizing under non-stringent conditions while reactions occurring under stringent conditions of hybridization were considered positive for HPV-16. The distribution of HPV sequences in biopsies obtained from the patients is shown in Table I. HPV sequences were found in 4/6 (67%) condylomatous lesions. The occurrence of specimens containing HPV DNA increased from 12% among non-neoplastic lesions to 50% among CIN lesions and 65% of invasive cancer. The occurrence of sequences appeared higher for clinical stage I and II disease (78%) than for more advanced disease (55%); however, this difference is not statistically significant.

One of four (25%) condylomatous lesions which had HPV DNA detected by the non-stringent assay had HPV-16 DNA. Within other diagnostic categories the ratio of HPV-16 DNA detected by stringent hybridization to HPV DNA defined under non-stringent conditions increased significantly with increasing severity of the disease. Neither of the 2 women with non-neoplastic disease and HPV DNA had HPV-16 as compared to 3/6 (50%) women with CIN, 7/7 (100%) with stage I or II invasive cervical cancer and 5/6 (83%) patients with stage III or IV disease.

Variation was observed in the patterns of HPV-DNA detected by hybridization of the EcoRI-digested DNA from biopsies (Fig. 1*b*). The HPV-16 genome used as probe has 2 EcoRI sites and digested circular genome should produce 2 fragments of about 6.95 and 0.66 kilobase pairs. Patterns containing bands of unit length HPV-16 genome or over (exemplified by lanes 1 and 2 in Fig. 1*b*) were most commonly observed in biopsies of non-invasive lesions. Patterns containing multiple bands, some of which were less than unit length (illustrated by lanes 3-5 in Fig. 1*b*) were commonly observed in biopsies of invasive cancers. None of the samples positive for HSV-2 sequences were positive for HPV sequences.

Epidemiologic risk factors

Although not designed as a case-control study, risk factors determined by interview were found to be consistent with reports of others. This is illustrated in Table II where the percentages of women with the risk factors are shown. For comparative purposes, the relative risks of disease associated with the risk factors are also shown. Although of comparable ages, the group of women with CIN were less educated, had had first coitus at earlier ages, were more likely to smoke and had a greater occurrence of anti-HSV-2 antibodies and HPV-DNA sequences than women with non-neoplastic lesions. However, with the numbers studied, only the greater occurrence of HPV-DNA sequences was statistically significant. Women with cervical cancer were older (46±11 yrs) than women with non-neoplastic lesions (33.6±99 yrs) and had significant excesses of all the risk factors (except education). As shown in Table II, the differences in cigarette smoking and HPV-DNA sequences were highly significant. Differences in the number of pregnancies are not shown in the Table. Four or more pregnancies had been experienced by 29% of women with non-neoplastic lesions, 58% of women with CIN and 89% of women with cervical cancer. The differences in parity were statistically significant.

The distribution of viral markers in relation to risk factors is shown in Table III. Since the numbers were small, patients with condylomatous lesions were included with the non-neoplastic group and the patients with CIN were pooled with the women with cervical cancer. Anti-HSV-2 antibodies were significantly associated with multiple sex partners (for entire sample 3/25 with one partner and 13/29 with more than one partner; $P < 0.05$). This was observed for women both with and without neoplastic lesions of the cervix (Table III). The presence of HPV-DNA was unrelated to sexual partner numbers among women with non-neoplastic lesions while among women with neoplastic lesions 46% with one partner were positive and 68% with multiple sex partners were positive. Early age at first coitus was associated with higher percentages of positivity for both viruses except for anti-HSV-2 antibodies among women with non-neoplastic lesions where only 3/23 were infected (Table III). HPV-DNA sequences were found more commonly among non-smokers than among smokers; however, only one of the women without neoplastic lesions smoked. There was no relation between the presence of anti-HSV-2 antibodies and HPV-DNA sequences.

