

Detection of Epstein–Barr genome in the lymph nodes of Hodgkin's disease

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Summary: We have used the enzymatic *in situ* hybridization method to investigate the presence of Epstein–Barr virus (EBV) genome in lymph node tissues from patients with Hodgkin's disease. Also, 11 patients with persistent human immuno-deficiency virus-associated generalized lymphadenopathy as well as seven autopsy cases with no Hodgkin's disease, formed part of these studies.

EBV DNA-positive reaction was demonstrated in Reed–Sternberg cells and variants in seven of 16 cases and in the small accompanying lymphocyte cell population in 14 of the 16 cases. It was also found in eight of the 11 cases with persistent generalized lymphadenopathy but in none of the lymph nodes from negative selective autopsy cases.

Our results indicate that the colorimetric *in situ* hybridization technique is useful in EBV nucleic acid detection and cell-type localization in Hodgkin's disease. Additionally, the detection of EBV genome, not only in the diagnostic cells but in the small lymphocyte cell components, could provide new insights into the potential role of this agent in the pathogenesis of Hodgkin's disease.

Introduction

It is known that Epstein–Barr virus (EBV) is clearly associated with endemic Burkitt's lymphoma and lymphomas developed in immuno-suppressed patients, as revealed by the presence of EBV genome in the malignant clone of proliferating cells.^{1–4} By using an EBV terminal-region DNA probe, it has been recently demonstrated that EBV DNA can be detected in approximately 20% of patients with Hodgkin's disease, and that these viral genomes are monoclonal in origin.^{5–7} More recently, using polymerase chain reaction (PCR), EBV DNA sequences were detected in 58% of cases of Hodgkin's disease.⁸ Furthermore, in the studies carried out to date using isotopic *in situ* hybridization methods, the EBV genome has only been encountered in the diagnostic RS cells and variants.^{5,8,9}

In this paper, we have used the enzymatic *in situ* hybridization method to study the presence and cell localization of EBV genome in a group of patients with Hodgkin's disease. We identified EBV DNA in the diagnostic cells of seven out of 16 cases of Hodgkin's disease lymph nodes and, more importantly in our opinion, found this DNA in lymphoid cells not belonging to the diagnostic cell population

in 14 out of 16 Hodgkin's disease lymph nodes. The presence of EBV-infected cells in cases of Hodgkin's disease and its relationship with malignancy is discussed.

Materials and methods

Sixteen cases of Hodgkin's disease (nine males and seven females, aged 12–65 years; mean 29), were selected from the files of the Pathology Department at the Hospital Ramon y Cajal, based on the existence of serum samples obtained at the time of lymph nodes biopsies. All patients showed positive IgG anti-Epstein–Barr nuclear antigen (anti-EBNA) antibodies titres. The 16 Hodgkin's disease patients studied were classified as follows: two lymphocyte predominant, three mixed cellularity, 10 nodular sclerosing and one lymphoid depletion.

Lymph node biopsy specimens obtained from the above patients were fixed in 10% buffered formaldehyde. Routine histopathology (H&E, PAS, Giemsa and reticulin) was performed on paraffin-embedded tissues. Hybridization was done as described elsewhere.¹⁰ Briefly, sections were covered with 20 µl of the hybridization cocktail containing biotinylated EBV DNA probe (Bioprobe, Enzo Diagnostics, Inc., New York, USA). The probe used was the Bam HI 'V' (internal repeat 1) region of the EBV genome cloned into pBR322¹¹ containing a 3.1 kb sequence which is reiterated 11–14 times in the EBV genome. The

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reaction was visualized after incubation with avidin–biotin–peroxidase complex (Enzo) with the use of hydrogen peroxide and aminoethyl carbazole (Sigma). Controls included the omission of the specific viral probe and the replacement of the EBV probe by biotinylated cytomegalovirus DNA (Enzo). Also, an oral hairy leukoplakia lesion from a human immunodeficiency virus (HIV) positive patient as well as the EBV-infected cell line B95-8 were used as positive controls. In addition, several normal lymph nodes derived from different sites at autopsy were obtained from seven patients without Hodgkin's disease and without clinical, serological and histological evidence suggestive of EBV infection. Finally, lymph node tissues from 11 patients with persistent HIV-associated generalized lymphadenopathy, which are part of a previously reported work,¹² were also used (employing the same 'in situ' technology) to test the role of immunosuppression in our results.

Results

An EBV-positive reaction was observed in the nucleus of the Reed–Sternberg cells with a localization, generally, around the nucleoli as well as the nuclear membrane, and exhibiting a round and homogeneous pattern. Hybridization with the EBV probe also showed positive signals in a small proportion of the accompanying non-malignant lymphoid cell population, meanwhile other lymph node cells appeared negative. The majority of Reed–Sternberg cells and variants as well as the non-neoplastic lymphoid cells showed a single positive hybridization site, being few in number. The slides could be interpreted best with high power magnification.

