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A SERO-EPIDEMIOLOGICAL STUDY OF THE RELATIONSHIP BETWEEN SEXUALLY TRANSMITTED AGENTS AND CERVICAL CANCER IN HONDURAS

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To investigate a possible cause-and-effect relationship between sexually transmitted diseases and cervical cancer, we performed a sero-epidemiological study on the presence of antibodies against a number of sexually transmitted agents (STAs) in patients with cervical cancer and their matched controls. In this study, we used serological techniques to investigate the presence of antibodies to cytomegalovirus, herpes simplex virus type 2, human immunodeficiency virus, *Chlamydia trachomatis, Treponema pallidum* and human papillomavirus (HPV) early protein E7 in sera from patients with cervical cancer, cervical intra-epithelial neoplasia and individually matched, healthy controls. The presence of antibodies to infectious agents other than HPV appeared not to be associated with risk of cervical neoplasia in either univariate or multivariate analysis. After adjustment for cytology, schooling and presence of HPV DNA in cervical scrapes, there was a significantly higher prevalence of antibodies to HPV-16 E7 protein in sera from patients with cervical cancer (OR = 3.6, 95% CI 1.0–12.9) than in healthy controls. The highest antibody prevalence was found among HPV-16 DNA–positive cervical cancer patients (33%). Our results indicate that in these study groups past infections with the STA considered seems to be of no apparent relevance for cervical carcinogenesis and that the HPV-16 anti-E7 response appears to be associated with cervical cancer. *Int. J. Cancer* 73:781–785, 1997.
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Carcinoma of the cervix is the second most common type of cancer affecting women worldwide and the leading cause of death from cancer among women in developing countries (CDC, 1989). Worldwide death estimates of 450,000–500,000 women have been reported, of which almost 80% occur in developing countries.

Well-documented epidemiological studies have shown that early age at first sexual intercourse, a history of multiple partners, increased parity and a history of sexually transmitted diseases (STDs) are among the strongest risk factors associated with cervical intra-epithelial neoplasia (CIN) and cancer (Bosch *et al.*, 1992; Schiffman, 1992).

This association of cervical cancer with sexual activity has been recognized since the early nineteenth century. The work of Beral (1974) presented important new observations and provided additional evidence that exposure to sexually transmitted infection was an important determinant for cervical cancer. In the 1960s and 1970s, herpes simplex virus type 2 (HSV-2) infections were implicated directly in human cervical carcinogenesis; however, the lack of association of this virus with the development of cervical neoplasia in prospective epidemiological studies shifted investigations to human papillomavirus (HPV) (Vonka *et al.*, 1984).

Epidemiological studies using PCR-based techniques have demonstrated clearly that certain types of HPV, mainly HPV-16, are the primary causative agents of cervical cancer and its precursor lesions (Melchers *et al.*, 1994; Muñoz *et al.*, 1992). HPV DNA is detected in 80–90% of cervical carcinomas, and the majority of those cases harbor HPV-16.

Although a large number of women without cervical abnormalities are infected with HPV, only a minority of these women will ultimately develop cervical cancer. Consequently, one or more additional events are necessary besides HPV infection for progression to malignancy (Bosch *et al.*, 1992).

Several sexually transmitted agents (STAs) other than HPV have been suggested independently to be implicated in the initiation of carcinogenesis, including *Neisseria gonorrhoeae, Treponema pallidum, Chlamydia trachomatis,* HSV-2, cytomegalovirus (CMV) and *Trichomonas vaginalis* (Jha *et al.*, 1993), but the significance of these associations remains to be established. Some studies have reported that women infected with human immunodeficiency virus (HIV) are more likely to develop cervical dysplasia (Ferrera *et al.*, 1997), and this association is stronger for women with a low $CD4^+$ T-lymphocyte count (Maiman *et al.*, 1990).

Epidemiological data show associations in which cervical cancer and its precursor lesions are promoted by at least one and perhaps several STDs. Serological methods which accurately reflect the presence or absence of past exposures to these individual agents are an ideal way to assess exposure in epidemiological studies of cervical neoplasia.

