Oncogenic Herpesviruses

Volume II

Editor

Dr. Fred Rapp

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FOREWORD

This book attempts to coalesce current information regarding the oncogenic properties of those human and animal herpesviruses which have been studied in some detail. Massive advances have been made within the past few years using modern technologies in studies designed to characterize the interaction of viruses and host cells. Investigations at the cellular and molecular level have often out-distanced those pertaining to the biology of the viruses and their role in disease. For this reason, this book begins with chapters by Dr. Hyman and by Dr. Roizman comparing the structure of herpesvirus DNAs and relating the structure to the function of some regions of that DNA. The function of various structural proteins is then examined by Dr. Spear. It is obvious that work in this area of research has taken great strides within the past few years. This is followed by a series of chapters dealing with herpesviruses specifically involved in a variety of animal neoplasias, including the naturally occurring neoplasia of the frog, covered by Dr. Granoff, and that of the chicken, elaborated on by Dr. Calnek. Isolation from nonhuman primates of herpesviruses with demonstrable oncogenic properties is discussed by Dr. Falk.

The general problem of herpesvirus latency, especially latency in experimental models and in man, is reviewed by Dr. Stevens. Drs. zur Hausen, Nahmias, and Geder then discuss the relationship of various herpesviruses to human neoplasias. The association linking Epstein-Barr virus with African Burkitt lymphoma and nasopharyngeal carcinoma is overpowering; yet, as these chapters point out, definitive proof of etiology remains elusive, a problem common when attempting to demonstrate virus involvement in the causation of cancer.

The interaction of herpesviruses with retroviruses is discussed by Dr. Hsiung and the final chapter by Dr. Tenser concerns itself with the long-range prospects for control of herpesviruses, including therapeutic agents which have been useful in the past, and such future expectations as potential vaccine therapy.

The increased emphasis placed on herpesviruses is reflected by the ever growing number of articles published in primary journals. Their obvious participation in a variety of human illnesses, including venereally transmitted diseases, congenital infection of the newborn, infectious mononucleosis, chicken pox, shingles, severe encephalitis, and keratoconjunctivitis, makes them objects of intense interest regardless of their role in cancer. Their recurrence in individuals long after primary infection, in terms of both physical and psychiatric manifestations, elevates these viruses to a major public health problem of our era. Already, their use in gene transfer studies makes them valuable tools for molecular studies. Clearly they deserve far more study. Equally clear is the fact that despite the reasons for their study, as information accrues it is likely to change medical practice. This should lead to better control of those diseases now considered infectious as well as rarer diseases that often represent more serious medical problems, such as encephalitis and possibly cancer.

THE EDITOR

Fred Rapp is Professor and Chairman of the Department of Microbiology at The Milton S. Hershey Medical Center of The Pennsylvania State University, where he also serves as director of the Specialized Cancer Research Center. He is a University Evan Pugh Professor of Microbiology and a Research Professor of Virology of the American Cancer Society. He received his doctorate degree in medical microbiology from the University of Southern California and is recognized as a leading cancer virologist. His current research focuses on the oncology, genetics, immunology, and latency of herpesviruses and regulation of virus gene expression. He participates as a member of the American delegation on viral oncology to the Soviet Union and France, and serves on the boards of numerous professional organizations, committees of the National Institutes of Health, and as editor or associate editor for a number of scientific journals. He received the first Ciba-Geigy Drew Award in Biomedical Research, and lectures widely in the United States and elsewhere.

