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Herpes Simplex Virus and Human Cancer. II. Search for Relationship Between Labial Tumours and Herpes Simplex Type 1*

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Abstract—*Cellular proliferation was obtained from* 30 *out of* 58 *labial tumours examined. None of the cellular cultures obtained presented Herpes Simplex Virus type* 1 *(HSV-*1*) specific antigens. Tumour cell cultures demonstrated the same susceptibility to HSV-*1 *as cells from normal lip tissue, HEp-*2 *and BHK cells. The presence of HSV-*1 *DNA was investigated in* 12 *labial tumours by the blotting technique. The sensitivity of the technique made it possible to reveal* 0.5 *viral genome equivalent per cell. Viral DNA was not detected in any of the tumours tested.*

INTRODUCTION

HERPES Simplex Viruses (HSV) have been divided into two types: type 1 (HSV-1) which is normally responsible for oral lesions and type 2 (HSV-2) which causes genital lesions. HSV-1 infections are usually acquired during early childhood by the oral–respiratory route [1] and more than 90% of adults have evidence of past HSV-1 infections [2]. On the other hand, HSV-2 infections are generally acquired venereally [3]. HSV infections are not necessarily lytic, culminating in the destruction of the cell and in elimination of virus. Both HSV-1 and HSV-2 can persist in a non-lytic form in some tissues such as the sensory ganglia [4]. Moreover, there are several data supporting a relationship between HSV and human tumours. The association between HSV-2 and cervical cancer is suggested by a number of studies. The first evidence comes from reports that the incidence of neutralizing antibodies to HSV-2 is higher in patients with cervical carcinoma than in matched controls [5–8]. Secondly, human cervical tumour cells contain HSV-2 RNA [9], HSV-2 DNA and RNA [10]. Thirdly, HSV-2 specific antigens are present in cervical carcinoma extracts [11–16]. The possible correlation between cervical carcinoma and HSV-2 stimulated us to search for a similar correlation between HSV-1 and lip tumours. With regard to this subject it has to be noted that the scientific literature is very scarce and only a few studies have been undertaken, especially concerning the epidemiologic and clinical aspects. It has been reported that often a tumour arises in the same location of herpetic recurrences [17]. It seems also that the immune response to HSV-1 is higher in preneoplastic stages, is reduced during oncogenesis and is restimulated after tumour excision [18]. Also the short time lag between the herpetic lesion and some tumour growth supports the hypothesis of a HSV-1 oncogenic role [19].

This paper reports data concerning (i) the search of specific antigens in cells derived from different labial tumours and cultivated *in vitro*, (ii) the susceptibility of these cells to HSV-1 infections and (iii) the search for the presence of viral DNA in tumour cells employing the blotting technique originally described by Southern [20].

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MATERIALS AND METHODS

Tumours and anamnesis

Fifty-eight patients presenting ulcerous–nodular lesions of the lip (spinocellular epitheliomas) were subjected to anamnestic inquiry before surgery. Our questions were directed towards previous herpetic recurrences and their eventual sites.

Research of antibodies to HSV-1

Two blood specimens were obtained from each patient, the first at the time of surgery and the second 3–4 months post surgery. Sera were titrated for HSV-1 antibodies by the microtiter complement fixation (CF) test [21] with antigens obtained from cytoplasmic extracts of cells infected with HSV-1 (F) [22].

Cell cultures

Tumours, obtained by biopsy, were finely cut in small Petri dishes. Some fragments were laid in part on the bottom of 30 ml Falcon plastic tissue culture flasks, in part on cover slips placed in 3.5 cm Falcon tissue culture dishes. The remaining tumour fragments were utilized for DNA extraction. For cell growth Eagle's minimal essential medium supplemented with 10% foetal bovine serum (ESV-10) was added. When confluence was reached, cells were dissociated by trypsin-EDTA and transplanted; cover slips, on which almost always both epithelial-like and fibroblast-like cells were present, were directly used for detection of HSV-1 specific antigens by indirect immunofluorescence.

Susceptibility of cells derived from tumours

HSV-1 (F) and HSV-2 (G) were used. These strains were kindly supplied by Prof. Roizman (Chicago). Cell susceptibility to HSV was determined by the number of plaques produced in cell cultures infected with 10^{-4}PFU/cell.

