

## ATTEMPTS TO DETECT VIRUS-SPECIFIC DNA IN HUMAN TUMORS. II. NUCLEIC ACID HYBRIDIZATIONS WITH COMPLEMENTARY RNA OF HUMAN HERPES GROUP VIRUSES

by

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*DNA derived from various human malignant and non-malignant tissues was hybridized with radioactive complementary RNA (cRNA) synthesized in vitro with the aid of E.coli-RNA-polymerase by using DNA of human herpes group viruses as templates. Epstein-Barr virus (EBV)-specific cRNA annealed significantly with DNA from nasopharyngeal carcinoma biopsies as well as with DNA preparations from leukocytes, bone marrow, lymph node and spleen of some patients with infectious mononucleosis. No significant hybridization was observed with either herpes simplex type 2 or type 1 cRNA and DNA from ten cervical carcinoma biopsies. cRNA of human cytomegalovirus and varicellazoster virus did not hybridize with DNA from Kaposi's sarcoma or DNA from heavily infiltrated spleens of patients with Hodgkin's disease. These data do not exclude a role of these herpes viruses in the etiology of cervical carcinoma, Kaposi's sarcoma and Hodgkin's disease. They show, however, that such a relationship (if it exists) must differ quantitatively to a considerable extent from the one observed with EBV in EBV-associated tumors.*

During the past 10 years the oncogenic potential of some members of the herpes virus group has been clearly established. A herpes virus was shown to be responsible for a lymphomatosis in chickens, Marek's disease (Churchill and Biggs, 1967; Biggs *et al.*, 1968); adenocarcinomas of the American leopard frog are most probably due to a herpes virus infection (Mizell *et al.*, 1969); herpes virus saimiri and herpes virus ateles are highly oncogenic in certain species of New World primates (Meléndez *et al.*, 1969; Meléndez *et al.*, 1972); a rabbit lymphoma virus has been isolated from cottontail rabbits (Hinze, 1969). In man

there exists substantial evidence for a role of Epstein-Barr virus (EBV) in the induction of Burkitt's lymphoma (reviewed by Epstein, 1970; Klein, 1972; zur Hausen 1972) and nasopharyngeal carcinoma (Wolf *et al.*, 1973). Another of the human herpes viruses, herpes simplex type 2, has frequently been observed in association with cervical carcinomas (Rawls *et al.*, 1969; Nahmias *et al.*, 1970). *In vitro* experiments revealed the transforming activity of EBV, partially inactivated herpes simplex types 1 and 2 (Duff and Rapp, 1971), and a fourth human-pathogenic herpes virus, cytomegalovirus (Albrecht and Rapp,

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1973). Whereas EBV transforms human lymphocytes, the three latter apparently induce malignant transformation in primary hamster cells.

The following experiments were devised in order to study further the role of herpes-group viruses in the induction of human malignancies. Complementary RNA (cRNA) was used as a probe for the presence of persisting viral DNA, since it represents a convenient tool for assessing the role of herpes viruses in human cancer (zur Hausen, 1972; zur Hausen and Schulte-Holthausen, 1972; zur Hausen *et al.*, 1973).

#### MATERIAL AND METHODS

##### Cells

P3HR-1 cells were kept in medium RPMI 1640 (GIBCO) supplemented with 10% calf serum.

The Vero line of African green monkey cells was maintained in MEM containing 10% calf serum.

Primary human fibroblasts were obtained from human embryonic skin or lung cells, prepared in this laboratory. These cells were also kept in MEM supplemented with 10% calf serum. All tissue culture media contained 100 µg/ml streptomycin and 100 µg/ml penicillin G.

##### Tumor materials

Biopsy material from various tumors was frozen immediately after surgical removal and kept at -20° C until DNA extraction. Cellular DNA was extracted as described previously (zur Hausen *et al.*, 1970).

##### Viruses and viral purification

*Epstein-Barr Virus (EBV)*. EBV was concentrated from the supernatant fluid of aged P3HR-1 cells as described before (Schulte-Holthausen and zur Hausen, 1970). The virus-producing cells were kept at 34° C during the whole incubation period.

