American Journal of Pathology, Vol. 139, No. 1, July 1991

Rapid Communication

Epstein–Barr Viral Genome in Lymph Nodes from Patients with Hodgkin's Disease May Not Be Specific to Reed–Sternberg Cells

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A possible etiologic role for Epstein–Barr virus (EBV) in Hodgkin's disease (HD) was investigated by probing for EBV genome in 52 biopsy specimens involved with HD and 43 hyperplastic lymph node specimens. Using dot-blot hybridization (Bam HIW probe), Southern blot hybridization (Xho I probe), and polymerase chain reaction analyses, 27%, 27%, and 58% of the nodes with HD were positive for EBV genome, respectively, as compared to 16%, 14%, and 43% in the hyperplastic lymph nodes. Clonal and nonclonal episomal EBV and linear replicating EBV genome were present in both conditions. Immunoglobulin heavy chain gene rearrangements were found in two clonal and two nonclonal EBV-positive HD cases, but not in the lymphoid hyperplasia cases. These findings and other recent reports showing EBV genome in benign lymphoid cells by in situ hybridization in Hodgkin's disease suggest that the characteristics of EBV infection in HD could be explained by the reactive cellular milieu, especially in the setting of defective immunity. The identification of EBV genome in Reed–Sternberg cells may, therefore, be a nonspecific phenomenon. (Am J Pathol 1991, 139:37–43)

Extensive investigative efforts have failed to disclose the etiology of Hodgkin's disease (HD), the cellular origin of the Reed–Sternberg (RS) cell and the significance of the reactive cells of HD. Patients with HD show a higher frequency and titers of antibodies to Epstein–Barr virus (EBV) than do patients with non-Hodgkin's lymphoma.[1–4] Recent reports of clonal immunoglobulin (Ig) and T-cell receptor gene rearrangements in HD have fueled speculation about a possible lymphoid origin for the RS cell.[5–10]

Hybridization studies performed during the 1970s failed to detect EBV genome in HD.[11] However filter hybridization, the polymerase chain reaction (PCR) assay, and *in situ* hybridization (ISH) have disclosed EBV genome in HD.[12–18] For example, 17% to 29% of HD biopsy specimens have been shown to contain EBV genome by dot-blot or Southern blot analyses,[12–16] and 58% were positive by PCR.[18]

Whether the presence of EBV genome and clonal antigen receptor gene rearrangements in HD involve RS cells, the reactive cellular component of HD, or both is unclear. Reports claiming exclusive localization of EBV genome to RS cells by isotopic ISH are questionable because this technique gives high background signals. Thus detection of low levels of EBV genome in the reactive cells may be precluded. To examine this issue, we probed 52 HD biopsy specimens and 43 hyperplastic lymph nodes using dot-blot, Southern blot, and PCR DNA hybridization techniques for EBV genome and Ig gene rearrangements. All cases of known infectious mononucleosis or post-transplant lymphoproliferation were excluded, as were cases with atypical features. Nine cases of HD initially examined by colorimetric ISH[19] failed to

Supported in part by Public Health Service grants CA30196 and CA36727, awarded by The National Cancer Institute, Department of Health and Human Services, the State of Nebraska Department of Health, LB506, and the Lymphoproliferative Research Fund.

Accepted for publication May 10, 1991.

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Table 1. *Presence of Epstein–Barr Virus Genome in Hodgkin's disease and Hyperplastic Lymph Nodes*

	No. cases	Hybridization method		
		Dot blot (%)	Southern analysis (%)	PCR %
Hodgkin's Disease				
Nodular sclerosis	21	24	24	52
Lymphocyte predominance, diffuse	6	17	17	34
Lymphocyte predominance, nodular	6	0	0	50
Mixed cellularity	13	46	46	69
Lymphocyte depletion	6	30	30	83
Total	52	27	27	58
Hyperplastic lymph nodes				
Follicular hyperplasia	21	14	19	43
Interfollicular hyperplasia	22	19	10	43
Total	43	16	14	43

reveal EBV genome in either the RS cells or reactive lymphoid cells; however optimization of this method is in progress. We found a comparable frequency of EBV genome in both hyperplastic lymph nodes and tissues involved by HD. Furthermore clonal and nonclonal episomal EBV genome, and linear replicating EBV genome were present in both conditions, and all cases of HD with Ig rearrangements were EBV positive. The possible significance of these findings is discussed.

