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Rapid Communication

Presence of Epstein–Barr Virus in Hodgkin's Disease Is Not Exclusive to Reed–Sternberg Cells

G. Khan,* P. J. Coates,* R. K. Gupta,† H. O. Kangro,‡ and G. Slavin*

From the Departments of Histopathology, Medical Oncology,† and Virology,‡ St. Bartholomew's Hospital Medical College, West Smithfield, London, United Kingdom*

Thirty-three cases of Hodgkin's disease (HD) have been studied for the presence of Epstein–Barr virus (EBV) using a novel nonisotopic* in situ *hybridization procedure, based on the detection of Epstein–Barr encoded RNAs with oligonucleotide probes. An intense and morphologically distinct nuclear staining, sparing the nucleolus was seen in a total of 12 cases (36%). In six of these cases, the signal was located to the Hodgkin and Reed–Sternberg cells (HR-S); in the other six positive cases, the signal was observed only in the non-neoplastic small lymphocytes. These lymphocytes were few in number and immunocytochemistry results were consistent with a B-cell phenotype. The presence of EBV in those cases characterized by nuclear staining of small lymphocytes was confirmed by the polymerase chain reaction (PCR) analysis. The authors report the detection of EBV in small lymphocytes in HD by* in situ *hybridization and discuss the implications of these findings in relation to the proposed etiologic association between EBV and HD. (Am J Pathol 1992, 140:757–762)

Hodgkin's disease (HD) is a unique malignant lymphoproliferative disorder in which the Reed–Sternberg cells, the characteristic neoplastic giant cells are dispersed among a predominant non-neoplastic inflammatory infiltrate. Although more than 150 years have elapsed since Thomas Hodgkin described the disease that later bore his name, both the etiology and the origin of the Reed–Sternberg cells remains an enigma.[1–3] Hodgkin's disease occurs throughout the world and a bimodal age incidence is well recognized in most countries, with an initial peak occurring in young adults in their late twenties and a second peak occurring in older persons in their seventh decade.[4,5] MacMahon[4] noted that in the elderly the disease had many of the characteristics of neoplasia and showed several epidemiologic features in common with other lymphomas. On the other hand, several features of the disease in young adults were more suggestive of an infective process than of a neoplasm.

An infective etiology of HD has long been suspected[4–7] and Epstein–Barr virus (EBV) has consistently been implicated as playing a role. Raised antibody titers to EBV viral capsid antigen have been demonstrated both pre- and postonset of the disease[8–10] and persons with prior infectious mononucleosis have been shown to be at increased risk of HD.[11] Furthermore, viral DNA has been detected in 20–44% of HD cases by Southern blotting[12–14] and in up to 79% using polymerase chain reaction (PCR) analysis.[2,15,16] However, in studies that involved the localization of the virus to the malignant cells by *in situ* hybridization (ISH) techniques, a much lower incidence has been reported.[2,17,18] Two possible explanations for the apparent discrepancies exist: 1) EBV may be present in the malignant cells in all cases that are PCR positive, but due to the lack of sensitivity of ISH procedures, the virus is not detected in all cases; 2) EBV may be present in cells other than HR-S in some cases, which is detected by PCR analysis but not by ISH methods, due to the small number of positive cells. This would mean that EBV plays a role in only a proportion of the cases in which viral DNA can be detected by PCR analysis.

Clarification of the two possibilities is essential in determining the percentage of HD cases that may be EBV associated. In the past, the lack of sensitivity of the ISH

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Address reprint requests to Dr. G. Khan, Department of Histopathology, St. Bartholomew's Hospital, West Smithfield, London EC1A 7BE, UK.

procedures has prevented this. We have developed a sensitive ISH technique based on the detection of Epstein–Barr encoded RNAs (EBERs) using digoxigenin-labelled oligonucleotide probes.[19] Using this technique, we have examined 33 cases of HD for the presence of EBV. A morphologically distinct hybridization signal was seen in 12 cases (36%), but localized to Hodgkin and Reed–Sternberg cells (HR-S) in only six cases. In the other six cases, the signal was located in small lymphocytes, which were generally scarce and dispersed throughout the section.

Methods and Materials

Tissues

Formalin-fixed paraffin-embedded blocks from 33 cases of HD (24 males, 9 females, age range: 15–68 years) were retrieved from the departmental files. These consisted of nodular sclerosing (n = 26), mixed cellularity (n = 3), lymphocyte predominant (n = 2), lymphocyte depleted (n = 2). Formalin-fixed paraffin-embedded B95-8 cells (EBV producer cell line), EBV-transformed lymphoblastoid cell line, and a case of HD known to be EBV-positive by NISH[19] were used as positive controls and sections of Ramos (EBV-negative cell line) and normal tonsil as negative controls.

