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Replication of Herpes Simplex Virus Type 2 in Normal Dysplastic and Neoplastic Human Cervical Epithelia*

ROSEMONDE MANDEVILLE,† ARTHUR HOLLOWAY,‡ STUART C. LAUCHLAN§ and RENÉ SIMARD¶

Institut du Cancer de Montréal, Centre Hospitalier Notre Dame, 1560 Sherbrooke est, Montréal, Canada H2L 4M1

Abstract—*The organ culture method was used to compare the susceptibility of normal, dysplastic and neoplastic epithelia (CIS and invasive cancer) of human cervical epithelia to HSV-2 infection as well as the study of the induced cytopathological alterations. Viability of these explants was first assessed by preservation of cellular morphology and growth characteristics established by (^{3}H)-TdR incorporation into cells and the observation of mitotic figures in all explants at different times after culture. Inoculation with Herpes simplex type 2 virus caused a productive infection and pathognomonic cytopathological changes in normal, dysplastic as well as in neoplastic cells, producing lesions basically very similar to those observed in exfoliative cytology of Herpes cervicitis. Virus particles were visualized by electron microscopy and infective virus released in the growth medium was demonstrated by an increase in virus titer confirming histopathological findings.*

INTRODUCTION

THE SPECTRUM of histopathological changes involving carcinoma of the human cervix includes dysplasia (mild, moderate and severe), carcinoma *in situ* (CIS) and invasive cancer [1]. It is highly probable that each neoplasm of the cervix passes through some or all of these sequences in its development. The dichotomy between normal tissue and invasive cancer is divided here into two categories, dysplasia, and CIS, which occupy the more benign and malignant parts of this spectrum, respectively. During the past several years, data have been steadily increasing which establish a relationship between Herpes simplex type 2 virus (HSV-2) and epidermoid carcinoma of the human cervix [2–4]. This relationship is a complex one and was originally recognized by seroepidemiologic studies [5–7] and supported by several lines of evidence [8, 9] mainly *in vitro* transformation studies [10–13]. Contributing factors to the importance of such a role for HSV-2 are its ubiquity and its occurrence in the male as a penile lesion [13]. Thus, by far, most of this evidence is circumstantial [for review articles 1, 2] and there seems little doubt that an association between HSV-2 and cervical cancer exists. However, pertinent data establishing a causal relationship are still missing. We have developed an organ culture model [14] which allows the maintenance of normal, dysplastic and neoplastic cervices of a long period of time (up to 2 months) and the comparative study of the susceptibility to HSV-2 infection of these explants.

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†Present address: Lady Davis Institute for Medical Research, Jewish General Hospital, 3755 Côte Saint Catherine Road, Montréal, Québec, Canada H3T 1E2.

‡Present address: Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada.

§Present address: Women and Infants Hospital of Rhode Island, 50 Maude Street, Providence, Rhode Island 02908, U.S.A.

¶Present address: Institut du Cancer de Montréal, Centre Hospitalier Notre-Dame, 1560 Sherbrooke est, Montréal, Canada H2L 4M1.

MATERIALS AND METHODS

Human uterine endo- and ectocervices were initiated from 3 groups of patients. 1. Normal patients undergoing hysterectomy for non-malignant pathology; 2. individuals with intraepithelial neoplasia (atypia), i.e., dysplasia and CIS. These were obtained by

punch biopsy from within the area of pathological epithelium under colposcopic guidance. The histologic lesions were graded, prior to the growth in culture, as mild, moderate or severe dysplasia or CIS according to established principles [15, 16]; 3. patients with histologically diagnosed and untreated invasive squamous carcinoma of the cervix immediately before ^{137}Caesium implantation and from whom punch biopsies were obtained. In these studies, none of the patients had a clinical history of recent or post herpetic cervicites.

The method used for the preparation of cultures was as follows: Biopsies collected in phosphate buffered salt solution (PBS), were processed within 1–4 hr. After several washes in PBS, gross connective tissue and mucous material were removed and specimens were then cut into fragments of about 1–2 mm^3, these fragments were then explanted in 35 mm plastic Petri dishes (Falcon Plastic Co., Oxnard, CA) containing 2 ml of culture medium and incubated at 37°C in 5% CO_2 atmosphere. Six to ten explants were placed in each dish. Each explant studied contained layers of epithelium, parabasal and underlying stromal tissues.

