Clonality, expression and methylation patterns of the Epstein–Barr virus genomes in lethal midline granulomas classified as peripheral angiocentric T cell lymphomas

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We analysed the terminal repeats of Epstein–Barr virus (EBV) in DNAs isolated from six lethal midline granuloma (LMG) biopsies. A single fused terminal fragment could be detected in each case, indicating that these angiocentric peripheral T cell lymphomas represent clonal proliferations of cells infected with EBV on a single occasion. Using reverse transcriptase-PCR, we detected EBV nuclear antigen (EBNA) 1 and latent membrane protein (LMP) 1, but not EBNA 2 messages in LMG biopsy RNAs. The splicing pattern of the EBNA 1 message was consistent with the usage of a promoter localized in the BamHI F fragment (F promoter). The BamHI W fragment repeats and LMP-coding sequences were highly methylated in all cases. In contrast, the LMP regulatory sequences were found to be hypomethylated or partially methylated, as in LMP-expressing nasopharyngeal carcinomas.

Introduction

The three main Epstein–Barr virus (EBV)-carrying proliferating cell types, lymphoblastoid cell lines transformed by the virus in vitro, Burkitt’s lymphoma cells and nasopharyngeal carcinoma (NPC) cells, regulate the expression of the viral genomes in non-lytically infected cells in different ways. Lymphoblastoid cell lines express six nuclear antigens (EBNAs 1 to 6) and two membrane antigens (LMP 1 and 2). Burkitt’s lymphoma biopsy cells and EBV-carrying group I Burkitt’s lymphoma cells that maintain a biopsy-like phenotype in vitro express only EBNA 1, a DNA-binding protein required for the maintenance of the viral episomes (Rowe et al., 1987). NPC cells also express EBNA 1 regularly. They do not express EBNAs 2 to 6, but LMP 1 is present in about 65% of tumours (Fähraeus et al., 1988). Group I Burkitt’s lymphoma and NPC cells initiate their EBNA 1 transcript at a promoter localized in the BamHI F fragment of EBV DNA (F promoter; Fp) (Sample et al., 1991; Schaefer et al., 1991; Smith & Griffin, 1992). Subsequent splicing of the primary transcript joins two small exons encoded by the BamHI F and Q fragments, and by the BamHI U fragment, respectively, with the EBNA 1 coding sequences encoded by the BamHI K fragment. The LMP 1 message is initiated at a different promoter (localized in the BamHI N fragment) in NPC cells (Baer et al., 1984).

In lymphoblastoid cell lines and in Burkitt’s lymphoma group III cells that have drifted to an immunoblastic phenotype during in vitro propagation, EBNAs 1 to 6 are all spliced from a single giant precursor RNA, initiated either at the BCR2, or more rarely the BWR1, promoters localized in the BamHI C and BamHI W fragments, respectively (Bodescot et al., 1987; Sample et al., 1986; Woisetschlaeger et al., 1989, 1990). The splicing pattern of the EBNA 1 transcript is different from that of the exclusive Fp users, such as group I Burkitt’s lymphomas and NPCs. The FQ exon is absent. Splice sites between BCR2/BWR1 and the silent F promoter are used to generate EBNA 2 and 5 messages and the splice sites downstream from Fp (not utilized in Fp user cells) contribute to generating EBNA 3, 4 and 6 messages.

Previously, we observed that hypomethylation of the 5’ flanking sequence of BNLF I, the LMP 1-encoding gene (also designated LRS, the LMP 1 regulatory sequence; Fähraeus et al., 1990), correlates with the expression of LMP 1 in NPC biopsies (Hu et al., 1991). In addition, at least one CpG pair was demethylated upstream of the BCR2 promoter when the group I Burkitt’s lymphoma Mutu cells drifted to a group III

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phenotype and BCR2 was activated (Altiok et al., 1992). This suggests that hypomethylation of regulatory regions may be correlated with the activation of certain EBV promoters in NPC and Burkitt’s lymphoma cells that carry highly methylated viral genomes.

In the present study we analysed the expression of EBNA1s and LMP1 in a special form of peripheral T cell lymphoma which is designated lethal midline granuloma (LMG) (Harabuchi et al., 1990). These tumours can be regarded as malignant manifestations of angioimmunoblastic proliferative disorders (Lipford et al., 1988); they grow progressively in the nasal cavity and midline facial tissues, with ulceration and necrotizing granuloma formation as the result. In two patients, clonality of the proliferating T cells was shown by demonstration of a rearranged T cell receptor gene (Gaulard et al., 1988), a reliable marker of lineage and clonality in human lymphoid neoplasms (Waldman et al., 1985).

