JOURNAL OF EXPERIMENTAL PATHOLOGY
Volume 2, Number 2, 1985
Mary Ann Liebert, Inc., Publishers

Detection of Herpes Simplex Virus Type-2 Antigen(s) in Biopsies from Carcinoma of the Uterine Cervix

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ABSTRACT

Indirect immunofluorescence technique was employed to detect herpes simplex virus type-2 (HSV-2) antigens in tumor biopsies from 215 patients with carcinoma of the uterine cervix. A total of 169 samples (79%) revealed brilliant nuclear fluorescence. Inflammatory cells infiltrating the tumor mass were positive to 60 of the 215 patients (28%). Samples showed no significant variation in the degree of fluorescence or proportion of cells binding HSV antibody with advancement in the clinical stage of the disease. Fluorescence was totally abolished when incubated with HSV-2 antiserum absorbed with a specific homologous virus. Among controls, there was fluorescence in 27% of cervical scrapings from normal women and 34% (42/124) among patients with gynecological disorders other than cervical malignancy. In cervical dysplasia 23 out of 40 patients (58%) expressed herpes virus-associated antigens. There was membrane fluorescence in live malignant cell preparations in 3 of 28 patients (11%). Normal cervix tissue from hysterectomy specimens and breast cancer cells were negative for herpes simplex virus-related antigens. Pre-immune serum and PBS showed nonspecific fluorescence in 25% and 23% of sera, respectively.

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INTRODUCTION

Epidemiological studies indicate that cancer of the female genitalia is second only to that of the oropharynx in India (1). Present knowledge regarding the cause of cancer of the uterine cervix favors a multifactorial theory, like any other neoplasia. Most of the sero-epidemiological studies suggest that low socioeconomic status, early age at marriage and pregnancy, multiple pregnancies, and high sexual activity are some of the risk factors associated with the development of cervical neoplasia (2,3). In addition to chemicals, chemotherapeutic agents and hormones, herpes simplex virus type-2 (HSV-2) has been incriminated as one of the infectious agents in the etiology of cervical cancer. In the present study, biopsy materials from patients with this neoplasia have been examined for the presence of herpes simplex viral antigens by an immunofluorescent technique.

MATERIALS AND METHODS

Punch biopsies from clinically diagnosed and histopathologically proved cases of cervical carcinoma were collected in sterile tissue culture medium (MEM with 5% fetal calf serum). The tissues were processed within 1 to 3 hours after collection. They were washed with Hank's BSS to remove blood clots and mucus, cut into small bits using sterile forceps and scalpels and gently minced to obtain a single cell suspension. Use of cell dispersing enzymes like trypsin and pronase were avoided since they have been found to alter the cellular antigens (4,5) and other surface characteristics. The cell suspension was centrifuged and washed three times. The supernatant was discarded and the cell button resuspended in a few drops of phosphate buffered saline (pH 7.2). Smears were prepared, dried in air and fixed in cold acetone for 10 minutes. All the slides were preserved at -20°C until staining (5). Duplicate smears were stained by the Papanicolaou method. The remaining portion of the cells were inoculated into tubes containing tissue culture medium supplemented with 20% fetal calf serum (DIFCO Laboratories), and incubated for 1 to 4 weeks. The cells were smeared on slides and fixed in acetone. Fresh live cells were examined for membrane fluorescence (6). Cervical scrapings were collected from the same patients and smears prepared.

The following controls were included for the study: normal cervix tissue from hysterectomy specimens; cervical scrapings from patients attending the gynecology clinic for other disorders (bleeding, cervicitis, vaginitis, cervical erosion, prolapsed uterus) and tissue specimens from the endometrium and body of the uterus from hysterectomy specimens from patients with cervical cancer who underwent surgery. Breast cancer biopsies were prepared as indicated above. Duplicate smears were examined by the Papanicolaou staining method, in both study and control groups.

For positive controls, HEP-2 monolayers were infected with herpes simplex virus type-2 strain, HV-219 (12) (received from the National Institute of Virology, Pune, India) and incubated at 37°C for 24 to 36 hours. When 2+ to 3+ cytopathic effect (CPE) developed, the cells were scraped off into the medium and centrifuged at 1000 rpm for 10 minutes at 4°C. The cells were resuspended in phosphate-buffered saline (PBS) (pH 7.2). Smears were prepared for both cytology and immunofluorescent staining (7). Noninfected HEP-2 cells prepared similarly served as the negative control.