Immunofluorescence technique

Cover slips of cell cultures from lip tumours, of uninfected HEp-2 cells and HEp-2 cells productively infected with HSV-1 were tested by indirect immunofluorescence with specific rabbit sera and fluorescein-conjugated anti-rabbit IgG.

DNA purification from tumour cells

Tumours, already cut in small fragments, were minced with scissors, homogenized in TD (25 mM Tris–HCl pH 7.4, 137 mM NaCl, 0.7 mM Na_2HPO_4, 5 mM D-glucose) [23] in a Dounce homogenizer equipped with a loose fitting piston, and digested from 6 to 10 hr at 37°C with 50 μg/ml Proteinase K (Merck) in the presence of 20 mM EDTA and 0.5% sodium dodecylsulfate (SDS). The viscous lysate was then gently extracted with phenol–chloroform mixture (60 g of phenol and 40 ml of chloroform), dialyzed first against 10 mM Tris–HCl pH 7.5, 5 mM EDTA, 1 M NaCl and then against the same buffer without NaCl. RNA was degraded by treatment with 40 μg/ml of RNase A (Worthington) at 37°C for 1 hr, followed by Proteinase K digestion, two more phenol–chloroform extractions and dialysis as above.

^{32}P-labeled HSV-1 DNA probe

HSV-1 (F strain) viral nucleocapsids were purified from cytoplasmic extracts of infected HEp-2 cells by velocity sedimentation in sucrose gradients [24], disrupted with 0.5% SDS, 20 mM EDTA and digested with Proteinase K. After two extractions with phenol–chloroform, viral DNA was furthermore purified by one cycle of equilibrium centrifugation in a CsCl gradient. *In vitro* labeling by nick translation was performed as follows. ^{32}P-labeling was carried out in a reaction mixture containing: 20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 μg bovine serum albumin (Sigma, fraction V), 80 μCi each of the four ^{32}P-deoxyribonucleoside triphosphates (The Radiochemical Centre, Amersham; specific activity 350 Ci/mM), 4 μg of HSV-2 DNA and 20 units of *E. coli* DNA polymerase I (grade I, Boehringer, Mannheim). Incubation at 18°C for 45 min was terminated by the addition of EDTA. Incorporated radioactivity (35–40% of input) was separated from residual triphosphates by Sephadex gel filtration (Pharmacia). The ^{32}P-DNA probe had a specific activity of 1×10^8 to 2×10^8 cpm/μg.

Blot-transfer hybridization

Cellular DNA (20 μg) was digested with ECO RI restriction endonuclease (Miles) in the appropriate reaction mixture at 37°C for 3 hr. The reaction was stopped with EDTA and DNA was precipitated with ethanol, dissolved in a small volume of five-fold diluted electrophoresis buffer (E. buffer: 40 mM Tris–HCl pH 7.8, 5 mM sodium acetate, 1 mM EDTA) [25]. Electrophoresis was performed on 0.6% agarose gels (Biorad) in horizontal slabs 0.5 cm thick and 20 cm long, using 20 μg of DNA for each sample. Electrophoresis was at 1.5 V/cm for 15 hr at room temperature. After electrophoresis, DNA was denaturated in the gel and transferred to a nitrocellulose

