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Direct Detection of Epstein-Barr Virus DNA from a Single Reed-Sternberg Cell of Hodgkin's Disease by Polymerase Chain Reaction

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Eleven cases of Hodgkin's disease (HD) were examined for the presence of the Epstein-Barr virus (EBV) genome, using the polymerase chain reaction (PCR) to detect EBV DNA in whole paraffin-embedded tissue specimens and in single cells picked out from the specimens with a micromanipulator. The EBV genome was detected in 5 of the 11 cases by conventional PCR. Single cell PCR demonstrated the EBV genome in Reed-Sternberg cells from all the EBV-positive cases, but not from any of the EBV-negative cases. Background lymphocytes and lysozyme-positive histiocytes from EBV-positive cases did not contain the EBV genome. These results indicate an etiological association of EBV with some cases of HD.

Key words: PCR – Epstein-Barr virus — Hodgkin's disease — Micromanipulator

The Epstein-Barr virus (EBV) has been clearly associated with Hodgkin's disease (HD)[1-5] and DNA filter hybridization studies have demonstrated the presence of EBV DNA in 17% to 44% of HD lesions.[1, 3, 5] However, this type of analysis may not be sensitive enough to detect EBV DNA in some HD cases, since Reed-Sternberg (RS) cells and their mononuclear variants are often scarce. The high sensitivity of the polymerase chain reaction (PCR) increased the proportion of detected EBV-positive HD cases.[4] PCR of DNA extracted from tissues does not identify the cellular source of EBV DNA and possibly detects EBV DNA from non-tumor cells in some cases. An *in situ* hybridization technique overcame this problem and revealed the exclusive localization of EBV DNA in RS cells.[2, 4, 5] However this technique is less sensitive than the PCR method and using it, we might not be able to get positive signals from cells infected with only a few copies of EBV.

In this study, we used a micromanipulator to collect microscopically identified RS cells from paraffin-embedded sections on a glass slide, and we applied PCR to these cells. This procedure could detect a few copies of EBV gene fragments in a morphologically and immunologically identified diagnostic RS cell.

Eleven HD biopsy specimens were fixed in B5 solution for 3 h and embedded in paraffin wax. They had been stored in a cold room (4°C) until they were used. The histological subtypes of 6 of the 11 cases studied were classified as mixed cellularity, 2 as nodular sclerosis, 2 as lymphocyte predominance and 1 as lymphocyte depleted (Table I). In none of the cases was there a history of previous or concomitant EBV infection; serological examinations were not available. Immunogenotypic analysis of the cases by Southern blot hybridization revealed no conspicuous immunoglobulin heavy chain or T cell receptor β chain rearrangement. To detect EBV DNA, conventional PCR amplification was applied to total DNA extracted from 9-μm-thick paraffin sections by a previously described method.[6] The conventional PCR method revealed EBV DNA in 5 of the 11 specimens (Table I). For PCR of DNA obtained from microscopically identified cells, 6-μm-thick sections were used; these were stained with hematoxylin only or hematoxylin-eosin (HE). Immunohistochemical staining with a monoclonal antibody Ber-H2 (Dakopatts, diluted 1:10) and anti-lysozyme (Dakopatts, diluted 1:500) was also employed using an avidin-biotin-complex peroxidase method. Ber-H2 was raised against an RS cell line and reacted with Ki-1 antigen (CD30), which is well known to be a good marker of RS cells,[7] in paraffin sections. Stained sections mounted on slides were observed microscopically, and RS cells, histiocytes and lymphocytes were identified and photographed at high, middle, and low magnification. After the coverglasses were removed, it was difficult to observe the histology and to find the target cell, but the low and middle magnification photographs helped us to reach it. The target cell, on a demounted dried slide, was picked out with a micromanipulator without touching the other surrounding cells. The cell was drawn up by a micropipette; this was observed microscopically to determine whether the procedure was successful. The drawn-up cell was then

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Table I. Patients' Data and Detection of EBV DNA Sequences by PCR in Whole Paraffin-embedded Tissue Sections