Materials and Methods

Clinical Materials

The lymph node specimens we studied were snap-frozen tissue in liquid nitrogen and stored at −72°C for molecular studies. Table 1 summarizes the subtypes of HD[20] that we studied. The lymphoid hyperplasia cases selected for study included only those cases with nonspecific reactive features, including cases with either predominantly follicular or interfollicular hyperplasia (Table 1). Cases with atypical features or morphologic evidence of AIDS-related complex (ARC), angioimmunoblastic lymphadenopathy (AILD), infectious mononucleosis (IM), necrotizing lymphadenitis, Kikuchi's disease, florid immunoblastic proliferation, or post-transplant lymphoproliferative disease were excluded.

DNA Extraction

High–molecular-weight total cellular DNA from each snap-frozen tissue sample was extracted by cell lysis-proteinase K digestion, phenol-sevag extraction, and ethanol precipitation using standard procedures.[21,22]

Southern Blot Analysis

Ten-microgram aliquots of DNA were digested with either Eco R1, Hind III, or Bam H1 restriction enzymes (Bethesda Research Laboratories, Bethesda, MD), electrophoretically separated by size in a 0.8% agarose gel, and transferred to a nylon filter by the method of Southern.[19] A probe to the Ig heavy chain joining (J_H) region (Oncogene Science, Inc., Manhasset, NY) was used to detect clonal B-cell populations. To evaluate for EBV genome structure (ie, episomal *versus* linear form) and to detect EBV clonality, the filters with Bam H1-digested DNA were hybridized with the 1.9-Kb Xho 1 fragment of the EBV genome (a gift of Dr. Raab-Traub). All probes were labeled with ^{32}p nucleotides by the random priming method.[23] Following hybridization with the appropriate probe, the filters were washed to stringency and exposed for 48 to 78 hours at −70°C on Kodak X-Omat AR film (Rochester, NY) using intensifying screens. Figures 2, 3, and 4 show the controls used.

Dot-blot Hybridization for EBV Genome

Five micrograms of high–molecular-weight DNA from each sample and control was applied to nylon filters (Sureblat, Oncor, Gaithersburg, MD) using a vacuum dot-blot apparatus. Hybridization was performed using a 2.1-Kb Bam H1W EBV-specific DNA probe (labeled by random priming to a specific activity of 2×10^6 cpm/ml) in standard hybridization buffer (10 ml) at 45°C for 10 hours, followed by stringent washing and exposure of the filters for 48 hours in Kodak X-Omat AR autoradiographic grids at −70°C. Negative controls included human placental DNA and DNA from the EBV-negative Burkitt's lymphoma (Ramos) cell line. Positive EBV controls included the EBV-positive Burkitt's lymphoma cell lines, Namalwa, and Raji.

DNA Amplification by Polymerase Chain Reaction

One microgram of DNA from each sample was used in a total reaction volume of 50 μl containing 1.5 units of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT), reaction buffer (Perkin-Elmer-Cetus), 0.2 mmol/l each of dATP, dCTP, dGPT, TTP and 50 pmol/per reaction of each primer. The PCR reaction mixture was covered with three drops of mineral oil and subjected to 35 cycles of amplification using a turbocycle format consisting of cycling at 94°C for 1 second followed by 50°C for 1 second and 95°C for 2 seconds. Precautions taken to avoid or detect possible contamination included the use of positive displacement pipettes, preparation of reaction mixtures in a ventilated hood, and the use of several positive (Namalwa cell line) and negative (Ramos cell line) controls interspersed among the clinical samples. In addition, PCR was performed twice on all samples to ensure consistency of results.

The PCR primers were complementary to the EBV EBNA 2 gene and had the following nucleotide sequence: 5′ TCGCACTCCTATGCATTTCC 3′ AND 5′ CTACTTCGCCATGTTTCTTA 3′. The specificity of the expected 260 base pair (bp) amplification product was confirmed by isotopic Southern hybridization with a 5′ end-labelled internal olignucleotide probe with the following nucleotide sequence: 5′ ACTTTTACCCCAGTCCCAACC 3′. Figure 1 shows controls used.

