Are Viruses Important in Carcinogenesis?

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The role of viruses in the etiology of animal cancers is fairly certain. Information derived under both natural and experimental conditions supports the concept that either DNA- or RNA-containing viruses can fulfill this function. The DNA-containing herpesviruses, especially the Epstein-Barr virus, are currently the primary objects of intense investigation concerning their role in human cancer. This article will focus on the properties of counterpart herpesviruses in lower animals as well as the human virus candidates with an assessment of the observations concerning their oncogenic potential (Am J Pathol 77:85–102, 1974).

In 1970, Gross [1] described Peyrilhe's uncanny perception, in 1773, of the causal relationship of viruses and cancer. Since then, investigators have been juggling with the etiology of neoplasia, with viruses remaining the most suspicious candidates for this role.

The etiologic role of viruses in certain animal cancers has been clearly established. For many, Koch's postulates have been fulfilled. The noted similarities of virus-induced animal tumors to neoplasias in man has served to stimulate the current surge for circumstantial evidence implicating viruses as etiologic agents in certain human tumors (Table 1). As herpesviruses are the nearest thing to primary candidate human cancer viruses presently available, this article will focus on the counterpart viruses in lower animals and on the human virus candidates, with an assessment of the proliferating knowledge concerning their oncogenic properties. Despite development of molecular technics and the excellent observations establishing the etiologic role of some RNA-containing tumor viruses in animals, the current evidence of a related RNA human cancer virus is too circumstantial to warrant strong consideration. However, some of the problems encountered during the RNA virus search that are unique to the human system should serve as a reminder of the complexity of the problems to be solved.[2]

Presented at the Fifty-eighth Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, NJ, April 7–12, 1974.

Supported by Contract 70-2024 within the Virus Cancer Program of the National Cancer Institute, National Institutes of Health.

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Table 1—Evidence for the Oncogenic Properties of Herpesviruses

Virus	Natural host	Cells transformed	Type of cancer
Marek's disease virus	Chicken	Leukocytes	Lymphoma (chicken)
Lucké frog virus	Frog	Unknown (has not been replicated in vitro)	Adenocarcinoma (frog)
Herpesvirus sylvilagus	Rabbit	Leukocytes	Lymphoma (rabbit)
Guinea pig herpesvirus	Guinea pig	Leukocytes; hamster embryo fibroblasts in vitro	Suspected leukemia (guinea pig)
Herpesviruses saimiri and **ateles**	Monkey	Leukocytes	Lymphoma (monkey)
Epstein-Barr virus	Man	Leukocytes	Suspected lymphoma (man)
Herpes simplex viruses	Man	Hamster, rat and human fibroblasts	Adenocarcinoma and fibrosarcoma (hamster)
Cytomegalovirus	Man	Hamster embryo fibroblasts	Fibrosarcoma (hamster)

Marek's Disease

Fulfillment of Koch's postulates has been demonstrated for herpesviruses in two animal lymphomas. The first to fall into this category was the Marek's disease herpesvirus (MDV), responsible for lymphoproliferative and neuropathic disorders in chickens. An association of this lymphoid tumor to the herpesvirus was not established until 1967. Independent research teams [3–5] cocultivated the chicken tumor cells with permissive (chicken kidney or duck embryo fibroblast) cells and observed herpes-type cytopathology. Unenveloped herpes-type particles in the cultures seen with the electron microscope supported these observations. Presence of virus-specific antigens in the feather follicle epithelium [6] inspired detection of complete virus replication which occurs only in the follicle cells.[7] Experimentally, cell-free transmission of MDV isolates rapidly produce Marek's disease in young chickens.[8] In nature, the infection is transmitted to susceptible chickens by dust and dander and is initiated in the respiratory tract. Although the virus is ubiquitous in nature, only a low percentage of infected chickens develop the lymphoma. Strains of MDV vary in the form of disease produced,[9] while the genetic constitution of the chicken influences the incidence of the disease.[10] Cumulatively, these observations provide proof for the etiologic role of a herpesvirus in cancer. New data have emerged recently claiming biologic and biochemical evidence for an *in vivo* interaction between Marek's disease herpesvirus and an avian leukosis virus.[11] Obviously, further speculation is unwarranted until this observation has been confirmed and extended.