Cervical cancer is the most prevalent cancer of women in Honduras, and STDs are highly prevalent in the country. Early exposure of the immature cervical epithelium to STDs, the trauma of repeated childbirth and multiple sexual partners in women whose defense factors are impaired by malnutrition add to the risk of developing cervical dysplasias in the country.

To investigate a possible cause-and-effect relationship between STDs and cervical cancer, we performed a sero-epidemiological study on the presence of antibodies against a number of STAs in patients with cervical cancer and their matched controls.

METHODS

Study group

This study is part of a larger case-control study on cervical cancer in Honduras. Women participating in the study were derived from public hospitals, communal health-care centers, family-planning and gynecology clinics serving the population of Tegucigalpa, the capital city of Honduras. Women with cervical lesions were referred to San Felipe General Hospital, a referral center for such patients in the country, where they were enrolled into the study. Cases were women aged 20–65 years with different grades of histologically confirmed CIN and invasive cervical cancer diagnosed between 1993 and 1995. Controls with a normal cervix, two per case, were selected from the clinic where the case was first seen and matched to cases according to a 5-year interval of

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age, with a maximum recruitment time of 1 month from the date of case diagnosis. These women attended these clinics to receive routine cervical cytological screening. In general, women either may be referred for screening by their doctor or may request screening on their own.

In both groups, after formal consent, a smear was made for cytological examination, which was classified according to the CIN classification system (OPS, 1990). The remaining material and an additional scrape were collected in 5 ml PBS, 0.05% merthiolate, pH 7.5, and used for DNA extraction and PCR HPV assays. A 10–15 ml blood sample was taken from each woman to measure antibodies against the most common STA. Sera were separated, divided into aliquots and stored at −20°C.

All study subjects were interviewed using a structured questionnaire given by specially trained personnel for the project. Information was obtained on demographic variables, sexual and reproductive histories and previous cytological screening. Screening events for the analysis were computed by reducing by 1 if the most recent cytological screening was reported to have taken place within 12 months prior to the interview.

HPV markers

DNA extraction and HPV-PCR assays. HPV DNA sequences were sought in cytological specimens obtained by cervical scraping by PCR amplification. Briefly, scrapes were vortexed, centrifuged, washed, pelleted and resuspended in 0.5 ml PBS. DNA extraction was performed according to the standard SDS-proteinase K-phenol-chloroform method, as previously described (Melchers *et al.*, 1989).

All samples were pre-screened with the β-globin primers PCO3/PCO4 (Saiki *et al.*, 1985) to assess sample integrity. Then, a general primer-mediated PCR (GP-PCR) strategy was followed for analysis of samples for the presence of HPV DNA. The overall presence of HPV was assessed using a general primer set (MY11, primer for the positive strand, and MY09, primer for the negative strand) directed against the L1 open reading frame, which amplifies a fragment of about 450 bp of at least 25 distinct genital HPVs as well as unidentified HPV types (HPV X) (Manos *et al.*, 1989). After low-stringency Southern blot analysis with a generic probe mix, PCR-positive specimens were subjected to consecutive HPV type-specific PCRs (TS-PCRs) (Melchers *et al.*, 1989). Each amplification test in both GP-PCR and TS-PCR included one tube with Siha cells as a positive control and one tube with distilled water as a negative control. To prevent contamination, strict spatial partitioning of the different technical steps of the PCR was done, and the recommendations of Kwok and Higuchi (1989) were followed.

Enzyme immunoassay with synthetic peptide. A synthetic peptide of HPV-16 E7 protein (amino acids 6–35) was used in an ELISA to screen a subset of 147 CINs and invasive cervical cancers and 294 controls for antibodies against HPV-16 E7 (Baay *et al.*, 1995). The cut-off value was obtained from the testing of 29 children's sera on E7/6–35, using the mean absorption plus three times the standard deviation. Each sample was tested in triplicate, and known positive, negative and borderline-positive sera were included in each plate. Results were expressed as a ratio (mean absorbance of patient serum divided by mean absorbance of cut-off serum). Sera showing a ratio higher than 1.0 were considered positive.

