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CLONALITY AND METHYLATION STATUS OF THE EPSTEIN-BARR VIRUS (EBV) GENOMES IN *IN VIVO*-INFECTED EBV-CARRYING CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELL LINES

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Directly growing Epstein-Barr virus (EBV)-carrying cell lines were established from a chronic lymphocytic leukemia (CLL) patient (PG) on repeated occasions. The lines carried the same ring chromosome 15 as the leukemia cells *in vivo* and were similarly trisomic for chromosome 12. They all showed the same J_H rearrangement, indicating that they had arisen from the same B-cell progenitor. They also had the same single EBV-terminal repeat (TR), indicating that they had been generated by a single EBV infection event. It may be surmised that a single CLL cell had been infected by EBV *in vivo* and established itself subsequently as a subclone within the CLL population. This subpopulation persists *in vivo* but does not appear to expand with time. After explantation, it transforms into lymphoblastoid cells and proliferates selectively as immortalized lines. The leukemia-representative CLL lines were phenotypically indistinguishable from the B95-8 virus-transformed normal diploid cells of the patient, established in parallel by *in vitro* infection. They grew as typical LCL clusters and expressed the same B-cell activation markers. The methylation status of EBV-DNA was different in the CLL lines and the B95-8-virus-transformed LCLs. When HpaII- and MspI- digested DNA was probed with *Bam*HI C, E, H and W fragments, the CLL lines showed a mixture of methylated and unmethylated restriction fragments as in certain EBV-carrying Burkitt lymphoma (BL) lines. In contrast, the EBV-DNA of B95-8 virus-transformed normal diploid cells was completely unmethylated, as in other LCLs.

Chronic lymphocytic leukemia (CLL) is not an EBV-associated disease, although some patients exhibit elevated EBV antibody titers (Johansson *et al.*, 1971). Neither EBV-DNA or EBNA has been detected in CLL cells *in vivo* (Lindahl *et al.*, 1974). CLL cells often resemble mature resting B cells (Carlsson *et al.*, 1988). They are often resistant to EBV-immortalization, although some EBV-carrying CLL lines could be obtained after experimental infection, but only exceptionally and with difficulty (Karande *et al.*, 1980; Najfeld *et al.*, 1980; Walls *et al.*, 1989).

We have established leukemia-representative, *in vivo*-infected EBV-carrying CLL lines from the blood lymphocyte populations of one CLL patient, PG (Lewin *et al.*, 1988). The vast majority of the CLL cells of this patient were EBNA-negative but a small minority (<0.1%) were EBNA-positive *in vivo*. To prevent virus release and new infection of previously uninfected cells, the cells were explanted in the presence of a viral inhibitor, phosphonoformate (PFA) and neutralizing antibody. EBNA-positive cell lines were grown from both high-density and low-density fractions of blood lymphocytes on 2 separate occasions with an interval of 3 months. They carried the same ring chromosome 15 marker and extra chromosome 12 as the corresponding CLL cells *in vivo*. All 4 PG-CLL cell lines studied in the present report (designated A1, A2, B1 and B2) grew in clusters like ordinary lymphoblastoid cell lines (LCLs). All 4 EBV-carrying leukemia lines carried EBV-DNA with an identically sized *Bam*HI K fragment.

Following the infection of B cells, the linear EBV-genome becomes circularized, due to the fusion of the terminal regions (TR) (Adam and Lindahl, 1975; Andersson-Anvret and Lindahl, 1978; Lindahl *et al.*, 1976; Hurley and Thorley-Lawson, 1988). Each new circularization event leads to a differently sized TR fragment (Given *et al.*, 1979; Kintner and Sugden, 1979; Raab-Traub and Flynn, 1986). Only one such fragment is present in each clonal cell line, established by a single infectious event (Brown *et al.*, 1988; Hurley and Thorley-Lawson, 1988).