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ONCOGENIC HERPESVIRUSES

Chapter 1

HERPETIC LATENCY AND REACTIVATION

Jack G. Stevens

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I. INTRODUCTION*

Herpesviruses have long been considered classic examples of agents that establish latent infections and, although it has not been conclusively demonstrated in all cases, it seems likely that all of these viruses possess this property.** As reviewed elsewhere,[1] most of the agents are harbored in either the nervous or lymphoid systems. The general "state of the art" can be sufficiently reviewed using herpes simplex and Epstein-Barr viruses (HSV and EBV, respectively) as the most well-studied agents involving either the nervous system or lymphoid system. This discussion will be limited to these two agents. In addition, although HSV and Varicella-Zoster virus (VZV) were the first herpetic agents considered to establish latent infections, knowledge concerning the fundamental nature of this phenomenon has only recently been gathered on these viruses. Therefore, most biochemical aspects of the present discussion will concern the more recently discovered EBV, where virus-cell interactions during the latent state are much better understood. It can be noted that the principal reason for this disparity in knowledge is that an in vitro (i.e., cell culture) latency system is not yet available for studying the classic neurotropic herpesviruses, but was available at the outset for the Epstein-Barr agent. Most cells in the lymphoblastoid cell culture where the EBV was first identified harbored the agent in a latent state.[1]

II. HERPES SIMPLEX VIRUS

A. General

It now seems almost certain that the hypothesis formally proposed by E. W. Goodpasture 50 years ago regarding the pathogenesis of recurrent herpetic disease is the correct one. According to Goodpasture,[2] following primary infection of the external surface of the body, the virus would ascend through associated sensory nerves, establish a latent infection in the corresponding sensory ganglia, later be reactivated (probably from neurons), pass in the same nerves to the body surface, and produce lesions in areas served by the nerves. Evidence gathered over the years which documents the general correctness of this pathogenetic mechanism has been reviewed in detail[1,3,4] and will not be discussed here. Rather, the latent infection itself will be considered.

B. Tissues and Cells Involved in the Latent State

Experimental work indicates that latent infections are characteristically confined to the nervous system; the most comprehensive study documenting this association was carried out in mice.[5] In these experiments, a generalized infection was introduced by intravenous inoculation of virus, and this was followed by in vitro cultivation of a variety of tissues (a procedure that "induces" the latent infection to become an active one[6]). Following this "induction" procedure, infectious virus could only be recovered from nervous tissues; this included adrenal medulla. In addition, earlier experiments first performed in my laboratories[6,7] and subsequently in others,[8-12] indicated that the virus could be induced and recovered from sensory and sympathetic ganglia serving certain external areas initially infected with the virus, but not from the areas themselves after the primary infection had subsided. The one possible exception to the present general conclusion that the nervous system is uniquely able to harbor latent virus stems

* The literature review for this contribution was completed in 1978.

** As stated earlier,[1] the latent state is defined here as one in which the virus cannot be demonstrated by "conventional" virologic means between episodes of acute disease. This implies that the agent is harbored in some nonreplicating form during this period.

from results derived from guinea pigs inoculated with virus in the rear footpad.[13] There the agent was found to persist for prolonged periods at the peripheral site of inoculation, even when it could not be recovered from the ipsilateral lumbosacral spinal ganglia. It will be of interest to determine whether more extensive investigations will show that this is a true latent infection completely unrelated to the nervous system.

The cell type(s) involved in maintaining the latent virus genome in the nervous system has not been positively identified but several indirect experiments point rather strongly to the neurons. The most compelling evidence is the selective finding of virus DNA in neurons when latently infected spinal ganglia from mice and rabbits are subjected to *in situ* hybridization methods using virus-specific cRNA as a probe.[3,14,15] However, there is one recent report that suggests satellite cells are involved. In an imaginative study involving the use of a temperature-sensitive virus mutant blocked at a late stage of replication, McLennon and Darby[16] have found that when "reactivation" is attempted by cultivation of latently infected murine ganglia at restrictive temperatures, only satellite cells contain virus antigens (detected by immunofluorescent methods). When the reactivation temperature employed was nonrestrictive, neurons were found to contain virus-specific antigens. Although other interpretations are possible, it could be suggested that under normal conditions satellite cells harbor virus genetic information. When reactivation occurs, this information is passed to neurons where amplification and subsequent passage to the surface of the body ensues.

Finally, throughout this discussion the assumption has been made that the infection is a true latent one; although this has not been conclusively proved, several considerations detailed earlier[1] suggest rather strongly that this is, in fact, the case. In addition, a recent report[17] in which virus DNA, but not RNA, was detected in latently infected ganglia is consistent with this suggestion. In that study, liquid phase hybridization techniques with a sensitivity of one virus genome equivalent of RNA per 2000 diploid cell equivalents of DNA were employed.

