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Class-specific herpes simplex virus antibodies in sera and cervical secretions from patients with cervical neoplasia: a multi-group comparison

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SUMMARY

Serum and cervical secretions were collected from patients with cervical dysplasia, carcinoma-in-situ (CIS), squamous cell carcinoma (cervical SCC), and controls with normal cervices, attending clinics within the West Lambeth Health District, London. Enzyme-linked immunosorbent assays were used to examine cervical secretory IgA (sIgA) and serum IgG and IgA antibodies to herpes simplex virus (HSV). Sexual and demographic factors were considered during data analysis, which involved fitting multiple linear or multiple logistic regressions to HSV antibody levels. Prevalence of sIgA-HSV and levels of serum antibodies to HSV in all groups were compared with those of gynaecology controls. Caucasian women with mild dysplasia had a significantly higher prevalence of sIgA-HSV. Serum IgG levels to HSV (IgG-HSV) were significantly elevated in women with mild dysplasia and severe dysplasia/CIS. Serum IgA levels to HSV1 (IgG-HSV1) were significantly higher in women with cervical SCC (after adjusting for smoking habits) and other genital tumours. Significantly higher levels of serum IgA to HSV2 (IgA-HSV2) were also found among Caucasian women with cervical SCC. The possible role of HSV as a co-factor in cervical carcinogenesis is discussed.

INTRODUCTION

Numerous sereoepidemiological studies have shown that patients with cervical SCC have an increased prevalence and higher titres of antibodies to herpes simplex virus type 2 (HSV2) than controls (reviewed by Nahmias & Sawanabori, 1978; Rawls & Campione-Piccardo, 1981) suggesting that HSV2 may play a role in the aetiology of cervical cancer. Previous studies at St Thomas's Hospital showed that

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a significantly higher proportion of such patients from Britain, Sri Lanka and Malawi had HSV2 antibodies when compared to controls matched for age, ethnic origin and social class. In addition, patients with cervical dysplasia or neoplasia had a significantly higher prevalence and higher titres of serum IgA antibodies to membrane antigens of HSV2-infected cells than matched controls (Mendis, Best & Banatvala, 1981; Mendis *et al.* 1981). Serum IgA antibodies may reflect local IgA antibody production, but few studies have examined the local IgA response to HSV in cervical secretions from patients with cervical dysplasia and neoplasia. Dent & Bienenstock (1974), using indirect immunofluorescence, were unable to detect HSV-specific IgA or IgM in the cervicovaginal washings of patients with cervical SCC, although IgG-HSV was detectable in the washings from 37% of patients and 32% of controls. More recently, IgA antibodies to HSV2 were detected by radioimmunoassay in cervical secretions from 21% of patients with cervical dysplasia and 57% of patients with cervical cancer, but not in normal controls (Kalimo *et al.* 1981).

We have examined cervical sIgA, serum IgG, and serum IgA antibodies to HSV in patients with cervical dysplasia, neoplasia, and controls. The IgA responses to HSV-1 and HSV-2 antigens were measured separately as it has been suggested that the IgA response to HSV may be more type-specific than the IgG response (Mendis, Best & Banatvala, 1981). HSV-1, as well as HSV-2, has been isolated from genital lesions (Guinan, Wolinsky & Reichman, 1985); it is therefore possible that HSV-1 may be involved in the aetiology of some cervical cancers. Factors associated with increased risk of cervical cancer, such as low social class, early age at first intercourse and multiple sexual partners (Rawls *et al.* 1976; Harris *et al.* 1980; Graham *et al.* 1982), have been taken into account during data analysis, as well as age, ethnic origin, number of pregnancies and age at first pregnancy. All such factors have not always been included in previous studies. Since it was not possible to match patients and controls directly for so many factors, analysis of covariance was used to analyse the results.

MATERIALS AND METHODS

Study population

Sera and cervical secretions were collected from women attending clinics at St Thomas's Hospital (STH) and in the West Lambeth Health District, London. The majority of the specimens were collected between 1982 and 1984.

Specimens were obtained from patients with mild dysplasia, severe dysplasia or CIS (confirmed by colposcopically directed biopsy) and cervical SCC (histologically confirmed) (Table 1). Specimens were also collected from patients with gynaecological malignancies other than cervical SCC (Table 1).

Specimens were collected from control women with normal cervical smears attending gynaecology clinics with conditions other than neoplasia (gynaecology controls), women attending a family planning clinic (family planning controls) and women attending genito-urinary (GU) medicine clinics for conditions other than genital herpes (Table 1).