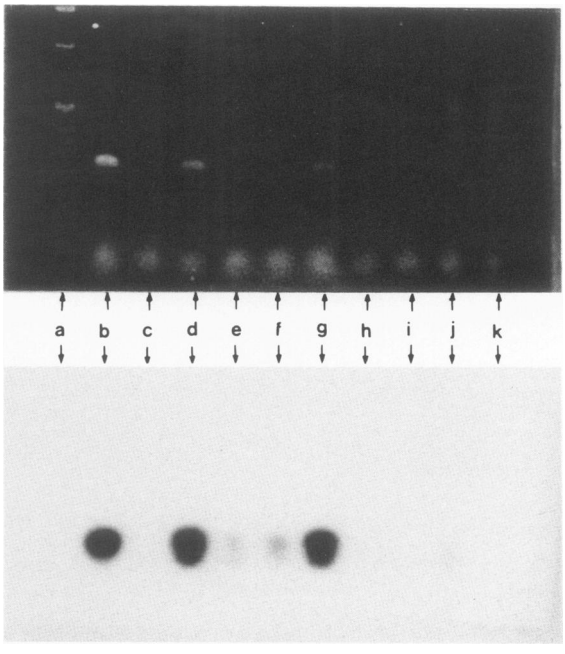

Figure 1. *Polymerase chain reaction detection of EBV genome in HD. Upper panel, ethidium bromide-stained 2% agarose minigel containing electrophoresed PCR amplification products; lower panel, corresponding confirmatory isotopic Southern hybridization of PCR products. Lane a, Hind III λ DNA size marker; lane b, EBV positive control (Namalwa cell line); lane c, EBV negative control (Ramos cell line); lanes d–k, representative HD cases. EBV genome is detectable in clinical samples D, E, F, G and J.*

Results

EBV Genome Detection

The prevalence of EBV genome in the cases of HD (Table 1) was 27%, 27%, and 58% using DB, SB, and PCR assays, respectively. The concordance of positive results between the DB, SB, and PCR analyses was 100%. Epstein–Barr virus was found most commonly in the mixed cellularity and lymphocyte depletion subtypes, although the increased frequency was not statistically significant. Epstein-Barr virus was detected in 16%, 14%, and 43% by DB, SB, and PCR analyses, respectively, in the hyperplastic lymph nodes. A comparison of follicular and interfollicular cases is shown in Table 1.

EBV Clonality and Genome Structure Analyses (Table 2)

Of the 13 cases of HD EBV detectable by Southern blot analysis, episomal EBV[24,25] was identified in nine cases, seven of which contained a single high–molecular-weight band genome (8–15 Kb) consistent with clonal EBV (Figure 2). Two HD cases (lanes d and h of Figure 2) and two control cases (lanes 3 and f of Figure 3) demonstrated a single band below the 8-Kb threshhold characteristic of episomal EBV genome. While it is unclear whether these cases represent linear genome in which the additional bands are below the detection limit (our interpretation) or variant low–molecular-weight episomal forms, for purposes of our study, the most important element is consistency in the interpretation of these cases. The presence of lower molecular-weight (2–6 Kb) hybridization bands indicating linear viral genome was identified in eight cases of HD, three of which also contained episomal EBV DNA. Four of the six cases of lymphoid hyperplasia positive for EBV by Southern analysis contained episomal EBV genome (Figure 3). Clonal EBV was

Table 2. *Epstein–Barr Virus (EBV) Clonality and Genome Composition in Hodgkin's Disease and Lymphoid Hyperplasia*

	Number of cases	Episomal EBV		Linear EBV
		Clonal	Nonclonal	
Hodgkin's disease	13	7	2	8
Lymphoid hyperplasia	6	2	2	4

