Epstein-Barr Virus Burden in Hodgkin’s Disease Is Related to Latent Membrane Protein Gene Expression But Not to Active Viral Replication

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The Epstein-Barr virus (EBV) has been increasingly detected in Hodgkin’s disease (HD), but its role in pathogenesis remains uncertain. We analyzed 20 specimens of HD known to contain EBV DNA by a sensitive reverse transcriptase polymerase chain reaction (RT-PCR). The cases were assessed for the presence of RNA transcripts of the BNLF1 gene (coding for the viral latent membrane protein [LMP]) and the late replicative gene BLLF1 (coding for the principle envelope glycoprotein [gp 220/350]). LMP RNA transcripts were found in 9 of 20 (45%) cases, mostly those containing many copies of viral DNA and of mixed cellularity (MC) histological subtype. Only one LMP RNA-positive case was also positive for RNA transcripts of the active replication gene BLLF1. Our results show that viral burden in HD is not primarily related to active viral replication, but is associated with LMP gene expression.

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MATERIALS AND METHODS

Tissue selection. Snap-frozen samples of 20 diagnostic lymph node biopsies from patients with HD (16 male, 4 female; age range, 6 to 81 years; mean age, 36.5) were collected over a period of 9 years and stored at −70°C. Details of these cases have been reported previously. Histological subtypes represented were: lymphocyte predominant–diffuse (LPD), four cases; nodular sclerosing (NS), five cases; and MC, 11 cases. All specimens were positive for EBV DNA by PCR and the number of EBV DNA copies and the degree of infiltration by RS cells determined in each case were as previously reported. Reverse transcriptase (RT)-PCRs and PCRs in this study were performed by one of us (D.J.) without prior knowledge of the EBV status of the cases.

Extraction of RNA. Total cytoplasmic RNA was extracted using the cesium chloride ultracentrifugation method with minor modifications. RNA solutions were adjusted to a concentration of 200 ng/μL in 2 mmol/L Tris (pH 7.5) EDTA, 0.4 mmol/L, and stored at −70°C until use.

RT-PCR. RT-PCR was performed with the GeneAmp rTth RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). The manufacturer’s protocol was applied with the following modifications: reverse transcription was performed on 200 ng of target RNA in a final volume of 10 μL with a final concentration of the downstream primer of 1.25 μmol/L. Subsequently, PCR was performed in a total volume of 50 μL with a final concentration of each primer of 0.25 μmol/L, a final concentration of MgCl₂ of 2.25 mmol/L, and 2.5 U rTth DNA polymerase. After 25 cycles, a 5-μL sample was taken and replaced by fresh rTth DNA polymerase (final concentration still 2.5 U/50 μL). Twenty-five more cycles were completed. All reactions were performed with a HYBAID Intelligent Heating Block, model IHB 101 (Hybaid, Teddington, Middlesex, UK). To ensure that positive results originated from RNA, control PCR was performed with AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) on all RNA specimens (see Interpretation of results). Technical details regarding PCR protocols, prevention of contamination, gel preparation, and Southern transfer were as previously reported.

Cycling. Reverse transcription was at 70°C for 5 minutes, then 60°C for 5 minutes, and then 70°C for 10 minutes. The optimal annealing temperature (Tm) for quantitation (DM152) is 70°C, and the Ts for the downstream primers for LMP and BLLF1 (Dhet B and HK 26, respectively) are 60°C and 58°C. PCR of the...
cDNA was then performed as follows: precycle at 95°C for 2 minutes; and cycles 1 to 25 and 26 to 50 at 93°C for 1 minute, 63°C for 30 seconds, and 72°C for 1 minute. Between cycles 25 and 26, the reaction was held at 72°C for 5 minutes.

**Primer:** The GeneAmp RNA PCR kit contains primers and control RNA for the interleukin (IL) gene incorporated into a vector. Thus, we used DM 152, 5'-CATGTCAAATTTCACT-3', identical to the IL-1α 3' primer, and DM 151, 5'-GTCTCTGTAACAGAAATGATC-3', identical to the IL-1α 5' primer, to semiquantitate the primers. For the LMP gene we used Dhet B, 5'-AAAGAGCAGAGATGG3' (downstream), and Dhet A, 5'-CATGTCATAGGCTGCTG-3' (upstream). They delineate a 939-bp segment extending from nucleotide sequence 1340 to 1939 of the transcribed BNLFl gene within the EcoRI Dhet fragment of the EBV genome as previously published. Primers for the BLLFl gene were HK 27, 5'-TTAAAGTGGCACAAGTTG-3' (upstream), and HK 25, 5'-TTCCCTGATTCCTAGTG-3' (downstream) and HK 25, 5'-AGCCTGAACTTCTGATGTG-3' (upstream). They delineate a 335-bp sequence of the BLLFl fragment of the EBV genome, and correspond to nucleotide positions 3801 to 3820 and 4135 to 4116, respectively.