Ernberg *et al.* (1989) have found that EBV-genomes are highly methylated in a Burkitt lymphoma (BL) line and in a nude-mouse-passaged NPC tumor. In contrast, LCLs derived from BL-EBV or NPC-EBV-transformed normal B cells *in vitro* carried unmethylated viral DNA, like all other LCLs tested. There was a certain relationship between the methylation of critical control regions and the expression of the corresponding EBV-gene (Ernberg *et al.*, 1989).

We have analyzed the clonality of the PG-CLL cell lines with regard to immunoglobulin gene rearrangement, the configuration of EBV-TR carrying restriction fragments and the methylation of various EBV-DNA regions, in comparison with an LCL established from the same patient by *in vitro* transformation of normal diploid B cells with the B95-8 substrain of EBV.

MATERIAL AND METHODS

Cell lines

As described previously (Lewin *et al.*, 1988), we have established 4 cell lines designated A1, A2, B1 and B2, from the CLL patient PG. They grew out from the explanted leukemic cells in the presence of the viral inhibitor phosphonoformate (PFA) and neutralizing antibodies on 2 occasions (A and B), with an interval of 3 months. A1 and B1 were derived from the low-density, and A2 and B2 from the high-density fraction of blood lymphocytes. Karyotyping of these cell lines showed the presence of ring chromosome 15 and of trisomy 12, characteristic of the original CLL in the PG blood (Lewin *et al.*, 1988).

For comparative studies, we have also established a cell line from the PG blood by *in vitro* infection with the B95-8 substrain of EBV.

Immunoblotting

Whole-cell extracts from CLL lines and the LCL line were prepared for immunoblotting as described by Gratama *et al.* (1988).

Surface markers

Monoclonal antibodies (MAbs) B1, 35.1C5, MHM6, 38.13 and J5 were used. B1 detects CD20, a pan-B-cell marker; 35.1 C5 detects CD45, a lymph-node follicle mantle zone and splenic marginal zone marker; MHM6 detects CD23, expressed on activated B cells; 38.13 detects CD77, a Burkitt lymphoma-associated antigen (BLA), also expressed on ger-

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minal center B cells; J5 detects CD10, the common acute lymphoid leukemia antigen (CALLA). Standard indirect immunofluorescence reactions were performed with all antibodies.

DNA preparation and analysis

High-molecular-weight DNA was extracted from late-passage CLL cell lines (A1, A2, B1 and B2) and the B95-8-transformed line PG/B95-8.

*Hind*III-digested DNA was used for the analysis of immunoglobulin gene rearrangement and *Bam*HI-*Eco*RI double-digested DNA was used for analysis of the viral TR. The methylation-sensitive *Hpa*II and the methylation-insensitive *Msp*I enzymes that recognize CCGG were used for the methylation studies (Waalwijk and Flavell, 1978).

The digested DNAs were size fractionated by agarose gel electrophoresis and transferred to nitrocellulose filter (Southern 1975).

The human IgH-J_H probe was a kind gift of Dr. P. Leder. It is a 5.6-kb *Bam*HI and *Hind*III fragment of the human IgH-J_H region.

Cloned EBV-DNA fragments were the kind gift of Dr. L. Rymo. A 1.9-kb *Xho*I subfragment of the *Eco*RI-Dhet fragment was used for TR probing (Brown *et al.*, 1988). A 0.9-kb *Sac*II-*Bam*HI subfragment of the *Bam*HI C fragment and the *Bam*HI W, H and E fragments were used to assess the methylation status of the viral genome. In our previous studies, these probes have revealed a consistent difference between highly methylated BLs or NPCs and the unmethylated LCLs (Ernberg *et al.*, 1989).

The probes were radiolabelled with (^{32}P)d CTP by nick translation (Rigby *et al.*, 1977). Pre-hybridization, hybridization, washing and autoradiography were as described by Wahl *et al.* (1979).

RESULTS

Characterization of EBV-carrying CLL lines and B95-8 virus-transformed LCL cell line

The PG lymphocytes, early and late passages of CLL and the B95-8 virus-transformed cell lines were tested for expression of the markers CD20, CD45, CD23, CD10 and CD77 defined by the B1, 35.1C5, MHM6, J5 and 38.13 MAbs, respectively (Table I).