The culture medium consisted of Minimum Essential Medium (MEM) supplemented with with 1.5% carboxy methyl cellulose (4000 centipoise, Sigma Chemical Co., St. Louis, MO), 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) and antibiotics (penicillin 100 i.u./ml, streptomycin 100 mg/ml and fungizone 2.5 μg/ml). After fixation of tissue in Bouin's solution, histological examinations were performed on hematoxylin and eosin-stained sections.

(^{3}H)-*Thymidine incorporation*

At different times after the initiation of cultures 10 μCi/ml of (^{3}H)-TdR (New England Nuclear, Boston, MA, specific activity 13.4 Ci/mmole) were added to different dishes, each of which contained 4–6 explants. Incubation with the labelled compound proceeded for 24 hr, at which time several explants from each dish were removed, washed extensively and incubated overnight in non-radioactive culture medium. The labelling was measured by autoradiography on 1.0 μm thick sections of explants fixed in Bouin's solution. Slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 in distilled H_2O) air dried for 2 hr at room temperature and stored in the dark at 4°C for 2 weeks. Autoradiographs were developed in Kodak D-19 developer for 2 min at 18°C. The preparations were then stained with hematoxylin and eosin.

Electron microscopy

Specimens were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 at 4°C for 30 min, washed twice in the same buffer, postfixed for 30 min with 1% OsO_4, dehydrated and embedded in Epon 812. In infected explants, areas of epithelium were chosen for thin sectioning by examining 0.5 μm sections stained with toluidine blue. Ultrathin sections were stained with 5% uranyl acetate and lead citrate, and examined with a Siemens "Elmiskip 101".

Viral techniques

Infection of explants. The MS strains of HSV-2 (American Type Culture Collection, Rockville, MD) used in this study was propagated on primary cultures of rabbit kidney cells. Explants were infected by suspension in virus containing medium (5×10^6 PFU per ml) at 37°C for 2 hr. After adsorption, the cultures were washed with fresh medium and cultured at 37°C in medium containing methyl cellulose and 2% fetal calf serum.

Virus assay

Virus infectivity was titrated on primary rabbit kidney cells, by the plaque method [17] under usual MEM medium containing 5% fetal calf serum, antibiotics and 0.6% agarose. To visualize the plaques, dishes were stained 2 days later with neutral red (1:7500 in PBS).

RESULTS

Tissues from 250 biopsies were used in this study. These included 30 dysplastic, 15 CIS and 20 invasive cancer patients. The remaining cervices were normal by conventional histological and clinical criteria [15, 16]. Results of cultivation were evaluated from findings in serial histological slides. When evaluating survival, the best preserved explants in a particular group in a Petri dish were considered. This method was chosen because it eliminates those explants which were damaged by technical mishandling or in which epithelium was not included. In preliminary experiments, we have used several culture media, and various sera. Results of

cultivation proved unsatisfactory as explants showed marked or total necrosis. Media of high viscosity that could support the growth of loosely architectured cells were then tested. Good results (80% survival) were obtained with methylcellulose, at a concentration of 1.5% as a supplement to MEM as compared to no survival in agar and agarose supplement to MEM.

Growth characteristics

The optimal conditions for maintainance of human cervical epithelium thus established, the sequential study of normal explants (Fig. 1) permitted us to study the growth pattern of these epithelia. In the first day of culture, ectocervical epithelium exhibits a splitting at the level of the parabasal layer (Fig. 2) and a desquamation of the intermediate and superficial layers of the epithelium. Only the basal and parabasal cell layers remain attached. During the subsequent 5–6 days, the basal cells flatten and grow to surround the entire explant (Fig. 3).

In endocervical epithelia, columnar cells loose their mucous secreting capacity and grow to surround the explant at about the same time (Fig. 4). Subsequently, growing epithelial cells from ecto- and endocervical explants form a few layers that continue to divide (mitotic figures were often seen), but in the majority of instances remained simplified and poorly differentiated.