Details of age, ethnic origin, social class, age at first intercourse, number of sexual partners, age at first pregnancy and total number of pregnancies were

Table 1. *Study population and specimens collected*

Groups	Number of individuals	Sera and cervical secretions available	Cervical secretions only available
A. Cervical dysplasia and neoplasia			
Mild dysplasia	38	34	4
Severe dysplasia carcinoma in situ	49	41	8
Invasive squamous cell carcinoma	54	29	25
Total	141	104	37
B. Controls			
1. Other genital tumours			
Adenocarcinoma of cervix	6	4	2
Adenocarcinoma of the endometrium	15	10	5
Others	7	3	4
Total	28	17	11
2. Women without tumours			
Gynaecology controls	99	67	32
Family planning controls	32	17	15
G-U medicine controls	42	24	18
Total	173	108	65
Total studied	342	229	113

obtained from all patients and controls using a standard questionnaire. Information on smoking habits was obtained from 288 of the 342 women (84%) in the study.

Specimens

Cervical secretions were collected by insertion of a cellulose sponge into the external cervical os for approximately 15 s (Treharne *et al.* 1978). The sponges were then stored at −70 °C. The secretions were eluted from the sponges with 0·7 ml phosphate buffered saline (PBS) before testing. This eluate was taken as a 1 in 8 dilution. Cervical secretions were tested for the presence of blood using Haematest Reagent tablets (Miles Ames Division, Miles Laboratories Inc, IN, USA).

Enzyme-linked Immunosorbent Assays (ELISAs)

Antigens were prepared by Triton X-100 extraction of HEp-2 cells infected with HSV1 (F strain) or HSV2 (G strain). Control antigen was prepared from uninfected HEp-2 cells (Coleman *et al.* 1983).

Serum IgG antibodies to HSV (IgG-HSV)

A modification of the ELISA described by Coleman and colleagues (1983) was used. Wells of microtitre plates were coated with 100 μl of HSV or control antigens diluted in carbonate buffer pH 9·6 and incubated at 4 °C overnight in a humidified chamber. The antigen-coated plates were then washed three times with PBS (pH 7·3) containing 0·05% Tween 20 (PBS–Tween).

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Sera were tested at a dilution of 1/50 in PBS–Tween containing 1% bovine serum albumin (PBS–Tween BSA). Two HSV antibody-positive and three negative control sera were included on each plate. A 100 μl volume of each diluted serum was added to two wells coated with control antigen and two wells coated with viral antigen and incubated at 37 °C for 2 h in the humidified chamber. Plates were then washed three times as before. 100 μl volumes of horseradish peroxidase (HRP)-conjugated goat antihuman IgG specific for γ-chains (Miles Laboratories Inc, Elkhart, IN, USA), diluted 1/3000 in PBS–Tween BSA, were added to each well and incubated at 37 °C for 45 min. After washing the plates four times with PBS–Tween, 100 μl of substrate solution (40 mg *o*-phenylene diamine dihydrochloride (OPD) and 100 μl of 30% H_2O_2 in 100 ml of substrate buffer containing 36·8 mM citric acid and 50·1 mM $Na_2HPO_4.12H_2O$) were added to each well. The reaction was terminated with 50 μl 2M H_2SO_4 after 10 min. The absorbance of each well was measured at 492 nm on a Titertek Multiscan (Flow Laboratories, Rickmansworth).

*Serum IgA antibodies to HSV*1 (*IgA-HSV*1) *and HSV*2 (*IgA-HSV*2)

The ELISA was modified to detect IgA-HSV1 and IgA-HSV2 separately, by employing HRP-conjugated goat antihuman IgA specific for γ-chains (Miles-Yeda Ltd, Miles Laboratories Inc, Elkhart, IN, USA).

Total sIgA in cervical secretions

Wells of microtitre plates were coated with rabbit antihuman IgA (Behring Diagnostics, Hoechst UK Ltd, Hounslow). After washing, diluted cervical specimens were added, and incubated at 37 °C for 3 h. Following further washing, HRP-conjugated rabbit antihuman secretory component (Dako Ltd, High Wycombe) was added and incubated at 37 °C for 1 h. The plates were again washed, the substrate solution added, and the reaction terminated as described above.