An enticing fact for the cancer virologist is the successful vaccination of chickens against Marek's disease. The vaccine virus, an apathogenic herpesvirus of turkeys or an attenuated strain of MDV, replicates in the host and elicits the synthesis of humoral antibodies.[12,13] However, the mechanisms involved in preventing development of Marek's disease lesions are unclear at this time. The prophylactic procedure is also supportive of the virus etiology of malignant transformation. If a vaccine can prevent the neoplastic process in chickens, the possibility exists that similar measures might be successfully used in man once etiology is established.

Amphibian Renal Adenocarcinoma

Latency, a characteristic feature of herpesviruses, has made the Lucké tumor of *Rana pipiens* a valuable, though often frustrating, system for the study of virus-induced neoplasms. Since the original association of a virus in the etiology of the frog tumor,[14] evidence has been accumulating to suggest that a temperature-sensitive herpesvirus is responsible for converting normal frog kidney tissue into a renal adenocarcinoma. Direct proof of the oncogenicity of the Lucké herpesvirus (LHV) awaits the development of a cell culture system in which the virus can replicate.

Circumstantial evidence gained momentum when a herpesvirus was detected by the presence of inclusion bodies in tumor cells from frogs that had been captured during the winter.[15] Description of the virus particles found only in cells with inclusions was first advanced by Fawcett.[15] Injections of cytoplasmic fractions of "cold" tumors containing herpesvirus into *R pipiens* embryos resulted in virus-free renal tumors in frogs held at 20 C, providing evidence for a virus etiology.

The "winter" phase or virus-containing tumor is found in frogs held in the laboratory at 4 to 9 C or in frogs captured during the hibernation months of winter and spring. Conversely, "summer" phase or virus-free tumors are carried by frogs whose environment, either in laboratory or in nature, is warm (20 to 26 C) (Table 2).

Temperature-shift experiments have demonstrated the latent state that a virus can assume in its host. Latency implies that the virus genome, or a portion of it, is present within the apparently normal cell but that virus particles and structural components are not being produced. Recent studies have demonstrated that when summer tumor fragments are transplanted into the anterior eye chamber of healthy leopard frogs or frogs not known to carry the virus, Lucké virus particles develop in tumor implants of the frogs held at 7.5 C.[17-19]

Table 2—Lucké Herpesvirus Temperature Sensitivity

	4 to 9 C	20 to 26 C
Virus particles	Yes	No
Nuclear inclusions	Yes	No
Depressed mitotic activity	Yes	No
Virus-specific mRNA	Yes	Yes
Virus-specific antigens	Yes	No

Further information came from the demonstration that when summer tumor explants are incubated on agar slants at 7.5 C virus induction occurs,[20] implying that the virus activation is independent of the intact host. Winter tumor explants held at the warm temperatures lose evidence of viral replication.[21] Molecular confirmation of the latent state of the virus in these cells has been obtained through recent hybridization data demonstrating the presence of virus-specific messenger RNA in summer tumor cells.[22]

The existence of various types of virus particles in tumor cells further complicates defining the etiologic role of viruses in neoplasms. Such is the case for the Lucké tumor in which two other viruses reside with the LHV. Morphologically indistinguishable from the Lucké virus, the tumor isolate referred to as FV 4[23] was able to replicate in leopard frog cell cultures but was shown not to be tumorigenic.[24] Differentiation of FV 4 and LHV also may be made antigenically and genetically.[25] In addition, a papova-like virus is found associated with tumor cells.[26] The possible requirement of this virus as a cofactor for Lucké virus replication and oncogenesis has not been ruled out despite its own lack of tumorigenicity.[26]

The fact that the Lucké tumor occurs in nature, and is therefore not a laboratory phenomenon, adds importance of the virus to cancer study. The naturally temperature-sensitive virus offers a probe for studying the functions required for the initiation and maintenance of the transformed state. In addition, the virus contrasts with most other DNA and RNA viruses in producing a carcinoma rather than a lymphoproliferative disorder or a sarcoma. This difference along with its particular sensitivity to temperature is especially relevant to the current status of herpes simplex virus type 2 and cervical carcinoma.