Recently, EBV DNA was detected in five out of five LMG biopsies by in situ hybridization and in three out of three by Southern blotting (Harabuchi et al., 1990). On the basis of terminal repeat analysis, i.e. examination of the products of EBV DNA circularization, we now report that all six LMG biopsies analysed in the present study are monochlonal proliferations of EBV-carrying cells that have acquired the virus in a single infectious event at the onset of the clonal proliferation or before. By reverse transcriptase (RT)-PCR we detected EBNA1 messages in the LMG biopsies, processed according to the Q/U/K splicing pattern. We have also found the LMP1 transcripts in all five biopsies examined. We could not detect any transcripts indicating C or W promoter (CpG/Wp) usage. The viral genomes were highly methylated in the BamHI W and LMP coding regions whereas the LMP regulatory region was found to be unmethylated in five cases and only partially methylated in one case.

Methods

**LMG biopsy samples.** The samples were taken from six Japanese patients of either sex. Five nasal tumours (LMG 1a, 2, 4, 5, 6), a skin metastasis derived from one of them (LMG 1b) and a recidive of a nasal tumour (LMG 7) were analysed. The biopsies designated LMG 1a, 2, 4, 5 and 6 in the present study were characterized earlier by Harabuchi et al. (1990). LMG 7 is a fresh biopsy. All cases were indicated histologically and phenotypically as peripheral T cell lymphomas, with ulceration and necrotizing granuloma formation as the result. In two patients, clonality of the proliferating T cells was shown by demonstration of a rearranged T cell receptor gene (Gaulard et al., 1988), a reliable marker of lineage and clonality in human lymphoid neoplasms (Waldman et al., 1985).

**DNA isolation and DNA methylation analysis.** High M₇ DNA was isolated from the LMG biopsy samples (LMG 1a, 1b, 2, 4, 5 and 6) as described previously (Ernberg et al., 1989). Aliquots containing 10 μg DNA were digested with excess amounts of either Hpall (a CpG methylation-sensitive enzyme) or MspI (CpG methylation-insensitive) as in a previous study (Minarovits et al., 1991). Both enzymes recognize the sequence CCGG (Waalwijk & Flavell, 1978). Separation of digested DNA fragments by agarose gel electrophoresis, transfer to Hybond N membranes (Amersham) and hybridization with[³²P]dCTP-labelled, cloned EBV fragments were done according to standard procedures (Mamatis et al., 1982). Fuji filters were used for autoradiography. The probes were removed from the filters by repeated washing (10 min each) in boiling 0.1 x SSC, 0.1% SDS. Complete removal of label was confirmed by autoradiography before hybridization with a new probe.

**Probes.** Cloned EBV DNA fragments were kindly provided by Dr Lars Rymo, Gothenburg, Sweden. The following probes were used (see also Fig. 1): an EcoRI-BamHI subfragment of the BamHI C fragment [nucleotides (nt) 7315 to 13215], covering oriP (Yates et al., 1984) and BCR2; a 1258 bp MspI subfragment of the BamHI C fragment carrying BCR2; the BamHI W fragment, a 0.8 kb BglI subfragment (nt 169449 to 170290) of the BamHI N fragment covering regulatory sequences of the LMP1 gene; a 1.6 kb Smal subfragment of the BamHI N fragment covering LMP 1 coding sequences.

**Clonotypic analysis of EBV genomes.** A Southern blot of BamHI-digested LMG biopsy DNAs was hybridized to the LMP 1-coding Smal subfragment (nt 167918 to 169598) of the BamHI N fragment,

![Fig. 1. Regions of the EBV genome analysed in this study](image)

- **(a)** Regions of the EBV genome analysed in this study (a) and MspI cleavage sites in the EBV DNA fragments used as probes (b), based on the DNA sequence of the B95-8 genome (Baer et al., 1984). (b) Numbers above the lines indicate nucleotides in the B95-8 sequence; numbers below the lines indicate major MspI fragments in base pairs. Thick lines indicate important coding or regulatory sequences within the regions probed. The following probes were used: (i) an EcoRI-BamHI subfragment from the BamHI C fragment (nucleotides 7315 to 13215) covering oriP, the latent origin of EBV replication (Yates et al., 1984) and one of the promoters for EBNA1 transcription (BCR2, indicated by a triangle); the BCR2-carrying 1258 bp MspI fragment was also used separately as a probe; (ii) the BamHI W fragment containing a B cell-specific enhancer (W ENH) (Ricksten et al., 1988), (iii) a Smal subfragment (nucleotides 167918 to 169598) of the BamHI N fragment covering LMP 1 coding sequences; (iv) a 0.8 kb BglI subfragment of the BamHI N fragment covering the LRS (Fähræus et al., 1990).
Expression and methylation of EBV DNA in LMG

