Proceedings of the National Academy of Sciences
Vol. 67, No. 1, pp. 204–212, September 1970

Immunofluorescent Detection of Herpesvirus Antigens in Exfoliated Cells from Human Cervical Carcinoma

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Communicated by W. Barry Wood, Jr., June 11, 1970

Abstract. Exfoliated cells from patients with squamous carcinoma of the cervix contain antigens related to herpesvirus subtype 2, as revealed by direct or indirect immunofluorescent techniques. Normal squamous cells from the same subjects and from controls without the disease, and cells from a small number of tumors at sites other than the cervix, did not react with anti-herpesvirus subtype 2 serum. Antisera to adenovirus 18 or mycoplasma orale did not react with the exfoliated cells.

Two subtypes of herpes simplex virus, biologically and antigenically distinct,[1,2] have been isolated from humans: herpesvirus subtype 1, associated with facial lesions, and herpesvirus subtype 2 (also called genital herpes) isolated from smegma and cervical lesions[3,4] and shown to be venereally transmitted.[5,6] Sero-epidemiologic studies have indicated a significantly higher prevalence of antibody to herpesvirus subtype 2 in sera of patients with invasive[4,7,16] and preinvasive[7] carcinoma of the cervix than in a matched control population.

The present studies demonstrate that some exfoliated dyskaryotic cells from women with cervical neoplasia react with rabbit antiserum against herpesvirus subtype 2 as revealed by immunofluorescence; normal squamous cells obtained from the same patients or from healthy controls do not react.

Materials and Methods. Virus: Prototypes of herpesvirus subtype 1 (HSV-1) and subtype 2 (HSV-2), the properties of which have been described,[8] were obtained from Dr. B. Roizman, University of Chicago, Chicago, Ill., and propagated in our laboratory in human epidermoid carcinoma No. 2 (HEp-2) cells.

Preparation of antisera: The sera used in the direct immunofluorescent tests were obtained from Dr. B. Roizman, and consisted of fluorescein-conjugated rabbit anti-HSV-2 and anti-HSV-1 sera, the preparation, absorption, and conjugation of which have been described.[8,9] The sources of antibody for the indirect immunofluorescent staining were: (*i*) pooled commercial human gammaglobulin (Lederle) containing neutralizing antibody to HSV-1 and designated γG; (*ii*) two human sera, from control subjects, containing neutralizing antibody to HSV-2 (Hu-2) and HSV-1 (Hu-1), respectively;[10] (*iii*) rabbit immune sera against HSV-2- or HSV-1-infected HEp-2 cell debris and respectively designated Ra-2 and Ra-1. The preparation of Ra-2 and Ra-1 sera differed from that used for the direct conjugates and consisted in the subcutaneous injection, twice a week for 4 weeks, of 1.5 ml of HSV-2- or HSV-1-infected HEp-2 cells harvested 24 hr after infection and mixed with complete Freund's adjuvant (Difco) to yield a 50% (v/v) suspension. These were followed, 8 days later, by one intravenous injection of 2 ml of the infected cell debris without adjuvant. The sera obtained 8 days after the last injection were decomplemented by heating at 56°C for 30 min, then ab-

sorbed at 37°C with uninfected HEp-2 cells until they failed to stain uninfected HEp-2 cells.

Study groups: The following groups of patients reporting to the Johns Hopkins Hospital were studied: (1) twenty-six patients seen in the outpatient cervical clinic and diagnosed as preinvasive cervical neoplasia (atypia and carcinoma *in situ*) confirmed cytologically, colposcopically, and histologically;[12] (2) ten patients with invasive carcinoma from the gynecology ward diagnosed histologically; (3) ten control subjects reporting to the outpatient cervical clinic with no evidence of cervical carcinoma and negative Papanicolaou smears, including three patients with squamous metaplasia, selected on the basis of a visible transformation zone not considered atypic, and two patients with herpetic cervicitis diagnosed cytologically; (4) one patient with mesodermal tumor of the endometrium and four patients with carcinoma of the breast from the gynecology and surgery wards. At the time of cell collection all subjects, with the exception of those with herpetic cervicitis, were reported free of active genital herpetic infection detected by cytology and pelvic examination.