Specific antiserum to herpes simplex virus-type 1 and 2, rabbit pre-immunization serum and FITC (fluorescein isothiocyanate) labeled goat anti-rabbit antibody were supplied by Kallestad Laboratories Incorporated.

To check the specificity of the reaction the HSV-2 antiserum was absorbed with HSV-1-infected HEP-2 cells, and one portion with HSV-2-infected cells. The antiserum and antigens were mixed in equal volumes and kept at 4°C for 18 hours. After centrifugation at 2000 rpm for 10 minutes at 4°C, the supernatant was used for the test (8). The anti-HSV-2 serum was also absorbed with homogenized normal cervical tissue, to remove nonspecific antibodies.

Staining

In every set of tests, known positive and negative smears were included. The method employed was an indirect immunofluorescent staining. The smears were covered with specific rabbit anti-HSV-2 serum and incubated at 37°C for 30 minutes. The slides were gently rinsed in phosphate-buffered saline (PBS) (pH 7.2) for 10 minutes and dried in air. This was followed by staining with FITC-conjugated goat anti-rabbit antiserum at 37°C for 30 minutes. The smears were washed in PBS, dried, and mounted in 1:1 glycerol-PBS mountant.

Controls

Smears were incubated with PBS and pre-immunization rabbit serum in parallel sets instead of the specific anti-HSV-2 serum, followed by FITC-labeled antiserum to rabbit immunoglobulin. In a third series, cervical cancer cells were tested with anti-HSV-2 serum absorbed with HSV-2 antigen.

The mounted slides were examined immediately under a Leitz Orthoplan fluorescent microscope and graded as 1+, 2+, or 3+ based on the intensity of fluorescence.

RESULTS

A summary of the observations is presented in Tables and figures (Tables 1-4 and Figs. 1-4).

Known positive controls exhibited brilliant fluorescence on the nucleus as well as the cytoplasm. Noninfected HEP-2 cells were completely negative. Good membrane fluorescence was detected in live cell preparations of HSV-2-infected HEP-2 cells. Among 15 normal healthy females, fluorescence was observed in four biopsies (27%). Both anti-HSV-1 and HSV-2 sera failed to bind to normal cervical cells from postsurgical specimens (Table 1). The cervical scrapings from patients with gynecological disorders other than cervical malignancy or premalignant lesions showed 34% positivity (Table 1). Based on cytology these controls could be allocated to the following groups: inflammation (66 cases), Trichomonas vaginalis infection (6 cases), moderate and severe dysplasia (40 cases), and normal (12 cases), which exhibited bright fluorescence in 21 out of 66 patients (32%), 2 out of 6 (33%), 23 out of 40 (58%), and 3 out of 12 patients (25%), respectively. In cases of inflammation, fluorescence was largely distributed on the infiltrating inflammatory cells and immunocytes. Characteristic herpes-like cytological changes were noted only in 2 out of 124 cases and they showed nuclear and cytoplasmic fluorescence.

TABLE 1

Indirect Immunofluorescent Staining with HSV-2 Specific Antibody Followed by FITC-Conjugated Anti-Immunoglobulin

SL. NO.	SPECIMEN	TOTAL NO. TESTED	NO. (+)VE	% POSITIVE
1.	Cervical scrapes normal women	15	4	26.6
2.	Nonmalignant gynecological disorders—cervical	124	42	33.8
	Cytology:			
	a. Inflammation	66	21	31.8
	b. Dysplasia	40	23	57.5
	c. T.V. Infection	6	2	33.3
	d. Normal	12	3	25.0

To exclude the possibility of false positivity or nonspecific reactivity, the smears were incubated in parallel with PBS. The percentage of cells reacting with specific HSV-2 antibody was assessed by subtracting the proportion of cells stained when PBS was substituted for specific antiserum. With PBS 49 out of 215 cervical cancer cell preparations (23%) were positive (Table 2) though the percentage of cells was rather low (1 to 11%). Pre-immunization serum used at the same dilution as the specific HSV-2 antibody produced fluorescence only in 5% of the cases.