Table 1. Primers and conditions for PCR reactions used for detection of LMP 1, EBNA 1 and 2 transcripts and usage of the F, C and W promoters*

<table>
<thead>
<tr>
<th>Designation</th>
<th>Primer</th>
<th>PCR cycle</th>
<th>Mg²⁺ (mm)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LMP 1</strong></td>
<td>5' CTGAGGATGGGAACAGGACTTGA (169456)</td>
<td>94 °C 30 s; 45 °C 1 min; 72 °C 2 min;</td>
<td>3-5</td>
<td>446 bp</td>
</tr>
<tr>
<td></td>
<td>5' TGACGAGGATGGAGCTTAGG (168858)</td>
<td>30 cycles</td>
<td>1-5</td>
<td>128 bp</td>
</tr>
<tr>
<td></td>
<td>5' TTG/TGTA/CTACTCTGCTGATGATG (169124)</td>
<td>94 °C 30 s; 68 °C 1 min; 72 °C 1 min;</td>
<td>3-5</td>
<td>469 bp</td>
</tr>
<tr>
<td></td>
<td>5' AGTATGTGGACAGCAGGCT (169045)</td>
<td>25 cycles</td>
<td>1-5</td>
<td>293 bp</td>
</tr>
<tr>
<td><strong>Fp, EBNA 1</strong></td>
<td>5' GTGCCGATCGGATGGCC (62440)</td>
<td>94 °C 30 s; 60 °C 1 min; 72 °C 2 min;</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td></td>
<td>5' CAGCTGAATGGCATAGAGACAAGGAC (11337)</td>
<td>35 cycles</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td></td>
<td>5' TGAAGGATGGCAGCGTTTACCA (14829)</td>
<td>35 cycles</td>
<td>1-5</td>
<td>300 bp</td>
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<tr>
<td></td>
<td>5' ACACCGAGACCCCAAGAGC (11360)</td>
<td>35 cycles</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td></td>
<td>5' AGTATGGAGGAGGCTTACCA (11451)</td>
<td>35 cycles</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td><strong>Cp</strong></td>
<td>5' GTAGGGATTCGAGGGAATTACTGA (48613)</td>
<td>94 °C 45 s; 63 °C 1 min; 72 °C 2 min;</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td></td>
<td>5' CATAGAAGAAGAAGAGGATGAAGA (47954)</td>
<td>30 cycles</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td><strong>Wp</strong></td>
<td>5' GTAGGGATTCGAGGGAATTACTGA (48613)</td>
<td>94 °C 45 s; 63 °C 1 min; 72 °C 2 min;</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td></td>
<td>5' CATAGAAGAAGAAGAGGATGAAGA (47954)</td>
<td>30 cycles</td>
<td>1-5</td>
<td>300 bp</td>
</tr>
<tr>
<td><strong>EBNA 2</strong></td>
<td>5' ACTTTGAGCCACCACTAGATACCA (47911)</td>
<td>94 °C 30 s; 63 °C 1 min; 72 °C 2 min;</td>
<td>2-5</td>
<td>327 bp</td>
</tr>
<tr>
<td></td>
<td>5' TGGAGGATGGCAGCGTTTACCA (48637)</td>
<td>30 cycles</td>
<td>2-5</td>
<td>327 bp</td>
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<tr>
<td></td>
<td>5' CATAGAAGAAGAAGAGGATGAAGA (47954)</td>
<td>30 cycles</td>
<td>2-5</td>
<td>327 bp</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate positions of the 5' nucleotides of the primers on the B95-8 sequence (Baer et al., 1984). Exon-exon boundaries are indicated by black triangles. In the case of LMP 1 primers, equimolar amounts of the sequence variants indicated by '⁄' were used.

localization of the right terminal region of the viral genome adjacent to the 500 bp tandem repetitive sequence.