Cell collection: Exfoliated cervical cells were obtained by the impression of a small sponge directly to the surface of the atypic lesion, and by the irrigation method described by Davis.[13] The cells were washed in 0.1 M phosphate-buffered saline, pH 7.2 (PBS) by 3 cycles of centrifugation at 2000 rpm for 5 min and resuspended in PBS. Slides prepared from these cell suspensions were air dried, fixed in cold methanol (−40°C), and stained within 3 days after cell collection. Other fixatives tested with essentially similar results include ethanol, acetone, and Pro-Fixx (Lerner Laboratories). Impressions of breast carcinoma and scrapes of squamous buccal cells from patients with cervical carcinoma were also tested.

Fluorescent staining: In the indirect immunofluorescent-staining procedure[14] slides were washed in PBS for 10 min, covered with an appropriate dilution of antiserum, and incubated for 60 min in a moist chamber at 37°C. At this time they were removed, washed with PBS for 3-min periods, covered with fluorescein-conjugated serum, and reincubated at 37°C for 60 min. The fluorescein conjugates used consisted of sheep anti-rabbit gammaglobulin (Grand Island Biological), goat anti-rabbit gammaglobulin, and anti-human gammaglobulin (Microbiological Associates) absorbed with human liver powder. Slides stained by the direct immunofluorescent method[15] were washed, covered with fluorescein-conjugated anti-HSV-2 serum, and incubated as above. After three cycles of washing in PBS, the slides were mounted and examined with a Zeiss fluorescent microscope. The source of ultraviolet light was an Osram HBO 200W, super pressure, mercury lamp. A BG12 exciter filter and Zeiss OG3 barrier filter were used. Photomicrographs were taken on Kodak high speed Ektachrome film.

Cell identification and assessment of fluorescence: Duplicates of slides stained by the immunofluorescent procedure were stained by a modified Papanicolaou method using hematoxylin, eosin, and orange G[13]. Three types of cells were detected in varying proportions on all smears, i.e., normal squamous differentiated, polymorphonuclear, and dyskaryotic cells (Fig. 4). In the fluorescein-stained smears the dyskaryotic cells were identified by the relatively large nucleus and the high nucleus/cytoplasm (N/C) ratio. They were easily differentiated from the normal squamous cells which have relatively small nuclei, and low N/C ratios, and from the polymorphonuclear cells by their size and the shape of their nuclei (see, for example, Fig. 4). A few columnar cells were sometimes observed. The proportion of each cell type showing fluorescence was determined by scanning the entire slide by UV microscopy and counting approximately 100 dyskaryotic cells in invasive carcinoma cases and as few as 25 in cases of atypia. A cell was accepted as showing positive fluorescence if it is sufficiently bright to be clearly visible in the reduced illumination produced when the beam-splitting slide was set in the position for photography. The reliability in assessing fluorescence was determined by a count of fluorescent cells from 15 patients done by an independent observer, whereas the reliability of identifying dyskaryotic cells was tested by marking such cells, restaining by the modified Papanicolaou stain and allowing a cytologist to examine the marked cells. Virtually 100% agreement was obtained.

Results. Immunologic reactivity of HSV-2 antigen in infected HEp-2 cells: In order to test the specificity of the reagents used in this study, HSV-2-infected and uninfected HEp-2 cells were stained with Ra-2, Ra-1, Hu-2, Hu-1, and γG sera by the indirect immunofluorescent procedure and with fluorescein-conjugated rabbit anti-HSV-2 and HSV-1 sera. Similar results were obtained with both techniques. The homologous antibody stained a larger proportion of cells (Fig. 3) than the heterologous one (Fig. 2); however, all sera failed to stain uninfected HEp-2 cells (Figs. 1–3). Reactivity could be specifically removed by absorbing these sera with HSV-2-infected HEp-2 cells.