**Internal oligonucleotide probes.** The specificity of the amplified products obtained with the LMP set of primers was confirmed with the LMP A internal oligonucleotide probe, 5'-TATCCACTGAAGTGTG-3', which corresponds to nucleotide positions 1379 to 1399 of the LMP gene. The specificity of the BLLFl set of primers was confirmed using the HK 27 internal probe, 5'-TATCCACTGAAGTGTG-3', identical to the IL-la 3' primer, and DM 151, 5'-GTCTCTGTAACAGAAATGATC-3', to semiquantitate the primers. The concentrations of target RNA per microgram of RNA amplification products giving a visible band of expected size under UV light, when specificity of this band was subsequently proven by prolonged exposure, several cases showed very weak signals (cases 4, 6, 8, Fig 2B). Figure 3 shows the results obtained with control PCR in the same cases, confirming the positive result in case 5.

**Interpretation of results.** In the control reaction, an input RNA of 10^2 copies still gave a visible band of appropriate size when viewed under UV light after 50 amplification cycles. This was the limit of positivity. A positive result was defined by the PCR amplification products giving a visible band of expected size under UV light, when specificity of this band was subsequently proven by hybridization with the internal oligonucleotide probe. Ampli-Taq PCR was performed on the RNA, also for 50 cycles, and any resulting amplification product indicated the presence of DNA, because Ampli-Taq has no RT activity. Thus, a positive result from the corresponding RT-PCR was interpreted as falsely positive, because the template for duplication may have been genomic DNA, rather than cDNA transcribed from RNA in the RT reaction.

**RESULTS**

Of the 20 cases, nine (45%) were positive for RNA transcripts of the BNLFl gene ("LMP RNA-positive") (Table 1 and Fig 1). A double hybridization signal was seen due to the presence of ssDNA generated in the PCR, as noted previously. The proportions in each histological subtype that were LMP RNA-positive were as follows: LPD, 1 of 4; NS, 1 of 5; MC, 7 of 11. The mean age of the positive cases was 32.4 years; the mean age of the negative cases was 39.9 years. The approximate numbers of viral copies found in the LMP RNA-positive cases are given in Table 1. In the 11 LMP-negative cases, viral loads were 10^2 (2 cases), 10^3 (5 cases), 10^4 (1 case), 10^5 (2 cases), and 10^6 (1 case).

**DISCUSSION**

EBV DNA quantitation of the cases selected for this study was established by PCR with the BMRFl set of primers, which detects two EBV copies per 10^7 diploid human genomes. We now report the results for the detection of RNA expression of two EBV genes: the first, BNLFl, coding for a portion of LMP RNA and, the second, BLLFl, coding for a protein expressed in replicative lytic infection. Using an RT-PCR technique that detects approximately 500 copies of target RNA per microgram of RNA, nine of 20 cases (45%) were LMP RNA-positive, whereas only one expressed the viral capsid gp 220/350 transcript.

In the nine LMP RNA-positive cases, the hybridization signal was very strong, indicative of abundant RNA transcripts of this gene. These cases tended to harbor more copies of the virus than those negative for LMP RNA transcripts. Thus, eight of nine positive cases contained 10^4 or more viral copies per microgram of DNA, as compared with only three of 11 negative cases (P < .05, chi^2 test with Yates correction).
EBV RNA in small lymphocytes, but not SR cells in some cases, and because LMP protein expression in HD has been seen exclusively in RS cells, this difference may be due to the ability of PCR to detect low numbers of EBV genome-positive, LMP-negative reactive lymphocytes in those cases with EBV genome-negative RS cells.

The expression of LMP may correlate with histological subtype in HD. In the large series of Pallesen et al., 23 of 24 specimens of MC subtype were LMP-positive. Despite this, no clinical or prognostic factor was subsequently linked with LMP expression by that group. We found LMP expression mainly confined to the MC subtype, with some positive NS and/or LPB cases, although in our series, like others, statistical significance was not reached.

In contrast with the expression of LMP RNA, we found abundant BLLF1 RNA transcripts in only one LMP RNA-positive case. Those cases showing very low levels of BLLF1 transcripts could represent minimal or illegitimate transcription. We conclude that, at the RNA level, late markers of viral replication are rarely present in HD. We suggest that the amount of virus present in the tissues of HD is therefore not related to active viral replication.

Our observation, that oncogenic LMP expression occurs
preferentially in cases with large amounts of EBV DNA, is in accord with previous observations that the in situ hybridization signal elicited from EBV nucleic acids in RS cells is far stronger than that seen in reactive lymphocytes,\(^3,10\) and that LMP has only been detected in tumor cells.\(^4,6\) Taken together, these findings suggest that tumor cells in HD are far more likely to be the source of measurable LMP expression than EBV genome-positive reactive lymphocytes, and that LMP plays a role in the pathogenesis of some cases of HD.

REFERENCES

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