Since no standard preparation of cervical secretory IgA was available, a pool of cervical secretions obtained from 10 patients with high levels of total sIgA was used to construct a standard curve, plotting absorbance against secretion pool diluted in PBS–Tween/BSA. Test specimens with specific absorbance higher than that given by a 1/4096 dilution of the secretion pool were considered positive for total sIgA.

Secretory IgA antibodies to HSV (*sIgA-HSV*) *in cervical secretions*

A modified ELISA was performed based on that described for IgG-HSV. Cervical secretions were incubated on the antigen-coated wells for 3 h at 37 °C. Diluted HRP-conjugated rabbit antihuman secretory component (SC) (Dako Ltd, High Wycombe, UK) was added to each well and incubated at 37 °C for 1 h. The reaction with substrate solution was terminated after 30 min.

The specificity of the conjugated antiserum for secretory component was confirmed by testing 20 sera which were known to be positive for IgA-HSV. None of these sera gave positive reactions when anti-SC conjugate was used, confirming that this conjugate does not cross-react with the heavy chain of IgA.

Calculation of results

Specific absorbance for each specimen was calculated as the mean absorbance given by the serum with the HSV antigen minus the mean absorbance given with the control antigen. Three negative control specimens were used to determine a 'cut-off' value, defined as the mean specific absorbance of these three negative control specimens plus three standard deviations from this mean. Specimens giving a specific absorbance above the cut-off value were regarded as positive and those at or below the cut-off value as negative. For serum antibodies, ELISA values (E values) were taken as the specific absorbance minus the 'cut-off' value.

Statistical analysis

Analysis of covariance was used to compare the HSV antibody levels of the seven study groups allowing for the possible effect of other independent variables. The independent variables included in the analysis were age (quantitative variable), ethnic origin (Caucasian/Black/Asian/other), social class (non-manual/unemployed/student), age at first intercourse (< 18, 18–24, $\geqslant 25$, never, not known), number of sexual partners (none, 1, 2–5, 6–9, $\geqslant 10$, not known), age at first pregnancy (< 18, 18–24, $\geqslant 25$, never pregnant, not known), number of pregnances (quantitative variable). Information on smoking habits was collected from 288 to 342 of patients (84%) in this study, who were classed as current smokers, past smokers and those who had never smoked. The data were reanalysed for this group of women allowing for effects of all significant independent variables.

The analysis was performed using the computer programme GLIM (Baker & Nelder, 1978). This involved fitting a multiple linear regression to the HSV antibody levels, with the exception of sIgA to HSV where multiple logistic regression was used.

Interactions between study groups and the independent variables were tested to determine any differing effects of the independent variables on the HSV antibody levels within the seven study groups. Those independent variables which did not have an interaction with group, but had a significant effect, at the 5% level, on the HSV antibody levels, were then taken into account when comparisons between groups were made. All groups were compared to the gynaecology control group during data analysis.

RESULTS

Serum IgG-HSV, IgA-HSV1, IgA-HSV2

IgG-HSV was detected in sera from 83–95% of women tested (Table 2). Serum IgA-HSV1 or IgA-HSV2 were detected in 76–93% of women tested (Table 2).

Levels of IgG-HSV were found to increase with age overall. After allowing for independent variables, patients with mild dysplasia and severe dysplasia/CIS had significantly higher levels of IgG-HSV than gynaecology controls ($P < 0{\cdot}05$) (Table 3).

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Table 2. *Prevalence of serum antibodies to HSV antigens**

Groups	No. positive/No. tested (%) IgG	IgA
A. Cervical dysplasia and neoplasia		
Mild dysplasia	29/34 (85)	26/34 (76)
Severe dysplasia carcinoma in situ	39/41 (95)	38/41 (93)
Invasive squamous cell carcinoma	27/29 (93)	27/29 (93)
B. Controls		
1. Genital tumours (Adenocarcinoma of endometrium and other genital tumours)	15/77 (88)	15/17 (88)
2. Women without tumours		
Gynaecology controls	60/67 (90)	54/67 (81)
Family planning controls	15/17 (88)	14/17 (82)
G-U medicine controls	20/24 (83)	22/24 (92)

* For IgG combined HSV1 and HSV2 antigen was used. For IgA, antigens were tested separately; the values given are for HSV2 antigen, since prevalences of antibodies to HSV1 antigen were similar.