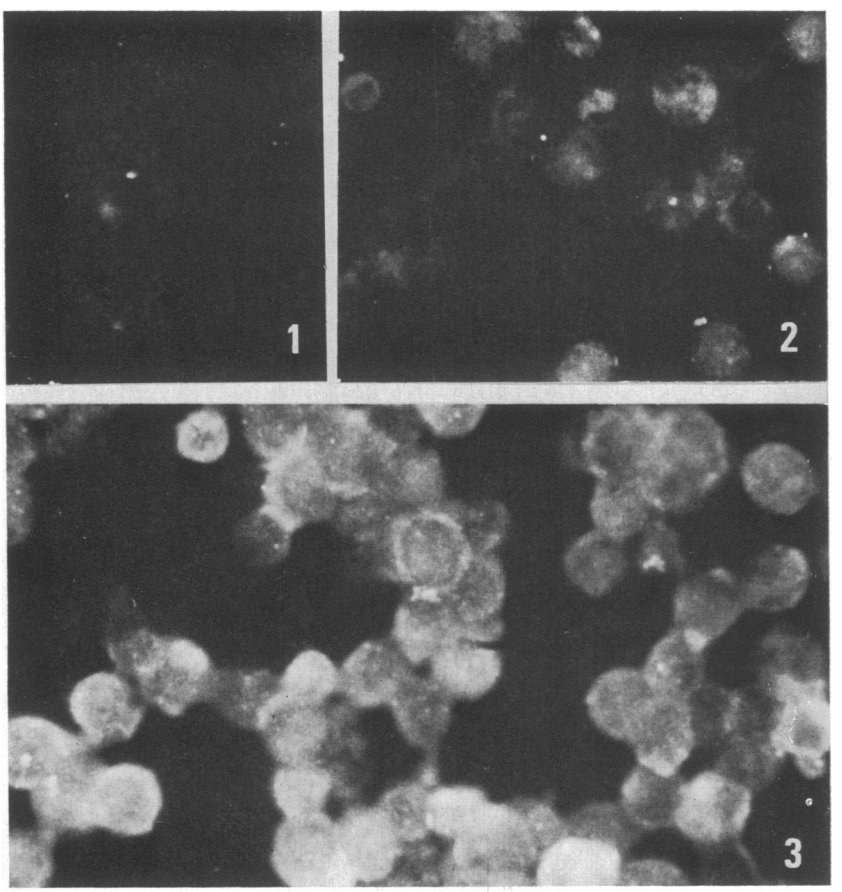

FIG. 1. Uninfected HEp-2 cells stained with Ra-2 serum (×320).
FIG. 2. HSV-2-infected HEp-2 cells stained with Ra-1 serum (×320).
FIG. 3. HSV-2-infected HEp-2 cells stained with Ra-2 serum (×320).

Immunologic reactivity of exfoliated cervical cells: Duplicate slides of exfoliated cells were stained by the modified Papanicolaou stain and by the indirect immunofluorescent procedure using: (1) Ra-2 serum and (2) PBS instead of antibody. Brilliant fluorescence with Ra-2 serum was observed in dyskaryotic but not in normal squamous cells from all cases of cervical carcinoma.

TABLE 1. *Proportion of dyskaryotic cells reacting with Ra-2 serum by the indirect immunofluorescent procedure using fluorescein-conjugated sheep anti-rabbit gammaglobulin.*

Case	Avg no. of dyskaryotic cells counted	Per cent staining with Ra-2 serum	Per cent staining with PBS control	Per cent staining specifically
1. Atypia				
252	25	20	4	16
337	25	25	6	19
338	50	40	12	28
339	50	30	7	23
443	50	45	4	41
230	30	25	9	16
457	40	35	12	23
466	50	30	20	10
481	50	25	4	21
Mean	41	27	8	19
2. Carcinoma *in situ*				
257	50	35	4	31
233	60	22	6	16
433	50	25	8	17
442	50	30	4	26
434	25	40	16	24
462	100	30	9	21
441	25	40	4	36
465	30	35	12	23
467	25	35	15	20
469	60	38	8	30
477	25	40	12	28
499	30	25	4	21
Mean	44	28	7	21
3. Invasive carcinoma				
11	100	45	16	29
4	100	38	18	20
255	100	48	0	48
268	30	30	10	20
463	50	45	18	27
482	150	35	10	25
506	200	40	14	26
Mean	104	42	11	31

The results obtained for 29 patients who had 25 or more dyskaryotic cells per smear are summarized in Table 1. The proportion of staining dyskaryotic cells varied with the patient and ranged between 20 and 50%; however, approximately 5–20% of the dyskaryotic cells displayed fluorescence when stained with PBS instead of antibody. Since the proportion of cells staining with PBS depended on the source of the fluorescein-conjugated gammaglobulin, and could not be removed by absorption with human liver powder, it is suggested that staining with PBS may be due to a heterophile antibody.[19] The proportion of cells staining specifically for herpes antigens was estimated by subtracting the proportion of cells staining with PBS from that staining with Ra-2 serum. This value ranged between 10 and 40% of dyskaryotic cells. The fluorescence observed with Ra-2 serum took the form of a diffuse cytoplasmic mass (Figs. 5, 8), sometimes covering the nucleus (Fig. 6). Occasionally, distinct perinuclear fluorescence (Fig. 6, 7), as well as fluorescent intranuclear granules (Figs. 5–7), were also observed.