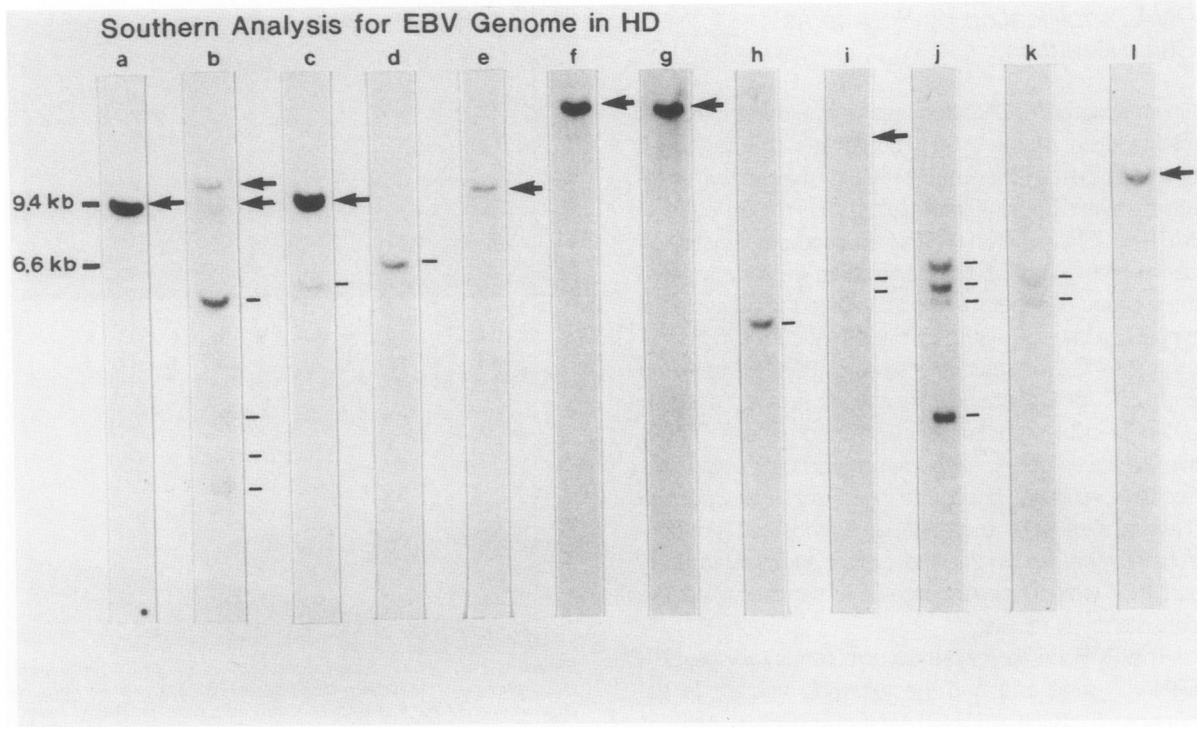

Figure 2. *Southern analysis for EBV genome in HD. Lane a, EBV-positive Burkitt's lymphoma (Namalwa); lanes b–l, representative cases of HD. Episomal (arrowhead) and linear (dash) EBV genome are indicated. Clonal episomal EBV is presented in lanes c, e, f, g, i, and l, whereas lane b shows episomal nonclonal EBV. Note presence of both episomal and linear EBV in lanes b and i (Enzyme, Bam HI, Probe, Xho I).*

present in two of these cases. Linear viral genome was observed in a total of four hyerplastic lymph node specimens (Figure 3). The six individuals in the control group with EBV genome detectable by Southern analysis included a 64-year-old woman with myelodysplastic syndrome, a 2-year-old female liver transplant recipient, a 5-year-old boy with bilateral cervical adenopathy and serology indicating past EBV infection, a 4-year-old girl with chronic tonsillitis and massive left cervical lymphadenopathy whose lymph node histology showed only follicular hyperplasia, a 66-year-old man with a sclerotic inguinal lymph node, and a 53-year-old woman with an enlarged inguinal lymph node showing dermatopathic features. There was no history of malignant lymphoma in any of these patients.

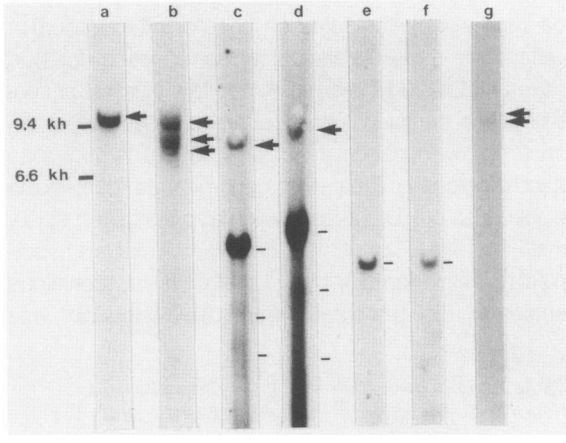

Figure 3. *Southern analysis for EBV genome in hyperplastic lymph nodes. Lane a, EBV positive Burkitt's lymphoma (Namalwa); lanes b–g hyperplastic LN specimens. Episomal (arrowhead) and linear (dash) EBV genome are indicated. Clonal episomal EBV is present in samples c and d, whereas nonclonal episomal EBV is present in samples b and g. Note presence of both clonal episomal and linear replicating EBV in samples c and d (Enzyme, Bam HI; Probe, Xho I).*

