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Serum antibodies to *Herpes simplex* Virus type-2 in carcinoma of the Uterine Cervix.

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Sera from 400 patients with uterine cervical cancer and 250 normal females were tested for HSV-2 antibodies by Indirect haemagglutination test. 890 samples from various patients control groups—women with skin and venereal diseases, breast cancer, oral cancer, cancer of other sites, gynaecological disorders other than malignancy, cervical dysplasia and carcinoma-in-situ were also tested. Compared to normal healthy females (36%), significantly higher percentage (70%) of cervical cancer patients had high HSV-2 antibody titres ($p < 0.01$). All other patient controls, except those with dysplasia and CIS ($p > 0.01$) revealed a prevalence comparable to normal females. Among cases the occurrence of high antibody titres was not age dependent while the normals showed a steady increase from the first to the fourth decade in life. No significant variation existed among the clinical stages of the disease. Fifty of the cervical cancer patients, sera were tested by the indirect immunofluorescent technique using *Herpes simplex* Virus type-2 infected Vero cells fixed in acetone and FITC–conjugated anti-human Immunoglobulin which showed 84% positivity with brilliant fluorescence.

Herpes viruses have been associated with neoplasia in humans and animals. In Burkitt's lymphoma (BL) the role of Epstein-Barr Virus (EBV) has been suggested[1]. An aetiological role of EBV in BL and Nasopharyngeal carcinoma (NPC) has been implied but not well established. A close association between *Herpes simplex* Virus type—2 and human uterine cervical cancer has been suggested[2,3]. Epidemiological studies on incidence of cancer by site from different countries indicate that cancer of the female genitalia is second only to that of oropharynx in India[4]. The ICMR Tumour Registry at the Regional Cancer Centre, Trivandrum has reported cervical cancer (26.4%) as the most frequent site of cancer among females. Detailed studies on the possible aetiological role of *Herpes simplex* Virus type-2 in cervical cancer have not been reported from this region.

Materials and Methods

The cervical cancer cases were selected from the patients attending the Regional Cancer Centre, other patient controls from the S.A.T. Hospital Gynaecology and

Obstetrics department, Skin and V. D. Department of Medical College Hospital, Trivandrum and normal healthy controls from Blood donors and hospital staff.

(a) The HSV-1 and 2 antigens were prepared in Vero Cell cultures as per Standard methods[7] and stored at —70°C. They were standardized using specific antisera (Flow Laboratories, U. S. A.). (b) Vero Cells infected with HSV-1 and 2 at 2+ to 3+ cytopathic effect were prepared as smears, air-dried, acetone fixed and preserved at —20°C till use[5], for immunofluorescence staining. (c) Blood samples were collected under aseptic conditions from cervical cancer patients (before and after therapy), other patient control groups and normal age and sex matched control population. Sera were stored at —20°C.

Serological tests

Essentially the techniques employed were as described earlier[6] the Indirect Haemagglutination test using HSV-1 and HSV-2 antigens. The type specific antibody reactivity was determined by using the II/I index at a threshold value of 85[7].

Immunofluorescent staining

Fifty sera samples from cervical cancer patients were tested on HSV-2 infected Vero cells, followed by incubation with Fluorescein—isothiocyanate labelled antibody to human total immunoglobulin. The smears were mounted with glycerol-phosphate buffered saline (1 : 9) mountant and examined under an orthoplan fluorescent microscope. The results were graded as 1+, 2+ or 3+ depending on the intensity of fluorescence.

Statistical analysis

Results were analysed by the Chi Square test and Students 't' distribution test.