In cervical explants derived from atypias (dysplasias and CIS), the results of cultivation show good preservation of cell morphology for up to a few weeks (Fig. 5) with typical pathognomonic features of the dysplastic stage. While 80% of normal, dysplastic and CIS explants could be maintained in culture, invasive cancer explants were very difficult to culture; out of a total of 20 explanted carcinomas, we evaluated 10 as having good preservation of malignant cells. These explants showed foci of cells with cytological signs of viability for up to 4–7 days (Fig. 6). However, we could not extend the cultivation of invasive cancer tissue for more than 10 days.

Histological studies of these biopsies maintained in organ culture showed that cells from normal, dysplastic and neoplastic epithelia resemble their *in vivo* counterparts in that they have the same morphology by classical histology and by electron microscopy. Mitosis was a regular finding most oftenly seen in neoplastic epithelia even after a week in culture.

DNA synthesis

The incorporation of (^{3}H)-TdR into cells was studied in a total of 10 normal and 15 pathological cervices. In normal ectocervical preparations, labelling immediately after explanation, showed that proliferative cells were confined to the parabasal and occasionally to the basal layers of the squamous epithelium (Fig. 7). Most labelled cells were in the first and second parabasal layers, indicating this area to be the only active progenitor compartment. Cells in the basal layer were also labelled, but less frequently than in the parabasal layer and there was no uptake by the intermediate and superficial layers. In endocervical epithelia only few columnar cells were labelled, on the other hand, stromal cells in both the endo- and ectocervix were not labelled.

In normal explants from endo- and ectocervix, grown for a few days in organ culture, the incorporation of (^{3}H)-TdR showed labelling of 95% or more of cells surrounding the explants (Fig. 8). In the stroma, however, very few cells were labelled (5–6 cells around blood vessels).

Explants of atypias labelled before and during culture showed labelling in more than 95% of the epithelial cells of the explants, but this was especially marked in layers clothing the explant (Fig. 9). Invasive carcinomas could also be labelled and showed heavy incorporation of (^{3}H)-TdR before and during culture. However, this incorporation was focal, and in the latter case not more than 15% of the cells were labelled.

Cytopathological changes induced by HSV-2 infection

As illustrated in Table 2 with an input multiplicity of 5×10^6 PFU/ml, morphological alterations were detectable only 4 days post infection in about 70% of the epithelial cells in normal ecto- and endocervical cells. Histologically, the first signs were hypertrophy and rounding up of individual cells (Fig. 10) with irregularity of nuclear outlines and focal cell fusion. Later in infection (5–6 days post infection) the enlarged pale nuclei were packed with small acidophilic particles, so that a distinct homogenous granular ground glass texture could be observed under low magnification. This was accompanied by peripheral condensation of the chromatin (Fig. 10). Some nuclei contained a single, central, coarsely granular acidophilic inclusion ("Cowdry A inclusion

Table 1. Proportion of HSV-2 infected cells according to the type of explant studied*

Type of explant	No. of infected explants	Time† post-infection (days)	% of cells showing CPE	Characteristic features
Normal	30/30	4	70	—
Dysplastic	20/20	2	70	—
CIS	10/10	2	50	polykaryocytes
Invasive cancer	10/10	2	50	polykaryocytes

*Virus input 5×10^6 PFU/ml for all explants studied.
†Time of appearance of first morphological signs of infection.

Table 2. Release of infectious virus by HSV-2 infected normal explants*

Time post infection (hr)	CPE‡	Virus† yield (PFU/ml) Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 4
0	—	0	0	0	0
4	—	0	0	0	0
24	—	0	0	0	0
48	—	0	0	0	0
72	—	7×10^3	2×10^3	9×10^2	5×10^3
96	+	9×10^3	8×10^3	7×10^3	9×10^3

*Virus input 5×10^6 PFU/ml.
†Virus released in supernatant fluid and assayed by plaque method (material and method) on primary rabbit kidney cells.
‡Cytopathological changes detected in normal explants infected with HSV-2 by histological examination.

body"), surrounded by a clear halo with the nuclear chromatin peripherally arranged. Only in these instances could viral infection be specifically diagnosed. Infected cells then separated from the neighboring normal cells and exfoliated rapidly and degenerative changes occurred shortly thereafter.