In Situ *Hybridization*

In situ hybridization was carried out on a 5 μm paraffin sections as described in detail,[19,20] using a combination of EBER-1 and EBER-2 oligonucleotide probes at a final concentration of 100 ng/ml. For oligonucleotide EBER probes, two sequences of 30 nucleotides each, corresponding to positions 90-119 and 82-111 of EBER-1 and EBER-2, respectively, were selected from their published sequences[21] and end-labelled with digoxigenin 11-dUTP using terminal transferase as described.[19] A 30mer oligonucleotide, unrelated to EBER sequence, but having similar G-C content (60%) was also synthesized and labelled as for EBER probes. This was used as a negative control for each case of HD, at a concentration of 500 ng/ml. All tissue sections were digested with proteinase K at a concentration of 50 μg/ml before hybridization. The hybridization signal was detected by a three-layer ABC-peroxidase technique (Vector laboratories, Peterborough, UK).

Double Labeling

Those cases showing hybridization for EBV in small lymphocytes, the authenticity of the signal was confirmed by RNase treatment of sections before hybridization.[20] To determine the phenotype of the EBV-infected lymphocytes, a combination of immunocytochemical analyses followed by ISH was performed on these cases. Immunocytochemical analysis was carried out using monoclonal antibodies L26 (B-cell marker) and UCHL-1 (T-cell marker) (Dako Ltd, High Wycombe, UK) and ABC-peroxidase/diaminobenzidine tetrahydrochloride (DAB) detection system.[22] After the immunostaining, the slides were dehydrated in ethanol and air-dried. The sections were subjected to 50 μg/ml of proteinase K digestion and ISH procedure followed as described. A single-step detection system using anti-digoxin alkaline phosphatase and NBT-BCIP substrate was used.

DNA Amplification

As an independent confirmatory test for the presence of EBV in those cases that showed positive hybridization in small lymphocytes, the sensitive technique of PCR was performed. The DNA was extracted from paraffin-embedded material from five of the six cases using 0.5 mg/ml of proteinase K incubated at 37°C for 5 days.[23] DNA obtained from a human EBV transformed cell line (Akiba, ICRF, London, UK) was used as a positive control. The EBV primers were selected from the unique sequence coding for BZLF, flanking a region of 286-base pair fragment: 1) 5′ ACA GTA GAA TTG TCT CCA GG 3′; and 2) 5′ GAC CAA GCT ACC AGA GTC TA 3′.

PCR final reaction volume of 100 μl consisted of 0.1 μg–1 μg of extracted DNA, 0.5 μmol/l of each oligonucleotide primer, 2.5 U Taq (Promega, Southampton, UK), 1× Taq buffer (Promega), and 0.2 mmol/l nucleotide triphosphates (dNTP). Reactions were subjected to 40 amplification cycles consisting of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C and extension for 1 minute at 72°C. The amplified products (8 μl) were analyzed by electrophoresis on 2.5% agarose gel and stained with ethidium bromide.

Results

In Situ *Hybridization*

Thirty-three cases of HD were examined for the presence of EBV by ISH techniques using digoxigenin-labeled oligonucleotide probes that were targeted against EBERs. A strong hybridization signal was seen in EBV-positive controls (B95-8, lymphoblastoid cell lines and EBV-positive case of HD). Sections of normal tonsil and EBV-negative Ramos cells were consistently negative.

Of the 33 cases of HD examined, a strong nuclear

hybridization signal was seen in 12 cases (36%). However, the hybridization signal was seen in HR-S cells in only six of these cases (18%) (Figure 1a); four of these cases were nodular sclerosing subtype and two were mixed cellularity. The hybridization signal was intense with a diffuse pattern of staining confined to the nucleus (Figure 1b). In the remaining six cases, the hybridization signal was seen in non-neoplastic small lymphocytes only. These lymphocytes were few in number, less than 12 cells in an entire section (Figure 2). No hybridization signal was seen with the 30mer (negative control probe), in any of the 33 cases that were examined.

To confirm the specificity of the EBV-positive signal that was seen in small lymphocytes, the sections were subjected to RNase treatment before hybridization. This treatment completely abolished the signal.