C. Nature of Latency

As suggested, basic phenomena associated with establishment, maintenance, and reactivation of latent infection must await development of manipulatable in vitro systems (involving virus and neurons) amenable to quantitative biochemical study. However, through the use of temperature-sensitive virus mutants with defined phenotypes in vivo (mouse brains), some useful information concerning the phenomenon has recently been derived. As described elsewhere[1,18,19] given some assumptions, it may be hypothesized that mutants that establish latent infections possess lesions in genes, the products of which are critical for establishment of latency. Mutants restricted at 38°C (normal mouse temperature) in cell culture systems are available and many have been screened for their capacity to establish latent infections in the central and peripheral nervous systems of mice. This work is still in progress and is of a long-term nature. The results to date can be summarized as follows:

1. The mutants can be divided into two groups that differ in the capacity to establish latent infections.
2. Both DNA^+ and DNA^- mutants can establish latent infections, suggesting (but not proving) that DNA replication is irrelevant to establishment of latent infection.
3. Mutants that produce the fewest identifiable virus structural components during acute infection at restrictive temperature in "differentiated" neuroblastoma cells maintained in vitro are the most likely to establish latent infection in vivo. However, this correlation does not extend to individual virus proteins since viruses

with the most altered patterns of polypeptide synthesis are not necessarily the most likely to establish latent infections. These latter results are encouraging findings that imply specificity of involved functions.

4. Peptides, in addition to "immediate" early (α) polypeptides, are necessary for establishment of latent infections.
5. Genes involved in latent infection are not clustered on the genetic or physical map of the virus genome. It is hoped that analysis and comparison of these and other mutants by more refined techniques will allow us to identify polypeptides related to establishment of latent infections; studies of the functions of these peptides can then be initiated.

Investigations of the mechanism(s) involved in maintaining latent infection are just beginning, but there is a suggestion that antiviral IgG may play an important role. The evidence supporting this statement and its extensions are presented elsewhere.[14] In brief, it is based upon demonstrating that virus DNA synthesis is inhibited in latently infected neurons populating ganglia transplanted in Millipore® chambers to equal uninfected mice passively immunized with specific immune IgG, but not in mice given nonimmune IgG. How antiviral antibody functions in this inhibition is not known, but a model consistent with data derived from this and possibly related systems would involve specific interactions between the IgG and virus-specific antigens on the surface of neurons. Through some yet undefined intracellular effector molecule, this interaction could repress complete expression of the virus genome. In this regard, it is also of interest to note that engagement of the virus-induced Fc receptor (in Vero or retinoblastoma cells) by the Fc fragment of IgG also leads to inhibition of virus replication[20] and a selective inhibition of virus DNA synthesis.[21]

D. Reactivation of Active Infection

Finally, as shown on Table 1, reactivation of virus from latently infected ganglia has been achieved by physical manipulations,[10,22-25] superimposed bacterial infections,[26] or immunosuppressive measures[27] in two different animal species. In at least three of these systems, infectious virus has been shown to reappear at the body surface at a predictable interval after the manipulation,[22,23,25] and clinically apparent lesions have been detected in two instances.[22,23]

In one of the latter systems,[25] irritation of the dura mater of latently infected rabbit trigeminal ganglia resulted in reappearance of virus in the ipsilateral eye, where the primary infection had been established. In the others, ultraviolet irradiation[22] or mechanical irritation[23] of the ear surface of mice harboring latent infection in cervical spinal ganglia was followed by both reappearance of the virus and erythematous lesions on the skin of the ear. The common biochemical alterations associated with the reactivation induced by these diverse procedures are of great importance to define. Unfortunately, no information is yet available. One problem is that following most of these procedures, reactivation of virus does not occur in the majority of manipulated animals.