Table 3. *Adjusted E values of serum IgG antibodies to HSV antigens*

	Adjusted mean E values allowing for independent variables (standard error)
A. Cervical neoplasia	
Mild dysplasia	1·94* (0·17)
Severe dysplasia carcinoma in situ	1·91* (0·16)
Invasive squamous cell carcinoma	1·67 (0·23)
B. Controls	
1. Genital tumours (Adenocarcinoma of endometrium and other genital tumours)	1·30 (0·28)
2. Women without tumours	
Gynaecology controls	1·50 (0·15)
Family planning controls	1·53 (0·24)
G-U medicine controls	1·24 (0·20)

* Significantly higher mean E values when compared to gynaecology controls ($P < 0·05$).

When serum IgA-HSV1 levels were analysed, the interaction of study group with age was found to be significant, that is, the relation of specific serum IgA-HSV levels with age differed between study groups. Further analysis revealed that for patients with severe dysplasia/CIS and cervical SCC, IgA-HSV1 levels significantly increased with age; IgA-HSV1 levels were not associated with age in the other study groups. Of the groups for which no association of IgA-HSV1 with age was found, patients with genital tumours other than cervical cancer had a significantly higher mean level of serum IgA-HSV1 than gynaecology controls

Table 4. *Adjusted levels of serum IgA antibodies to HSV antigens*

	IgA-HSV1 Adjusted mean E value allowing for independent variables (standard error)	IgA-HSV2 Adjusted mean E value for Caucasians allowing for independent variables (standard error)
A. Cervical neoplasia		
Mild dysplasia	0·15 (0·03)	0·18 (0·06)
Severe dysplasia carcinoma in situ	0·21* (0·03)	0·21 (0·05)
Invasive squamous cell carcinoma	0·10* (0·06)	0·33† (0·05)
B. Controls		
1. Genital tumours (Adenocarcinoma of endometrium and other genital tumours)	0·36† (0·07)	0·27 (0·06)
2. Women without tumours		
Gynaecology controls	0·18 (0·03)	0·17 (0·03)
Family planning controls	0·20 (0·05)	0·17 (0·07)
G-U medicine controls	0·13 (0·04)	0·10 (0·07)

* In these groups there was a highly significant positive association of IgA-HSV1 levels seen in other groups; it was not possible to give an overall comparison of IgA-HSV1.

† Significantly higher mean E values when compared to gynaecology controls ($P < 0·05$).

($P < 0·05$). When investigated separately the 10 patients in this group with adenocarcinoma of the endometrium from whom sera were obtained were not found to have significantly different levels if IgA-HSV1 compared to gynaecology controls.

When IgA-HSV levels were re-analysed for the 288 patients for whom smoking data were available, the interaction of group with age was no longer significant and IgA-HSV1 levels could be compared in all groups. Patients with both cervical SCC and other genital tumours were then found to have significantly higher levels of IgA-HSV1 than gynaecology controls ($P < 0·05$).

When serum IgA-HSV2 levels were analysed, a significant interaction between group and ethnic origin was found. Further analysis was therefore performed for Caucasians only, since the number of non-Caucasian patients was small. After allowing for the effects of other independent variables, patients with cervical SCC were found to have significantly higher levels of IgA-HSV2 than gynaecology controls (Table 4).

Total sIgA and sIgA-HSV in cervical secretions

Only 31 of 54 specimens from patients with cervical SSC contained detectable total sIgA, although total sIgA was detectable in most specimens from women in the other groups. Specimens in which no sIgA was detected were eliminated from the study.

Analysis of sIgA-HSV showed significant interactions between group and ethnic group, age at first intercourse and age at first pregnancy. As the variables related to sexual experience are associated, one of these was omitted. When the data were reanalysed with exclusion of age at first pregnancy from the model, the only

Table 5. *Prevalence of cervical secretory IgA (sIgA) to HSV antigens among Caucasians*

	Number positive / Number with total s IgA (%)
A. Cervical neoplasia	
Mild dysplasia	16/25 (64)*
Severe dysplasia carcinoma in situ	15/38 (39)
Invasive squamous cell carcinoma	4/27 (15)†
B. Controls	
1. Genital tumours (Adenocarcinoma of endometrium and other genital tumours)	5/22 (23)
2. Women without tumours	
Gynaecology controls	30/76 (39)
Family planning controls	5/22 (23)
G-U medicine controls	13/23 (57)

* Significantly higher prevalence than gynaecology controls ($P < 0{\cdot}05$).
† Significantly lower prevalence than gynaecology controls ($P < 0{\cdot}05$).

significant interaction remaining again was that of study group with ethnic group. The analysis was therefore performed for Caucasians only.