The pan-B-cell marker CD20 was expressed similarly in the PG blood lymphocytes and the derived cell lines except for the early passages of the PG/B95-8 line. The expression of lymph-node follicle mantle zone and splenic marginal zone marker, CD45, was higher in PG blood lymphocytes and CLL cell lines than in the normal B-cell-derived line. The expression of CD23, a marker for activated B cells, was similarly high in the early passages of CLL and PG/B95-8 lines and declined after cultivation. CD77 (BLA), a Burkitt-lymphoma-associated antigen, was expressed only on the early-passage CLL cell lines. Similarly, CALLA, the common acute lymphoid leukemia antigen, was expressed on the *ex vivo* and early passage of CLL cells but was lost in later passages.

The markers thus show that the CLL cells acquired an LCL-like phenotype *in vitro*. One difference was still maintained, however, since the CLL cell lines expressed higher levels of CD45.

EBNA pattern of the EBV-carrying CLL cell lines and LCL line

Immunoblots from all 4 CLL cell lines (A1, A2, B1 and B2) and LCL cell line, PG/B95-8, were probed with an EBV-seropositive poly-specific human serum.

Judging by the EBNA size pattern, all the CLL lines (A1, A2, B1 and B2) carried the same virus that differed from the B95-8 virus (Fig. 1).

Clonal origin of the EBV-carrying CLL cell lines (Fig. 2)

*Hind*III-digested DNAs from the late passage of 4 CLL cell lines (A1, A2, B1 and B2) and the LCL cell line (PG/B95-8) were hybridized with the J_H probe.

All 4 CLL cell lines showed 2 rearranged J_H patterns at 5.8 and 4.2 kb. No corresponding rearranged J_H patterns were detected in the LCL line, PG/B95-8.

TR-fragment analysis (Fig. 3)

*Bam*HI-*Eco*RI double-digested filters were hybridized with the *Eco*RI-Dhet terminus probe. A single TR fragment was detected in all 4 CLL lines. No corresponding fragment was detected in the PG/B95-8 line.

*Analysis of EBV-DNA methylation (Fig. 4*a, b, c *and* d*)*

DNA was digested with the isoschizomers *Hpa*II (CpG methylation-sensitive) and *Msp*I (CpG methylation-insensitive). The EBV-DNA probed with *Bam*HI C, W, H and E showed a mixture of methylated and unmethylated restriction fragments in all 4 CLL lines but it was completely unmethylated in the B95-8 virus-transformed LCL (PG/B95-8).

DISCUSSION

The identical J_H rearrangement in all 4 CLL cell lines isolated from the patient confirms the monoclonal origin of the leukemic cells *in vivo* and the representativeness of the derived CLL lines.

The single and identical TR band in all 4 CLL lines shows that these were derived from a single infection event. Since virus release was inhibited by PFA after explantation and the transfer of preformed virus was inhibited by neutralizing antibodies, this infectious event must have taken place *in vivo*.

Based on our previous studies on the establishment of directly outgrowing LCLs, we have postulated that latent EBV

TABLE I – SURFACE MARKER EXPRESSION OF PG BLOOD LYMPHOCYTES (PG LYMPH), EARLY AND LATE PASSAGES OF THE DIRECTLY OUTGROWING CLL LINE AND OF THE B95-8 TRANSFORMED NORMAL B-CELL LINE (PG/B95-8)

Antigen	MAb[1]	% positive cells[2]				
		PG lymph	CLL lines[3]		PG/B95-8	
			Early (3–4 months)	Late (>2 years)	Early (3–4 months)	Late (>2 years)
CD20	B1	37 ± 6	35 ± 3	30 ± 2	98 ± 2	29 ± 1
CD45	35.1C5	62 ± 19	78 ± 10	52 ± 7	26 ± 4	16 ± 8
CD23	MHM6	39 ± 9	84 ± 11	17 ± 4	94 ± 2	47 ± 10
CD77	38.13	3 ± 3	20 ± 17	0	0	0
CD10	J5	36 ± 11	78 ± 15	0	2 ± 2	2 ± 2

[1]Monoclonal antibody.–[2]Mean ± SD of at least 3 experiments.–[3]All the 4 CLL lines were analysed. There were no significant differences among the CLL cell lines derived from low- or high-density fractions.