Guinea Pig and Rabbit Herpesviruses

Ubiquitous herpesviruses have been associated with other animal neoplasias, and undoubtedly the list of viruses will continue to grow. Bovine herpesviruses have most recently become a prospective member of the list.[27] *Herpesvirus sylvilagus* can produce a malignant lymphoma

in its natural host, the cottontail rabbit.[28,29] Freshly isolated leukocytes appear to contain no infectious virus but, following *in vitro* cultivation, the virus becomes detectable in a small percentage of cells. Incomplete replication and persistence in proliferating lymphoid cells recall similarities to Marek's disease virus.

Rarely detected in fresh tumor tissue, a latent guinea pig herpesvirus (GPHV) can be expressed in lymphoblasts or spleen tissue cultured *in vitro* from leukemic or normal strain 2 guinea pigs.[30–32] The nature of the herpesvirus and a C-type particle also present in leukemic cells are unknown. Athough GPHV demonstrated *in vitro* transformation of hamster cells and leukocytes,[33,34] neither of the two viruses, the herpesvirus or the C-type particles, has shown indications of independent neoplastic potential when inoculated into guinea pigs.

Herpesviruses in Nonhuman Primates

Association of viruses with human cancer has increased the importance of nonhuman primate virology. Two oncogenic viruses have been isolated from monkeys with the potential to produce fatal neoplasms in several species other than the natural host.[35–37] *Herpesvirus saimiri* (HVS) fulfills Koch's postulates; it normally replicates in healthy squirrel monkeys but is the causative agent of malignant lymphomas in marmoset monkeys.[38] The natural host for *Herpesvirus ateles* (HVA) is the spider monkey.

Although no virus is found in HVS tumor–bearing animals, cultivation of the neoplastic cells stimulates derepression of the virus genome with concomitant production of infectious virus and virus antigens within 24 to 72 hours.[39] Permanent cell lines have also been established.[40]

The prospective research potential of HVS (already demonstrated to be a cancer virus) and of HVA lies in similarities to EBV [41] and in the immunologic response the host manifests against the different viruses. The oncogenic potential of these viruses in the natural host is intriguing. The squirrel monkey's rapid development of antibodies after HVS infection appears to correlate with the resistance to the oncogenic properties of the virus.[42] Lymphomas develop in rabbits inoculated with HVS with a concomitant low level of antibody, while HVA-infected rabbits produce high levels of antibody and remain tumor free.[43]

Epstein-Barr Virus—A Prime Suspect

The chief contender for position as the first virus demonstrated to be involved in the etiology of a human neoplasia is the Epstein-Barr

virus (EBV), another herpesvirus. Due to lack of an acceptable host for reimplantation of the suspected agent, the case cannot be proven directly. However, circumstantial evidence continues to be generated that implicates not only EBV but other herpesviruses, such as herpes simplex (HSV) and human cytomegalovirus (CMV), in human cancer.

Questions to be considered include: a) Is the state of the host's immune system decisive? b) Do different routes of virus entry imply different diseases? c) Is the involvement of a cofactor necessary for the disease? and d) Is a genetic predisposition of the host or virus required?

EBV has been firmly associated with two lymphoproliferative diseases—one malignant (Burkitt's lymphoma), the other self-limiting (infectious mononucleosis). Less strongly, correlations have also been made to nasopharyngeal carcinoma[44,45] and to the Guillain-Barré syndrome.[46]

The case of EBV and infectious mononucleosis (IM) was established primarily on seroepidemiologic grounds as reviewed by Epstein and Achong.[47] Oral transmission of the virus is suggested by the presence of EBV in throat washings from patients with mononucleosis.[48,49] A filterable agent from the patients transformed fetal and adult leukocytes[50] and replicated in marmoset leukocytes with the release of virus.[51] Demonstration of the oncogenic potential of these leukocytes will increase the case for EBV as an oncogenic agent.