Double Labeling

A combination of immunocytochemical analysis and ISH was carried out to determine the phenotype of the EBV-positive lymphocytes. In two of the six cases analyzed, EBV-positive lymphocytes were also labeled with the monoclonal antibody L26, suggesting a B-cell phenotype (Figure 3). The results of these studies were difficult to interpret, partly due to the scarcity of the EBV-positive cells. In addition, many EBV-positive cells were seen to be surrounded by L26-positive lymphocytes, and it was difficult to determine whether these cells were themselves L26-positive or whether the staining was due to the surrounding lymphocytes. No EBV-positive lymphocytes were identified as being positive for UCHL1.

DNA Amplification

As an independent confirmatory test for the presence of EBV in small lymphocytes, five of the six cases were available for PCR analysis. A specific amplification product of 286-base pair was visualized after ethidium-bromide staining in all five cases, which indicated the presence of EBV. No amplification was seen in the negative control (Figure 4).

Discussion

EBV is a lymphotropic herpesvirus, well recognized for its oncogenic properties.[24,25] This ubiquitous virus has

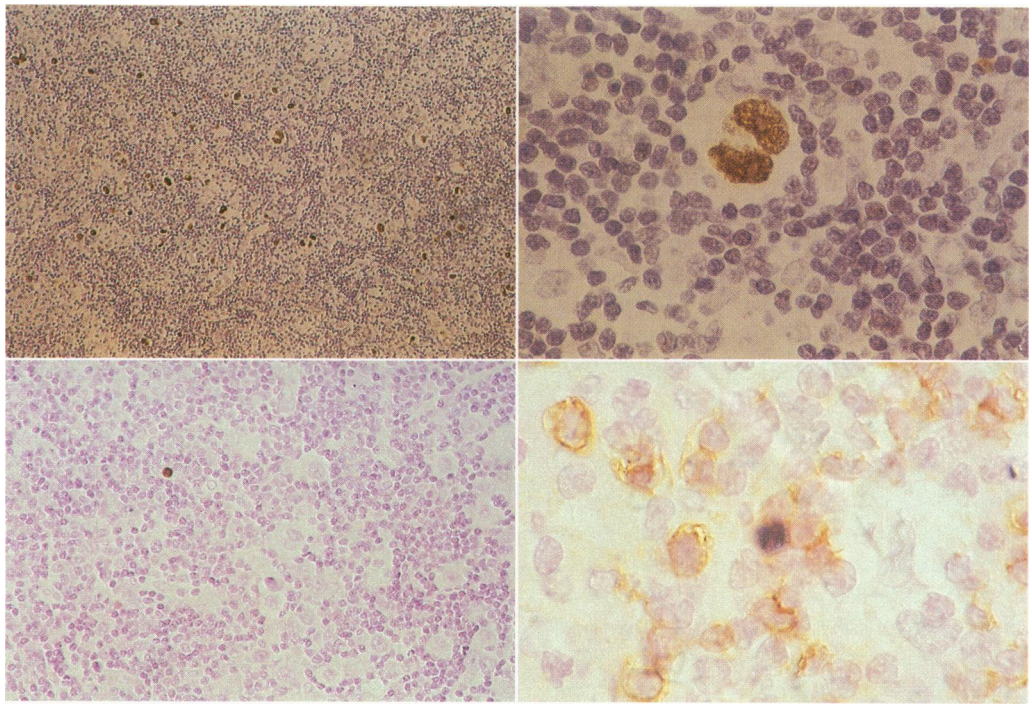

Figure 1. *A case of HD with EBV in HR-S cells.* **A** *(top left): An intense nuclear hybridization signal localized to HR-S cells. Almost all of the morphologically malignant cells are positive for EBV.* **B** *(top right); High power of* **(A)** *showing a classical Reed–Sternberg cell with nuclear hybridization with sparing of the nucleolus.*
Figure 2 *(bottom left). A case of HD with EBV in small lymphocytes. Hybridization is localized to a small lymphocyte. The malignant cells are clearly negative.*
Figure 3 *(bottom right). Double-staining in a case of HD with EBV in small lymphocytes. CD20 (B-cell marker) is detected using ABC-peroxidase method (brown) and EBV is detected by ISH using direct alkaline phosphatase system (blue).*