III. EPSTEIN-BARR VIRUS (EBV)

A. In Vivo Studies

EBV is the only herpesvirus to be investigated in relative depth with respect to the latent state, and thus represents the model against which the others will be compared. Therefore, those aspects of EBV replication that relate to the latent state will be presented.

Table 1
EXPERIMENTAL REACTIVATION OF LATENT HERPES SIMPLEX VIRUS FROM SENSORY AND SYMPATHETIC GANGLIA

Animal used	Method of inoculation, tissue harboring latent infection	Method of reactivation	Reappearance of virus at body surface	Ref.
Mouse	Intradermal, rear footpad, lumbosacral spinal ganglia	Neurectomy	—	10
	Intradermal, rear footpad, lumbosacral spinal ganglia	Pneumococcal pneumonia	No	26
	Intraocular, superior cervical ganglia	Neurectomy	—	24
	Intradermal, ear, cervical spinal ganglia	Ultraviolet light*	Yes	22
	Intradermal, ear, cervical spinal ganglia	Physical irritation of skin*	Yes	23
	Corneal scarification, trigeminal ganglia	Cyclophosphamide, X-irradiation	Yes	27
Rabbit	Corneal scarification, trigeminal ganglia	Physical irritation of ganglia*	Yes	25

Infectious virus was only searched for at the body surface, although for these models, pertinent sensory ganglia have previously been shown by the authors to harbor latent virus.[7,28]

It is now well established that latent EBV infections involve B-lymphocytes to the exclusion of other mononuclear cell types.[1,29] In addition, although virus-cell interaction has been little studied (no in vitro system is yet available), malignant (and normal?) epithelial cells of the nasopharynx also harbor EBV genetic information. In malignant cells taken directly from patients with Burkitt's lymphoma (or in the case of nasopharyngeal carcinoma, in relevant epidermal cells passed once in nude mice to select against contaminating nonneoplastic cells), EBV DNA has been shown to be present in multiple copies (10 to approximately 100) consisting mostly of covalently closed circular molecules.[30] In addition, it appears that a minor portion of the virus sequences in these cells is integrated into the cellular DNA. Tumor cells of both lymphoid[31] and epidermal[32] origin, and lymphocytes derived from mononucleosis patients[33] express a virus-associated, and probably virus DNA-encoded, intranuclear antigen routinely demonstrated by anticomplement immunofluorescent methods.[34] This antigen, termed EBNA (Epstein-Barr nuclear antigen), has now been characterized as having a molecular weight of 174,000 daltons and an affinity for interphase and metaphase eukaryotic chromosomes.[35] In addition, the EBV genome-carrying lymphocytes have also been reported to express a virus-associated antigen(s) localized in the cellular membrane.[36]

B. Latent Infections In Vitro

Most of the "in vivo" results summarized in the preceding section were extensions of earlier studies performed on EBV genome-carrying lymphoblastoid cell lines maintained in culture. Those studies and subsequent investigations with these cell types have been more extensive and instructive than work with cells taken directly from EBV-infected individuals. Therefore, the following discussion will concern pertinent characteristics of certain EBV genome-carrying lymphoblastoid cell lines.

1. Latent Virus DNA

The initial search for virus DNA, was carried out on Raji cells, a lymphoblastoid line derived from a Burkitt's tumor.[37,38] As a result of studies performed in several

laboratories in the early 1970s, it is now generally accepted that each cell of this line contains approximately 50 genome equivalents of virus DNA and that this number remains remarkably constant over time, even when the cells are cloned and grown into sublines. Whether the complete EBV genome is present in these cells is not definitively known, but two types of experiments suggest that it is. First, when DNA from either Raji cells or purified EBV is used to drive a radioactive EBV DNA probe into hybrids in kinetic DNA-DNA reassociation experiments, the shape of the curves generated is identical.[39,40] Second, treatment of the Raji line with 5-bromodeoxyuridine results in the induction (in a very few cells) of virus particles which are morphologically intact[41] and infectious.[42] As in the tumors, it appears that in this cell line most virus DNA is present as closed circular molecules and a minor portion seems to be covalently associated with host cell DNA.[43-46]