Extraction of RNA and RNA-RT-PCR. Total RNA was extracted from the LMG biopsies 1b, 2, 4, 5, 6 and 7 using a modified acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi, 1987). One to 2 μg of total RNA was used for the synthesis of first strand cDNA by avian myeloblastosis virus (AMV) RT. Briefly, the extracted RNA was dissolved in 20 μl reaction buffer containing 50 mM-Tris–HCl pH 8.5, 145 mM-KCl, 10 mM-MgCl₂, 4 mM-DTT, 125 μM-dNTP, 1 μM-14-mer random primer, 0.5 μl of ribonuclease inhibitor (Pharmacia, 50 units/μl) and 5 units of AMV RT (Pharmacia) and incubated for 60 min at 42 °C followed by 5 min at 95 °C to denature the enzyme. The reaction was performed in an automatic Thermal Cycler (Techne PHC-2). The cDNA was stored at -20 °C until PCR amplification.

Three μl cDNA was used as template for PCR. The 50 μl reaction mixture contained 10 mM-Tris–HCl pH 8.5, 40 mM-KCl, 1 to 8 μM-MgCl₂ (according to titrated optimum), 1 μM of primers and 50 μM of each of the four nucleotides dATP, dGTP, dCTP and dTTP. One unit of Taq polymerase (Perkin-Elmer Cetus) was added to each reaction. The samples were overlaid with two or three drops of mineral oil and then subjected to amplification in the thermal cycler. The number of cycles, melting and reannealing conditions, incubation times and the number of the primers used are shown in Table 1. All the primers were synthesized as 17 to 25 nt oligonucleotides by Scandinavian Gene Synthesis Systems. The PCR products were separated by electrophoresis in 1 to 2% agarose gels, stained with ethidium bromide and photographed using a Polaroid camera.

**Results**

*Clonotypic analysis of EBV genomes*

The linear form of replicative EBV DNA contains a variable number of direct tandem 500 bp repeats (terminal repeats) at each end. Circularization by joining of the termini generates the circular episomal form carried in growth-transformed cells. Since all episomes are generated by amplification of a single initial circle in the infected cell, analysis of the fused terminal fragments (which vary by increments of 500 bp) may indicate the mono-, oligo- or polyclonality of EBV-carrying cell populations (Raab-Traub & Flynn, 1986). *BamHI-*
digested DNA from each LMG biopsy was probed with a SmaI subfragment of the BamHI N fragment. A single terminal repeat band was obtained in each case (Fig. 2). The size of the fused terminal fragment was different for each tumour, but identical for the nasal tumour LMG 1a and its skin metastasis LMG 1b. All LMG tumours can therefore be regarded as clonal proliferations of cells that had been infected with EBV on a single occasion.

Fig. 3. Methylation analysis of the EBV DNA in LMG biopsies. Total cellular DNA was digested with HpaII (odd-numbered lanes) or MspI (even-numbered lanes). The resulting DNA fragments were separated on a 1.5% agarose gel, blotted to a Hybond N membrane and hybridized with the BamHI W fragment (a), a 1.6 kb SmaI subfragment of the BamHI N fragment covering LMP coding sequences (b), a 0.8 kb subfragment of the BamHI N fragment covering the LRS region (c), an EcoRI–BamHI subfragment of the BamHI C fragment (d), or a 1258 bp MspI fragment carrying the BCR2 promoter (e). The following DNAs were analysed in (a) and (b): LMG 1a (lanes 1 and 2); LMG 1b (lanes 3 and 4); LMG 2 (lanes 5 and 6); LMG 6 (lanes 7 and 8). In (c), (d) and (e) the following DNAs are shown: LMG 1a (lanes 1 and 2); LMG 1b (lanes 3 and 4); LMG 2 (lanes 5 and 6); LMG 4 (lanes 7 and 8); LMG 5 (lanes 9 and 10); LMG 6 (lanes 11 and 12). In (e), a faint band indicating the presence of partially unmethylated HpaII sites is indicated by a black triangle (lane 5).
Expression and methylation of EBV DNA in LMG

Moreover, the absence of multiple fragments of less than 8 kb, which are diagnostic of linear viral DNA (see B95-8 in Fig. 2), indicates that the EBV genome does not enter its replicative cycle in LMG cells.