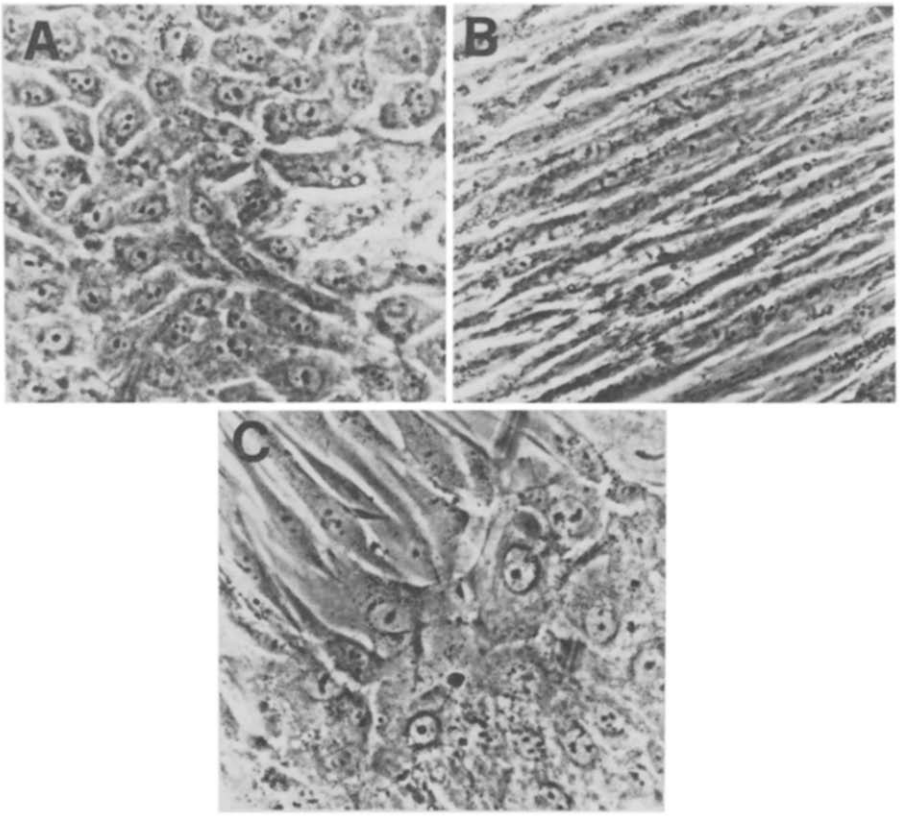

Fig. 1. *Cells proliferated from labial tumour fragments: Epithelium-like cells* (*A*), *fibroblast-like cells* (*B*) *and mixed cell population* (*C*). *Live, unstained,* 22 days *old cultures are photographed under an inverted microscope at approximately* 500 × *magnification.*

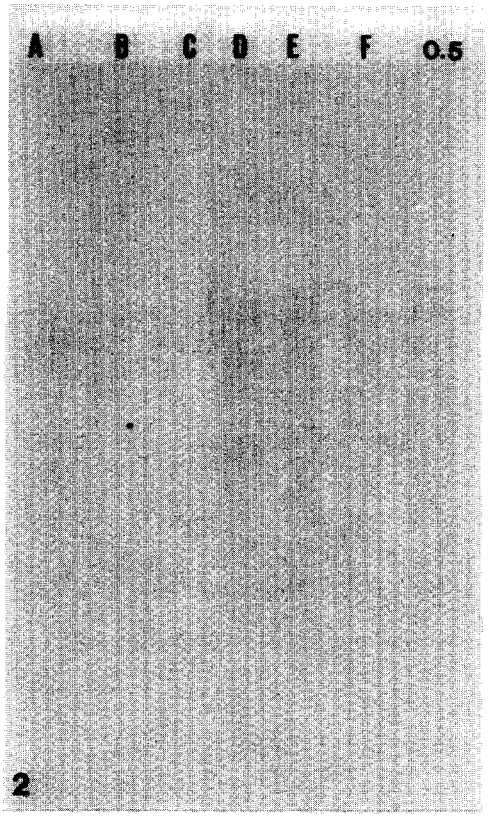

Fig. 2. Autoradiogram of blot hybridization: The last blot represents control hybridized with 0.5 HSV-1 *genome equivalent per* HEp-2 *cell genome; other blots (A–F) show DNAs extracted from tumour cells and hybridized with* ^{32}P HSV-1 *DNA.*