*Herpes simplex virus*. Herpes simplex viruses type 1 (Thea) and 2 (Haase) were kindly provided by Dr. Schneeweis, Bonn, and grown on Vero cells. Vero cells were infected at an input multiplicity of 2-3 and incubated for 24-48 h. Virus was isolated from the supernatant fluid as described before (Schulte-Holthausen and zur Hausen, 1970) or from infected cells. In the latter case cells were sedimented by low-speed centrifugation, resuspended in 0.01 M Tris (pH 7.2)

containing 0.5% NP40 and 8% sucrose as described by Weinberg and Becker (1969), incubated for 15 min at 37° C and again subjected to low-speed centrifugation. The resulting supernatant was layered on a sucrose gradient (20-35%) and centrifuged at 30,000 × g in the SW 27 rotor for 70 min at 4° C. The virus bands under these conditions in the lower third of the gradient.

*Cytomegalovirus*. Human cytomegalovirus (AD-169) was grown on human skin or lung fibroblasts. Virus was transferred by cell-free infection after filtration of the supernatant through 1.2 µ Sartorius membrane filters (Sartorius, Göttingen). The resulting preparation contained between 10<sup>4</sup> and 10<sup>5</sup> plaque-forming units per ml. For purification, the virus was harvested from cell-free supernatants of aged cultures approximately 7 days after infection. The purification conformed with the one reported for EBV (Schulte-Holthausen and zur Hausen, 1970).

*Varicella-Zoster virus*. The stock virus was isolated from a patient who suffered simultaneously from varicella and zoster eruptions. The virus was serologically identified as varicella-zoster virus and serially propagated on human embryonic lung or skin fibroblasts. Virus purification procedures were successful only from supernatants after long-term maintenance (12 days) of the infected cells in the same medium. The concentration procedures were identical with the ones reported for EBV.

##### Extraction of viral DNA

Viral DNA was extracted from viral concentrates as described previously (zur Hausen and Schulte-Holthausen, 1970). In brief, the virus was layered on top of preformed sucrose-SDS gradients, lysed by the addition of 1 mg/ml of pronase B and 0.5% SDS and sedimented for 14 h at 53,000 × g in the SW 27 rotor at 25° C. The material banding at about 58 s was either pelleted in the 50 Ti rotor at 135,000 × g for 15 h (20° C) or dialyzed. Thereafter it was adjusted with CsCl to a density of 1,710 g/cm<sup>3</sup> and spun to equilibrium in the 50 Ti rotor for 72 h at 60,000 × g. Fractions containing the viral DNA were pooled and dialyzed.

##### Transcription of viral DNA

Transcription of 1-2 µg of purified viral DNA was performed with *E. coli* RNA polymerase as

described in the preceding paper (zur Hausen *et al.*, 1974).

#### Hybridization methods

The technology of nucleic acid hybridizations was essentially identical with the one described in the previous report (zur Hausen *et al.*, 1974).

### RESULTS

#### Hybridizations with EBV-cRNA

Fifty  $\mu\text{g}$  of DNA from biopsies of nasopharyngeal carcinoma patients (obtained from Dr. G. Klein, Stockholm), from materials derived from patients with infectious mononucleosis, as well as DNA from other human tumors, together with control tissues, were subjected to hybridizations with EBV-specific cRNA. The result is shown in Figure 1.

Most of the DNA from nasopharyngeal carcinoma biopsies annealed with EBV-cRNA significantly above the background of skin fibroblast DNA. A substantial proportion hybridized above the level of Raji cell DNA which was shown in previous experiments to harbor approximately 50 EBV genome equivalents per cell (zur Hausen *et al.*, 1972). Three negative nasopharyngeal carcinomas were carefully re-examined histologically. Two of these biopsies did not contain any tumorous tissue, the third biopsy represented an adenocarcinoma.

In infectious mononucleosis, DNA preparations from the peripheral blood of four patients showed a different picture. Two of them, both from severe clinical cases, annealed with 1,800 and 400 cpm, respectively. DNA from the bone marrow of the former also hybridized clearly with EBV-cRNA, although to a lesser extent than DNA prepared from the peripheral blood. DNA from the peripheral blood of two other patients with mild infectious mononucleosis was negative under the conditions of this test. In all four patients the diagnosis of IM was confirmed serologically (seroconversion of EBV-VCA antibodies). From a fifth patient with severe and long-lasting IM, DNA from spleen and lymph-node cells was obtained. Both preparations hybridized with EBV-cRNA (400 and 850 cpm, respectively). Thus three of five patients with IM revealed the presence of viral DNA in cells of either peripheral blood and bone marrow or of lymph nodes and spleens. All other material tested, in particular

DNA from spleen cells of Hodgkin patients, DNA from Kaposi sarcomas and from human skin fibroblasts, showed no significant annealing with EBV-cRNA.

