Activation of Epstein-Barr Virus Replication in Hodgkin and Reed-Sternberg Cells

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Recent evidence has shown that Hodgkin’s disease (HD) is associated with Epstein-Barr virus (EBV) infection. This association was originally suggested by increased EBV antibody titers in sera of HD patients, an increased risk of developing HD after infectious mononucleosis, and by epidemiology. Furthermore, EBV genomes have been demonstrated in DNA extracts in 17% to 44% of tumors using filter nucleic acid hybridization and in 35% and 58% by the polymerase chain reaction technique. However, with these methods the cellular source of EBV-specific DNA sequences is not identified.

More direct evidence of a possible causal relationship between EBV and HD has been indicated by the immunocytochemical demonstration of EB nuclear antigen (EBNA) in Reed-Sternberg (RS) cells of one case of HD that developed after an infectious mononucleosis syndrome, by the detection of viral genomes in RS cells using in situ hybridization (ISH) and by analyses of the terminal portions of EBV genomes that have indicated a monoclonal or oligoclonal proliferation of EBV-infected cells in HD. Moreover, the most recent studies have suggested that EBV is not merely a silent passenger in the tumor cells because some latent gene products that may have a role in growth regulation are transcribed and translated in RS cells. Thus, Pallesen et al found strong expression of latent membrane protein BZLF1, which controls the switch between EBV latency and replication, and also to LMP. LMP was demonstrated in RS cells in 47 cases (49%). Three of the LMP-positive cases (8%), but none of the LMP-negative cases, expressed the BZLF1 protein. BZLF1 positivity was confined to rare RS cells. These three cases showed no detectable early, virus capsid, or membrane antigens. Our findings show that activation of EBV immediate early genes occurs only infrequently in RS cells, indicating that control of viral latency is not severely impaired in HD patients.

It has generally been assumed that the EBV genome remains latent and that replication does not occur in epithelial and particularly in lymphoid tumor cells infected with the virus. However, recent studies have shown that spontaneous replication in tumors may sometimes occur as demonstrated by the presence of whole virions by electron microscopy, of linear EBV DNA by Southern blotting, and of proteins associated with the early or late viral productive cycle using immunocytochemistry.

To further study the EBV-tumor cell interaction in HD in vivo we performed an immunohistologic investigation of tumor tissues to examine if activation of the EBV productive cycle occurs in this disease.

MATERIALS AND METHODS

We studied tumor tissue from 96 HD cases stored frozen at −70°C for up to 10 years at the Laboratory of Immunohistology (Aarhus, Denmark). They were subclassified as mixed cellularity (n = 27), nodular sclerosis (n = 59), and lymphocytic predominance (n = 10). Lymphocytic depletion subtype was excluded from this study because of its controversial nature. The major part of this material is similar to that used in our previous study on EBV latent gene expression in HD.

Expression of LMP was detected by four pooled monoclonal antibodies (MoAbs), CS.1-4, reactive with at least three different epitopes of LMP. The BZLF1 protein, which is capable of disrupting latency in EBV-infected lymphoid cells, was detected by a new MoAb, designated BZ1, raised against a fusion protein containing part of Staphylococcus aureus protein A at its N-terminus and joined to the full-length BZLF1 sequence (pREX-BZLF1). BZ1 recognizes both the active, dimeric form and the inactive, monomeric form of the BZLF1 protein. MoAbs used against early replication-associated antigens (EA) were: 37G11, 38G6 (courtesy H. Wolf, Munich, Germany) and anti-EBV EA-D (product no. 9240; DuPont, Billerica, MA). Late productive-cycle proteins were labeled using 2F2 (H. Wolf) and product no. 9246

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Fig 1. EBV gene and gene expression in RS cells of HD (A through C). (A) Alkaline phosphatase anti-alkaline phosphatase (APAAP) immunohistological staining on frozen tissue section using MoAb BZ1 against the EBV-encoded BZLF1 switch protein. This is a case of HD, mixed cellularity type. Rare RS cells show intense nuclear staining. The small lymphocytes are negative. (B) Same case, paraffin section immunostained with CS.14 MoAbs against EBV latent membrane protein. Strong membrane and cytoplasmic staining is restricted to RS cells. (C) The same case. In situ hybridization on paraffin sections using a 35S-labeled EBV DNA probe shows a strong signal over the nuclei of RS cells. (D) Positive control case of oral hairy leukoplakia from the tongue of an AIDS patient. Intense nuclear staining is seen in most keratinocyte nuclei of the upper epithelial layers whereas the basal layers are negative. Note characteristic negative intranuclear vacuoles in most positively stained nuclei.