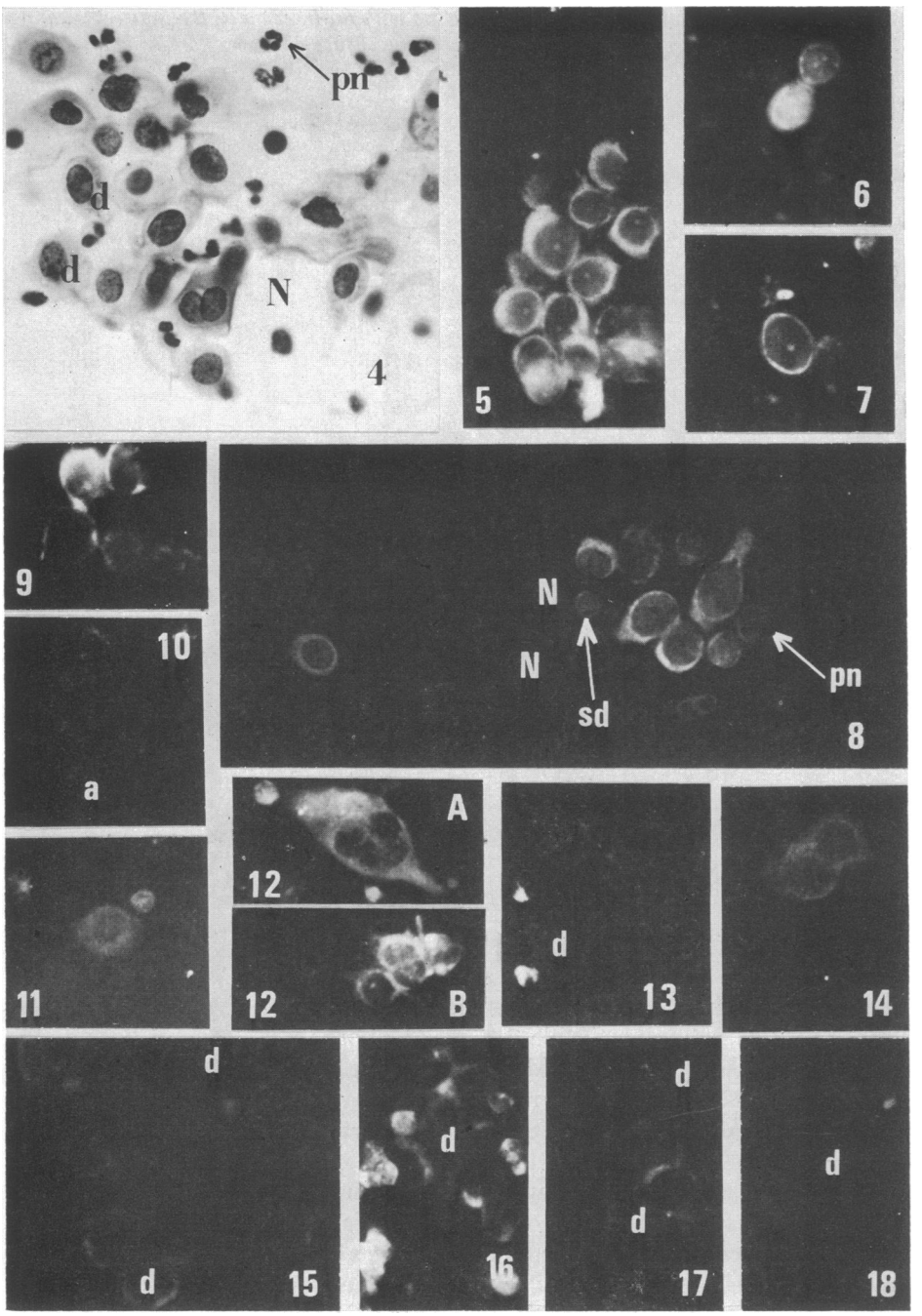

FIG. 4. Exfoliated cells from a patient with invasive cervical carcinoma, including normal cells (N), dyskaryotic cells (*d*), and polymorphonuclear cells (pn). Papanicolaou stain (×600).

FIGS. 5–7. Fluorescence in exfoliated dyskaryotic cells from patients with invasive carcinoma of the cervix. Stained with Ra-2 serum (×480). Note intranuclear granules in Fig. 6, and perinuclear fluorescence in Fig. 7.