Using the same viral titer (5×10^6 PFU/ml), replication of HSV-2 in pathological explants was also studied. Cervical dysplasias showed an early and severe response to infection. Thus, explants derived from cervical dysplasias and infected just before explantation showed all the sequential cytological manifestations of HSV-2 infection described for normal epithelium infected by HSV-2 but only 1 or 2 days earlier than in normal explants (Table 1).

When infected with HSV-2, CIS and invasive cancer explants responded in an identical way. In addition to the characteristic lesions seen in normal dysplastic explants, CIS (Fig. 11) and invasive cancer (Fig. 12) showed a large number of polykaryocytes. These giant cells possessed 5–8 nuclei each in which margination of the chromatin and A-inclusion bodies could be seen (Figs. 11 and 12). These cells were only seen in CIS and invasive cancer explants, i.e., polykaryocytes were not detected in normal or dysplastic explants (Table 1) infected with HSV-2. In all cases of control explants initiated from normal or pathological cervices, no changes compatible with HSV-2 infected cells could be observed although some explants were kept for up to 2 months.

Electron microscopy made it possible to study the nuclear and cytoplasmic changes in much greater detail and to demonstrate virus particles in infected explants (Figs. 13, 14 and 15) from both normal and pathological cervices. Infected cells showed peripheral condensation of the chromatin, reduplication or quadruplication of the inner nuclear membrane (Fig. 13, arrows) and accumulation of naked viral particles in the nucleoplasm. The envelopment of naked particles appeared to take place at the nuclear membrane, and occasional figures of nuclear "budding" could be visualized (Fig. 15). Mature virions accumulated in the cytoplasm either infraform or in perinuclear cisternae or within membrane bound vacuoles (Fig. 14). Virions were characterized by a dense viral core, a relatively opaque and thick capsid and an outer membranous envelope. Sometimes, we observed empty capsids in the nucleus (Fig. 13).