In contrast, the possible virus involvement in Burkitt's lymphoma results in a jaw tumor of children in Africa, where it is endemic. A clinically and pathologically similar disease is found in America but only rarely. Whether or not EBV is potentially responsible for the American version is under investigation.[52–53]

The instigation of intense EBV cancer research began with the observation of herpesvirus particles in a cell culture derived from Burkitt's lymphoma, a mere decade ago.[54] The association of EBV to Burkitt's lymphoma has been substantiated by the following.

Normal peripheral white cells or fetal lymphoid cells exposed to cells carrying EBV or to EBV concentrates transform into immortal lymphoblasts.[55–59] These *in vitro* transformed cells, as well as Burkitt's lymphoma cells, demonstrated EBV-specific antigens.[60,61] In addition, established Burkitt lymphoblastoid cell lines that are antigen negative can be induced to synthesize antigens and subsequently virus particles by exposure of the cells to halogenated pyrimidines.[62,63] Fusion of a virus-producing lymphoblastoid line with human cells results in repression of the virus in the somatic cell hybrid. Following exposure

to idoxuridine (IUDR) or dibutyryl cyclic AMP, these "negative" cells express the virus.[64,65] Sera from patients with Burkitt's tumor exhibit high anti-EBV titer.[66] Molecular hybridization has detected the presence of EBV DNA in all Burkitt lymphoblastoid lines as well as in the tumors.[67,68] The physical state of the DNA in these cells is controversial. Nonoyama and Pagano [69] have suggested that the virus genome may be carried in a plasmid-like state, while recent evidence by Adams *et al* [70] indicates that the EBV DNA is linearly integrated with host cell DNA.

The possible existence of other virus genomes in the tumors has made the proposed involvement of EBV less clear-cut. Recent molecular hybridization studies reveal that Burkitt tumor cells contain RNA related to the murine leukemia virus.[71] Particles that band in sucrose gradients at 1.16 to 1.19 g/ml and that possess both 60 to 70S RNA as well as RNA-directed DNA polymerase have also been reported.[72] Whether these particles are cofactors in the disease or merely passengers remains to be demonstrated.

Other complications in clearly defining the system are malaria and the sickle cell trait. The endemic area of Burkitt's lymphoma prompted Burkitt to propose that malaria, occupying a similar geographic location, is a cofactor in the disease.[73] Malaria's stimulation of the reticuloendothelial system may alter the host's defense against the lymphoproliferative power of EBV. Secondly, genetics may be involved in susceptibility since the sickle cell trait confers partial protection against malaria, and children with the trait have a reduced tumor incidence.[74] That EBV is not a passenger seems established by recent findings that other (non-African) lymphomas do not contain the virus genome. This suggests a special case for Burkitt's lymphoma, and a cofactor unique for African children in the endemic area in which these tumors are found.

Herpes Simplex Viruses

Mounting evidence is associating other herpesviruses with a variety of human cancers. Demonstration of the transforming potential of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) in hamster embryo fibroblasts and the oncogenic potential of the resultant cells has served to classify these ubiquitous viruses as potential carcinogens in humans. HSV-1 is generally isolated from nongenital regions, while HSV-2 is venereally transmitted and associated with anogenital areas. The two viruses are distinguishable by biologic, biochemical and biophysical parameters.[75]