Case No.	Subtype[a]	Sex	Age (year)	Prognosis (month)[b]		EBV genome
1	NS	F	41	Alive with tumor	(44)	−
2	MC	M	72	Alive without tumor	(32)	−
3	MC	M	73	Alive without tumor	(30)	+
4	LP	M	59	Alive without tumor	(30)	+
5	NS	M	19	Alive without tumor	(28)	−
6	LP	M	13	Alive without tumor	(28)	−
7	MC	M	58	Died of tumor	(12)	+
8	LD	F	71	Died	(14)	−
9	MC	M	26	Alive with tumor	(19)	+
10	MC	M	38	Alive without tumor	(9)	−
11	MC	F	63	Alive without tumor	(6)	+

a) MC, NS, LP and LD represent mixed cellularity, nodular sclerosis, lymphocyte predominance, and lymphocyte depleted, respectively.
b) Number of months from diagnosis to the present, or to death.

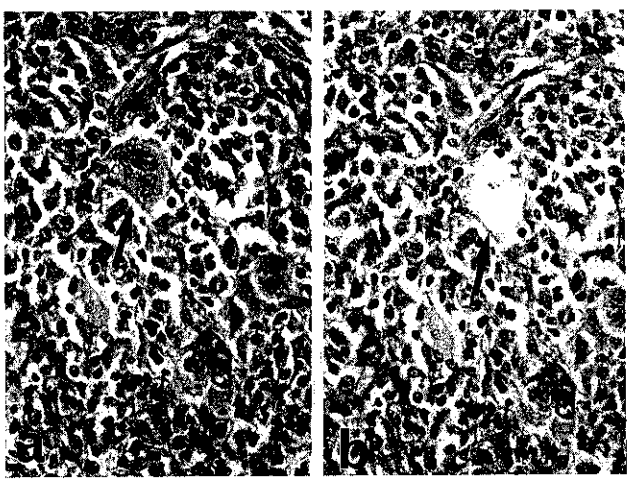

Fig. 1. Picking out of an RS cell from paraffin-embedded, HE-stained section; (a) shows the RS cell before picking out (arrow), (b) shows the section after picking out of the RS cell (arrow).

transferred to a 600-μl microtube for PCR. The portion of the specimen from which the target cell had been picked out was photographed and the photographs taken before and after this procedure were compared to exclude the possibility of contamination. Figure 1 shows an RS cell before and after picking out. One each of HE-stained RS cells, Ki-1-positive RS cells, lysozyme-negative RS cells, lysozyme-positive plump histiocytes, hematoxylin-stained RS cells and lymphocytes was picked out and used for PCR analysis. The cells were digested with 1 mg/ml proteinase K at 37°C for 2 h in 30 μl of digestion buffer; 10 m*M* Tris-HCl (pH 7.6), 0.45% NP-40 and 0.45% Tween 20. After inactivation of proteinase K by boiling at 97°C, the solution was made up to 100 μl of PCR mixture; 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.3), 2.5 m*M* $MgCl_2$, 0.02% porcine gelatin (Sigma, USA), 200 n*M* dNTPs, 200 μM primer pair, and 2.5 unit of Taq polymerase (Perkin-Elmer Cetus, USA). Oligonucleotides corresponding to specific sequences within the *Bam*HI W fragment[8] (TC60=CCAGAGGTAAGTGGACCT, TC61=GACCGGTGCCTTCTTAGG) and within the *Eco*RI B fragment[6] (primer 1 = GTGTGCGTCGTGCCGGGGCAGCCAC, primer 2 =ACCTGGGAGGGCCATCGCAAGCTCC) were used as primer pairs. On setting up the amplification reactions, all the procedures were done in a clean bench which was used only for this experiment. The annealing temperature for PCR amplification was 51°C for primers TC60 and TC61, and 59°C for primers primer 1 and primer 2. Forty-seven PCR cycles were performed for DNA from a single cell and 35 cycles were performed for 1 μg of DNA from the whole tissue specimen (conventional PCR). One μg of DNA is equivalent to the DNA of 1.7×10^5 human cells.[9] PCR with TC60 and TC61 has detected one EBV-infected cell among 1.0×10^5 non-infected cells.[8] Single cell PCR was carried out with primers for the *Bam*HI W fragment because of the short length of its target sequence (125 bp).[8] The shorter the target length is, the better sensitivity can be anticipated, because DNA in paraffin blocks is expected to be degraded into fragments. Besides, the *Bam*HI W fragment is apt to be amplified, because it is repeated 10 times in the EBV genome.