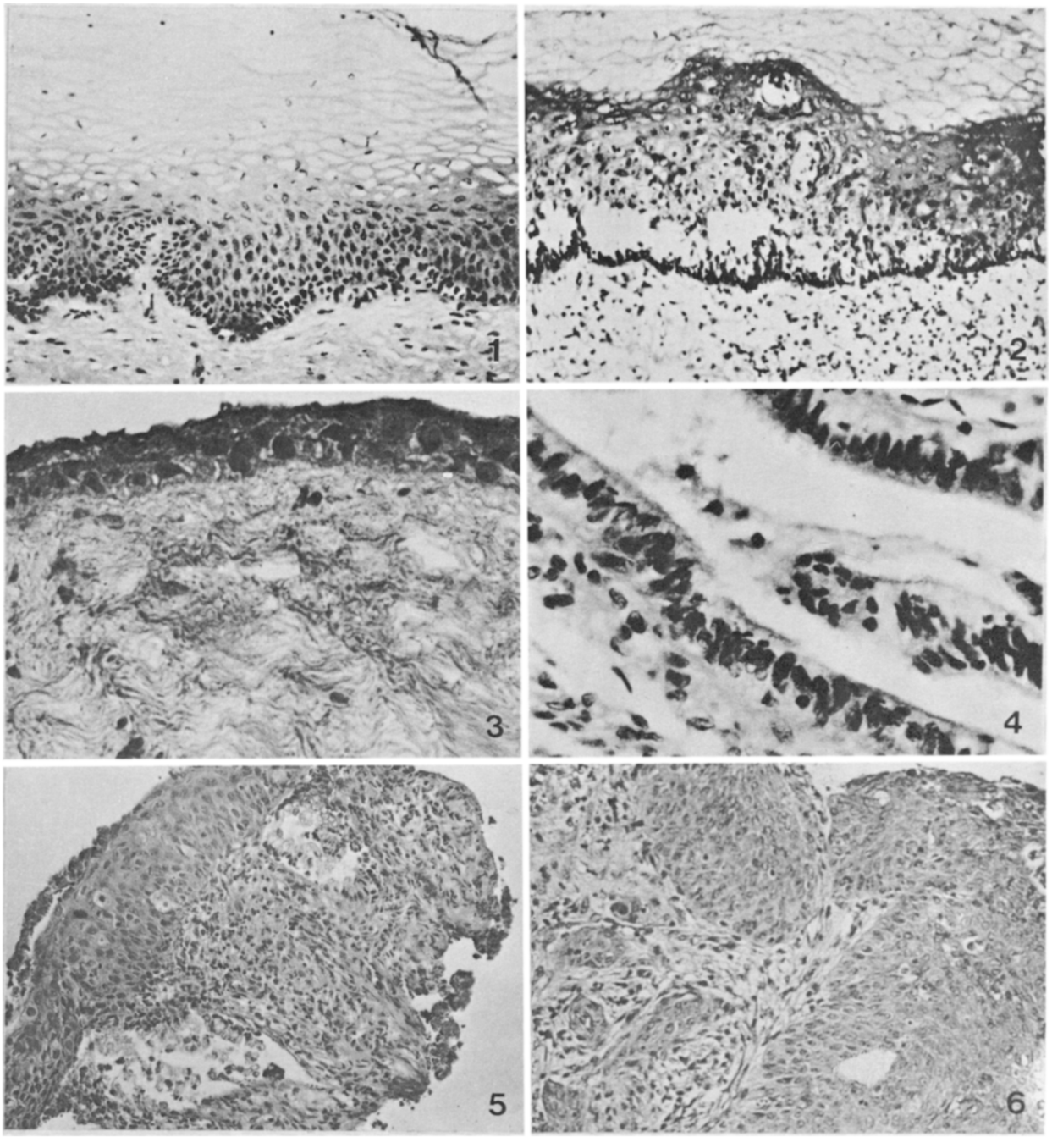


Fig. 1. *Normal human cervical epithelium before explantation; untreated ectocervix.* × 100 (*hematoxilin and eosin.*)

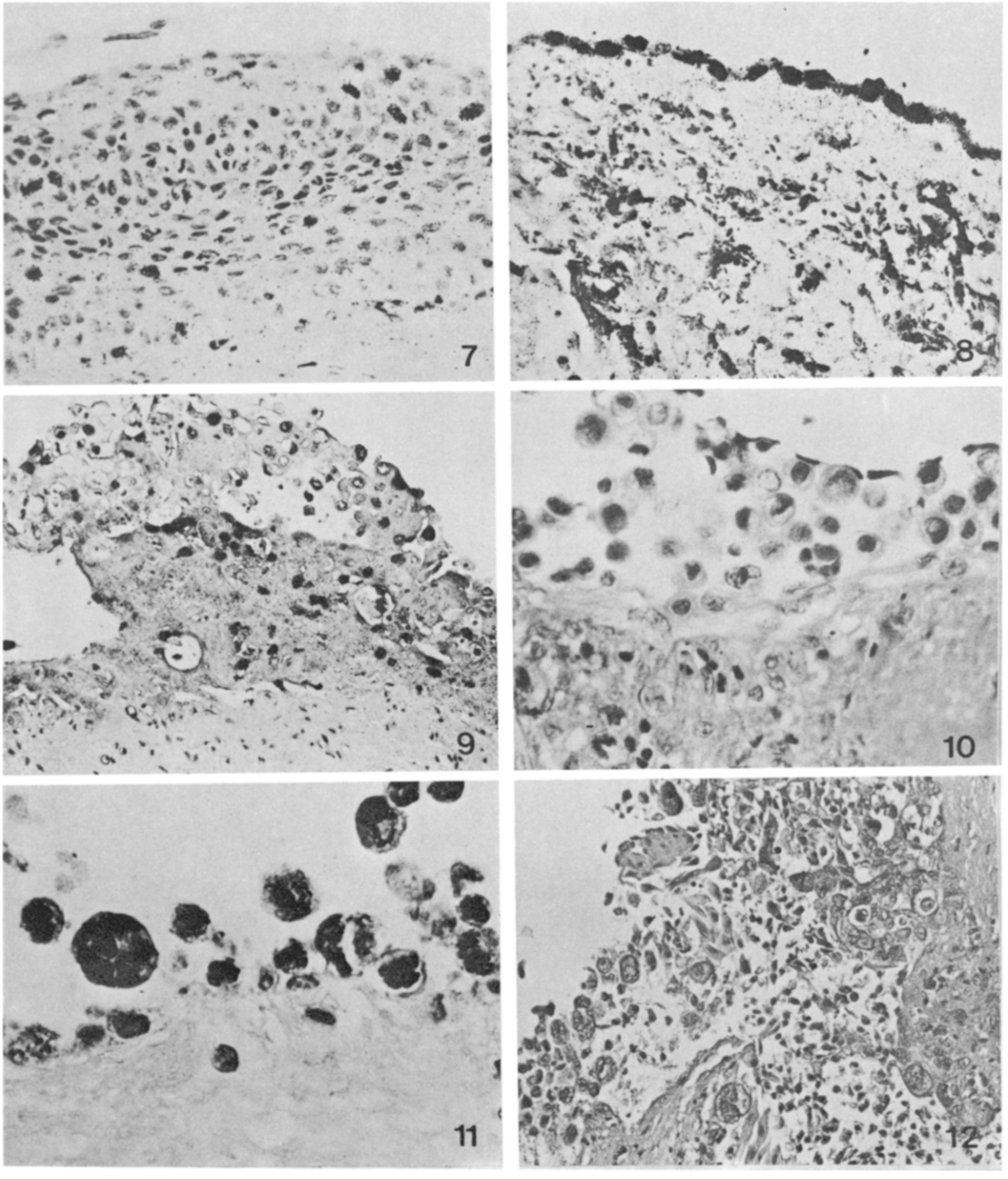
Fig. 2. *Human cervical tissue, ectocervix, after* 1 day *in organ culture. Note the lifting off of the intermediate and superficial layers of the epithelium.* × 100 (*hematoxilin and eosin*).