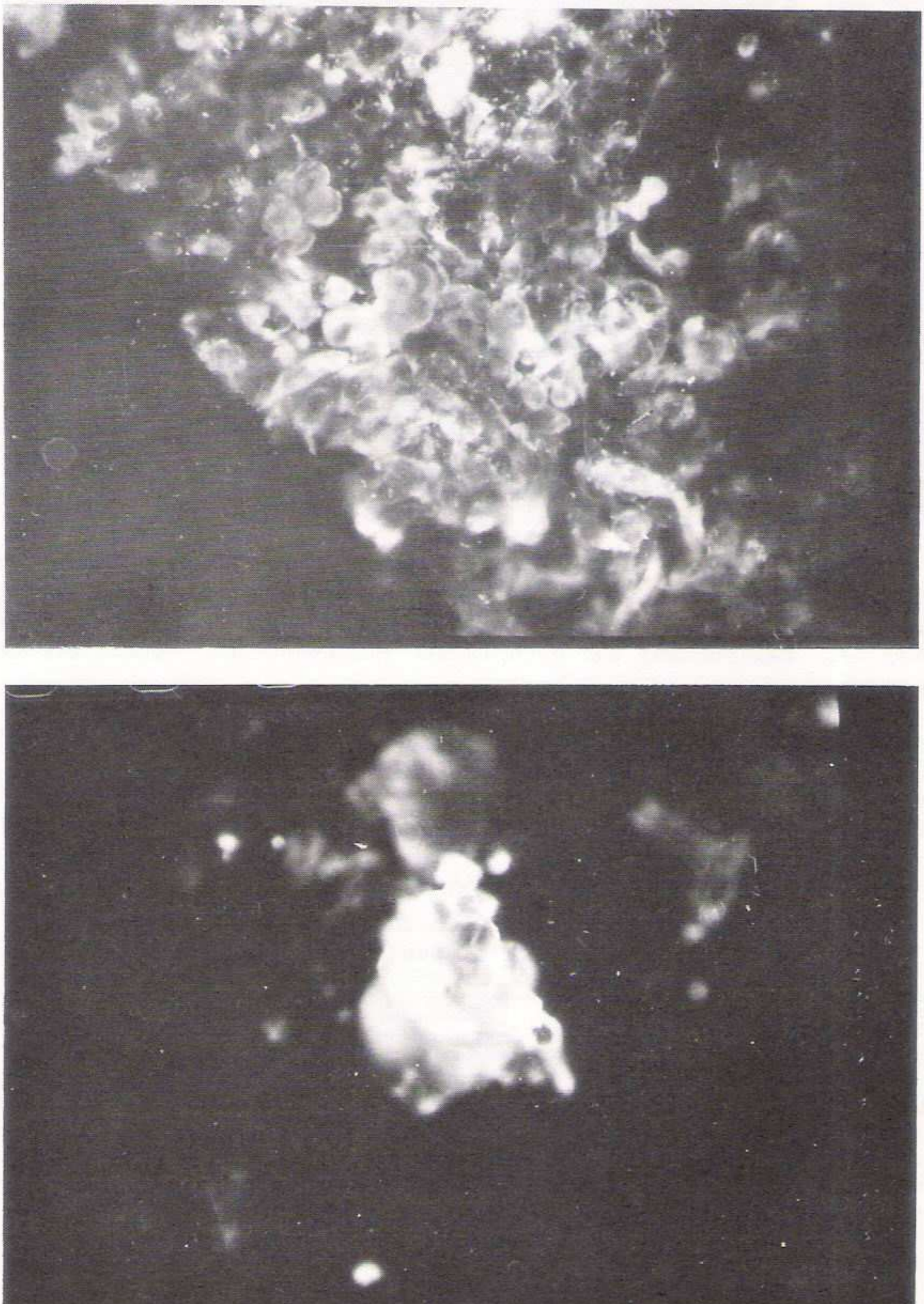
TABLE 2

Indirect Immunofluorescence Staining of Acetone Fixed Cervical Cancer Biopsy Cells

SL. NO.	REAGENTS USED	NO. TESTED	NO. & % (+)VE	DISTRIBUTION OF FLUORESCENCE	
1.	Phosphate-buffered saline + FITC - Anti-Ig	215	49 (22.7)	Tumor cells and immunocytes	
2.	Specific HSV-2 antibody + FITC - Anti-Ig	215	169 (78.6)	Nuclear ---	169/215
				Nuclear and cytoplasmic ---	68/169
				Perinuclear ---	54/169
		60 (28)		Inflammatory cells	