Results

Results are summarized in Tables 1 to 5. The specific HSV-2 antibody reactivity was measured by determination of the II/I index using the formula II/I index[7]

$$= \frac{\log 10 \text{ antibody titre to HSV—2}}{\log 10 \text{ antibody titre to HSV—1}} \times 100$$

The difference between normal control groups and cervical cancer patients was quite significant ($p<0.01$). No significant difference was noted among the various age-groups among cases ($p>0.05$) while a gradual increase in the percentage of normal females possessing HSV-2 antibodies could be perceived. There was statistically significant difference in the antibody prevalence between age-groups

Table 1 : Reciprocal of Titres of HSV-2 Antibodies in Cervical Cancer Cases and Controls

Study Group and No. tested	No. +ve and %	< 8	8	16	32	64	128	256	512	AMT	GMT
Cervical Cancer 400	282 (70)	55 (14)	8 (2.8)	22 (7.8)	32 (11.3)	39 (13.8)	54 (19.1)	66 (23.4)	61 (21.6)	324	123.90
Normal 250	91 (36)	59 (24)	21 (22)	28 (31.1)	15 (16.6)	13 (14.4)	6 (6.6)	4 (4.4)	4 (4.4)	63	28.09

Figures in brackets—Percentage
(AMT—Arithmetic Titre)—Mean
(GMT—Geometric Titre)—Mean

21—30 and 31—40 ($p<0.01$), 31—40 & 41—50 ($p<0.05$), 21—30 and 41—50 ($p<0.001$) and 31—40 and 51—60 ($p<0.01$). Such age — dependancy was not evident in the cancer patients in any age-group ($p>0.05$). A greater percentage of the patients had very high titres unlike the normals among whom only very few had higher antibody titres. The difference in the number of cases and control with no detectable HSV—2 antibodies is striking 14% and 24% respectively ($p<0.01$). (Tables 1, 2). The prevalence of HSV-2 antibodies in various other control groups is presented. Though 44% of females with skin and non-herpetic veneral diseases and 43% of women with non-malignant gynaecological disorders (like bleeding per vagina, cervicitis, vaginitis, cervical erosion, monilial and *Trichomonas vaginalis* infections, prolapse uterus etc.) had HSV−2 antibodies the difference was not statistically significant. The antibody profile was similar in patients with breast cancer (39%), oral cancer (43%) and cancers of other sites (39%) p-value being >0.5, except in women with various degrees of cervical dysplasia (58%) $p>0.01$. (Table 3). Follow-up studies on forty patients showed a definite decrease in the antibody titres in 54% while 23% had four-fold increase in the values when tested 16 to 20 weeks after radiotherapy (Table 4). The clinical stage of the disease showed no correlation with occurrence of HSV antibodies. (Table 5). Information on extra-marital relationship was lacking and therefore, the relation between incidence or development of cancer of the uterine cervix with this factor and other sex-related attributes could not be assessed.

Table 2 : Age-wise Distribution of HSV-2 Antibody Titres in Cacx and Controls (with II/I Index > 85)

Age-Groups	Study Groups	Reciprocal of serum dilutions							G M T (log)
		8	16	32	64	128	256	512	
21—30	a	—	—	—	1	1	1	—	2.1070
	b	3	—	—	—	—	—	—	0 9030
31—40	a	8	—	1	7	7	7	8	1 9644
	b	—	2	3	—	3	3	4	2.0869
41—50	a	—	6	19	14	26	32	35	2.1799
	b	8	13	6	7	1	1	—	1.3628
51—60	a	—	16	12	17	20	26	18	2.0324
	b	9	13	6	6	2	—	—	1.3294

a. — Cervical Cancer Cases
b. — Normal Females

G. M. T. — Geometric Mean Titre

Table 3 : Prevalence of HSV-2 antibodies in various control groups

Patient Groups	No. tested	No. (+) ve	%	p-value
1. Normal	250	91	36	
2. Pregnant	53	20	38	>0.05
3. Females with skin and venereal diseases (non-herpetic)	270	119	44	>0.05
4. Non-malignant gynaecological disorders	112	48	43	>0.05
5. Females with Ca Breast	100	39	39	>0.05
6. Females with other (non-cervical) cancers	170	66	39	>0.05
7. Cervical atypia/dysplasia	40	23	58	>0.01
8. Females with oral cancer	145	62	43	>0.05

