

Short Communication

Demonstration of a Unique Epstein–Barr Virus-Positive Cellular Clone in Metachronous Multiple Localizations of Hodgkin's Disease

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The recent detection of clonal episomes of Epstein–Barr virus (EBV) in a significant proportion of Hodgkin's disease (HD) cases has suggested a re-evaluation of the possible pathogenetic role of EBV in the development of the disease. Here we report that in two EBV-positive HD, arisen in human immunodeficiency virus-1-infected drug users, a unique episomal EBV genome was detected in multiple metachronous HD lesions of each patient. These findings demonstrated that the same EBV-positive cellular clone was present in multiple localizations of HD as well as in specimens taken at different times. Combined in situ hybridization and immunohistochemical analyses evidenced EBV genome and EBV-encoded latent membrane protein-1 on Reed–Sternberg cells. Therefore, the data strongly support the possibility of a causal role for EBV in the pathogenesis of HD. (Am J Pathol 1993, 142:33–38)

Although its malignant nature is widely accepted, Hodgkin's disease (HD) displays features very similar to those of infectious diseases. In particular, the observation of numerous clusters of HD^{1,2} as well as epidemiological characteristics^{3,4} have suggested an infectious, possibly viral, pathogenesis. A possible pathogenetic role of Epstein–Barr virus (EBV)

was originally suggested by the observation that persons with a history of infectious mononucleosis⁵ or with elevated antibody titers toward EBV antigens^{6,7} have an increased risk of HD. Renewed interest for such association has arisen from prospective serological studies that revealed that elevated anti-EBV antibody levels are present before the diagnosis of the disease.⁸ In addition, clonal episomal EBV genomes were recently detected in a significant fraction (17% to 41%) of HD cases.^{9–11} These last findings indicate that a clonal cell population harboring EBV is present in pathological tissues from a portion of HD patients. Moreover, *in situ* hybridization (ISH) analyses revealed that EBV is prevalently found in Reed–Sternberg cells (RSCs) and their variants.^{10,12} Further studies, employing polymerase chain reaction (PCR) or analysis of EBV latent membrane protein-1 (LMP) expression, have demonstrated a higher association of EBV with mixed cellularity^{13–15} and cellular phase nodular sclerosis¹⁵ with respect to the other subtypes. Taken together, these results indicate that, in at least a group of HD cases, EBV may play a pathogenetic role in the development of the disease.

With the aim to characterize better the relationship between EBV and HD, we determined whether a unique EBV-positive cellular population may be constantly present in anatomically separated metachronous localizations of the disease. Here we report that in two EBV-positive HD cases, arisen in human immunodeficiency virus-1 (HIV-1)-infected drug

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users, each of the pathological specimens obtained from multiple localizations and at different times displayed the presence of the same viral clone.

Materials and Methods

Tissue Samples

Tissue samples for histopathological and immunohistological studies were obtained from both the patients by biopsy procedures performed for diagnostic purposes and from one patient also during the autopsy study. Frozen tissues from the following specimens were analyzed for presence of EBV genome: a left cervical lymph node with HD and another involved left cervical lymph node, removed 6 months later, from the first patient; a left cervical lymph node with a reactive histological pattern and a right cervical lymph node with HD, biopsied 5 months later, from the second patient. Autopsy specimens from the latter patient were further analyzed; they included two superficial and two abdominal lymph nodes from different sites, all involved by Hodgkin's lymphoma tissue, and a cerebellar tissue sample without neoplastic infiltration. All pathological specimens were classified according to the Rye modification of the Lukes and Butler classification.¹⁶

Immunohistological Analyses

Immunohistological analyses were performed on Bouin-fixed, paraffin-embedded tissue sections. The following monoclonal antibodies were used: leukocyte common antigen/LCA or CD45, BerH2/CD30, LeuM1/CD15, epithelial membrane antigen/EMA, vimentin, LN1/CDW75, LN2/CD74, L26/CD20, CD3, UCHL1/CD45RO, Leu22/CD43, MT1/CD43, and KP1/CD68. The immunoreactivity, the source of these commercially available antibodies, and the immunohistological technique used have been reported previously.¹⁷⁻¹⁹

Immunohistochemistry with Anti-LMP and anti-EBV Nuclear Antigen 2 Antibodies

Anti-LMP was available as a pool of four monoclonal antibodies (mAbs) (CS.1-4) (Dakopatts A/S, Glostrup, Denmark). Anti EBV nuclear antigen 2 (EBNA 2) was available as a single reagent (PE 2) reported to react with EBNA 2A and EBNA 2B antigens,²⁰ kindly provided by Dr. L. Young. Immunostaining was performed on Bouin-fixed, paraffin-embedded tissue sections by the alkaline phosphatase-antialkaline phosphatase method. Positive controls were

included in all runs and consisted of sections of LMP- and EBNA 2-expressing EBV-positive cell lines. Negative controls consisted of consecutive test sections in which the primary antibody was replaced with nonimmune serum of the same IgG subclass (Dakopatts).

