HSV-2 Antigens Absent from Biopsied Cervical Tumor Cells: A Model Consistent with Latency[1] (36468)

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Herpesvirus type 2 (HSV-2), isolated from cervical lesions (1) and shown to be venereally transmitted (2) has been associated with squamous carcinoma of the human cervix on the basis of seroepidemiologic data (3, 4). Immunofluorescence studies have shown that exfoliated anaplastic cells from patients with preinvasive and invasive carcinoma of the cervix contain HSV-2 antigens (5). More recently however, it has been reported that cervical tumor cells in tissue culture do not show the presence of viral antigens unless grown under suboptimal conditions consisting of a medium of high pH (6). These apparently contradictory observations, as well as the difference in the function of animal cells in the artificial *in vitro* environment of cell culture and in the whole animal (7), raise the question of the nature of the association of HSV-2 with neoplastic cells *in vivo*.

The objective of this communication is to report that *in vivo*, as well as in tissue culture, cervical tumor cells do not show the presence of HSV-2 antigens unless previously exposed to conditions of stress; however, virions are not observed. This study is made possible by the observation that prior to exfoliation tumor cells on the surface of the neoplastic lesions are exposed to glandular secretions of relatively high pH (8).

Materials and Methods. Cells and cell collection. Frozen sections and impressions of biopsy specimens (biopsied tumor cells), were obtained from patients with preinvasive and invasive cervical carcinoma. Exfoliated cells were collected at the same time, by the impression of a small wet sponge directly to the surface of the neoplastic lesion, or by the irrigation method of Davis (9), using 0.1 *M* phosphate buffered saline (PBS) pH 7.1. Slides were fixed in methanol (—40°) for immunofluorescent staining and in 4% glutaraldehyde for electron microscopy.

Antisera. The preparation of rabbit immune sera against HSV-2 (Ra-2) using debris of HEp-2 cells (human epidermoid carcinoma) infected with HSV-2 for 24 hr was as described (5). They were adsorbed with human liver powder and with uninfected HEp-2 cells until they failed to stain them. Their specificity was determined by staining uninfected and HSV-2 infected HEp-2 cells. All Ra-2 sera (made in different animals) stained cells infected with HSV-2 for 6–24 hr; however, they failed to stain uninfected cells (5) and cells infected with HSV-2 for less than 5 hr (Aurelian, unpublished data), suggesting that they contain antibody to structural HSV-2 proteins (10). They were used in indirect immunofluorescent tests with fluorescein-conjugated anti-rabbit gamma-globulin made in goats (Microbiological Associates) and adsorbed with human liver powder (11). Slides were examined with a Zeiss fluorescence microscope with an Osram HBO 200W pressure mercury lamp. A BG 12 exciter filter and Zeiss OG 3 barrier filter were used.

Cell identification and assessment of fluorescence. Exfoliated (normal squamous differentiated cells, anaplastic and polymorphonuclear cells) were identified on the basis of size, nucleus shape and cytoplasm to nucleus ratio (5). The proportion of each cell type showing fluorescence was determined by scanning the entire slide by UV microscopy. A cell was accepted as showing fluorescence if

[1] Supported in part by contract NIH-NCI-71-2121 from the special virus cancer program, NCI, and in part by grant VC-16 from the American Cancer Society.

it was sufficiently bright to be clearly visible in the reduced illumination produced when the beam-splitting slide was set in position for photography. Reliability in assessing fluorescence was determined by a count of fluorescent cells from eight patients done by an independent observer; that in identifying dyskaryotic cells by marking them, restaining by the Papanicolaou method (9), and allowing a cytologist to examine the marked cells. Virtually 100% agreement was obtained. Frozen sections were cut from areas adjacent to those used for histological diagnosis.

Study groups. Twenty-two patients seen in the outpatient cervical clinic of the Johns Hopkins Hospital diagnosed as preinvasive cervical neoplasia (atypia and carcinoma *in situ*) confirmed cytologically, colposcopically and histologically. Seven patients with invasive cancer from the gynecology ward diagnosed histologically. At the time of cell collection, all patients were reported free of active herpetic lesions, on the basis of cytology and pelvic examination.

Electron microscopy. Slides fixed in glutaraldehyde were stained in duplicate with Ra-2 serum. Fluorescent atypic cells were identified and the area surrounding them scraped free of cells. The slides were fixed in 1% osmium tetroxide, soaked in uranyl acetate for 15 min, dehydrated, and impregnated with Araldite 6005 for 15 min. Gelatin capsules filled with Araldite were inverted on the areas containing the atypic fluorescent cells identified previously and, following incubation at 60° for 11 hr, the blocks containing these cells were removed from the slides by the brief application of the slides to dry ice. The cells were located on the blocks and sections made with an MT-2 Porter Blum ultramicrotome. Sections were stained with lead citrate and examined with an RCA EMU-3G electron microscope.