Cryostat section, APAAP method.

(DuPont) against virus capsid antigens (VCA), and 11D7 (H. Wolf) and anti-EBV induced membrane antigen (product no. 9245, DuPont) against membrane antigens (MA). Because expression of the immediate-early BZLF1 protein is a prerequisite for the synthesis of EA and by implication for VCA and MA, the latter were only investigated if BZ1 showed labeling of Hodgkin and RS cells.

Antibodies were applied to frozen sections fixed in acetone at room temperature for 10 minutes using standard immunoperoxidase or (for BZ1) alkaline phosphatase anti-alkaline phosphatase (APAAP) methods. Fresh-frozen oral hairy leukoplakia lesions from acquired immunodeficiency syndrome (AIDS) patients and frozen pellets of the EBV-positive cell line P3HR-1, both of which manifest productive EBV infection, served as positive controls, whereas tonsils or lymph nodes with chronic nonspecific inflammation were negative controls. Presence of endogenous peroxidase or alkaline phosphatase was evaluated using buffer instead of primary antibody.

EBV genomes were detected using ISH on sections of formalin-fixed and paraffin-embedded tissue as described previously. The probe (BamHI-W-internal repetitive fragment of EBV strain M-ABA) was labeled with 35S-dCTP by nick translation. Hybrids were detected by autoradiography. In all experiments a 35S-labeled cytomegalovirus probe was used on adjacent sections as a negative probe control. Positive controls included sections of oral hairy leukoplakia and acute infectious mononucleosis tonsils.

RESULTS

LMP was detected in 47 of 96 cases (49%). The different subtypes of HD varied in their expression of LMP as described previously, with 96% of mixed cellularity, 34% of nodular sclerosis, and 10% of lymphocytic predominance containing LMP-positive RS cells. Labeling was restricted to the cytoplasm and cell membrane of Hodgkin and RS cells.

BZLF1 protein was detected in only 3 of 47 LMP-positive cases (6%) and in none of the LMP-negative cases. Two of the positive cases belonged to the mixed cellularity and one to the nodular sclerosis subtype. Labeling was localized to nuclei of a very few RS cells and was not seen in nuclei of the nontumor cell population. A BZ1-positive RS cell is shown in Fig 1A. Figure 1B and C illustrate LMP and EBV DNA in RS cells as detected by immunohistology and in situ hybridization, respectively (same case as Fig 1A). BZ1-staining was usually less intensive in RS cells than in
keratinocyte nuclei of oral hairy leukoplakia, where it was restricted to the upper epithelial layers in all cases (Fig 1D), and in P3HR-1 cells, in which 4% of cells were labeled (not illustrated).

The three BZ1-positive HD cases were subsequently immunostained with the MoAbs to EA, VCA, and MA, but these proteins were not detected in either RS cells or nontumor cells.

No specific staining was seen in the negative control tissues. Cross-reactivity with normal histiocytes resulting in weak to moderate cytoplasmic staining was observed with two commercially available MoAbs to VCA (DuPont, product no. 9246) and to MA (DuPont, product no. 9245), but not with the others.

DISCUSSION

EBV is a human herpesvirus that may follow either a latent or a productive (replicative) life cycle. Productive cycle gene expression is organized as a cascade of groups of genes. Expression of the immediate-early gene BZLF1 of EBV appears to play a key role in initiating this lytic cascade. BZLF1 is a gene regulatory molecule that may work partly by counteracting a cellular factor that represses viral transcription, thereby switching on the productive cycle partly by counteracting a cellular factor that represses genes. Expression of the immediate-early gene BZLF1 of EBV appears to play a key role in initiating this lytic cascade. BZLF1 is a gene regulatory molecule that may work partly by counteracting a cellular factor that represses viral transcription, thereby switching on the productive cycle. BZLF1 protein together with other transactivators can activate the promoter for the BMRFL gene that encodes the diffuse component of the EA. EA-D is an essential component for the viral DNA polymerase activity.