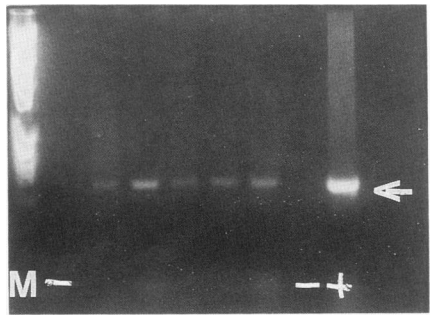

Figure 4. *PCR analysis on DNA extracted from HD cases with EBV in small lymphocytes. Positive (+) and negative (−) controls are indicated together with molecular weight marker M (1Kb ladder, Gibco BRL) and the specific amplification product of 286 base pair (arrow).*

been implicated in the etiology of a wide range of benign and malignant human diseases (reviewed in[26]). In recent years, substantial evidence has accumulated supporting a role for EBV in the pathogenesis of HD. The epidemiologic and histologic features of HD have long indicated a possible infective cause.[4–7] Furthermore, not only has EBV DNA been localized in HR-S cells[12,17,18] but the virus has been shown to be active in these cells and not merely a silent passenger.[27–29] In the present study, we show the presence of EBV genome in HR-S cells in 18% of HD cases using a sensitive nonisotopic ISH technique. A strong hybridization signal was seen in almost all of the HR-S cells in these cases. It is conceivable that a small number of EBER-positive small lymphocytes were present in these cases but were overlooked due to the predominance of EBER-positive HR-S cells. These and other results[12,17,18] suggest that infection with EBV may be a primary event, with the virus subsequently being passed from cell to cell during growth of the malignant clone. In contrast, if infection of HR-S cells had occurred during later stages of the disease process, it is unlikely that all cells would have become infected. EBV therefore, may be directly involved in the etiology of some cases of HD and is unlikely to be a coincidental post-transformational infection.[30]

The extent to which EBV is associated with HD is unclear. By PCR analysis, a high incidence of EBV genomes have been found in HD,[2,15–16,30] whereas ISH studies have consistently shown a much lower prevalence for the presence of EBV genomes in HR-S cells.[2,17,18] Such discrepancies have been attributed to the lack of sensitivity of ISH procedures and it has been proposed that the presence of EBV genome in HD is restricted or exclusive to HR-S cells.[12,15,31] Furthermore, some investigators have assumed a strong association between EBV and HD based on PCR analysis, without cellular localization of the virus.[16,32] However, since 90% of the general population is seropositive for EBV and the virus is known to remain latent in lymphocytes,[4] it is equally possible that the high prevalence of EBV seen in HD by PCR analysis is due to the detection of latent or reactive EBV and not associated with HD. To this end, we examined 33 cases of HD for the presence of EBV by a sensitive ISH technique based on the detection of EBERs[33] using digoxigenin-labeled oligonucleotide probes.[19]

The sensitivity of this method is apparent when one considers the high copy number of EBERs in latently EBV-infected cells (10^7 per cell).[21] Using this method, EBV has been detected in 100% of AIDS-related primary central nervous system lymphomas,[34] which is in contrast to previous studies reporting a much lower prevalence. The increased sensitivity of this method has enabled us to locate the virus to HR-S cells in 18% of the cases, consistent with other reports.[17,18] In a further 18%, the virus was located in small lymphocytes only and was absent from the neoplastic cells. These lymphocytes were scarce, but due to the strong nuclear hybridization signal, characteristic of EBER distribution,[35] they were easily identified. Tissue was available from five of the six lymphocyte positive cases for PCR analysis. All five cases were found to be EBV positive, confirming that these tissues contained EBV.

The presence of EBV in small lymphocytes in HD has important implications for the association of EBV in the etiology of HD. However, it is by no means surprising, since 90% of the general population is EBV seropositive and an estimated 1 in 10^6 peripheral blood lymphocytes are infected with the virus in normal carriers.[36] The EBV-positive lymphocytes in our six cases were scarce and may represent the carrier state. In support of this notion is our finding of EBV in small lymphocytes in some cases of T-cell malignancies which we are presently studying. Alternatively, the presence of EBV in these lymphocytes may represent EBV reactivation, possibly as a consequence of immunosuppression, which is well recognized in HD patients. In two of the cases with EBV in small lymphocytes, the infected cells were shown to be B-lymphocytes, the cells known to express the EBV receptor.[37] Our finding of the presence of EBV in non-neoplastic small lymphocytes in HD is supported by reports that show the presence of oligoclonal EBV in some cases of HD,[30,38,39] which suggests that more than one population of cells contain EBV.

In conclusion, the demonstration of EBV in HD tissue by PCR analysis, does not by itself indicate a role for the virus in the disease process. We have unequivocally shown that in a significant proportion of HD cases, EBV is present in the small lymphocytes exclusively and therefore unlikely to be directly involved in the pathogenesis of the disease. However in some cases, the virus is located to HR-S cells and may be involved in the etiology of these cases of HD. A sensitive ISH method is needed to deter-

mine the cellular localization of the virus before one can further investigate the etiologic role of EBV in HD.

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