Results. HSV-2 antigens in biopsied tumor cells. Neoplastic cells in biopsy specimens, frozen immediately upon collection, are not exposed to suboptimal environmental conditions. To inquire into the presence of HSV-2 antigens in these cells, frozen sections and impressions of biopsies, obtained from 29 patients with cervical cancer were stained in duplicate with Ra-2 serum and PBS instead of antibody. Fluorescence was not observed in biopsies from all patients studied in this series. Furthermore, electron microscopy of cells from patients 553 and 565 did not reveal virions and cytoplasmic changes associated with the synthesis of herpesvirus antigens (12).

HSV-2 antigens in exfoliated tumor cells. By virtue of their nature, exfoliated cells are exposed to suboptimal conditions of nutrition and pH (8, 13). Accordingly slides of exfoliated cells from the same patients were stained in duplicate with Ra-2 serum and PBS instead of antibody. The results, summarized in Table I, indicate that exfoliated tumor cells from 25 of the 29 (86%) patients studied in this series react with Ra-2 serum; the proportion of reactive cells is independent of the stage of the disease but varies with the patient and ranges between 0 and 40%. Fluorescence appeared as a diffuse cytoplasmic mass, sometimes with granules (Fig. 1); nuclear fluorescence was not observed. Normal squamous differentiated cells did not react with Ra-2 serum. Fluorescence was not observed with preimmunized rabbit serum adsorbed with uninfected HEp-2 cells and with antisera to Adenovirus 18 and *mycoplasma orale.* Adsorption of Ra-2 serum with HSV-2 infected HEp-2 cells until it failed to stain infected HEp-2 cells, also removed its activity for anaplastic cells of patients 506, 533 and 542. Atypic cells from these patients reacted with two control human sera containing neutralizing antibody to HSV-2 but not with a human serum devoid of antibody to either herpesvirus type, and a smaller proportion of cells reacted with rabbit antiserum to HSV-1 (18). Twenty anaplastic cells (patients 565, 593A and 1149) containing fluorescent antigens and examined by electron microscopy did not reveal the presence of complete or incomplete virus particles. However, changes associated with the synthesis of herpesvirus antigens (12) and absent in tumor cells from biopsies were seen. These consisted of cytoplasmic vesicles, fragmentary membrane-like structures and

TABLE I. Proportion of Atypic Cells Reacting with Ra-2 Serum.

Case	Average number counted	Percent staining with		
		Ra-2	PBS	Specific[a]
	Atypia			
533	100	46	4	42
539	25	36	0	36
555	27	11	11	0
556	31	42	0	42
549	50	6	0	6
576	29	3	0	3
586	65	0	0	0
585	28	18	0	18
575	25	2	0	2
583	25	20	0	20
587	137	18	0	18
	In situ			
524	37	46	2	44
547	50	22	0	22
554	55	25	7	18
552	350	18	0	18
565	25	16	0	16
578	100	5	4	1
580	100	7	0	7
543	200	0	0	0
544	100	2	1	1
553	25	0	0	0
589	63	22	0	22
	Invasive			
506	200	20	0	20
542	150	29	2	27
591A	100	32	7	25
593A	25	32	0	32
1147	100	29	0	29
1149	145	14	8	6
541	50	14	2	12

[a] Calculated by the subtraction of the proportion of cells staining with PBS from that staining with Ra-2 serum.

irregular electron-dense granules, as well as masses of closely packed osmiophilic granules probably representing the fluorescent cytoplasmic granules (Fig. 2).

Discussion. The mechanisms of herpesvirus persistence and recurrence in patients with latent herpetic infections are still unknown (14). On the basis of work with cells grown in culture, the suggestion has been made that virus persistence is dependent on the arrest of virus multiplication in the infected cells at some point before it expresses the functions leading to inhibition of host macromolecular synthesis (7, 14), an essential prerequisite for the synthesis of structural components of the virus (15). The inadequacy of conclusions drawn from work with cells in culture as they relate to human infection is, however, evidenced by the observation that whereas EBV particles and cytoplasmic antigens are present in tumor cells grown in culture (16) they are not observed in tumor cells *in situ* (17).

The results described in this communication show that biopsied cervical tumor cells harbor the HSV-2 genome in a repressed state. Under naturally occurring suboptimal conditions (high pH), expression is initiated resulting in the presence of HSV-2 antigens in exfoliated tumor cells; however, unlike the cells removed from the tumor and grown in culture at a pH suboptimal for growth (6), exfoliated cells containing structural HSV-2 antigens do not show evidence of virus particles. Two points merit discussion with regard to these findings.