Two hundred and fifteen tissue specimens came from patients with moderate or poorly differentiated squamous cell carcinomas by histopathology. Of these 169 showed nuclear, 68 cytoplasmic and nuclear, and 54 definite perinuclear fluorescence. In 60 out of 215 smears (28%) there was intense fluorescence confined to the infiltrating inflammatory cells. The normal squamous cells were negative, whereas brilliant staining was observed in

the malignant cells only (Figs. 1-3). The percentage of positive cells per microscopic field varied from patient to patient between 25% and 60%. The HSV-2 antibody bound to a larger number of cells compared to anti-HSV-1 serum (Table 2).



FIGS. 1 AND 2. Acetone-fixed cells from biopsies of carcinoma of the cervix incubated with specific herpes simplex virus type-2 (rabbit) antibody, followed by FITC-conjugated goat antibody to rabbit antibody (magnification 400 X). Brilliant cytoplasmic and nuclear fluorescence is seen.

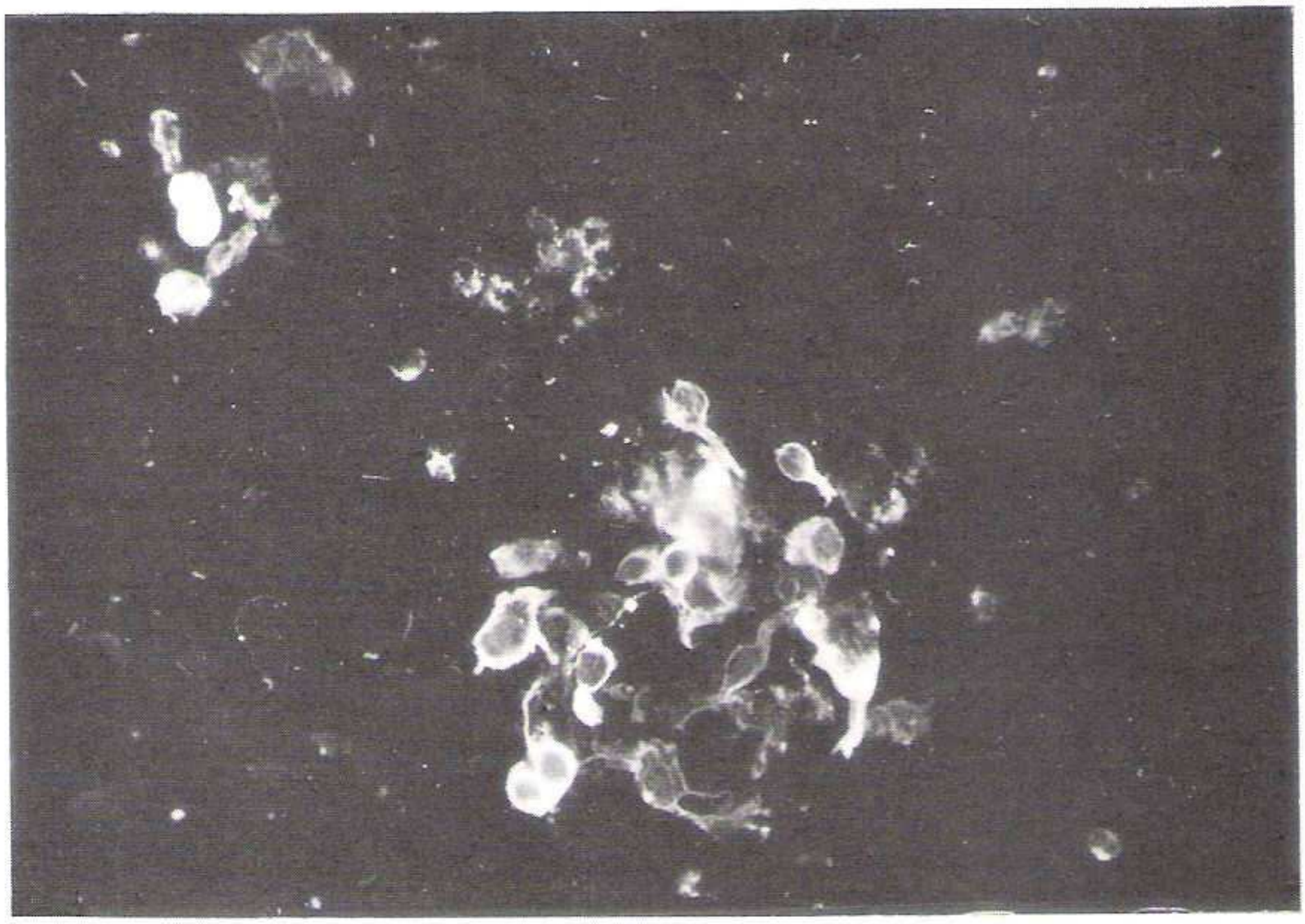


FIG. 3. Acetone-fixed cells from biopsies of carcinoma of the cervix incubated with specific herpes simplex virus type-2 (rabbit) antibody, followed by FITC-conjugated goat antibody to rabbit antibody (magnification 400 X). Perinuclear and cytoplasmic fluorescence. Nuclei are totally negative.

Acetone-fixed fresh exfoliated cells from cervical cancer cases, smears of cells derived from the biopsy material, and cells after incubation with tissue culture medium for varying periods revealed no notable differences in the degree of fluorescence or percentage of positive cells (Table 3).

TABLE 3

Comparison of Immunofluorescence of
Cervical Carcinoma Cells Based on the Preparation

	EXFOLIATED CELLS		FRESH BIOPSY CELL SUSPENSION		BIOPSY CELLS CULTURED	
REG NO.	NO. OF CELLS (+)VE/HPF*	DEGREE	NO. (+)/HPF	DEGREE	NO. (+)/HPF	DEGREE
S-1	5-8	+++	8-10	+++	6-8	+++
S-2	3-5	+	8-10	++	8-12	+
S-3	15-20	+++	15-20	+++	10-12	+++
S-4	20-30	+++	30-40	+++	30-40	+++
S-5	0-4	++	0-8	+++	2-5	+++
S-6	5-6	+	10-15	++	6-10	+++
S-7	2-5	+++	8-10	+++	5-10	+
S-8	0-1	+	1-2	+++	2-5	++
S-9	2-5	++	2-4	+++	0-3	+
S-10	3-10	+	20-30	++	10-15	+

*HPF = high-powered microscope field.