#### Hybridizations with herpes simplex virus cRNA

Purified HSV-1 (Thea) or HSV-2 (Haase)-DNA was transcribed *in vitro* and the resulting cRNA used for the detection of viral DNA sequences in human cervical carcinoma biopsies. Ten biopsies were carefully selected to represent in their majority tumor cells and annealed with type 1

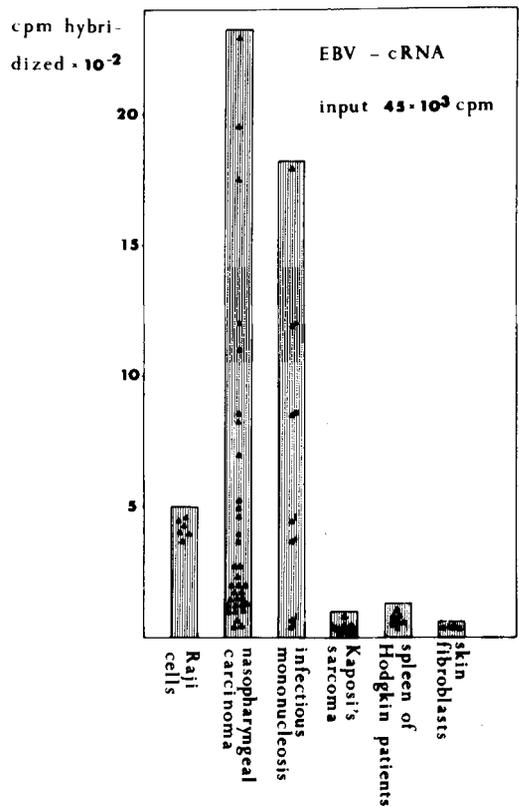


FIGURE 1

Hybridization of EBV-cRNA with DNA from human malignant and nonmalignant tissues. DNA from a non-virus-producing lymphoblastoid line Raji, which contains about 50 EBV genome equivalents per cell (zur Hausen *et al.*, 1972), served as positive control. The symbols in the column "infectious mononucleosis" represent: ' DNA from leukocytes of the peripheral blood, + DNA from a lymph node,  $\times$  DNA from cells of bone marrow,  $\bullet$  DNA from spleen cells.

and type 2 cRNA. DNA of HSV-1 or HSV-2-infected cells served as controls. The results are shown in Figures 2 and 3.

Although approximately 20% of both cRNAs annealed to the respective positive control (and showed a clearly significant cross-hybridization with the DNA derived from cells infected with the heterologous virus), no annealing was evident with DNA from cervical carcinomas. Additional DNA preparations which originated from Kaposi sarcoma biopsies, from spleen cells of Hodgkin's disease patients, and from normal skin fibro-

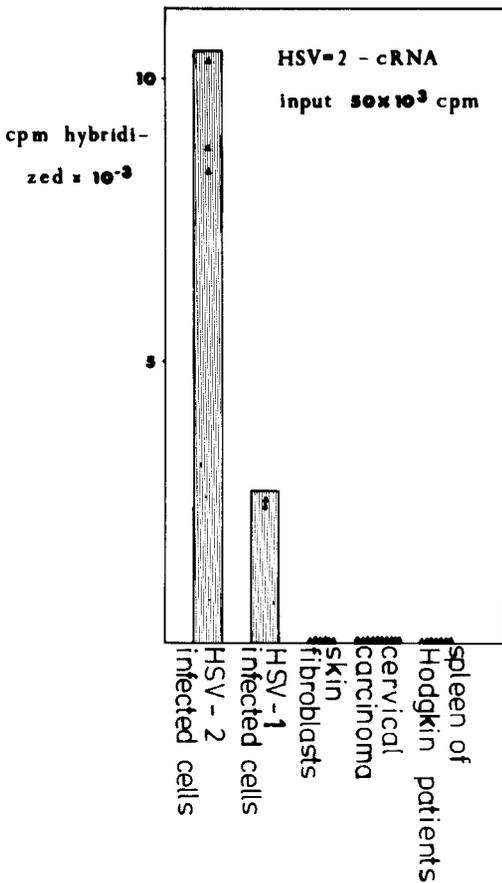


FIGURE 2

Hybridization of HSV-2-cRNA with DNA from cervical carcinoma biopsies and DNA from spleen cells of patients with Hodgkin's disease. DNA from HSV-2 and HSV-1-infected human cells served as positive control.