Serological detection of STDs

The presence of serum IgG antibodies (indicative of past infection) to the following infectious agents was determined by commercially available ELISA kits: *C. trachomatis* (Chlamydien IgG rELISA; Medac, Hamburg, Germany), which detects antibodies against *Chlamydia*-specific lipopolysaccharides (LPSs); CMV (Gull, Salt Lake City, UT), which shows high sensitivity and specificity; HSV-2 (Gull), which uses a purified HSV-2 antigen (St. Jeor strain); HIV-1 (HIVAB-HIV-1 EIA; Abbott, North Chicago, IL), with positive results confirmed by Western blot (Cambridge Biotech, Worcester, MA). Antibodies to syphilis were tested by a microhemagglutination assay (TPHA; Fujirebio, Tokyo, Japan) specific for antibodies against *T. pallidum* in human serum.

Statistical analysis

To estimate the association between cervical cancer and the different markers of STDs, we used conditional logistic regression to compare the prevalence of STAs in cases and controls and to calculate odds ratios (ORs) and 95% confidence intervals (CIs). A set of adjustment factors were included in the model to control for possible confounding effects.

RESULTS

A subset of serum samples from a total of 667 women included in the case-control study were analyzed for an association between antibodies to various STAs and cervical neoplasia. For the remaining women, not enough serum sample was available for testing. Patients and controls were similar regarding age. The mean age for cases was 45.9 years and that for controls, 45.2 years; less than 20% of cases and controls reported having had 4 or more sexual partners. Demographic data indicated that a large proportion of our study subjects were economically disadvantaged, with family incomes of less than $1,000 per year. In general, cases had not attained as high an educational level as controls and had a lower mean annual income.

Table I shows, for each of the 4 study groups, the total number of women examined and the percentages of seroreactivity to each of 5 antigens, as well as the antibody response to more than one STA.

TABLE I – SEROPREVALENCE OF INFECTIOUS AGENTS IN WOMEN WITH DYSPLASIA, CERVICAL CANCER AND CONTROLS

Agent	CIN I % seropositive (N)[1]		CIN II % seropositive (N)[1]		CIN III % seropositive (N)[1]		Cancer % seropositive (N)[1]	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
CMV	100 (9)	100 (18)	100 (8)	100 (15)	95.8 (24)	91.7 (48)	89.8 (49)	94.7 (95)
C. trachomatis	55.6 (9)	61.1 (18)	62.5 (8)	78.6 (14)	80 (25)	68 (50)	70 (50)	62.1 (95)
HSV-2	100 (9)	100 (18)	100 (8)	100 (15)	100 (25)	92 (50)	97.9 (48)	95.7 (93)
T. pallidum	16.3 (43)	9.3 (86)	17.1 (35)	9.1 (66)	8.9 (45)	7.0 (86)	18.3 (104)	11.9 (194)
HIV	4.7 (43)	0 (86)	5.6 (36)	0 (67)	0 (45)	2.3 (86)	0 (104)	0.5 (195)
Multiple infections[2]	11.1 (9)	0 (18)	37.5 (8)	7.1 (14)	4.0 (25)	4.0 (50)	14.3 (49)	11.6 (95)

[1]N = Total number of sera tested from patients and controls.–[2]*C. trachomatis* and *T. pallidum.*

Prevalence of STA antibodies was generally more common among cases than controls, the difference being more marked among the CIN III and invasive cancer groups (the exception being antibodies to HIV in both groups). Among cases and controls in all groups, the most common STA antibodies detected were CMV and HSV-2, with a positivity higher than 89.8% and 92%, respectively. Antibodies to *C. trachomatis* were higher in cases of CIN III and invasive cancer than in corresponding controls. Syphilis was not a rare infection in the entire studied group, with the highest seroprevalence encountered in the invasive cancer patients and their controls (18.3% and 11.9% for cases and controls, respectively). The presence of antibodies to HIV was more common among cases (1.8%) than controls (0.7%), with an overall seroreactivity of 1.1% in the studied group. Multiple seroreactivity to individual agents was exceptionally high for the 8 CIN II cases. However, this interactive effect decreased in CIN III and cervical cancers patients.