The data obtained from staining of control cells with Ra-2 serum are shown in Table 2. Metaplastic cells, cytologically differentiated from atypic cells on the

TABLE 2. *Proportion of control cells reacting with Ra-2 serum by the indirect immunofluorescent procedure using fluorescein-conjugated sheep anti-rabbit gamma-globulin.*

Case	Avg no. of dyskaryotic cells counted	Per cent staining with Ra-2 serum	Per cent staining with PBS control	Per cent staining specifically
Squamous metaplasia				
502	100	0	0	0
503	80	4	6	0
514	25	2	2	0
Carcinoma of endometrium				
519	50	0	0	0
Carcinoma of breast				
1	100	5	5	0
2	50	2	3	0
3	100	0	0	0
4	100	10	10	0
Herpetic cervicitis				
493	25	80	0	80
494	100	90	0	90

basis of the shape of their nuclei and the chromatin pattern, did not display fluorescent staining (Fig. 11), whereas rounded cells (Fig. 12*B*) and giant cells (Fig. 12*A*) from two cases of herpetic cervicitis displayed fluorescent staining with Ra-2 serum but not with PBS. Fluorescence was not detected in endocervical columnar cells and in buccal squamous epithelial cells from patients with cervical carcinoma and in neoplastic cells from breast carcinoma. Exfoliated cells from a case of mesodermal tumor of the endometrium, diagnosed histologically subsequent to staining with Ra-2 serum, displayed a complete absence of fluorescence (Fig. 10).

The proportion of staining dyskaryotic cells varied with the serum tested, possibly due to a different antibody content. Thus four out of nine sera specifically stained 25–30% of dyskaryotic cells from patients 482 and 506 (Table 1), whereas a somewhat lower proportion of cells from these patients reacted with the

FIG. 8. Exfoliated cells from a patient with carcinoma *in situ*, stained with Ra-2 serum. Note absence of fluorescence of normal (N) and polymorphonuclear (*pn*) cells, and ring-like fluorescence of small dyskaryotic (*sd*) cell on top of normal one (×480).

FIG. 9. Exfoliated cells from a patient with invasive carcinoma stained with Ra-1 serum. Note single fluorescent dyskaryotic cell (×480).

FIG. 10. Anaplastic cells from a case of mesodermal tumor of the endometrium. Note absence of fluorescence with Ra-2 serum (×600).

FIG. 11. Squamous metaplastic cell stained with Ra-2 serum (×480).

FIG. 12. Exfoliated cells from a case of herpetic cervicitis stained with Ra-2 serum. (*A*) Giant cell (*B*) Rounded cells (×480).

FIG. 13. Clump of dyskaryotic cells from an invasive carcinoma (no. 506) showing virtual absence of fluorescence in a blocking experiment using Ra-2 serum (×480).

FIG. 14. Dyskaryotic cells from a patient with invasive carcinoma (no. 255) stained with Ra-2 serum previously absorbed with HSV-2-infected HEp-2 cells (×480).

FIG. 15. Dyskaryotic cells stained with rabbit anti-adenovirus 18 serum (×800).

FIGS. 16–18. Dyskaryotic cells stained with anti-mycoplasma orale serum, PBS, and preimmunized rabbit serum respectively (×480).

other Ra-2 and Hu-2 sera. The direct immunofluorescence appears to be less sensitive; however, at a 5-times higher concentration than that used with Ra-2 serum, fluorescein-conjugated anti-HSV-2 serum stained approximately 30–35% of dyskaryotic cells of patients no. 482 and 506.

Specificity of immunofluorescence of dyskaryotic cells: The specificity of the fluorescence reaction of dyskaryotic cells from patients with cervical carcinoma was investigated by four series of experiments with cells from 10 patients who had a high percentage of dyskaroytic cells reacting specifically with Ra-2 serum. In the first series of experiments, exfoliated cells were stained with control sera previously shown to be negative for HSV-2-infected HEp-2 cells. These consisted of preimmunized rabbit serum, human serum without antibody to either herpesvirus subtype, rabbit anti-adenovirus-18 serum (prepared by subcutaneous injection of infected KB cells, harvested at 24 hr after infection), and rabbit anti-mycoplasma orale serum (Baltimore Biologicals). In the second series, the cells were stained with the cross-reacting Ra-1, Hu-1, and γG sera. In the third series, exfoliated cells were stained with Ra-2 serum that had previously been absorbed with HSV-2-infected HEp-2 cells until the Ra-2 serum failed to stain HSV-2-infected HEp-2 cells. Finally, exfoliated cells were stained with Ra-2 and Hu-2 sera before restaining with the fluorescein-conjugated rabbit anti-HSV-2 serum.