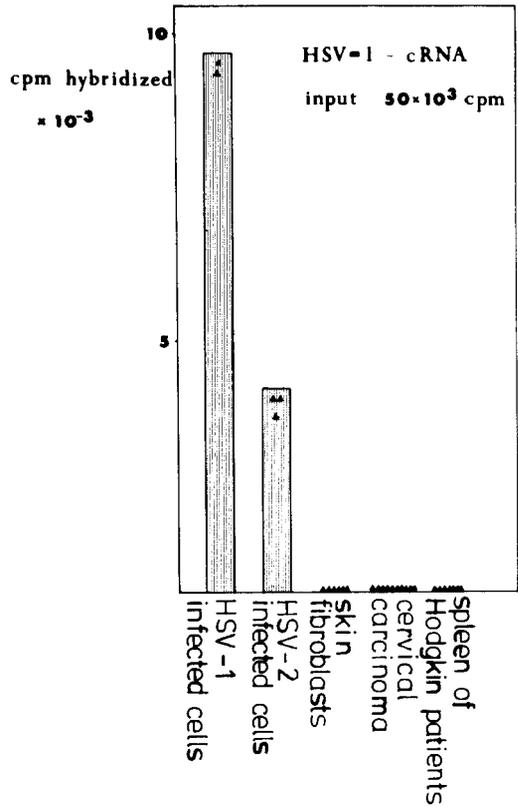


FIGURE 3

Hybridization of HSV-1-cRNA with DNA from cervical carcinoma biopsies and DNA from spleen cells of patients with Hodgkin's disease. DNA from HSV-1 and HSV-2-infected cells served as positive control.

blasts, were all negative under the conditions tested.

*Hybridizations with varicella-zoster virus-cRNA*

Since there exist reports on serological cross-reactivity of HSV and VZ-antigens (Kapsenberg, 1964; Ross *et al.*, 1965; Schmidt *et al.*, 1969) we investigated whether the reported serological results on HSV/cervical carcinoma interrelationship could be explained by the involvement of VZ virus in this disease.

In addition, in view of the frequent occurrence of zoster eruptions in Hodgkin's disease patients, attention was focused on the question of whether

virus-specific DNA could be discovered in cells of heavily infiltrated spleens of such patients.

VZ-cRNA, prepared from purified DNA of a laboratory strain of VZ virus, was used in these tests. The result is shown in Figure 4.

Whereas the positive control annealed with 12.5% of the input, no significant hybridization was observed with DNA from cervical carcinomas and from spleen cells of Hodgkin patients. A considerable number of other "control" material, *i.e.*, DNA from *condylomata acuminata*, laryngeal papillomas, Kaposi's sarcomas and skin fibroblasts, also reacted negatively.

*Hybridizations with CMV-cRNA*

In order to test the possible association of Kaposi's sarcoma with human cytomegalovirus

(Giraldo *et al.*, 1972), DNA from those tumors was annealed with CMV-cRNA. The result is shown in Figure 5. DNA from CMV-infected human fibroblasts annealed with 28% of the input. Kaposi's sarcoma DNA (total tumor) however, was entirely negative in these hybridizations. In addition, DNA from Hodgkin spleens and also DNA from human skin and human skin fibroblasts grown in tissue culture did not anneal with CMV-cRNA.