DISCUSSION

Frenkel *et al.* (1972) were the first to report HSV-2 DNA in cervical cancer tissue. This specimen con-

TABLE II – DISTRIBUTION OF RISK FACTORS

Risk factors	Percent of groups[1] with risk factor			Relative risk[2] for	
	Non-neoplastic	CIN	Cervical cancer	CIN	Cervical cancer
Primary education or less	53	75	75	2.7	2.7
First coitus before 18 years of age	24	50	58	3.3	4.5[3]
Two or more sex partners in lifetime	41	33	75	0.7	4.3[3]
Smoked cigarettes	6	17	50	3.2	16.0[4]
Anti-HSV-2 antibodies	12	25	42	3.8	5.5[3]
HPV-DNA sequences	12	50	65	7.5[3]	13.94

[1]Groups consisted of 17 patients with non-neoplastic lesions, 12 patients with CIN and 20 patients with cervical cancer. The means (± standard deviation) of the groups were 33.6 (± 9.9), 36 (± 10) and 46 (± 11) yrs, respectively. Data for all risk factors was available except for a single anti-HSV-2 antibody determination in the cervical cancer group.–[2]Relative risk of disease associated with risk factors calculated using the distribution of the factors among women with non-neoplastic lesions as the control group.–[3]Differences in distribution of risk factor statistically significant; $p < 0.05 > 0.01$.–[4]Difference in distribution of risk factor statistically significant; $p < 0.01$.

TABLE III – DISTRIBUTION OF VIRUS MARKERS IN RELATION TO RISK FACTORS

Risk factor	HPV-DNA sequences				Anti-HSV-2 antibodies			
	Non-neoplastic[1]		Neoplastic[2]		Non-neoplastic		Neoplastic	
Sex partner (number)								
1	3/12	25%	6/13	46%	0/12	0%	3/13	23%
⩾2	3/11	27%	13/19	68%	3/11	27%	10/18	56%
Age at first coitus								
⩽17 yrs	4/10	40%	11/17	65%	1/10	10%	9/17	53%
⩾18 yrs	2/13	15%	8/14	57%	2/13	15%	4/13	31%
Smoking								
Yes	0/1	—	5/12	42%	1/1	—	4/11	36%
No	6/22	7%	14/20	70%	2/22	9%	9/20	45%
Anti-HSV-2 antibody								
Yes	1/3	—	8/13	62%				
No	5/20	25%	11/18	61%				

[1]Non-neoplastic patients included those shown in Table II plus patients with condylomatous lesions of the cervix. –[2]Neoplastic patients included those with CIN and cervical cancer.
Results are presented as number positive/number tested and then % positive.

tained 39% of the viral genome and about 3.5 copies of viral DNA were present per diploid cell. Using similar methods, others were unable to confirm this observation in a limited number of samples. More recently, DNA from invasive cervical cancer tissue has been examined for HSV-2 DNA sequences by Southern blot hybridization using defined fragments of viral DNA as probes. Three of nine tumors were reported to contain viral DNA sequences in one report (Galloway *et al.*, 1983) while 1/8 tumors was positive for HSV-2 sequences in another study (Park *et al.*, 1983). Our observation of 2/13 samples with HSV-2 DNA sequences is in agreement with these reports, although our estimates of positivity may be low due to the number of tests excluded from analysis because of lack of positive hybridization with control HSV-2 DNA (0.3 genome equivalent). Park *et al.* (1983) found no HSV-2 sequences in DNA from normal cervices, human fetal tissue or ovarian cancer, using conditions similar to those employed to detect viral sequences in cervical cancer DNA. However, 1/12 samples from an inflammatory lesion of the cervix was found positive in our study and the occurrence of HSV-2 DNA sequences in cancer tissues was not significantly different from that of the control samples in the small numbers tested.

Several transforming regions of HSV-1 and HSV-2 genomes have been defined by *in vitro* experiments (Hayward *et al.*, 1983). For HSV-1, DNA located at 0.31-0.41 map units (mu) has been associated with transformation while, for HSV-2, DNA at 0.42-0.58 and at 0.58-0.62 mu can transform cells *in vitro*. Of the cancer specimens found to contain HSV sequences, all have reacted with probes representing at least one of these regions. The specimen reported by Park *et al.* (1983) reacted with a probe representing between 0.58 and 0.61 mu and 2 of the positive samples reported by Galloway and McDougall (1983) also reacted with probes from this genome region. These latter investigators also found positive reactions with probes representing between 0.32 and 0.40 mu which is compatible with the reactions we observed using the BglII J fragment (0.32-0.38 mu). These consistent findings suggest a unique interaction between these regions of the viral genomes and cells which could contribute to the neoplastic process. However, further investigation will be needed before the significance of the presence of these viral sequences is known.