The specificity of the fluorescence in the malignant cells was ascertained by incubating parallel sets of highly positive samples with specific anti-HSV-1 serum (absorbed with heterologous virus infected HEP-2 cells) and anti-HSV-2 serum absorbed with the homologous virus (HSV-2) infected cells. Excepting the 15-25% of positivity with anti-HSV-1 specific serum the fluorescence was almost totally abolished, with respect to both degree of fluorescence and the number of positive cells per field (Table 4).

TABLE 4

Indirect Immunofluorescence Using HSV-2 Antibody on Patients Controls

SL. NO.	GROUP	NO. TESTED	NO. AND % (+)VE	DISTRIBUTION OF FLUORESCENCE
1.	Living cervical carcinoma	27	3 - (11)	Membrane
2.	Breast tumor	5	0 - --	--
3.	Cervical carcinoma - body of uterus		0 - --	--
	Endocervical columnar cells	3	0 - --	--

The cultures cells were centrifuged and washed with Hank's BSS. Indirect immunofluorescence was performed on the live cell suspensions. In 3 out of 27 cases membrane fluorescence of 2+ degree of intensity was observed, though on relatively very few cells (Fig. 4).

Breast cancer cells and cells from other sites in patients who had undergone hysterectomy (body of the uterus, endocervix, etc.) had no notable fluorescence, indicating the absence of detectable herpes simplex antigens.

DISCUSSION

A review of the available literature on cancer of the uterine cervix implies a possible association of herpes simplex virus type-2 with this neoplasia. Most of the work has involved sero-epidemiological case control studies detecting and quantifying the specific HSV-2 antibodies in patients suffering from cervical cancer, by neutralization, complement fixation, and passive hemagglutination tests (9-12). Previous studies in our laboratory led to the finding that a larger percentage of women with invasive cervical malignancy possessed higher titers of antibodies to genital herpes simplex virus compared to the normal females. The outcome of the present work indicates the presence of HSV-2-associated antigens in such tumors in a significant proportion of the biopsies examined, and the fact that these were present on the malignant cells, but not in the normal squamous cell population in the tumor tissues. The findings seem to deserve attention and further study.