Methylation patterns of selected EBV regions in LMG biopsies

Fig. 3 (a) shows the methylation analysis of the BamHI W region in the nasal tumour LMG 1a, its skin metastasis (LMG 1b) and in the nasal tumours LMG 2 and 6. The HpaII fragments were larger than the corresponding MspI fragments in each case, indicating the presence of methylated CCGG sequences in this region. Fig. 3 (b) shows that due to restriction fragment length polymorphism, the MspI cleavage pattern of the LMP coding region differs in LMG biopsy DNAs from the one expected on the basis of the B95-8 sequence (see Fig. 1); the HpaII sites are highly methylated in this region as well.

On the contrary, as shown in Fig. 3 (c), the 5' flanking sequence of the LMP 1 gene, the LRS (Fähraeus et al., 1990), was predominantly unmethylated in all tumours except LMG 4 where a mixture of methylated and unmethylated sequences could be observed. Polymorphism of the MspI fragment sizes compared to the B95-8 prototype can be noticed in this region as well.

The methylation of the BamHI C region (Fig. 3 (d)) showed a mixed pattern: certain HpaII sites were methylated whereas others remained unmethylated in the nasal tumour LMG 1a, its skin metastasis LMG 1b, and in nasal tumours LMG 2, 4 and 5. There were no identical HpaII and MspI bands in this region in LMG 6 (Fig. 3 (d)). We noticed earlier that the HpaII sites flanking an approximately 0.9 kb fragment within oriP remain unmethylated in Rael, a Burkitt's lymphoma cell line carrying highly methylated EBV genomes (Ernberg et al., 1989). These sites were also unmethylated in all LMG cases with the exception of LMG 6, where at least one of them was methylated. The fragment of approx. 1250 bp, which includes BCR2 and a 1330 bp fragment, migrated close to each other; at least one of the HpaII sites flanking the 1250 bp fragment was methylated in LMG 1a, 1b, 5 and 6. Reprobing the blot with the BCR2-carrying MspI fragment revealed that the flanking HpaII sites were partially unmethylated in LMG 2 (though this is less obvious on the photo than on the original autoradiograph) but they were methylated in LMG 4 (Fig. 3 (c)).

Detection of LMP 1 transcripts by RT-PCR

LMP 1 transcripts could be detected in all biopsy samples available for RNA preparation (LMG 1b, 2, 4, 5, 6 and 7; Fig. 4a, Table 2).

Splicing pattern of the EBNA transcript in LMGs

As shown in Fig. 4, presence of the C1 and C2 exons in an mRNA indicates usage of the BCR2 promoter. The presence of the W0 exon proves BWR1 usage, and of the Q exon indicates Fp activity. Using the primer combinations listed in Table 1, we could distinguish between these three alternatives. The Q/U/K splicing pattern, corresponding to Fp usage, was present in all five LMG RNA samples analysed (Fig. 4b). We could not detect transcripts carrying exons C1 and C2 (Fig. 4c) or exon W0 (data not shown). Using primers specific for EBNA 2 coding sequences we could not detect EBNA 2 transcripts in the LMG biopsy RNAs (Fig. 4d); this is consistent with the usage of Fp, localized downstream from the EBNA 2 coding region.
Table 2. Summary of RT–PCR analyses of LMG biopsy RNAs

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>LMP 1</th>
<th>EBNA 1 (Q/U/K)</th>
<th>Cp</th>
<th>Wp</th>
<th>EBNA 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 1b</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LMG 2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LMG 4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LMG 5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LMG 6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LMG 7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B95-8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BJAB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P3HR-1</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

*ND, Not done.

Discussion

The two best known EBV-associated malignancies, Burkitt’s lymphoma and NPC, represent monoclonal proliferations of neoplastic cells. Terminal repeat analysis showed that the proliferating EBV-carrying cells have been infected on a single occasion in both tumours. This has been taken to suggest that these tumours originally arose from a single EBV-infected cell (Raab-Traub & Flynn, 1986). Recently, EBV genomes have been detected in other neoplasias, including a variety of T cell lymphomas, Hodgkin’s disease and lymphoproliferations of immunocompromised patients (Harabuchi et al., 1990; Ott et al., 1992; Weiss et al., 1987; Katz et al., 1989; Staal et al., 1989). The role of the virus in the T cell lymphomas and Hodgkin’s disease is unknown.

We have found that all LMG biopsies analysed contain EBV DNA from a single infectious event. Although T cells carrying the viral genome can be detected occasionally during the course of acute or chronic EBV infections (Kikuta et al., 1988; Yoneda et al., 1990; Mori et al., 1992), most normal T cells would be expected to remain uninfected even in patients with a heavy EBV load. This suggests that the probability of LMG development is much higher in an EBV-infected than in an uninfected T cell. This implies that the virus has contributed to the aetiology of these lymphomas. One may also speculate that T cells at specific anatomical locations (e.g. nasal sinuses) might be exceptionally prone to EBV infection.