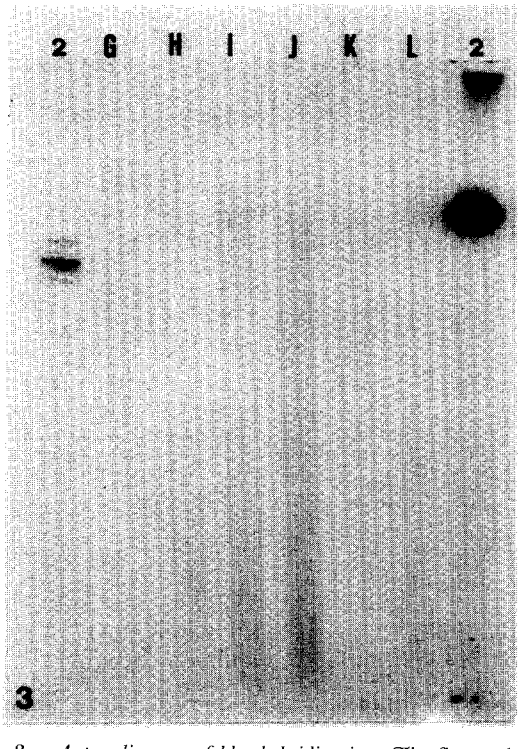

Fig. 3. Autoradiogram of blot hybridization: The first and the last blots show controls hybridized with 2 HSV-1 *genome equivalents per* HEp-2 *cell genome (the DNAs were treated with ECO RI, first blot, and not treated, last blot); other blots (G–L) show DNAs extracted from tumour cells and hybridized with* ^{32}P HSV-1 *DNA.*

membrane as essentially described by Southern [20] except for transfer of the whole gel. The nitrocellulose sheet was finally laid against a pre-flashed Kodak Royal X-O-Mat film in the presence of a $CaWO_4$ intensifying screen (Ilford Fast Tungstate) [26].

RESULTS

Frequency and sites of herpetic labial recurrences

Forty per cent of patients with labial tumours remembered previous herpetic recurrences. It was not always easy to determine a precise for the recurrences. When possible, 4% were located on the upper lip, 73% on the lower lip, 23% on either one. Only in few cases it was possible to ascertain that the tumour had appeared at the same site as the recurrences.

Search for antibodies to HSV-1

As already described by us in a previous work (Italian General Review of Dermatology, in press) we did not notice differences in antibody titers to HSV-1 between patients with lip cancer and controls without such abnormalities. Moreover, we did not observe any increase or decrease in immunological response to HSV-1 during oncogenesis and after excision of the tumour.

Cultivation of cells from labial tumour and normal tissues

It was possible to obtain cell proliferation from bioptic material in thirty out of fifty-eight tumours examined and in three out of four normal tissues. Epithelium-like cells, fibroblast-like cells or mixed cell populations began to appear 4–8 days after explantation (Fig. 1). Spontaneous degeneration was not observed, nor could any cytopathogenic agent be isolated.

Susceptibility of cells derived from tumour and normal lip tissue

Susceptibility to HSV was studied in six cell cultures: four derived from lip tumours and two derived from normal tissue. All the cultures were susceptible to HSV infection as HEp-2 and BHK cells, infected for comparison. Three observation supported such a conclusion: (1) essentially the same number of plaques was produced (Table 1); (2) the same yield of infectious virus was obtained; (3) cytopathic effect and plaques had the same morphologies in all the cultures. Susceptibility to HSV infection did not change after a number of passages.

Detection of HSV-1 antigens

By indirect immunofluorescence tests we investigated the presence of viral antigens in cells derived from tumour fragments. Thirty-five cases were tested and all of them were negative (data not shown). Fluorescence was also not detected in uninfected HEp-2 cell controls. On the other hand, HEp-2 cells and cells derived from tumours showed diffuse nuclear and cytoplasmatic fluorescence when infected with HSV-1.

Sensitivity of the hybridization technique

In order to assess the sensitivity of the blot hybridization technique, DNA from uninfected HEp-2 cells mixed with unlabeled HSV-1 DNA in the ratios of 0.5 and 2 genome equivalents per diploid cell genome was processed as described in Materials and Methods and hybridized to blots containing labeled HSV-1 DNA. The results of these hybridizations are shown in Figs. 2 and 3 and reveal that this approach has sufficient sensitivity to detect HSV-1 DNA corresponding to 0.5 copies of viral sequences equivalent to about a 9×10^6 mol-wt DNA fragment [27].