Table 4 : Serum HSV-2 Antibody titres in Follow-up Studies—16—20 weeks Post Radiotherapy

Clinical stage	No. tested	4—fold decrease in titres			4—fold increase in titres			Constant values	
		No.		%	No.		%	No.	%
Stage II	8	5	—	62.5	0			3	37.5
Stage III	19	10	—	52.6	5	—	26.3	4	21.0
Stage IV	13	7	—	53.8	2	—	15.3	4	30.7

Table 5 : Prevalence of HSV-2 antibodies in Ca cx patients— in different clinical stages

Clinical stage	No. tested	No. (+) ve	%	p. value
I	5	2	40	>0.05
II	105	66	63	>0.05
III	176	126	71	>0.05
IV	114	88	77	
Total :	400	282		

Immunofluorescent staining

Forty two sera out of the 50 samples tested (84%) showed brilliant staining of *Herpes simplex* virus type-2 infected acetone Vero cells. The fluorescence was found to be mainly on the nuclear area in the majority of the positive samples and in the remaining the cytoplasm and cell membrane exhibited good fluorescence. The quantitative analysis of the patients' sera gave positivity at *higher serum dilutions* than by the Indirect haemagglutination test showing greater sensitivity. Uninfected Vero cells prepared in a similar manner and incubated with patients' sera followed by FITC-labelled anti-human immunoglobulins produced no fluorescence, thus eliminating the possibility of non-specific false positivity. This was also confirmed by the complete absence of fluorescence in HSV-2 infected Vero cells incubated with phosphate buffered saline instead of patients' serum and stnined with FITC-labelled anti-human Ig. Thirty percent of the samples expressed positive staining with *Herpes simplex* Virus type-1 infected Vero cells.

Discussion

In the present study patients with cervical cancer had higher levels of HSV-2 antibodies compared to age-matched controls. This difference has been observed in previous works too[7,8]. All patient controls, except patients with abnormal cervical cytology, dysplasia and ca-in-situ had antibody levels similar to the normal females. High HSV-antibody titres have been reported in patients with cancer of the larynx[9] while higher frequencies of antibodies was observed in Hodgkin's disease but not in patients with nasopharyngeal cancer and controls[10]. Seven to thirty percent of women with venereal infections possessed antibodies to HSV-2 antigens[11], After 16-20 weeks after radiotherapy, 18% of patients revealed increasing titres. This could be indicative of the recurrence of the tumour[12].

Since infection by *Herpes simplex* virus type-2 has all epidemiological characteristics of a venereally transmitted agent, the association between this virus and cervical cancer could represent one of the co-variables of sexual promiscuity[13]. But the difference between the cervical cancer cases and controls with respect to HSV-2 antibody titres cannot be attributed to sexual factors alone[14].

There is a steady increase in the frequency of prevalence of HSV-2 antibodies from 36% in normals, 58% in precancerous lesions like dysplasia and carcinoma-in-situ to 70% in patients with invasive cervical cancer. Similar reports have been published[15].

Contrary to these, there are a few reports of serological investigations which do not lend support to the hypothesis that HSV-2 is involved in the pathogenesis of cervical neoplasia. Their results indicate no appreciable risk of developing cervical neoplasia in association with HSV-2 infection and there existed no significant difference in antibody prevalence between patients with carcinoma-in-situ or invasive carcinoma and matched controls[16] which differs from the findings of the majority of the case-control studies in different populations. Their data were compatible with the two explanations for the higher prevalence of HSV-2 antibody in cervical cancer patients that (1) the HSV-2 infection and the disease may be independent co-variables of sexual promiscuity and (2) increased sensitivity to or activation of the infection due to neoplastic changes[16]. *Herpes simplex* virus type-2 antigens have been detected directly in tumour tissues from invasive cervical cancer[17] (V. Thankamani et al 1985). The role of human papilloma virus and cytomegalovirus in association with *Herpes simplex* Virus type-2 or independently in the aetiology of human cervical cancer are being investigated.

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