Immunoglobulin Heavy Chain Gene Rearrangements

Immunoglobulin heavy chain (J_H) gene rearrangements were detected in four cases of HD (Figure 4). The rearrangements were monoclonal in two cases and oligoclonal in two cases (Table 3). All cases with J_H rearrangements were positive for EBV by both filter hybridization and PCR assays. Clonal EBV was present in one of the two cases with a monoclonal J_H rearrangement and one of the two cases with oligoclonal J_H rearrangements, whereas EBV was nonclonal in the other two cases. All of the cases of lymphoid hyperplasia, including those positive for EBV genome, were negative for J_H rearrangements.

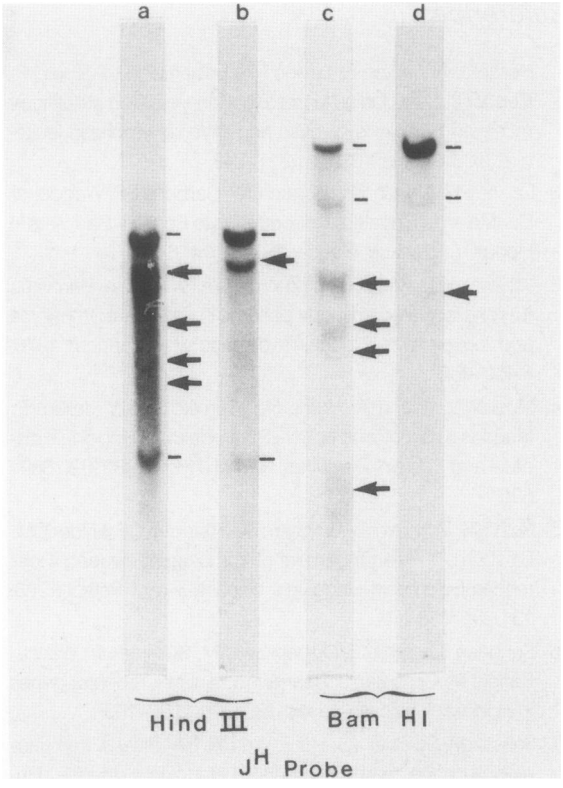

Figure 4. *Ig heavy chain gene rearrangements in HD. HD samples are represented in lanes A–d. Germline (dash) and rearranged (arrowhead) bands are indicated. Lanes a and c exhibit polyclonal whereas b and d exhibit monoclonal Ig heavy chain gene rearrangements.*

Discussion

Several epidemiologic studies revealed a 3.5-fold increased risk of developing HD following infectious mononucleosis (reviewed in Roth et al[5]), elevated EBV-specific antibodies in patients with Hodgkin's disease,[2–5] and RS-like cells in lymph nodes from patients with infectious mononucleosis.[26] Also EBV is associated with head and neck carcinomas and T- and B-cell non-Hodgkin's lymphomas.[27] However against an etiologic association of EBV with HD is the uncommon occurrence of HD in individuals with inherited immunodeficiencies and solid organ transplant recipients.[28] Furthermore HD rarely arises in extranodal sites, whereas EBV is strongly associated with extranodal lymphoproliferative diseases in immunocompromised patients.

Recently, using filter hybridization and PCR techniques, approximately one half of cases of HD were found to contain EBV genome.[12–18] Furthermore EBV genome was identified in RS cells by ISH.[16–18] Despite the evidence that EBV genome is not present in approximately one half of HD cases and many HD-derived cell lines,[29] the view prevails among some investigators[16,17,19] that EBV genome in HD is restricted or specific to the RS cell component, and is, therefore, possibly directly involved in the neoplastic transformation of RS cells. But these speculations have assumed a specific etiologically significant interaction of EBV with RS cells and an absence of EBV genome in the reactive cells that surround the RS cells in HD. To address these issues, we probed for EBV genome and evaluated its clonality and structure in HD and hyperplastic lymphoid tissues using the latter as a control for the reactive cellular milieu in HD. Thereby we sought to test the hypothesis that immunodeficiency[30] accompanying HD might allow latent virus to reactivate and drive polyclonal B-cell proliferation within the lymph nodes involved with HD.