In this study we have demonstrated that EBV-infected RS cells manifest a latent infection with strong expression of LMP, whereas replication-associated proteins are only rarely detected. The BZLF1 protein could be demonstrated in rare RS cells in three of the LMP-positive cases (6%) but in none of the LMP-negative cases. Gene products such as early (EA) and late (VCA, MA) replication antigens could not be detected in BZLF1-positive cases in our study. This suggests that further gene transcription is impaired, resulting in an abortive viral productive cycle. Alternatively, cells expressing late productive-cycle proteins may be so rare as to remain undetected even with the sensitive immunohistologic method used in this study.

In a recent immunohistologic study on EBV replication in lymphoma cells in tissue of AIDS-related non-Hodgkin’s lymphomas we found BZLF1 protein expression in 6 of 10 EBV-positive cases. EA expression was found in four, but VCA and MA in only one of these cases. This suggests that disruption of EBV latency with induction of abortive replication is a regular feature of lymphomas developing in this group of severely immunocompromised patients. This is consistent with evidence that EBV latency is maintained under strict host control. Although HD patients have impaired cellular immunity, this is not as severe or as well defined as in patients with AIDS, organ transplantation, or with inherited immune deficiency, all of whom are at greatly increased risk of developing high-grade EBV-associated B-cell non-Hodgkin’s lymphomas.

That factors other than host immunity may be important in maintaining EBV latency has been suggested by recent experiments by Gradoville et al. These workers have shown that the extent to which BZLF1 protein can induce activation of EBV replicative genes depends on the phenotype (differentiation) of the infected cell. Gradoville et al showed that in some cell lines the virus is blocked at the stage of EA gene expression, whereas other cell lines may be induced by BZLF1 protein and other transactivators to express late replication proteins and to produce virions. Therefore, phenotypic and differentiation-dependent host cell factors in RS cells may well be crucial in deciding the extent to which replication-associated genes are activated by BZLF1. The nature (phenotypic as well as genotypic) of Hodgkin and RS cells is still controversial, some cases suggesting an origin from B cells and others from T cells. However, in most cases no definite lineage-specific markers are expressed. It is conceivable that the low permissiveness for EBV replication by RS cells, as demonstrated in this study, may be a consequence both of the relatively intact immunity in HD patients and of the particular phenotype (differentiation) of Hodgkin and RS cells.

Our finding, at the protein level, that activation of the EBV productive cycle may occasionally occur in infected RS cells finds support from two reports in the literature on the presence of small linear EBV DNA fragments in Southern blots of DNA extracted from HD biopsies. Such linear fragments, detected with a probe containing unique right terminal region sequences of EBV, are believed to signify the presence of whole or defective virions. They were identified by Katz et al. in a case of HD developing in a patient with Crohn’s disease, and in one of seven EBV-positive HD cases studied by Anagnostopoulos et al. In both cases, however, the cellular source (RS cells vs residual B cells) remained uncertain. Our study documents that BZLF1 protein expression, when it occurs, is restricted to RS cells. Wu et al. have reported finding abundant expression of EBER RNA transcripts in RS cells in most cases of EBV-positive HD. It has recently been suggested that EBERs may be regarded as specific markers of latent EBV infection, a proposal that is consistent with our failure to detect BZLF1 expression in RS cells in the majority of cases of HD.

The clinical implications of our results are not clear. They suggest that the control of viral latency in HD is not severely impaired. This argues against the use of acyclovir in HD because this drug is only effective against the productive EBV cycle. However, it is possible that the partial activation of productive cycle genes seen in occasional HD cases may affect cell growth and such patients should be considered as candidates for antiviral therapy.

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