Fig. 3. *Human cervical explant after* 5 days *in organ culture. Epithelial cells showing all characteristics of normal viable surface epithelium.* × 250 (*hematoxilin and eosin*).

Fig. 4. *Human cervical explant after* 5 days *in organ culture. Columnar epithelium showing good preservation of viable cells.* × 250 (*hematoxilin and eosin*).

Fig. 5. *Explant of epidermoid carcinoma* in situ 7 days *in organ culture. Explant still showing characteristic pre-invasive cancer cells.* × 250 (*hematoxilin and eosin*).

Fig. 6. *Explant of invasive carcinoma* 5 days *in organ culture. Explants showing pearls of cancer cells, separated by stroma.* × 250 (*hematoxilin and eosin*).

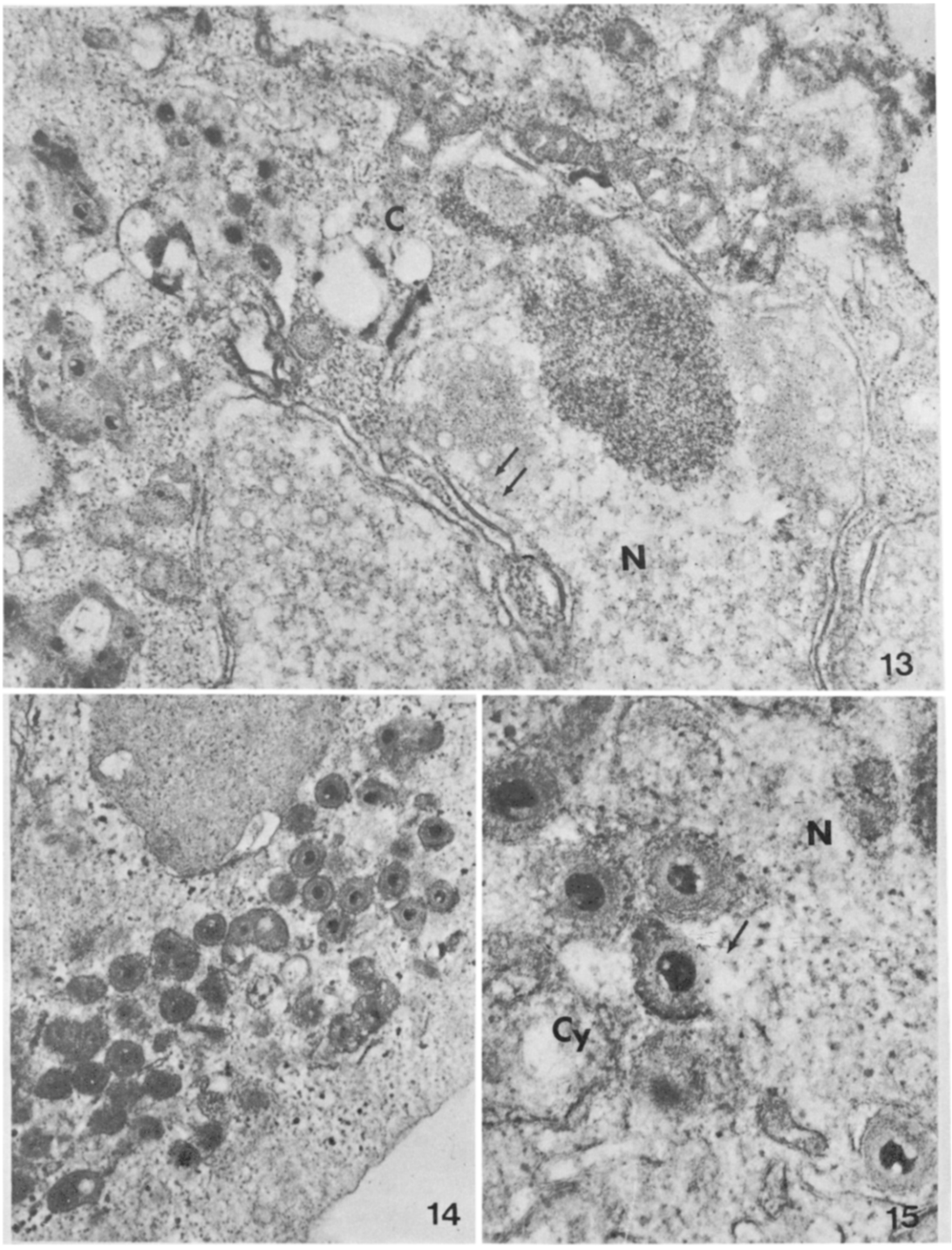


Figs. 7 *and* 8. *Autoradiograms of normal cervical explants labelled* (7) *before culture;* (8) *after* 32 days *in organ culture. Note labelling of few parabasal cells in the first day* (7) *and most of the cells surrounding the explant in* (8). *Exposure time* 2 weeks. × 100 (*hematoxilin and eosin*).

Fig. 9. *Autoradiograms of CIS grown for* 10 days *in organ culture. Note labelling throughout the full thickness of the epithelium.* × 100 (*hematoxilin and eosin*).

Fig. 10. *Explants of normal cervical epithelium and dysplastic;* 5 days *after infection with HSV-*2 (2.4×10^6 PFU/ml). *Obvious intranuclear inclusions, margination of chromatin, characteristics of HSV-*2 *infections can be recognized.* × 250 (*hematoxilin and eosin*).

Figs. 11 *and* 12. *Explants of CIS* (11) *and invasive carcinoma* (12) 2 days *after infection with HSV-*2 (2.4×10^6 *PFU/ml). Note multinucleated cells as a prominent feature* (*arrows*). (11) × 250 (*hematoxilin and eosin*). (12) × 100 (*hematoxilin and eosin*).



Figs. 13, 14 *and* 15. *Electron micrographs of explants of normal* (*Fig.* 13) *and dysplasia* (*Figs.* 14 *and* 15) *cervical epithelium* 5 days *after infection with HSV-2. Note budding* (*arrow*) *from the nucleus* (*N*) *to the cytoplasm* (*Cy*). *Protiferation of the nuclear membrane* (*double arrow*) *is a constant feature in HSV-2 infection. Fig.* 13 ×40,000; *Figs.* 14 *and* 15 ×80,000.

The cytopathologic changes observed by histological and electron microscopic techniques were highly reproducible and infective virus released by normal cervical explants infected with HSV-2 was assayed from the supernatant of cervical explants at various time intervals. The results presented in Table 2 show that infective virus could not be detected earlier than 72 hr post infection, the virus titer remains high after 96 hr reaching values of about 9.5×10^3 PFU/ml. In all experiments, the amount of infective virus decreased significantly later in the course of infection.