Table II shows the crude ORs and 95% CIs for the presence of antibodies to CMV, *C. trachomatis*, HSV-2 and *T. pallidum;* we also assessed any possible increased risk of developing disease after adjusting for HPV DNA and other probable confounding factors based on our analysis of risk factors (data not shown). This analysis gave us the minimal set of risk factors significantly related to disease status. This minimal set was different for CIN I/II, CIN III and invasive cancer.

The presence of antibodies to any of the infectious agents tested appeared not to be associated with risk of cervical malignancy in either univariate or multivariate analysis. A 2-fold increased risk of CIN III was observed for women with antibodies to *C. trachomatis* even after adjustment for HPV DNA, though it was statistically not significant. In regard to HSV-2 in the invasive cancer group, adjusting for HPV DNA alone made the association of HSV-2 and disease status disappear. Serological evidence of previous syphilis conveyed an increased risk of developing dysplasia and cervical cancer. In cervical cancer patients, the risk was still 2-fold higher after HPV infection was considered; however, it did not reach statistical significance.

To evaluate the association of antibodies against HPV-16 E7 protein with CIN I, II, III and invasive cancer patients and their matched controls, we tested a subset of their sera in a synthetic peptide ELISA (amino acids 6–35). Twenty of the controls did not give consistent results on repeated testing. These sera were scored as equivocal and excluded from statistical analysis.

On PCR analysis, there were 139 HPV-16-related-positive subjects (types 16, 31, 33, 35, 52, 58; 85 patients, 54 controls). Seventy-five subjects were positive for other HPVs (types 6, 11, 18, 45, 53, 55, 56, 59, 62, 66, 69, X; 31 patients, 44 controls). There were 207 subjects in the HPV-negative group (31 patients, 176 controls). Antibodies to synthetic peptide E7 were detected more frequently in HPV-16-related-positive patients than in those positive for other HPVs or HPV DNA-negative. There were 219 HPV-16-DNA-negative cases with serological response. The majority of control samples in which seroreactivity was revealed did not harbor HPV 16.

Table III presents the risk estimates for cervical dysplasias and invasive cancer in relation to reactivity to synthetic peptide E7 of HPV 16. ORs are presented, adjusted by HPV DNA, schooling and previous cytology. Although not statistically significant, the association between seroreactivity to HPV-16-derived peptide was related more clearly to CIN III and invasive cervical cancer than to the mild dysplasias (OR = 1.92, 95% CI = 0.4–8.6; OR = 1.6, 95% CI = 0.8–3.2; OR = 1.27, 95% CI = 0.3–4.8, respectively). The almost 8-fold increased risk conferred to the CIN III group after adjustment for the presence of HPV DNA can be explained by the fact that only 5 cases in this group had an HPV-negative PCR; consequently, this is without statistical relevance. On the contrary, a significant increased risk for cervical cancer was noted when cytology, schooling and presence of HPV DNA in cervical scrapes was taken into consideration (OR = 3.6, 95% CI = 1.0–12.9).

DISCUSSION

The increased awareness of the possible interaction of STDs with the development of cervical cancer has special significance for Honduras, where these diseases, whose effective control will significantly reduce the morbidity and mortality they cause, are highly prevalent.

Epidemiological studies have supported the idea that synergism between multiple infections may be a cause of the development of cervical cancer (Dillner *et al.*, 1994) and that long-standing infections will promote cervical malignancy through chronic irritation and, thus, a higher possibility of HPV infection. To gain insight for these observations in this country, we examined the role of several STAs in cervical neoplasms and the possible cooperation of HPV with other viruses and bacteria in developing genital lesions.