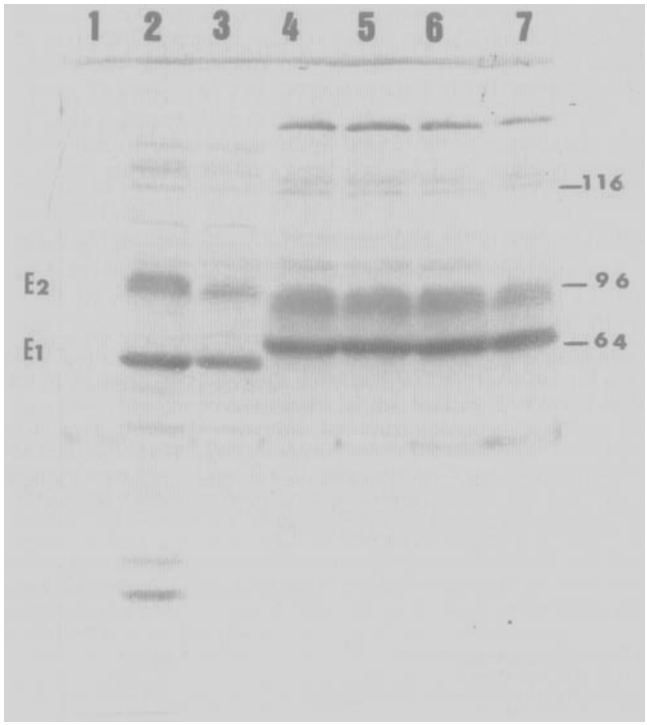

FIGURE 1 – EBNA pattern in EBV-negative control Bjab (1), EBV-positive control B95-8 (2) PG/B95-8 (3), A1 (4), A2 (5), B1 (6) and B2 (7) probed with a polyvalent human serum. Numbers on the right indicate molecular mass in kilodaltons (kDa). EBNA-1, -2 (E1,E2) were assigned to their corresponding mol. wt.

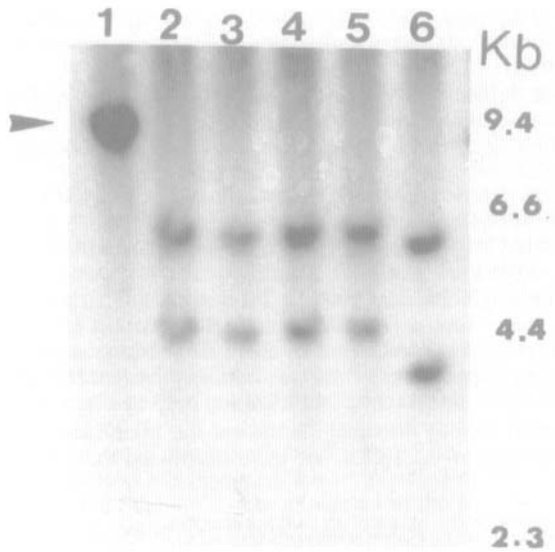

FIGURE 2 – J_H rearrangement in *Hind*III-digested DNA from placenta (1), A1 (2), A2 (3), B1 (4), B2 (5) and PG/B95-8 (6) by Southern blot. The germ-line band is indicated by an arrow-head.

persists largely in small, resting B cells localized in the high-density fraction (Lewin *et al.*, 1987, 1988). Since more than 90% of patient blood lymphocytes corresponded to resting B cells according to the density and less than 0.1% were EBNA-positive, it is likely that the EBV-carrying CLL cells correspond to the B-cell population in which EBV normally exists in a persistently latent form. Thus, the clonal expansion of the CLL cells was due to the leukemic process and the presence of