Analysis of DNA from tumour tissues for viral DNA sequences

No bands hybridizable to HSV-1 DNA was found in tumour DNAs by blot hybridization in the twelve tumours analysed. Experiments were performed only once in five cases, because of the scarce pathologic material, and twice in the other seven cases. The results of

Table 1. *HSV susceptibility of cell cultures derived from labial tumours, normal tissues,* HEp-2 *and BHK cells*

	Viral titer on cells							
Virus	1T	2T	3T	4T	5N	6N	HEp-2	BHK
F	8×10^8	1.2×10^9	2.1×10^9	6.3×10^8	8.7×10^8	1×10^9	1.6×10^9	2.5×10^9
G	7.5×10^8	5×10^8	9×10^8	7.8×10^8	1.2×10^9	6.7×10^8	1×10^9	1.3×10^9

T, from labial tumour; N, from normal labial tissue. Numbers of plaque forming units (PFU)/virus ml are reported (mean of two values).

the hybridization experiments are illustrated in Figs. 2 and 3. The last column of Fig. 2 represents HEp-2 cell DNA with 0.5 viral genome equivalent per diploid cell genome; the other columns show DNAs extracted from six tumours. DNAs from tumour cells do not demonstrate any hybridization band present in controls. In Fig. 3 autoradiograms of the other samples tested are shown. The first blot contained an ECO RI reconstruction mixture of HEp-2 cell DNA with two viral genome equivalents per cell. In the last blot this same reconstruction mixture was not treated with restriction enzymes. The middle blots show autoradiograms of DNA extracted from the remaining six tumours. Also in this instance no hybridization band between HSV-1 DNA and tumour cell DNA was present.

DISCUSSION

The importance of studying labial tumour cell susceptibility to HSV is represented by the fact that the relationship between HSV-1 infection and lip carcinoma could be linked not to a viral carcinogenic capability, but instead to an easier growth of virus in tumour or tumour-differentiating cells. A similar hypothesis was advanced also for the association between HSV-2 and cervical carcinoma [28]. Results reported in Table 1 show that at the multiplicity of infection we used there is no difference in susceptibility between cells from tumour, cells from normal tissues and cells normally used in the laboratory to cultivate HSVs. However, the conditions of our observation differed from those of natural infection both because viruses were extensively propagated on *in vitro* cultures, and because the cells grown and used were not necessarily identical to normal tissue or lip tumour cells.

Regarding the frequency and sites of herpetic labial recurrences, our research showed that (i) herpetic recurrences in patients with labial tumours had a similar frequency (40%) to the one reported in an epidemiological investigation [29] performed by anamnesis on 1838 people randomly chosen, where the frequency was about 38%; (ii) in contrast to earlier observations by Kvasnicka [17, 19], we found no apparent correlation between the site of tumour and the site of previous HSV lesions. However, the results on the frequency and the location of the recurrences have to be interpreted with caution, since they are in part based upon anamnestic data.

The lack of virus specific antigens in cells grown from lip tumour fragments do not exclude the presence of HSV-1 specific antigens in tumoral cells (or in some of them). In fact, similar studies conducted on cervical carcinoma cells showed that HSV-2 specific antigens were present only in exfoliated cells and not in cells obtained by biopsy [15]. Dreesman *et al.* [30] showed that a HSV-2 specific DNA-binding antigen was expressed in 38% of tissues with pathological findings of severe dysplasia or carcinoma.

Finally, with regard to the search of HSV-1 DNA in tumoural cells, the sensitivity of our technique could evidence as far as 0.5 copies of viral sequences equivalent to about a 9×10^6 mol. wt DNA fragment [27]. We were not able to detect viral DNA in any of the twelve tumours tested. Our failure in detecting HSV DNA in spinocellular epitheliomas could support the findings of McDougall *et al.* [9] of HSV RNA only in premalignant biopsies but not in invasive cancer. It must be pointed out that examination of tumour tissue is complicated by factors not encountered in experimental tissue culture systems. Tumour biopsy samples are usually very small and in most cases consist of both neoplastic and normal cells. Extraction of DNA from such samples will result in a dilution of the sequences from any particular cell type and could provide "false negatives". We think that the technique recently described by Leiden *et al.* [31] will be very useful to eliminate "false negatives". With this technique, in fact, it is possible to study only the transformed or neoplastic cell DNA portions with higher density, which presumably should contain HSV-1 DNA.

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