First, the specificity of the reaction with Ra-2 serum for cervical anaplastic cells was previously shown (5) and further ascertained by the following control experiments: (a) Ra-2 sera react with HEp-2 cells infected with HSV-2 for 6–24 hr but not with uninfected cells or cells infected with HSV-2 for 1–4 hr (5, Aurelian, unpublished data); (b) staining was not observed with PBS, preimmunized rabbit sera adsorbed with uninfected HEp-2 cells, antisera to Adenovirus 18 and *Mycoplasma orale,* whereas it was present in cells stained with antisera to HSV-1, displaying the expected cross-reactivity (18); (c) anaplastic cells reacted with two human sera from subjects without cancer but with neutralizing antibody to HSV-2 and not with a human serum devoid of antibody to either herpesvirus type, and (d) adsorption of Ra-2 sera with HSV-2 infected HEp-2 cells until they failed to stain the infected cells, also removed their reactivity for atypic cells. Nonspecific staining reported previously for a proportion of dyskaryotic cells stained with PBS (5) was absent in 90% of

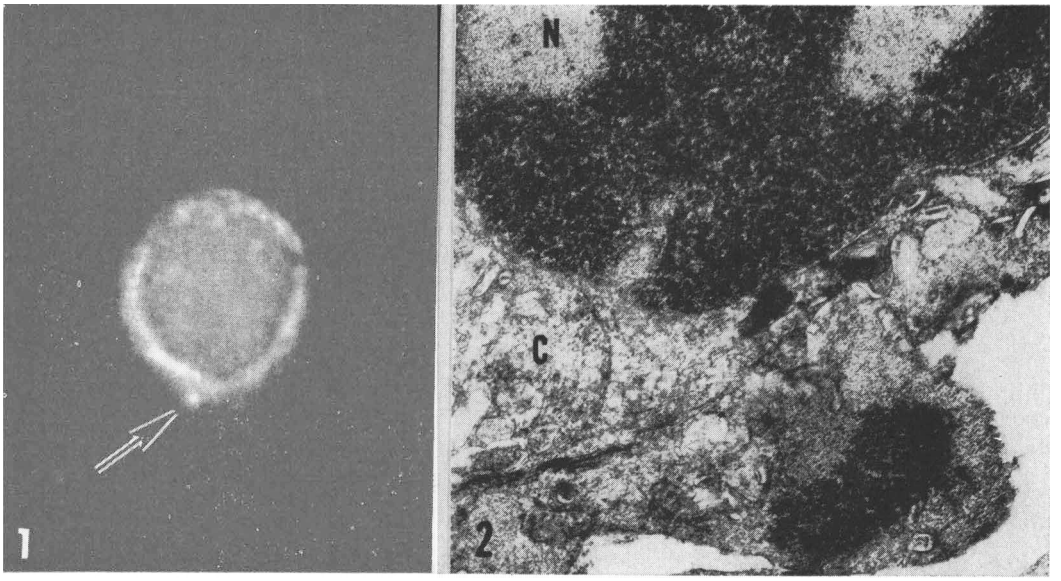

FIG. 1. Exfoliated anaplastic cell from patient 565 stained with Ra-2 serum, displays diffuse and granular (arrow) cytoplasmic fluorescence. (×1000).

FIG. 2. Dense granular accumulation (G) and disorganized membranous structures in cytoplasm from the fluorescent cell in Fig. 1. Nucleus (N), cytoplasm (C). (×25,700).

patients studied in this series.

Second, considering the likely hypothesis that the only difference between exfoliated tumor cells and those in biopsy specimens obtained from the same patients at the same time is the exposure of the tumor cells on the surface of the neoplastic lesions, prior to exfoliation, to glandular secretions of high pH (8), the presence of HSV-2 antigens in exfoliated but not in biopsied cells suggests that tumor cells harbor the viral genome in a repressed state. This interpretation is consistent with the observation that cells removed from the tumor biopsy and grown in culture also do not show the presence of HSV-2 antigens unless maintained in a medium of high pH (6). However, unlike the culture situation (5, 6, 19), exfoliated anaplastic cells do not show the presence of nuclear fluorescence with Ra-2 serum and virus particles are not observed by electron microscopy. The clinical significance of this difference is stressed by the observation that all patients studied in this series were free of active herpetic lesions at the time of collection of their exfoliated cells.

Since proteins specified by herpesviruses are made in the cytoplasm and migrate to the nucleus for virus assembly, it may be suggested that in anaplastic cells migration does not occur. It is noteworthy that arginine deprivation of herpesvirus infected cells causes the absence of nuclear fluorescence and inhibits formation of virus particles (20), although the major viral proteins are synthesized and accumulate in the cytoplasm; their migration to the nucleus does not occur (21).

We wish to express our gratitude to Mrs. B. Schumann and Mrs. C. Burl for excellent technical assistance and to Mrs. J. Arnick for help with the manuscript.

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Received Jan. 26, 1972. P.S.E.B.M., 1972, Vol. 140.