Even though many investigations have focused on STAs and their implication in carcinogenesis, no consistent picture has emerged for an etiological role of infectious agents other than HPV in the development of cervical cancer (Brinton, 1992). Indeed, our data suggest that seropositivity for CMV, *C. trachomatis,* HSV-2, *T. pallidum,* HIV and non-HPV multiple infections were not significantly more common in women with dysplasia and cervical cancer than in controls.

Our findings are similar to the prospective study of Hakama *et al.* (1993), which reported no association with risk of cervical malignancy for such infectious agents as CMV, HSV-2, Epstein-Barr virus and *N. gonorrhoeae,* though they showed a significant 5-fold increased risk for *C. trachomatis* and development of cervical cancer. Nevertheless, they claimed that this result might

TABLE II – ASSOCIATIONS OF INFECTIOUS AGENTS WITH RISK OF CERVICAL CANCER

Agent	CIN I/II OR (95% CI)	CIN III OR (95% CI)	Cervical cancer OR (95% CI)
CMV	—	2.3 (0.2–24.4)	0.3 (0.06–1.8)
		1.6 (0.1–22.6)[1]	0.42 (0.05–3.37)[1]
			0.19 (0.00–6.8)[2]
C. trachomatis	0.71 (0.23–2.23)	1.9 (0.6–5.8)	1.37 (0.67–2.82)
	0.28 (0.04–2.23)[1]	2.0 (0.5–7.9)[1]	0.95 (0.36–2.50)[1]
	0.24 (0.03–2.08)[3]		0.24 (0.04–1.45)[2]
HSV-2	—	—	2.3 (0.22–24.4)
			0.80 (0.04–15.1)[1]
			0.16 (0.0–36.6)[2]
T. pallidum	2.01 (0.88–4.6)	1.3 (0.3–4.9)	1.84 (0.89–3.8)
	1.93 (0.83–4.50)[1]	1.4 (0.2–9.4)[1]	2.13 (0.94–4.82)[1]
	1.73 (0.72–4.16)[3]		0.76 (0.20–2.82)[2]

[1]Adjusted for presence/absence of HPV.–[2]Adjusted for HPV, schooling and cytology.–[3]Adjusted for HPV and schooling.

TABLE III – ANTIBODIES TO HPV-16 E7 PROTEIN IN SERA OF CERVICAL NEOPLASIA CASES AND CONTROLS

	% Seropositive (N)[1]		OR (95% CI)	OR (95% CI)
	Cases	Control		
CIN I	11.8 (17)	12.1 (33)	1.27[2] (0.3–4.8)	1.91[2] (0.43–8.4)[3]
CIN II	16.7 (12)	4.5 (22)		
CIN III	15.2 (33)	10.3 (58)	1.92 (0.4–8.6)	7.7 (0.7–88.1)[4]
Cervical cancer	22.4 (85)	13.7 (161)	1.6 (0.8–3.2)	3.6 (1.0–12.9)[5]

[1]N = Total number of sera tested from patients and controls.–[2]CIN I/II.–[3]Adjusted for HPV and schooling.–[4]Adjusted for HPV.–[5]Adjusted for cytology, schooling and HPV.

have been biased by the *C. trachomatis* assay cross-reacting with antibodies to *C. psittaci/C. pneumoniae* and by the study population sample being too small. In our study, after taking HPV DNA status into consideration, a non-significant 2-fold increased risk was seen for women with *C. trachomatis* antibodies in the CIN III category. Our analysis could have been hampered by the IgG recombinant ELISA used since the *Chlamydia*-specific fragment of the LPS employed as antigen is genus- and not species-specific, and we cannot override cross-reaction with *C. psittaci/C. pneumoniae* as well.