Table II. Summary of Single Cell PCR of EBV DNA from RS and Other Cells in HD

Case No.	Hematoxylin-stained		HE-stained	Immunohistochemically stained		
	RS cells[a]	Lymphocytes[a]	RS cell[b]	Ki-1$^+$ RS cell[b]	LZM$^-$ RS cell[b]	LZM$^+$ histiocytes[b]
1	−	−	ND	ND	ND	ND
2	−	−	−	ND	−	−
3	+	+	+	NS[c]	+	−
4	+	−	+	+	+	−
5	−	−	ND	ND	ND	ND
6	−	−	ND	ND	ND	ND
7	+	−	+	NS[c]	+	−
8	−	−	ND	ND	ND	ND
9	+	−	+	+	+	−
10	−	−	−	ND	ND	ND
11	ND	ND	+	+	+	−

a) PCR was applied to four or five cells.
b) PCR was applied to a single cell.
c) PCR was not applied because RS cells were negative for Ki-1.
Abbreviations: RS cell, Reed-Sternberg cell; LZM, lysozyme; ND, not done.

After amplification, one-sixth of the PCR products were electrophoresed on a 4% NuSieve GTG agarose gel (FMC BioProduct, USA). The gels were stained with 1 μg/ml of ethidium bromide for an hour and examined under long-wavelength ultraviolet irradiation. The fragments in the gels were transferred onto a nylon membrane (Hybond-N+, Amersham, UK) by Southern's method,[10] and were probed with an internal oligonucleotide probe end-labeled with ^{32}P-ddATP by terminal deoxynucleotide transferase.[11] Hybridization conditions were; 6×SSC, 0.1% sodium phosphate, and 5 × Denhardt's solution at 42°C.

PCR amplification performed on DNA obtained from whole paraffin-embedded sections from 11 HD cases revealed that the samples from 5 cases (45%) contained EBV DNA (Table I). Both primer pairs yielded the same results, which are shown in Table I.

In the preliminary study, 4 or 5 RS cells were picked out from each hematoxylin-stained specimen and submitted to gene amplification of EBV DNA. Both ethidium bromide staining and Southern blot hybridization showed that RS cells in all 4 of the examined HD cases identified by conventional PCR as containing EBV were infected by the virus and that RS cells in EBV-negative cases did not contain EBV genome (Table II). By carrying out PCR amplification of DNA from single cells, we were able to detect EBV genome directly from an HE-stained RS cell in all EBV-positive HD cases by both ethidium bromide staining and Southern blot hybridization. Amplified DNA fragments of the EBV in RS cells are shown in Fig. 2. Since ordinary histiocytes sometimes show Hodgkin's cell-like characteristics, we carried out PCR on lysozyme-staining specimens to differentiate true RS or Hodgkin's cells from lysozyme-positive ordinary histiocytes. As was expected, EBV genomes were detected in lysozyme-negative RS and Hodgkin's cells, but not in lysozyme-positive histiocytes.