EBV Genome Detection

In Situ Hybridization

The probe for EBV consisted of 1 µg/ml biotinylated EBV DNA prepared from the 3.1-kb *Bam*HI-W (internal repeat 1) fragment of the EBV genome (Enzo Diagnostics, Inc., New York, NY) in hybridization mixture as suggested by the supplier. ISH studies were performed on Bouin-fixed, paraffin-embedded tissue sections using a modified procedure based on previously described methods.²¹⁻²³ Cells were scored positive for viral DNA if they showed deposition of grains in excess. Controls for ISH assay consisted of slides containing both a negative control well of fixed Ramos cells and a positive control well of fixed B95.8 cells (Enzo Diagnostics, Inc.). An additional negative control consisted of omission of the biotinylated probe from the hybridization mixture.

Southern blot analysis

Genomic DNA was extracted according to conventional methods. Southern blotting, molecular hybridization, and autoradiography were performed as previously described.²⁴ The following EBV-specific probes were used: a 1.9-kb *Xho*I fragment corresponding to fragment *Eco*RI D adjacent to EBV right-terminal repeats and a 4.1-kb *Eco*RI fragment corresponding to an *Eco*RI I fragment adjacent to the EBV left-terminal repeats.²⁵

Results

Report of the Cases

The first patient (AM), a white, 21-year-old man, was an intravenous drug user with HIV-1 infection who presented in April 1986 with a history of weight loss, unexplained fever, night sweats, and persistent generalized lymphadenopathy (PGL). Histological examination of a lymph node of the left side of the neck revealed a massive involvement by HD, "mixed cellularity" subtype. Combination chemotherapy was administered, with partial response. In October 1986 a new biopsy of two lymph nodes from the left neck area revealed a focal involvement by HD in one sample, and a florid follicular hyperplasia compatible

with PGL in the other lymph node sample. The patient was lost to follow-up.

The second patient (VM), a white, 20-year-old man, was an intravenous drug user with HIV-1 infection who presented in December 1986 with a history of PGL associated with night sweats. Two left cervical lymph nodes were biopsied. Histological examination of both the samples revealed a reactive lymph node pattern compatible with PGL. In May 1987 histological examination of several small lymph nodes removed from the right neck area showed a total involvement by HD, "lymphocyte depletion" subtype. Combination chemotherapy was then administered, but in the following few months opportunistic infections and bacterial pneumonia developed. The patient died in November 1987. The autopsy study revealed the persistence of Hodgkin's lymphoma in the superficial and abdominal lymph nodes associated with spleen involvement.

Histopathological, Immunophenotypical and Virological Findings

Two lymph node specimens from the first patient were interpreted as involved by "mixed cellularity" HD (Figure 1A). Another lymph node, which was removed together with that subsequently biopsied, showed follicular hyperplasia alone. RSCs immunoreacted with LeuM1/CD15, BerH2/CD30, LN2/CD74, L26/CD20, and vimentin; LCA/CD45, EMA, and the other tested antigens were not expressed.

The initial lymph node specimens from the second patient displayed typical features of florid follicular hyperplasia compatible with PGL,¹⁸ and lymph node samples biopsied 5 months later showed a complete replacement by lymphoma tissue with rare classic RSCs in a fibrous lymphocyte-depleted background (Figure 1, B and C). In the involved lymph nodes obtained at autopsy, the histopathologic patterns repeatedly showed a diffuse effacement of normal