Of particular interest is the relationship of risk to infection with HSV-2, which has been supported by numerous case-control studies examining the relationship between this virus and cervical cancer (Brinton, 1992). In a study of STDs and other risk factors for cervical dysplasia among southwestern Hispanic and non-Hispanic white women, Becker *et al.* (1994) found that antibodies to HSV-2 were associated significantly with dysplasia among non-Hispanic whites compared to a lack of association among Hispanic women. Muñoz *et al.* (1995) used type-specific glycoproteins in an HSV-2 ELISA and, contrary to the results of Hildesheim *et al.* (1991), concluded that the role of HSV-2 is merely marginal; their results did not support the hypothesis that recurrent HSV-2 infections are of importance for cervical neoplasia. In our study, the seroprevalence of HSV-2 and CMV was extremely high in both cases and controls and was not associated significantly with incidence of cervical neoplasms. The apparent association seen for HSV-2 and the risk of developing cervical cancer was entirely due to confounding by HPV DNA. We cannot rule out, however, a lack of specificity of the assay used to detect HSV-2 infection due to possible cross-reactivity with HSV-1.

Infections with *T. pallidum* are chronic and exhibit degrees of latency. Furthermore, the spectrum of these infections may elicit different patterns of antibody response. At present, none of the syphilis serological tests are 100% specific for antibodies to *T. pallidum* and positive reactions may be seen in patients infected with other pathogenic treponema, as in pinta and yaws, which are prevalent in Central America. In our group of subjects, the effect of syphilis on the risk of developing cervical cancer disappeared when adjustment for previous screening was introduced in the logistic model. We can speculate that, in general, women with syphilis are screened less often.

Contrary to the well-established association of certain HPV types with development of cervical cancer (Muñoz *et al.*, 1992), the humoral immune response against HPV remains to be clarified and fully characterized. As yet, there are no standardized serological assays to identify HPV infection.

The HPV-16 viral oncoproteins E6 and E7 bind to the products of tumor-suppressor genes and are the only viral proteins uniformly retained and expressed in cervical cancer cells, being capable of synergistically immortalizing human and rat cells *in vitro* (Watanabe *et al.*, 1989). In fact, several studies have shown that antibodies to HPV-16 E7 are present at higher prevalence in sera from cervical cancer patients than in those from healthy controls (Müller *et al.*, 1992). However, the clinical significance and HPV type specificity of these responses remain dubious.

In this study, we have analyzed sera from a subsample of cases with CIN and cervical carcinoma and from their control group for the presence of antibodies against a synthetic peptide containing known B-cell epitopes of HPV-16 E7, as well as for the presence of HPV DNA in cervical scrapes. In agreement with earlier studies (Müller *et al.*, 1992), our results indicate that anti-E7 antibodies were associated independently with cervical cancer cases. In this group, we detected an overall prevalence rate of antibodies against peptide E7/6–35 of 22.4% among cases *vs.* 13.7% among controls for an adjusted OR of 3.6 (95% CI 1.0–12.9). No significant differences were found for seroreactivity between patients with different grades of CIN. Baay *et al.* (1995), using HPV-16 E7 synthetic peptide in an ELISA identical to ours, found a prevalence rate of 17.7% in patients with cervical cancer in a study correlating clinicopathological parameters and antibody status to HPV-16 E7 protein. In a similar way, Müller *et al.* (1992), using a synthetic peptide also comprising amino acids 6–35, reported a prevalence rate of 37% in HPV-16 DNA–positive cervical cancer patients. In our current study, despite the higher prevalence of E7 reactivity among HPV-16 DNA–positive cervical cancer patients (33%), the correlation between HPV DNA status and the presence of anti-HPV-16 antibodies remained low (data not shown). We can infer from this last finding that only a proportion of HPV-16-infected individuals generate antibodies to this type-specific reactive epitope of the E7 protein, which in turn reflects the still unclear dynamics of seroreactivity to HPV infections.

Even though antibodies to *C. trachomatis* and *T. pallidum* showed the strongest associations, although statistically not significant with cervical cancer, our results are not consistent enough for drawing conclusions as to the STA other than HPV with which cervical cancer is associated most specifically. However, it cannot be excluded that genital infections with other STAs may play a separate etiological role in cervical carcinogenesis, and more definite conclusions would require a study of much larger populations. Furthermore, our data imply that even though E7 antibodies can help discriminate between cancer patients and minor dysplasias, further studies are needed to map more specific epitopes that will define more clearly clinical and HPV type specificity.

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