A number of HPV types have been delineated by the relatedness of their genomes as determined by DNA-DNA hybridization (reviewed by Pfister, 1984). Different HPV types have been associated with certain body sites of infection and with the nature of lesions produced. HPV types 6, 11, 16 and 18 commonly infect the genital tract (Boshart *et al.*, 1984). Flat warts of the uterine cervix may contain cytological abnormalities considered to represent intraepithelial neoplasia (Meisels, 1981) and it has been argued on histological grounds that invasive cervical cancer represents a progression of subclinical papillomavirus infection (Reid *et al.*, 1984). Indeed, a number of investigators have found HPV-DNA in DNA extracted from intraepithelial neoplastic lesions (Zachow *et al.*, 1983; Green *et al.*, 1982; Gissmann *et al.*, 1983; McCance *et al.*, 1983; Durst *et al.*, 1983; Lancaster *et al.*, 1983). In the present study we found HPV DNA sequences, as detected under non-stringent conditions of hybridization, in a large proportion of biopsies from condylomatous lesions. This finding is in keeping with reports of others (Lancaster *et al.*, 1983; Boshart *et al.*, 1984; Gissmann *et al.*, 1982; Crum *et al.*, 1984). We also found HPV DNA in intraepithelial neoplastic lesions of the cervix and the incidence (50%) was similar to that reported by others. We found 68% of the biopsies from invasive cervical cancers positive for HPV-DNA sequences, in agreement with the observations of Durst *et al.*, (1983) who found 53% biopsies positive using the same HPV-16 probe and similar relaxed hybridization conditions. In our study all but one of the positive biopsies from cervical cancer cases contained HPV-16 as defined by reaction under stringent conditions of hybridization. This occurrence was significantly greater than in the 4/11 biopsies of other lesions which contained HPV sequences. These findings suggest that the DNA sequences in the HPV-16 probe correlate with invasive neoplasia and are compatible with the concept that a subset of subclinical papillomavirus infections caused by HPV-16 may be those that progress to invasive cancer (Crum *et al.*, 1984).

The double-stranded DNA molecules of the papilloma viruses are usually found in a circular superhelical form with both strands of DNA covalently closed or in a circular relaxed form resulting from one single-strand nick. A linear form resulting from a double-strand break is rarely detected in DNA extracted from

virus particles although such linear forms can be created by endonuclease digestion restricted to a single site. Viral DNA extracted from benign proliferative lesions has generally been found in one or more of these three forms, an observation supporting the concept that the viral DNA normally exists as an episome (reviewed by Pfister, 1984). We also found viral DNA bands thought to represent a form of unit length DNA in extracts of most benign lesions. However, heterogeneous bands, in terms of size and intensity, were observed in many specimens obtained from invasive cancers. Similar findings have been noted by others. For example, heterogeneity of HPV-6 DNA molecules was noted in 2/3 samples of locally invasive Buschke-Lowenstein tumors (Gissmann *et al.*, 1982). Recently, heterogeneity of HPV-18 DNA molecules in cervical cancer tissue was also reported by Boshart *et al.* (1984). While there are a number of possible explanations for the presence in the tissues of HPV-DNA molecules larger or smaller than expected for circular episomal DNA of the HPV type used for their detection, further investigations will be required to clarify the significance of these observations.

Epidemiological studies have defined early age at first intercourse, multiple sex partners, anti-HSV-2 antibodies and smoking as pivotal risk factors (Rotkin, 1973; Kessler, 1976; Rawls and Campione-Piccardo, 1981; Winkelstein *et al.*, 1984). Since our study was not designed as a case-control study, caution must be exercised in interpreting our observations regarding risk factors and their interactions. Further caution is required because of the small numbers tested. However, comparison with women harboring non-neoplastic lesions of the cervix revealed that the women with neoplastic lesions had risk factors consistent with their disease. Significant relative risk of invasive cervical cancer was associated with first coitus before 18 years of age (rr = 4.5), more than 1 sex partner (rr = 4.3), anti-HSV-2 antibodies (rr = 5.5), the presence of HPV-16 DNA sequences (rr = 13.9) and cigarette smoking (rr = 16). The occurrence of anti-HSV-2 antibodies and HPV-DNA sequences appeared to be related to early age at first coitus and multiple sex partners. The presence of HPV-DNA sequences was more frequent among non-smokers than among smokers. No correlation was observed between anti-HSV-2 antibodies and HPV-DNA sequences. The findings suggest multiple etiologies rather than synergism between these factors as postulated by zur Hausen (1982). However, because of the small numbers studied and because of the nature of the women used for controls upon which to evaluate risk factors, no conclusions can be drawn about the possible contributions of the various factors to the genesis of neoplastic lesions.

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