Other cell lines derived by various techniques from various sources have been investigated by similar methods and several interesting findings have emerged. First, it seems that essentially all EBV-containing lymphoblastoid cell lines contain multiple genome equivalents of the virus DNA irrespective of whether they are derived from tumors, infectious mononucleosis patients, normal individuals, or fetal lymphocytes infected by the virus in vitro.[47] In addition, the studies completed to date indicate that virus DNA is present in both integrated and extrachromosomal states, with the latter in the greatest abundance. This is consistent with the suggestion that both integrated and nonintegrated virus DNA is obligatory to establishment of these cells, and possibly also to maintenance of the latent state. Studies that have attempted to dissect this problem further and possibly to establish which is necessary for either phenomenon have so far not given an unequivocal answer. One originally non-EBV-carrying B-cell line (Ramos), infected later with the virus in vitro, was shown to carry one integrated genome equivalent per cell.[48] However, cultures of human cord blood lymphocytes infected in vitro and then serially passed 30 to 50 times appear to carry 10 to 15 genome equivalents of DNA; this DNA is apparently not integrated.[49]

2. Transcription and Translation Products of Latent Virus DNA

From results derived by molecular hybridization experiments,[50,51] Kieff and coworkers have indicated that there can be at least three levels of virus DNA expression in EBV-carrying lymphoblastoid cell lines.[51] In the extreme case (the productive, cytocidal infection), a minority of cells in the population may spontaneously produce infectious virus and are destroyed in the process. In another instance, EBV genome-carrying cells superinfected with EBV may be abortively or productively infected and also destroyed. In this case, molecular hybridization experiments involving a radioactive probe consisting of EBV DNA have shown that virus transcript complexity approaches that present during productive infection. However, unlike productive infection, not all virus transcripts in these cells are polyadenylated. The third and most interesting case, termed restringent by Orellana and Kieff,[51] is obtained in latently infected cells. Here, employing RNA from five nonproductive and usually nonearly antigen-containing, but EBV DNA-containing cell lines in order to drive the molecular hybridization reaction, it was shown that 11 to 30% of the virus DNA was represented in transcripts, with about 5% also present in polyribosomal fractions.[50] In two cell lines studied further (Raji and Namalwa), the polyadenylated and polyribosomal fractions were enriched for the same class of EBV RNA. This class was coded for by about 5% of the EBV DNA, and the same DNA sequences encoded for these RNA species in the two cell lines.[51] This contrasted with 45% of the genome transcribed in productively infected cells with all transcripts being polyadenylated and appearing in polyribosomes.

Table 2
EBV PRODUCTS IN LATENTLY INFECTED B-LYMPHOCYTES (PROTOTYPE RAJI)

Cellular compartment	Virus product
Nucleus	Integrated and extrachromosomal DNA RNA transcripts from 5% of the virus genome EBNA*
Cytoplasm	Polyribosomes with viral message
Cytoplasmic membrane	LYDMA*

* Although these antigens are generally considered to be virus gene products, this has not been rigorously proved.

From these studies it can be concluded that: (1) EBV DNA is transcribed during the latent state, but not all sequences that appear during productive infection are transcribed during the latent state. (2) A subset of the RNA transcribed during the latent state is polyadenylated and appears in polyribosomes. The amount of RNA appearing in polyribosomes is sufficient to code for a peptide(s) consisting of at least 5000 amino acids, and at least one of the putative translation products seems likely to be the EBNA. In addition, an immunologically detected but biochemically undefined antigen term LYDMA (lymphocyte-detected membrane antigen)[52] has also been found on the surface of most EBV genome-carrying lymphoblastoid cell lines and appears to be a translation product of the virus message. The possible role played by either of these antigens in latency is presently unknown. A summary of virus products present in various cellular compartments of latently infected lymphocytes is presented in Table 2.