DISCUSSION

The present results show that HSV-2 replicates actively in both normal and pathological cervical cells. This study also provides evidence that normal, dysplastic and neoplastic epithelia of the human cervices can be grown in organ culture for up to a few weeks when the nutrient medium is supplemented with 1.5% methylcellulose. This is of great interest since normal cervical epithelium is very difficult to maintain as established cell lines (reviewed by Wilbanks, 1976). Only in one instance was Vesterinen *et al.* [18, 19] able to grow cells from explants as epithelial monolayer cultures.

It is our belief that this semi-viscous media (methylcellulose) is of importance since it helps preserve the active progenitor cells in the normal epithelia as indicated by the results of the study of *in vitro* DNA synthesis. Convincing data on the localization of proliferating cells in normal and pathological cervical epithelium is also provided. In normal epithelia, TdR-labelled nuclei were present in the deeper layers of the squamous epithelia while cells in the intermediate and superfical zones were not labelled. Proliferation of the lower layer of parabasal cells indicated the latter to be the main source of cell renewal, whereas the basal cells proper apparently serve as reserve cells. These results are in agreement with previous studies performed on normal epithelium [20, 21]. The uppermost layers of the squamous epithelium seem to have only a protective function, since there is no TdR-incorporation into these cells. Furthermore, they are first to desquamate when normal cervical squamous epithelium is explanted (Fig. 2). Their possible protective role is also supported by the observation that cytopathological changes in normal epithelium occur only late, i.e., 1–5 days post-infection (after the desquamation of the superficial layers). In cases of dysplasias and CIS, labelling of cells in the superficial zone reflects the disturbance in the rate of DNA replication in those cells. Similar findings were obtained also with invasive cancer cells.

Normal cervical epithelium maintained in organ culture showed sloughing of the superficial layers and outgrowth from germinal cells i.e., basal and parabasal layers. Viability of these cells was morphologically and autoradiographically illustrated. Pathological cervices showed a greater number of surviving cells of abnormal morphology that could be labelled with (^{3}H)-TdR.

Replication of HSV-2 in explants derived from normal ecto- and endocervical epithelium, showed characteristic changes of herpetic infection. These changes are very similar to those described in herpetic cervicites. On the other hand, we could not detect any specific lesion in stromal cells. This was believed to be due mainly to the low percentage of dividing cells as well as the poor preservation of stromal cells in culture. There is very little information [22] concerning the replication of HSV-2 in normal cervical epithelia, while no reports have been made on replication of HSV-2 in pathological cervical tissues. In our studies, dysplastic explants, mild, moderate or severe, when infected with HSV-2 showed essentially the same cellular alterations as normal cervical epithelium infected with HSV-2. On the other hand, CIS and invasive cancer cells, when infected revealed an earlier appearance (2–3 days post-infection) of cellular changes as well as a characteristic conspicuous appearance of multinucleated cells, i.e., polykaryocyte formation (Figs. 11 and 12). We believe that formation of polykaryocytes by HSV-2 in malignant explants and not in normal explants, is due to a neoplastic feature of the host cell since viral and environmental factors were rigorously the same as for both normal and pathological explants. Indeed, similar work with other viruses appears to indicate that malignant cells from established cell lines have a higher fusion capacity than primary and secondary diploid cells [23].

The fact that dysplastic and malignant epithelia (CIS and invasive cancers) could be infected by HSV-2 is of special importance in the pathogenesis of herpes genitalis. It is a common belief that cells transformed by RNA and DNA viruses are often resistant to reinfection by the homologous virus and especi-

ally since Raff and Li [24] have reported that cells transformed by u.v.-inactivated HSV-2 (333-8-9 cell line) were resistant to super infection by HSV-2. It might also lead to the assumption that HSV-2 is not etiologically related to cervical cancer.

In *résumé*: the evidence in this report confirms the usefulness of the organ culture model for studying the cytopathological changes accompanying HSV-2 replication in the human cervical epithelia. It also shows that pathological cervical tissue can replicate this virus; this is a new approach that may provide the basis for further investigation in which the effect of other lytic and transforming viruses as well as suspected carcinogenic agents can be quantitatively and systematically studied in an attempt to elucidate the causative agents in invasive cancer of the cervix. This technique could also be of value in the treatment of herpes genitalis as an *in vitro* assay for test and assessment of any new pharmaceutical product.

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