The EBV genome was detected in Reed–Sternberg cells and variants as well as in non-malignant lymphoid cells in seven cases: five cases were nodular sclerosis, one case was mixed cellularity and the last corresponded to lymphocyte predominance (Table I) (Figure 1). Seven other cases, in which EBV DNA was not found in the

Table I Detection of EBV genome in Hodgkin's disease

Histological type	EBV DNA	
	Reed–Sternberg cells	Accompanying cells
Nodular sclerosis	5/10 cases	8/10 cases
Mixed cellularity	1/3 cases	3/3 cases
Lymphocyte predominant	1/2 cases	2/2 cases
Lymphocyte depleted	0/1 case	1/1 case

diagnostic cells, presented positive hybridization in diffuse scattered non-malignant lymphoid cells (Figure 2). In the other two Hodgkin's disease cases, no hybridized EBV DNA-positive reaction was encountered.

None of the lymph node specimens without Hodgkin's disease and without clinical, serological and histological evidence of EBV infection showed EBV DNA-positive signals. Contrarily, between 20 and 30% of the B95-8 cells and approximately 60% of the keratinocytes cells from the oral hairy leukoplakia lesion, showed marked nuclear positivity. Also, eight of the 11 cases with persistent generalized lymphadenopathy showed EBV DNA-positive reaction in scattered lymph node lymphocyte cells.¹²

Discussion

In serological, epidemiological, immunohistochemical and molecular genetic studies, several

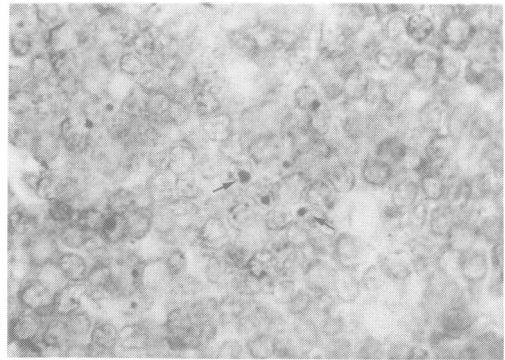


Figure 1 *In situ* hybridization with biotinylated EBV-DNA probe shows positivity in one Reed–Sternberg cell (arrow) as well as in some scattered small lymphoid cells (arrow) ($\times 1000$).

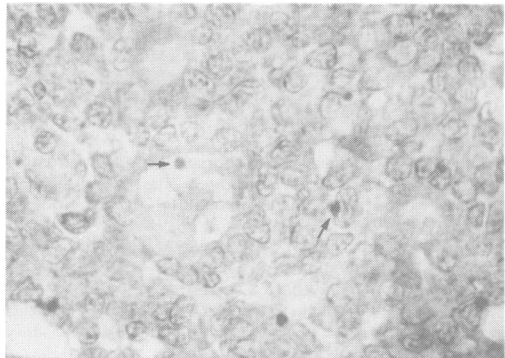


Figure 2 Positive hybridization only in the accompanying non-malignant small lymphocytes (arrows) ($\times 1000$).

groups of investigators have called attention to the possible relationship between EBV and Hodgkin's disease.^{5-7,9,13-15} A recent report indicates that using the PCR method, EBV genome can be detected in lymph node tissues from approximately 58% of patients.⁸ In addition, the application of isotopic *in situ* hybridization methods has shown that EBV nucleic acids were always exclusively confined to the nucleus of Reed-Sternberg cells and variants.^{5,8,9}

Using a colorimetric *in situ* hybridization method, which has been proved to be useful to detect EBV DNA,¹⁶ we were able to demonstrate the presence of this viral genome in the Reed-Sternberg cells and variants, in approximately 45% of our cases of Hodgkin's disease. Nevertheless, the most surprising observation was not only the high frequency of cases in which EBV DNA was found in the diagnostic cells, but the demonstration, in close to 85% of our cases, of such viral genome also localized in the nucleus of some accompanying small lymphocytes.

Our results lead us to believe that the application of colorimetric methods for *in situ* detection of hybridized EBV DNA, allows better morphological identification and cell localization of these nucleic acids. It is possible that the background detected by isotopic *in situ* hybridization, represented by scattered black grains, may not be discernible in some cases due to the small number

of viral copies. Furthermore, the positive-stained reaction and the lack of background obtained by our methods also permits a precise detection and cell-type identification of EBV DNA, including those very small nuclear positive signals.

The detection of EBV DNA in the accompanying lymphoid cells in addition to the Reed-Sternberg cells as well as in only half of the cases in the reactive cells, suggests the hypothesis that the immunodeficiency accompanying Hodgkin's disease might favour a latent EBV reactivation with expansion of lymphoid clones harbouring EBV^{7,17} with the presence of EBV within Reed-Sternberg cells being a non-specific phenomenon. The fact that we have previously found positive EBV signals in the lymph nodes of approximately 75% of immunosuppressed patients with persistent generalized lymphadenopathy¹² might support this hypothesis. In this regard and using a colorimetric *in situ* hybridization method the presence of EBV in the reactive cells in Hodgkin's disease has also been recently demonstrated.^{18,19} Furthermore, and with the PCR technique, Mashih *et al.*²⁰ have recently found a comparable frequency of EBV genome in both hyperplastic lymph nodes and tissues involved by Hodgkin's disease. These findings add support to the hypothesis that EBV infection could be directly related to the pathogenesis of Hodgkin's disease, at least in some cases.

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