DISCUSSION

Epstein-Barr virus DNA has been regularly observed in biopsies of Burkitt lymphomas (zur Hausen *et al.*, 1970) and nasopharyngeal carcinomas (zur Hausen *et al.*, 1970; Nonoyama and Pagano, 1973). The presented results underline

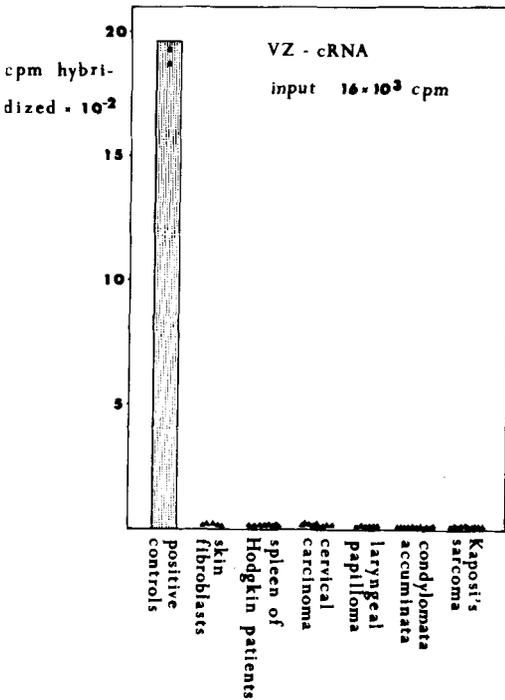


FIGURE 4

Hybridization of varicella-zoster virus cRNA with DNA from various human tumors, spleen cells of patients with Hodgkin's disease, and normal human skin fibroblasts. DNA from varicella-infected human fibroblasts served as positive control.

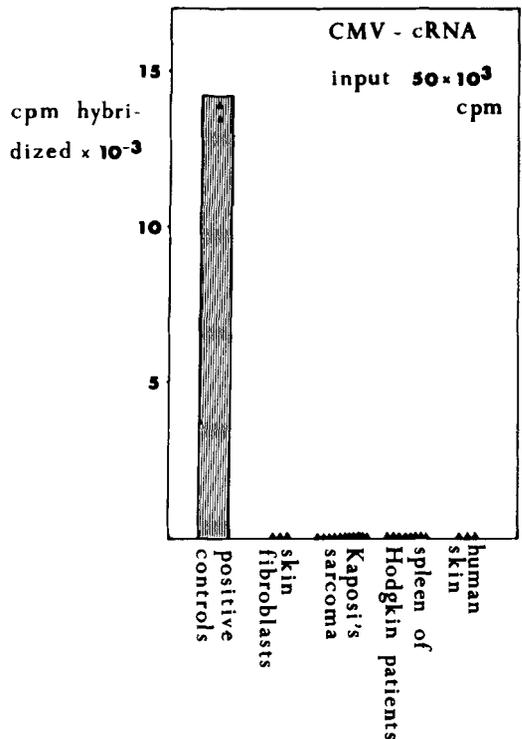


FIGURE 5

Hybridization of CMV-cRNA with DNA from Kaposi's sarcoma biopsies and spleen cells of Hodgkin patients. CMV-infected human skin fibroblasts served as positive control.

the specific role of EBV in nasopharyngeal carcinoma. At the same time they demonstrate the presence of virus-specific DNA within cells of the peripheral blood, bone marrow, lymph nodes and spleen of some patients with infectious mononucleosis. It appears that some of these cells, as well as cells of those nasopharyngeal carcinomas which anneal in the higher range, must be loaded with virus-specific DNA. This conforms with previous findings of multiple viral genome equivalents in cells of Burkitt lymphomas, nasopharyngeal carcinomas (zur Hausen, 1972), and of non-virus-producing lymphoblastoid lines (zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971; zur Hausen *et al.*, 1972).

The sensitivity of hybridizations with EBV-cRNA permits the detection of two or three viral genome equivalents per cell, as determined by reconstruction tests (zur Hausen, 1972). Despite this limitation, the *in vitro* product represents a convenient tool in ascertaining the involvement of EBV in Burkitt's lymphoma, nasopharyngeal carcinoma and infectious mononucleosis.

If additional herpes group viruses play a role in certain human malignancies, we hoped to reveal their presence within those tumor cells by applying the same technique of cRNA-hybridizations. The role of herpes simplex type 2 in cervical carcinoma and of cytomegalovirus in Kaposi's sarcoma was of particular interest. Reconstruction tests with cRNA of these human herpes viruses, synthesized in this laboratory, revealed that the sensitivity of the test slightly exceeded that of EBV-cRNA. It permitted the detection of approximately one genome equivalent per cell (zur Hausen *et al.*, to be published). When using this material in DNA-cRNA hybridizations we failed to find DNA homologous to the respective viruses in those tested malignancies which are suspected of being associated with herpes virus infections. This accounts in particular for the cervical carcinoma biopsies which did not contain detectable amounts of either HSV-2 or HSV-1 DNA.