Our studies demonstrated many similiarities between hyperplastic lymphoid processes and HD regarding the presence and structure of EBV genome (Table 4). Both conditions showed a relatively high prevalence of EBV genome, and contained episomal clonal, episomal nonclonal, and linear viral genome (Tables 1 and 2, Figures 2 and 3). These findings suggest that the characteristics of EBV infection in HD could be explained by the reactive cellular milieu of HD, especially in the setting of defective immunity. The poor correlation of numbers of RS cells with the quantity of EBV genome present further supports the lack of specificity of EBV for RS cells. For example, only 40% of lymphocyte-depleted HD cases positive for EBV by PCR were also positive by filter hybridization as compared with 67% of mixed cellularity HD cases (Table 1). In HD, the higher frequency and amount of EBV ge-

Table 3. *Presence of Epstein–Barr Virus (EBV) Genome and J_H Rearrangements in Hodgkin's Disease*

EBV status	No. cases	Monoclonal J_H rearrangements	Oligoclonal J_H rearrangements
Clonal episomal EBV	7	1*	1†
Nonclonal episomal EBV	2	1†	1‡
Linear EBV only	4	0	0
EBV genome detectable by PCR alone	17	0	0
EBV negative	20	0	0

* Nodular sclerosis
† Mixed cellularity
‡ Lymphocyte depletion
PCR, polymerase chain reaction

nome and clonal EBV, as well as the occasional occurrence of Ig heavy chain rearrangements in the presence of EBV genome may be due to immune defects in HD[30] that facilitate EBV reactivation within the reactive lymphoid cells and subsequent B-cell proliferation and clonal predominance. Weiss et al,[15] have also found Ig gene rearrangements in two cases of HD containing clonal EBV. Our recent finding of EBV-positive B-cell oligoclonal lymphoproliferative disease in SCID mice engrafted with peripheral blood leukocytes containing an estimated one to three EBV-infected B-cell/10^6 B cells[31,32] is in keeping with the view that latent EBV within B cell(s) of a lymph node can reactivate and produce a monoclonal or oligoclonal lymphoproliferative process such as we have observed in HD.

The presence of EBV in the reactive cells in HD, in addition to RS cells, was recently shown in studies using colorimetric ISH.[33,34] These data contradict the idea of "exclusive" localization of EBV genome to RS cells claimed by some investigators using isotopic ISH[16,17,19] and support our conclusion that EBV is not specific to RS cells in HD. It is possible that the high background signal and crude localization inherent in isotopic ISH may mask the signal from low-level EBV genome in the reactive lymphoid cells of HD.

The issue remains as to whether the EBV within RS cells is a primary or a secondary phenomenon. Given that RS cells lack EBV receptors,[35] it is likely that the virus is derived from the surrounding infected lymphoid cells through alternate routes, such as diffusion, phagocytosis, cell fusion, or membrane leakage. For example, Kadin et al[36] have shown that Ig can leak through the cell membrane of RS cells. Once inside the RS cell, EBV replication could proceed and result in expression of viral antigens, as has been demonstrated using fusigenic Sendai virus to infect artificially T cells with EBV.[37] Also EBV-carrying T-cell lymphoproliferative disorder has been described.[38] The concept that localization of EBV genome within cells is not synonymous with specificity is well illustrated in a recent report by Randhawa et al[39] describing the detection of EBV by ISH within hepatocytes and adrenal cortical cells in liver transplant recipients.

An unexpected finding was the presence of clonal EBV in hyperplastic lymph nodes. The presence of clonal EBV has also been reported in reactive lymph nodes from organ transplant recipients.[25,40] These findings bring into question the use of EBV clonality as a criterion to define a neoplastic cellular population, particularly when replicating virus is present. Further study of the pathobiology of EBV reactivation in immunosuppressed hosts is needed to clarify the clinical significance of EBV clonality and the role of EBV in HD.

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