Caucasians with mild dysplasia had a significantly higher prevalence of sIgA-HSV than gynaecology controls ($P < 0{\cdot}05$) (Table 5).

DISCUSSION

We have found significant differences in levels of class-specific antibodies to HSV among patients with cervical neoplasia and controls, after allowing for any significant effects of the independent variables (age, ethnic origin, social class, age at first intercourse, number of sexual partners, age at first pregnancy and total number of pregnancies). Patients with mild dysplasia and severe dysplasia/CIS had significantly higher levels of serum IgG-HSV than gynaecology controls ($P < 0{\cdot}05$). Levels of serum IgG-HSV increased with age in all our study groups, a trend which has been noted previously (Lycke, Norrby & Roos, 1974). This may be due to repeated antigen stimulation resulting from reactivation of latent virus.

Patients with genital tumours other than cervical SCC had significantly higher levels of serum IgA-HSV1 than gynaecology controls. This effect was not due to high antibody levels in the 10 patients with endometrial carcinoma. It is unfortunate that the significant interaction of study groups with age made it difficult for us to compare the serum IgA-HSV1 levels of patients with severe dysplasia/CIS and cervical SCC with other groups. However, when data were reanalysed for the women from whom information on smoking habits was available, women with cervical SCC as well as other genital tumours were found to have significantly higher levels of IgA-HSV1 than gynaecology controls ($P < 0{\cdot}05$). Caucasian women with cervical SCC also had significantly higher levels of IgA-HSV2 than gynaecology controls ($P < 0{\cdot}05$).

The significantly increased levels of serum IgA-HSV1 and IgA-HSV2 in patients with cervical SCC may reflect the elevated total serum IgA levels in these patients (G. E. Dale, J. M. Best, B. Slavin & E. Kearney, unpublished results). Dent & Bienenstock (1974) also found that patients with cervical carcinoma had significantly higher levels of total IgA in both serum and cervical secretions than controls. High levels of total serum IgA may be due to leakage into the serum of locally produced sIgA from the inflamed mucosal surface in the vicinity of the tumour.

Cervical sIgA-HSV was detected in secretions from Caucasian women with mild dysplasia significantly more frequently than in secretions from gynaecology controls. It is likely that the significantly lower prevalence of sIgA-HSV in women with cervical SCC compared to controls in our study reflects the difficulty of collecting secretions from these patients. Blood was detected in cervical specimens from all but one of the 54 patients with cervical SCC. A substantial amount of blood and thus little cervical secretion was collected from many of these women, evident from the absence of total sIgA in 23 of 54 specimens from women with cervical SCC. Contamination of specimens with blood made it necessary to use a conjugated antiserum specific for secretory component, in order to detect locally produced sIgA.

Few previous studies of HSV antibodies among patients with cervical neoplasia and controls have allowed for possible risk factors which influence HSV-2 seropositivity. Some studies in which age at first intercourse and number of sexual partners were considered have failed to show significant differences between patients and controls (Rawls, Adam & Melnick, 1972; Vonka *et al.* 1984). Other studies have shown significant differences, having allowed for such factors as ethnic origin, age, socioeconomic level, marital status, and number of pregnancies, but not age at first intercourse and number of sexual partners (Pasca *et al.* 1975; Peltonen, 1975; Seth, Prakash & Ghosh, 1978). A recent large seroepidemiological study of patients and controls from six countries with different rates of cervical cancer concluded that infection with HSV-2 was a covariable of venereal factors, although a role for HSV-2 in the genesis of a proportion of cervical cancers was not excluded (Rawls *et al.* 1986).

The development of cervical cancer is likely to involve multiple factors. Recent studies have shown that human papillomavirus (HPV) DNA is present in a high proportion of cervical SCCs and that HVP 16 DNA is integrated into the host genome of such tumours (McCance *et al.* 1985). Zur Hausen (1982) has postulated that the genesis of cervical cancer may be promoted by papillomaviruses and initiated by mutagens such as HSV and constituents of cigarette smoke. The significantly elevated levels of IgG-HSV in patients with mild dysplasia and severe dysplasia/CIS, and the increased prevalence of cervical sIgA-HSV in Caucasian patients with mild dysplasia that we have noted support the hypothesis that HSV may play a role in early events of cervical carcinogenesis.

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