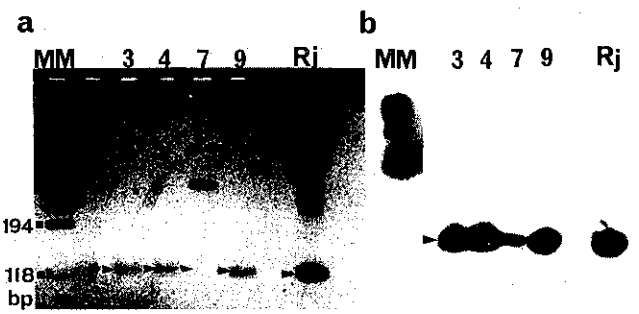

Fig. 2. After 47 PCR cycles of the EBV DNA sequence from an HE-stained RS cell, PCR products were analyzed by gel electrophoresis in 4% NuSieve gel (a) and by Southern blot hybridization (b). (a), Numbers cited above are the case numbers shown in Table I. MM represents the molecular marker $\phi\chi$174/*Hae*III digest. Cases 3, 4, 7 and 9 were positive. Rj represents an EBV-infected cell line, Raji-4, as a positive control. (b), PCR products were transferred to a nylon membrane and were hybridized with an internal sequence probe, TC62= TTCTGCTAAGCCCAAC. After autoradiography, the molecular marker was hybridized with $\phi\chi$174/*Hae*III labeled with ^{32}P. "High-molecular DNA" in case 7 in stained gel was an extra band obtained in PCR. It did not hybridize with internal probe, as shown.

At this time, 4 or 5 lymphocytes around RS cells were also examined by the single cell PCR. Each specimen was examined five times. Specimens from all but one case

(Case 3) were negative for EBV. In case 3, EBV DNA was detected in 2 of 5 sets of experiments.

Ki-1 antigen is expressed on EBV-transformed lymphocytes,[12] and some Ki-1-positive lymphoma cases have been shown to be EBV-positive.[13] In our study, Ki-1-positive RS cells were found in 6 of the 11 specimens examined; 3 specimens contained EBV-positive RS cells, while in 2 of the Ki-1-negative cases RS cells showed positive PCR signals (Table II). EBV DNA was exclusively amplified from RS cells in EBV-positive HD, but not from histiocytes or lymphocytes in the same cases, with a single exception.

PCR[14] is a very sensitive method and has demonstrated an association of EBV with HD in a high proportion of cases.[4] This ordinary PCR method, however, when applied to whole tissue specimens, does not identify the cellular source of EBV-specific DNA sequences. This study was carried out to confirm the presence of EBV genomes within RS cells, and we succeeded in amplifying EBV DNA from a single cell in all EBV-positive HD cases.

Regarding the sensitivity of single cell PCR, a positive PCR signal was obtained in a half of RS cells picked out singly. When 4 or 5 cells were employed, the sensitivity in this study was 100%. Because *Bam*HI W fragment of EBV is repeated 10 times,[8] we should be able to amplify the virus DNA successfully from a single RS cell even if the cell contained only one or a few copies of the genome. Therefore, the critical stage in determining the success of this method may be the transfer of a cell to a PCR tube by pipetting. The specificity was 100%, because no unexpected amplified fragments were found in any of the experiments. This high specificity was based on the following advantages. i) We did not use an EBV *Bam*HI W fragment-inserted plasmid until all the experiments were finished. ii) DNA purification procedures, which frequently cause cross-contamination, were not necessary because the samples contained only a very small quantity of admixtures such as protein and salt which might inhibit gene amplification.

In situ hybridization could identify EBV genomes in RS cells in some HD cases; this is sufficient to reveal the relationship between HD and EBV. However, this method is less sensitive than the single cell PCR method documented above, and the cell morphology is less distinct. This PCR method has several advantages. RS cells can be identified by HE-staining combined with immunohistochemical staining, and this enables the direct detection of the EBV genomes from histologically identified RS cells. A single, or few copies of, viral genomes can be detected by PCR of DNA from a single cell. Higher sensitivity can be expected than for ordinary PCR with conventionally extracted DNA, since even very few cells in the specimen can be selected microscopically and can be used for PCR.

The presence of EBV genome in RS cells strongly suggested an etiologic association of EBV with HD, although approximately one-half of the HD specimens did not contain the EBV genome. Some recent reports[15] implied that detection of EBV in some HD specimens could be explained by EBV infection in the reactive cellular milieu, in the setting of defective immunity associated with HD. This idea is not plausible in the light of the present study, since although our cases did not have HD with severe immunodeficiency, such as acquired immunodeficiency syndrome, the EBV genome was not demonstrated in lymphocytes around RS cells, or in lysozyme-positive histiocytes except for case 3 in which EBV DNA was detected in 2 of 5 background lymphocytes examined.

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