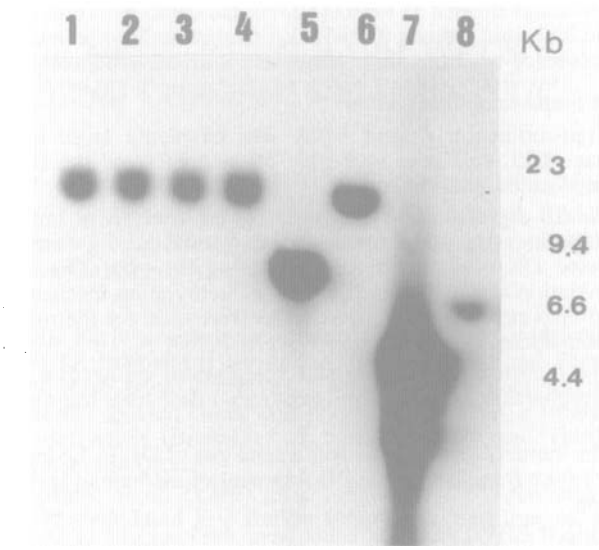

FIGURE 3 – Clonal variation in *Xho*I subfragment of the *Eco*RI-Dhet terminus of *Bam*HI-*Eco*RI double-digested DNA of A1 (1), A2 (2), B1 (3), B2 (4), Daudi (5), Raji (6), B95-8 (7) and PG/B95-8 (8).

the EBV-genome has obviously not provided their carrier cells with any advantage *in vivo*.

Type-1 BL cells express only EBNA1 but not EBNA 2-6 or LMP (Rowe *et al.*, 1987). They may represent the neoplastic counterpart of the *in vivo*-infected B cells that carried the latent virus. In these BL cells, EBV-genomes are methylated. In contrast, EBV genomes of the *in vitro*-transformed LCLs were unmethylated even in cases where the transforming virus had been rescued from the BL (Ernberg *et al.*, 1989).

In view of the fact that these CLL lines are representative of the *in vivo* EBV-infected leukemia cells, we have assessed their EBV-DNA methylation status, in comparison with B95-8-virus-transformed normal B cells derived from the same donor. In contrast to the indistinguishable cellular phenotype and surface-marker expression of the CLL and LCL lines, the viral genomes were partially methylated in the CLL but unmethylated in the LCL line.

On the basis of our earlier detection of latently infected EBV-carrying cells in the small, heavy lymphocyte fraction of healthy individuals (Lewin *et al.*, 1987) and our finding that bone-marrow replacement eradicates the resident virus (Gratama *et al.*, 1988), we have suggested that latent EBV persists in small, heavy B cells (Klein, 1989). BL cells are phenotypically more similar to resting than to activated B cells. We have also speculated that the EBV-DNA methylation and expression status of the BL cells may reflect the state of the viral genome in the corresponding normal B cells. The majority of CLL cells of the peripheral blood also resemble resting B cells with regard to density and surface-marker expression. The methylation status of the EBV genomes in the CLL cells is consistent with this assumption.

One potentially important difference between the BL and CLL lines, on the one hand, and *in vitro* EBV-transformed LCLs, on the other, lies in the fact that the former arise by the proliferation of *in vivo*-infected cells, whereas LCLs are immortalized by an *in vitro* infection that transforms resting B cells into blasts. This event might be accompanied by the activation of many genes. *In vivo*, EBV-carrying proliferating blasts are eliminated by the immune response and thus can only give rise to lymphoproliferative disease in immunodefective recipients (Hanto *et al.*, 1983). Since BL and CLL resemble resting cells, the virus may escape the immune response in healthy individuals by remaining latent in the small resting B