Although once considered to be associated primarily with sexual promiscuity, cervical cancer has been linked to all levels of the social scale and to HSV-2.[76,77] Seroepidemiologically, prevalence of neutralizing antibody to HSV-2 is higher in women with cervical cancer than in control groups.[78,79] A correlation of greater tumor advancement with a correspondingly higher incidence of HSV-specific antibody (anti–AG–4) compared to the less advanced tumors has also been reported.[80] Careful examination of the results suggests that a host cell antigen may play a part in the reaction observed. Complement-fixing antibodies present in the sera of patients with certain tumors, including cervical carcinoma, appear to be specific for a herpesvirus-labile antigen.[81–83] Herpesvirus particles have been observed in prostatic carcinoma cells [84] and isolated from degenerating cervical tumor cells grown in cell culture.[85]

Antigenic data also relate this virus to the cancer. On exfoliating squamous carcinoma cells, but not tumor biopsies, HSV-2 antigens were detected by immunofluorescence.[86–88] Frenkel *et al* [89] claim to have found a portion of HSV-2 DNA in cells of one cervical carcinoma. Confirmation of these results would strengthen the hypothesis that the virus genome or part of it is necessary for maintenance of the transformed state.

Returning to animal models for further support, the previously mentioned hamster-transformed cells arose when hamster embryo fibroblasts were exposed to HSV-2 or HSV-1 whose infectivity was inactivated by ultraviolet light.[90,91] Inoculation of these cells into newborn hamsters resulted in tumor production, often accompanied by metastases. The footprints of the virus, HSV-specific antigens, have been detected in the cytoplasm of transformants and on the corresponding unfixed cell membranes. Neutralizing antibody, specific for the transforming virus, has been found in tumor-bearing animals. Hybridization technics using RNA from the transformed cells and DNA from the virus revealed that at least 10% of the HSV-2 genome is in the HSV-2–transformed cells.[92]

Clinical interest was recently stimulated by the demonstration of enhanced lung metastases in weanling hamsters immunized against HSV-1 and then challeged with HSV-2–transformed cells.[93] The large prevalence of antibody to HSV-1 in the human population, and the possible role of blocking antibodies establishes need for continued study of the system.

Application of these experiments led to the transformation of thymidine kinase (TK)–negative cells by a virus carrying a TK gene.[94]

The transformed cells demonstrated virus TK activity, supporting the theory that the virus genome is stably conserved in transformants. A system analogous to the Lucké frog virus was reported with the transformation of human embryonic lung cells by HSV-2 at a temperature nonpermissive for virus replication.[95] Confirmation of such a system and development of one for transformation of cells by temperature-sensitive herpesvirus mutants would enhance study of the functions involved in virus-mediated transformation.

Utilizing the nonhuman primate system, investigators have sought to initiate in animals the observations seen in man. Mice occasionally develop cervical atypia or carcinoma when inoculated intravaginally with HSV-2.[96,97] Experimentally or venereally infected with HSV-2, Cebus monkeys have developed characteristic cervical and penile herpes lesions.[98,99] The recurrence of the infections is similar to that in man, indicating the latent presence of the virus. Of paramount interest would be the development of tumors in these animals.

The most recent evidence for involvement of herpes simplex viruses in the neoplastic process stems from the transformation of hamster embryo cells by HSV treated with a heterocyclic dye, neutral red, and subsequently inactivated for infectivity with ordinary light.[100] Some of the transformants are oncogenic in newborn hamsters (Figure 1).[101] This virus inactivation procedure is similar to the one followed by clinicians to treat HSV infections.[102,103] It is curious that one would want to transform an infectious population of viruses often causing a mild disease into a population with demonstrated potential to transform normal cells into cancer cells.

The Emergence of Cytomegalovirus

Until the past few years, little was known about the human cytomegaloviruses. In 1973, this herpesvirus was reported to have transformed hamster embryo fibroblasts.[104] More recently, demonstration of its ability to stimulate cell DNA synthesis has increased its importance in the struggle to characterize a human cancer virus.[105] Further investigation of the virus will most assuredly add to the understanding of virus–cell interactions and to the role of viruses in carcinogenesis.

Prospectus and Prospectives

Evidence has been presented demonstrating the transforming and oncogenic properties of both animal and human herpesviruses. The malignant potential of several animal herpesviruses is well documented

and accepted. Just how conclusively does the cumulative data implicate a herpesvirus as a human carcinogenic agent?