The fluorescence observed when incubated with anti-HSV-1 serum could be due to the common cross reacting antigens of the two types of the virus (13). The specificity of the reaction was confirmed by the abolition of the fluorescence, in a good number of the highly positive smears, when the HSV-2 antibody was extensively absorbed with the homologous virus.

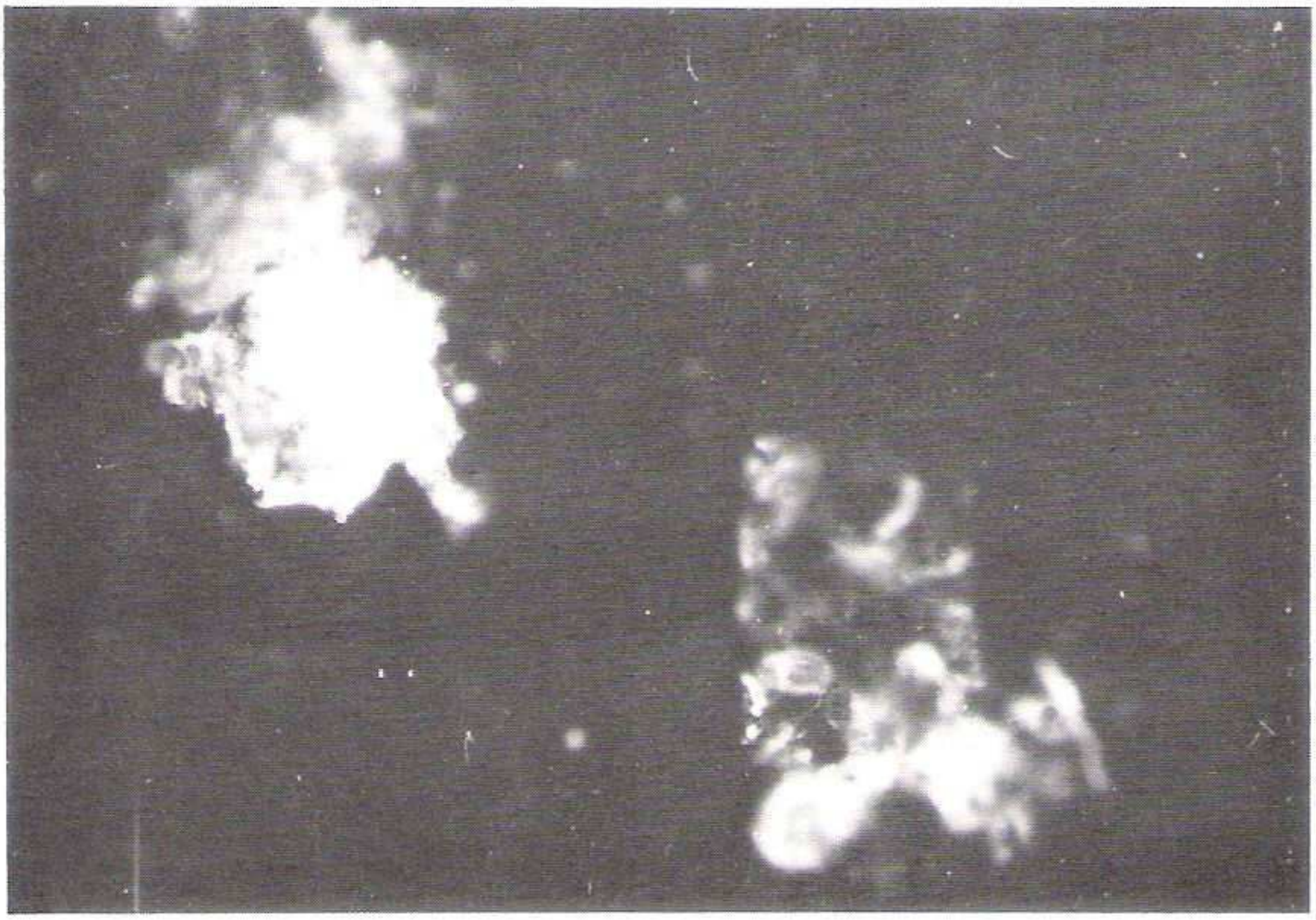


FIG. 4. Acetone-fixed cells from biopsies of carcinoma of the cervix incubated with specific herpes simplex virus type-2 (rabbit) antibody, followed by FITC-conjugated goat antibody to rabbit antibody (magnification 400 X). Indirect immunofluorescence of live (biopsy) cell suspension. Bright cytoplasmic membrane fluorescence is notable with absence of fluorescence in the nuclear region.

The fluorescence on infiltrating inflammatory cells in 28% of the biopsies may be due to the FC receptors present on the immunologically active cells. The nonspecific fluorescence when incubated with PBS and pre-immune serum may be attributed to the presence of heterophile antibodies in the conjugated antiserum preparation (14). It is also possible that this is due to HSV-specific antibodies absorbed from the patient's blood *in vivo* by the HSV-specific antigens present on the tumor cells. These antibodies may be functioning as blocking antibodies in tumor enhancement or involved in the cell-mediated destruction of the tumor cells (15). The wide range of patient to patient variation (25-60%) in the percentage of positive cells may be due to the variation in the quantity of HSV-2 antigens present on the tumor cells (14).

There have been many reports on the presence of HSV-2 antigens on exfoliated cells from cervical carcinoma and their absence in normal cells obtained from the same patients (14,16). Using immunofluorescence tests it has been noted that cells infected with herpes simplex virus develop new antigens which alter the antigenic specificity of the host cell membranes (17). Virus-induced, virus-specific antigens appear on HSV-infected BHK cells (18), and during infection structural components of the virus are synthesized and incorporated into membranes of infected cell surfaces (19). Aurelian and co-workers have isolated HSV-2 from cultured human cervical cancer cells (20), and a fragment of herpes simplex virus DNA has been detected in cells from a case of carcinoma cervix (21). Further, antibodies to AG-4, an early antigen from HEP-2 cultures infected with