EBNA 1 and two small EBV-encoded RNAs are expressed in a constitutive fashion in lymphoblastoid cell lines, Burkitt’s lymphomas and NPCs (Rowe et al., 1987; Fähraeus et al., 1988; Minarovits et al., 1992). LMP 1 is also constitutively expressed in epithelial cells and in the T cell line Molt-4 (R. Fähreus, unpublished). In cells of the B lineage it is expressed only if EBNA 2 is present (Fähraeus et al., 1990). EBNA 2 is expressed only in cells with a B immunoblastic phenotype, notably lymphoblastoid cell lines and group III Burkitt’s lymphoma cells (Rowe et al., 1987). Our data showed that EBNA 1 and LMP 1 but not EBNA 2 mRNAs are expressed in LMG biopsies. The splicing pattern of the EBNA 1 message is consistent with the usage of the Fp. This precludes transcription of the EBNA 2 exons. The EBNA 1 and LMP 1 expression and the absence of the EBNA 2 message, found in the LMG biopsies, is similar to the previously established pattern for NPC.

We observed earlier that the methylation pattern of EBV DNA depends on the host cell phenotype: it is highly methylated in Burkitt’s lymphoma and NPCs, but hypomethylated in lymphoblastoid cell lines transformed by the virus in vitro (Minarovits et al., 1991; Hu et al., 1991). The BamHI W repeats and LMP coding sequences were found to be highly methylated in LMG biopsies as well (Fig. 2). This implies that T lymphoma cells are also capable of methylating EBV DNA.

In earlier studies, the LMP coding sequences were found to be methylated in NPC biopsies and nude mouse-passaged NPC lines as well, independently of the expression of LMP protein (Hu et al., 1991; Alday et al., 1990). In contrast, the LRSs were hypomethylated in LMP-expressing NPCs and methylated in NPC biopsies in which LMP protein could not be detected by immunoblotting (Hu et al., 1991). LMP was detected by immunofluorescence in three out of three nasal tumour biopsies taken from LMG patients in an earlier study and by Northern blotting in one metastatic lesion (Harabuchi et al., 1990). This is consistent with our detection of LMP transcripts in five out of five LMG biopsies using RT–PCR in the present study. The hypomethylation of LRS in the LMP-expressing LMG biopsies (see Fig. 2d) is consistent with our earlier findings and suggests that promoter usage within highly methylated EBV genomes is associated with hypomethylated regulatory regions.

Previously we detected two unmethylated HpaII sites within oriP in Rael, a group I Burkitt’s lymphoma cell line that carries highly methylated EBV genomes (Ernberg et al., 1989). The same sites were also unmethylated in the LMG biopsies studied except LMG 6 where at least one of the two HpaII sites flanking a 0.9 kb fragment is methylated (Fig. 3d).

BCR2 promoter usage is associated with the demethylation of at least one HpaII site localized upstream of this promoter in group III clones of the Burkitt’s lymphoma cell line Mutu (Altiok et al., 1992). The corresponding group I Mutu clones that do not use the BCR2 promoter are methylated in the same region. This is consistent with the lack of BCR2 usage and methylation of at least one of the HpaII sites flanking the BCR2-carrying fragment in all LMG biopsies with the exception of LMG2 where
these sites are partially unmethylated. Absence of transcription from BCR2 in the latter case may be due to the absence of specific trans-activating factors (Gross & Garrard, 1988). Alternatively, it is possible that the RT-PCR used in this study is not sensitive enough to detect a low-abundance EBNA 2 message expressed in only a fraction of biopsy cells. Similarly, hypomethylation of certain HpaII sites in a minority of cells or EBV genomes could remain undetected by Southern blotting. Using immunofluorescence, Harabuchi et al. (1990) observed EBNA 2 expression in five LMG biopsies (also included in the present study). The percentage of EBNA 2-positive cells was low (5 to 6%), however, and EBNA 2 protein could not be detected in the same specimens by immunoblotting (T. Osato, unpublished results). Harabuchi et al. (1990) also detected EBNA 2 expression by Northern blotting in a metastatic lesion of a nasal lymphoma. Unfortunately there was no material left from that biopsy and therefore it could not be included in the present study. In the case of the other biopsies we could not confirm the presence of EBNA 2 by RT-PCR.

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