3. Regulation of the Latent State and Reactivation of Infectious Virus

It is well known that the latent state can be upset and "pushed" toward the lytic state by in vitro cultivation of lymphocytes,[53] treatment of these cells with halogenated deoxypyrimidines,[41,42,54] or by superinfection with a nontransforming strain of EBV.[55,56] How this happens is unclear, but induction by iododeoxyuridine requires incorporation of the compound into the cellular DNA.[54]

Beyond these induction experiments, the regulation of EBV genetic information in lymphoblastoid cell lines has been studied by scoring the appearance of virus gene products in various somatic cell hybrids which differ in the extent to which virus-related functions are expressed. Here, heterokaryons were formed between EBV genome-containing lymphoblastoid cell lines in which the virus replicative cycle was characteristically blocked at different points in the replicative cycle or between such cells and nongenome-containing cells of lymphoid or epidermal origin.

Unfortunately, the mechanisms involved in the maintenance of the latent state (i.e., nonlytic state in which only EBNA and LYDMA are known to be produced) and the possible role played by EBNA and LYDMA in the phenomenon have not been clarified by these methods. Cell hybridization studies have, however, given insight into the control mechanisms operating after the latent genome has been induced. These initial experiments and their extensions have been presented in considerable detail by Klein,[29] and are briefly introduced here. First, evidence for a positive control mechanism was shown when a nonproducer B-cell line was fused to a producer line and resulted in heterokaryons which were producers.[57,58] Second, a negative control mechanism has also been suggested. Here, fusion of EBV line Raji (a line in which virus genetic expression only rarely proceeds beyond the latent state in most cells) with the Daudi

or Jijoye lines (lines in which some cells replicate virus DNA and synthesize capsid antigens) blocks virus DNA synthesis.[57]

These results indicate that the host cell plays an important role in virus regulatory processes, and other findings are in accordance with this suggestion. Thus, indirect experiments with the selective DNA polymerase inhibitor phosphonoacetic acid indicate that replication of latent virus DNA is accomplished by the host cell polymerase.[56,60] It has also been established that latent virus DNA replication is synchronized with that of the host cell DNA.[61] In addition, lymphocytes from different sources transformed by the same virus substrain express virus-specific genetic information to different extents[29] and fusion of producer lymphocytes to other cell lines (i.e., HeLa cells) results in the disappearance of this property.[62,63] However, such a combination can be induced to produce virus capsid antigens if a nonproducer lymphocyte-epithelial cell combination is treated with iododeoxyuridine.[64,65]

Clearly then, although regulatory events operating during maintenance of the latent state remain largely undefined, information gathered from studies of "induced" lymphocytes indicates that they are complex. "Induction" appears to involve both positive and negative control mechanisms and the host cell has substantial influence on this process. How these findings relate to the transcriptional studies presented earlier, in which transcriptional and post-transcriptional control mechanisms were indicated, is not yet clear, and useful speculation will depend on additional information.

IV. CONCLUSIONS AND EXTENSIONS

The studies reviewed here begin to unravel the exquisite mechanisms by which two common herpesviruses have adapted to persist in the human population. Both persist as latent agents capable of being reactivated in cells harboring virus genetic information. However, the cells involved appear to be quite different. In the case of HSV, terminally differentiated, highly specialized cells of ectodermal origin harbor the agent. In the other, mitotically active, or potentially mitotically active cells of mesodermal origin are involved. In the case of EBV, however, the potential role of epidermal cells in the pathogenesis of infection has not been adequately defined. Certainly such cells harbor virus DNA[30,66,67] and are also capable of replicating the agent.[67]

Further incisive studies concerning the latent state of HSV are likely to depend upon the development of a suitable in vitro system, although indirect studies involving temperature-sensitive mutants in the mouse have been instructive. Studies of phenomena associated with latency in EBV infections will be considerably enhanced when a system is available in which latently infected cells can be induced to synchronously produce significant levels of infectious virus. Such a system would be useful in at least two ways. The virus produced could be used to synchronously infect B-lymphocytes and these could then be manipulated to analyze the biochemical steps involved in establishment of the latent state. In addition, the induced, previously latently infected cells could be employed to define the nature of the induction phenomenon.

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