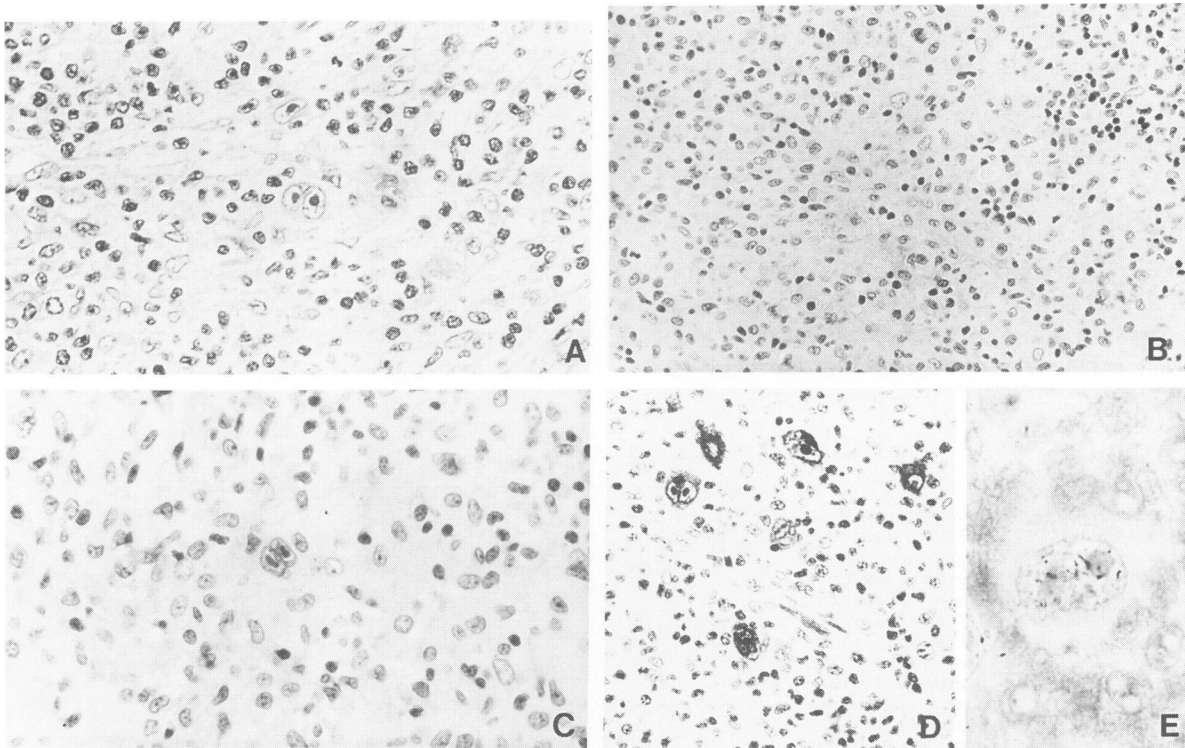


Figure 1. A: Left cervical lymph node from the first patient, showing "mixed cellularity" HD. The cellular infiltrate is relatively polymorphous with lymphocytes, histiocytes, plasma cells, a classic Reed-Sternberg cell (center), and a mononuclear variant (top center). (Hematoxylin and eosin; $\times 400$.) B: Right cervical lymph node from the second patient showing "lymphocyte depletion" HD. The normal structure is replaced by haphazardly arranged spindle cells and histiocytes, whereas lymphocytes are markedly reduced in number. An atypical Reed-Sternberg cell is seen (top center). (Hematoxylin and eosin, $\times 250$.) C: Same node as in B showing a Reed-Sternberg cell of classic type (center). (Hematoxylin and eosin, $\times 400$.) D: Same node as in A showing LMP staining within the cytoplasm of some Reed-Sternberg cells (Bouin-fixed, paraffin-embedded section, alkaline phosphatase-antialkaline phosphatase method, (Hematoxylin counterstain, $\times 400$)). E: Same node as in A showing that the ISH signal is present as dense grains over the nuclei of a Reed-Sternberg cell. (Biotinylated EBV probe, nuclear fast red counterstain, $\times 1000$ oil immersion.)

increased incidence of concurrent or subsequent EBV-positive non-Hodgkin's lymphoma but not with the development of HD.

The constant presence of a unique EBV-infected cellular clone, with morphological features of RSCs, in all different localizations of the disease suggests that such a cell population has a functional role in the pathological tissue and, therefore, that EBV has an etiopathogenetic role in EBV-positive HD cases.²⁹ Possibly, unusual virus-cell interactions or specific EBV mutants may confer on the infected cell peculiar antigenic properties able to elicit and sustain the reactive cellular components of the disease but to evade cytotoxic T cell recognition.³⁰ The immunohistochemical detection of LMP in HD-involved tissues is further evidence for an active role of EBV in the disease.^{13,31} The expression of this protein is of particular interest because in addition to promoting neoplastic transformation^{32,33} and inhibiting terminal differentiation,³⁴ LMP can serve as a target for T lymphocyte recognition.³⁵

In conclusion, the demonstration that the same EBV-infected clone is present in HD lesions from different anatomic sites and in metachronous localizations of the disease, in conjunction with the demonstration of EBV genome and EBV-encoded LMP expression in RSCs strongly supports a causal role for the virus in the pathogenesis of HD.

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