Specific fluorescence was not observed with any of the control sera (Figs. 15–18); however, approximately 5–15% of the anaplastic cells of patients 506, 482, 469, and 255 (Table 1) stained with Ra-I, Hu-1, and γG sera (Fig. 9), indicating that like HSV-2 infected cells, dyskaryotic cells stain with the cross-reacting antibody. As illustrated in Figure 14, absorbed Ra-2 serum did not stain atypic cells of patients 482, 441, 442, 443, 462, 255, and 469 (Table 1). With cells from patient 506, fluorescence was specifically blocked in the direct immunofluorescence test by prior staining with Ra-2 or Hu-2 sera (Fig. 13).

Discussion. The main conclusion of this study is that exfoliated dyskaryotic cells, from patients with histologically proved invasive and preinvasive cervical carcinoma, contain antigens related to those found in cells infected with HSV-2. This conclusion rests on the finding that antiserum made against HSV-2-infected HEp-2 cells, as well as human sera that contains neutralizing antibody to HSV-2, reacted with exfoliated dyskaryotic cells, as determined by direct or indirect immunofluorescence tests.

Although a substantial number of dyskaryotic cells stained "non-specifically," possibly due to fluorescein-conjugated heterophile antibody in the fluorescent sera,[19] the number that stained specifically was generally 2 to 5 times greater, and therefore readily quantitated. Moreover, the immunologic specificity of the reaction was shown by several control experiments. For example, fluorescence was not detected when cells were stained with preimmunized rabbit serum, rabbit anti-adenovirus 18, and anti-mycoplasma orale sera. Reactivity could be removed by specific absorption of Ra-2 serum with HSV-2 infected, but not with uninfected, HEp-2 cells. Also, prior reaction of dyskaryotic cells with antibody against HSV-2-infected cells blocked subsequent direct staining by fluorescein-conjugated-anti-HSV-2 serum. Antiserum made against HSV-1 infected cells

reacted with the dyskaryotic cells; however, it did so to a lesser extent than anti-HSV-2 serum, an observation consistent with the known cross reactivity of HSV-1 and HSV-2.[3,8,10,11,18]

All patients in this series, with preinvasive as well as invasive carcinoma of the cervix, yielded dyskaryotic cells which contained antigens related to HSV-2; however, the proportion of such cells varied and was generally lower than 50%. This variation may reflect differences in the amount of antigen present in neoplastic cells or may be due to the inability to distinguish a truly neoplastic cell from a normal young or metaplastic one. Exfoliated cervical cells from patients without carcinoma of the cervix did not show evidence of herpes antigens except for those diagnosed clinically as having herpetic cervicitis. The negative cases included three with squamous metaplasia of the cervix, one case of mesodermal tumor of the endometrium, and imprints of four cases of carcinoma of the breast, suggesting that the presence of herpes-related antigen in cervical carcinoma cells is relatively specific.

The significance of the detection of antigens related to herpesvirus subtype 2 in cells from cervical carcinoma is not clear at present. It is possible that some or all of these cells are infected with HSV-2, perhaps abortively. In that event, they should contain herpesvirus DNA and possibly complete or incomplete virions. Present experiments are directed towards exploring these possibilities, as well as towards a better definition of the viral antigens detected in these cells. More broadly, the findings presented in this paper strengthen the evidence for an association between HSV-2 and cervical carcinoma. Although it cannot yet be concluded whether or not the association is an etiological one, the fact that all patients with the earliest definable lesions have a significantly higher prevalence of anti-HSV-2 antibody than control subjects,[7,10,12] and yield exfoliated cells containing HSV-2 antigen, indicates that herpesvirus infection is an early event, possibly preceding neoplastic conversion. The precise relationship between HSV-2 infection and cervical carcinoma must, however, await further experimentation.

The authors wish to express their gratitude to Dr. Hugh J. Davis for his help in collecting cell samples, reviewing Papanicolaou smears and for his invaluable advice, and to Dr. Daniel Nathans for his interest and helpful criticism. They also thank Mrs. Y. Stein for technical assistance.

This investigation was supported by U.S. Public Health Service grant (FR 00130) and by grant 1N-11K from the American Cancer Society.

Abbreviations used: PBS, phosphate-buffered saline; N/C, size ratio of nucleus to cytoplasm.

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