To date, the Epstein-Barr virus has been indicted most often in association with a human neoplasia. Several basic criteria met by EBV, as previously discussed, include: a) ubiquity of the virus, b) virus genetic information is regularly found in the associated tumors, c) virus is capable of stable interactions with the host, d) virus converts normal cells into transformants with infinite replicative capabilities without impairing essential cell functions, and e) virus stimulates cell DNA synthesis. A summary of the herpesvirus–cell interactions are listed in Table 3.

Table 3—Herpesvirus–Cell Interactions

	Replication	Transformation	Latency
Synthesis of early virus RNA and proteins	+	± or +	±
Cell appears altered	+	+	—
Virus gene products expressed	+	+	—
Synthesis of virus DNA, late RNA and proteins	+	—	—
Assembly and release of virus particles	+	—	—

What have these years of research offered to the public? The early detection of cancer may be possible using serologic methods. Development of the finding of complement-fixing antibodies specific for herpesvirus nonvirion antigens in certain cancer patients may be of prognostic value. The demonstration of the transforming potential of photodynamically inactivated virus may prevent a higher risk for cancer in patients who would have been so treated for minor herpetic lesions. Vaccination of chickens against Marek's disease has saved the poultry industry, and ultimately the public, millions of dollars.

The fourth important area of direct clinical application to virus-cancer research is the potential vaccination of the public against certain cancers. Hopefully, these measures could eradicate EBV and HSV from the population. Success with vaccination of chickens against MD makes the venture optimistic. However, extreme caution must be exercised. Vaccines made by inactivating oncogenic viruses or by live virus [106] may be hazardous in at least three ways: [107,108] a) laboratory propagation of the virus may have created defective particles, b) inactivation may have increased the number of these potential

transformants, and c) the route of inoculation may expose non-permissive but transformable cells to the virus.

Vaccines made against structural components, such as virus antigen, offer somewhat more hope. Again, extensive safety tests and long range follow-up of vaccines will be necessary. None of this will be cheap in terms of manpower, time or money.

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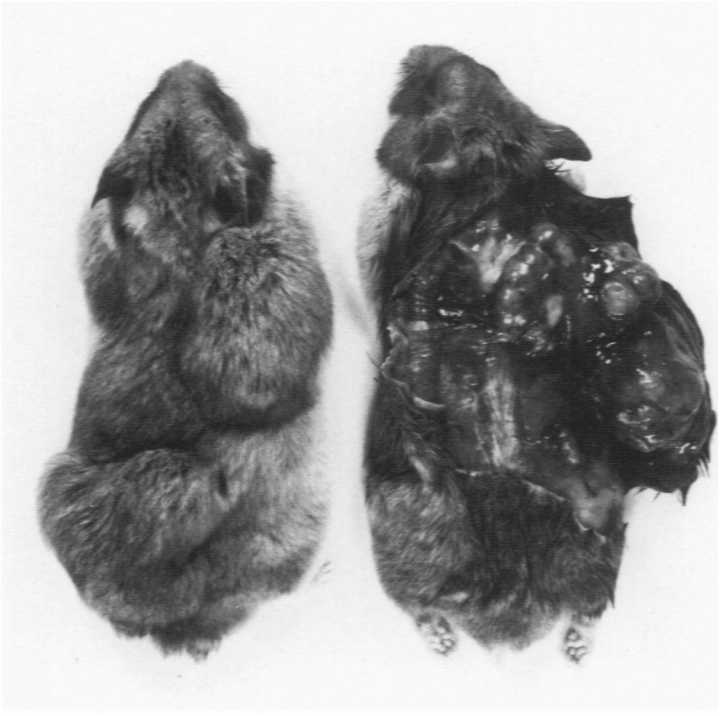

Fig 1—Tumor induced in a Syrian hamster inoculated with hamster cells transformed by herpes simplex virus type 2 inactivated by photodynamic treatment.

[End of Article]