Obviously, these results do not exclude the presence of fragments of HSV-2 DNA within such tumors, as observed in one biopsy by Frenkel *et al.* (1972). Negative results, however, were obtained in similar experiments with HSV-2 cRNA and DNA from approximately 30 additional cervical cancer biopsies (Schulte-Holthau-

sen, unpublished results; Petersen, personal communication). C<sub>0</sub>t-hybridizations with *in vitro* labelled HSV-2 DNA and DNA derived from five cervical carcinoma biopsies also failed to detect virus-specific DNA within the tumor cells (Pagano, personal communication). It is obvious, therefore, that the association of HSV-2 DNA with cervical carcinomas (if it exists) should be quantitatively vastly different as compared to EBV in EBV-associated tumors.

The same accounts for a possible role of VZ and human CMV in Hodgkin's disease and Kaposi's sarcoma. Here in particular the role of CMV in Kaposi's sarcoma deserves some discussion, since CMV has been isolated repeatedly from this tumor (Giraldo *et al.*, 1972). Preliminary results of C<sub>0</sub>t-hybridizations with *in vitro* labelled CMV-DNA and Kaposi tumor DNA (which would have permitted the detection of about 0.2 genome per cell) were also negative (Dörries *et al.*, unpublished results). Thus, molecular hybridization does not contribute further evidence for a role of CMV in this malignancy.

Hybridizations with cRNA suffer from the disadvantage that the *in vitro* product may not be representative of the whole viral genome. Thus, if fragments of viral DNA, not transcribed in the *in vitro* reaction, were present in multiple copies, they would still escape detection by this method. Comparison of cRNA-annealing with C<sub>0</sub>t-hybridizations, however, by using *in vitro* labelled EBV-DNA, basically yielded identical results (Nonoyama and Pagano, 1973). In addition, the use of cRNA proved to be a convenient tool in the detection of EBV-specific sequences in Burkitt's lymphoma and nasopharyngeal carcinoma biopsies, even by *in-situ* hybridizations (Wolf *et al.*, 1973; Wolf *et al.*, in preparation). By this same token, the annealing of cRNA of other human herpes group viruses with DNA from tumors suspected of being associated with such agents did not provide encouraging results. It should be emphasized, however, that these data do not exclude these viruses from the etiology of the respective tumors.

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TENTATIVES DE DÉTECTION DE L'ADN SPÉCIFIQUE DU VIRUS  
DANS LES TUMEURS HUMAINES. II. HYBRIDATIONS DE L'ACIDE  
NUCLÉIQUE AVEC L'ARN COMPLÉMENTAIRE DES VIRUS  
HERPÉTIQUES HUMAINS

L'ADN dérivé de divers tissus humains malins ou non a été hybridé avec l'ARN complémentaire (ARNc) radioactif synthétisé in vitro à l'aide de l'ARN polymérase d'*E. coli*, l'ADN des virus herpétiques humains servant d'indicateur. On observe une hybridation considérable de l'ARNc spécifique du virus d'Epstein-Barr (EBV) avec l'ADN provenant de biopsies d'épithélioma du rhino-pharynx et avec des préparations d'ADN provenant de leucocytes, de moëlle osseuse, de ganglions lymphatiques et de rate de quelques sujets atteints de mononucléose infectieuse. Aucune hybridation significative n'a été observée avec l'ARNc d'herpes simplex type 2 et 1 et l'ADN de dix biopsies de carcinome cervical. On ne constate pas non plus d'hybridation de l'ARNc de cytomégalo-virus humain et de virus de la varicelle et du zona avec l'ADN du sarcome de Kaposi ou l'ADN des rates fortement infiltrées de sujets atteints de maladie de Hodgkin. Ces observations n'excluent pas la possibilité d'un rôle de ces virus herpétiques dans l'étiologie des carcinomes cervicaux, des sarcomes de Kaposi et de la maladie de Hodgkin. Toutefois, elles montrent que cette relation (si elle existe) doit être sensiblement différente, au point de vue quantitatif, de celle que l'on observe avec l'EBV dans les tumeurs associées à ce virus.

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