HSV-2, have been shown to be present only in patients with cervical cancer and premalignant lesions but not in normal females (22). Patients with more advanced stages of this malignancy possessed higher levels of antibodies to HSV-2, which are possibly evoked by expression of HSV-2 antigens on tumor cells (23,24). Recently HSV-related antigens have been demonstrated on human cervical carcinoma cells by the immunoperoxidase technique (25,26). Previous workers have detected HSV-2-associated antigens in exfoliated cells and not in the deeper subsurface layers of the tumor suggesting that, prior to exfoliation, tumor cells on the surface of the neoplastic lesions are exposed to glandular secretions of relatively high pH and unfavorable nutritional conditions (27) and that cervical tumor in tissue cultures do not indicate the presence of viral antigens unless grown under suboptimal conditions, such as in a medium of high pH. This theory is consistent with the latency of the virus and its induction under artificial in vitro culture conditions (27). In our study culture of a few samples for varying periods did not reveal any significant variation in the fluorescence pattern.

The nature of the association between HSV-2 and human cervical carcinoma is not yet clear. These observations suggest the presence of specific HSV-2 antigens on tumor cells derived from biopsy material. They are supported by the findings of other workers and certainly underscore the possible etiological role of this virus in cervical cancer either independently or in combination with other agents like the human Papilloma virus (28).

ACKNOWLEDGMENTS

The authors record their sincere thanks to Dr. Pradeep Seth (All India Institute of Medical Sciences, New Delhi), Dr. D.V. Ablashi (National Cancer Institute, U.S.A.), and Dr. J. Shanmugam (Sri Chitra Tirunal Medical Centre, Trivandrum) for their helpful discussions. They wish to express their gratitude to Dr. Kaveri Gopalakrishnan (Department of Gynecology and Obstetrics, S.A.T. Hospital, Trivandrum) and Dr. N. Sreedevi Amma and Dr. M.K. Lalitha Bai (Department of Cancer Cytology, Regional Cancer Centre, Trivandrum) also for their help and full cooperation in the collection of specimens and discussion on the test results.

This work has been partly financed by grants of the Indian Council of Medical Research.

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