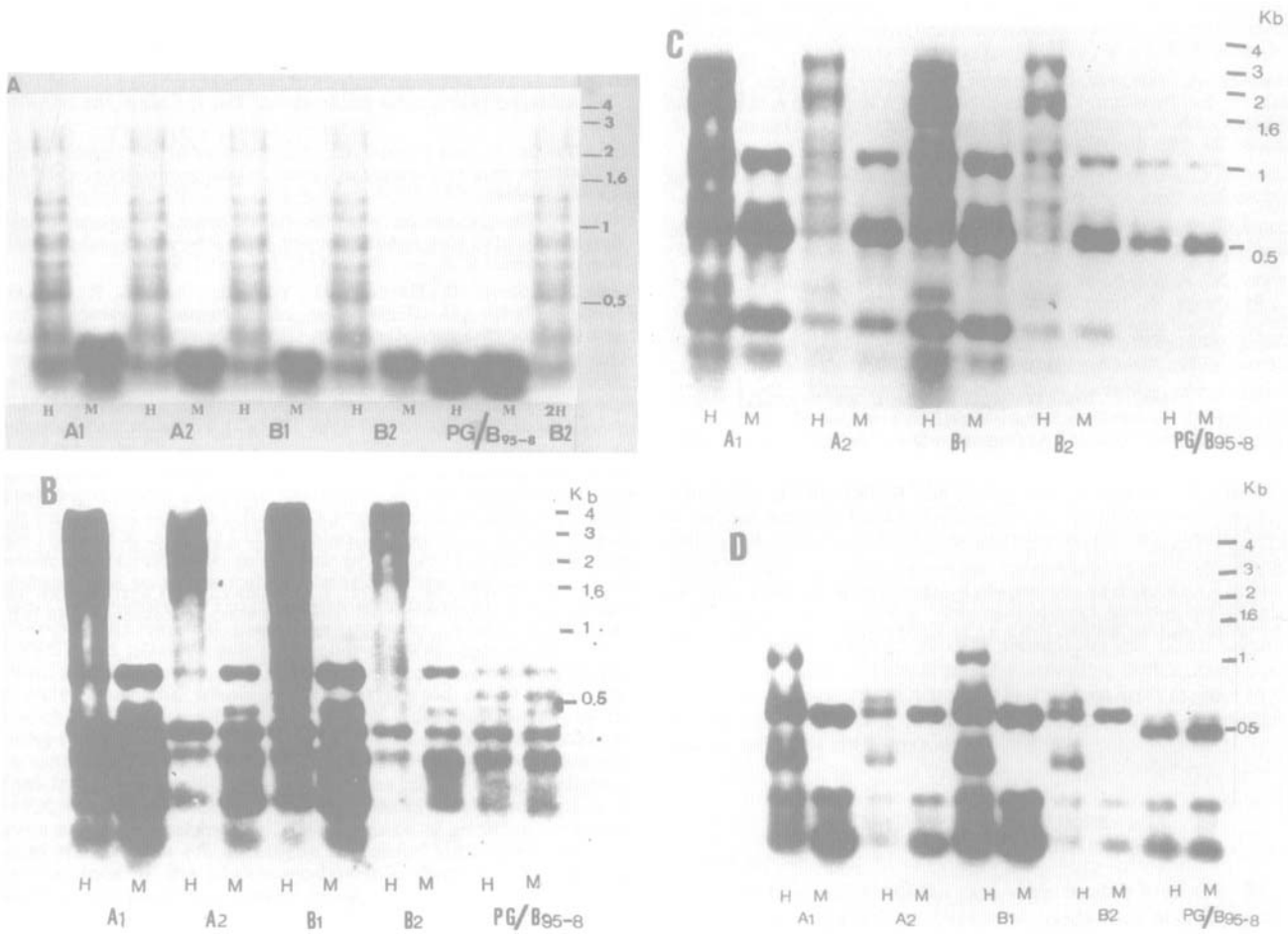

FIGURE 4 – Analysis of the methylation pattern in A1, A2, B1, B2 and PG/B95-8 cell line. Cellular DNAs were cleaved with *Hpa*II (H) or the isoschizomer *Msp*I (M) and probed with a 0.9-kb *Sac*II-*Bam*HI subfragment of the *Bam*HI C *(a)*, BamHI E *(b)*, *Bam*HI H *(c)* and *Bam*HI W *(d)* EBV-DNA fragments. CLL line B2 DNA digested with double amount of *Hpa*II (2 × *Hpa*II) is also shown in Figure 4*a*.

cells. In such cells, the virus may down-regulate its immunogenic proteins, by analogy with BL (Ernberg *et al.*, 1989; Rowe *et al.*, 1987). This may take place when previously expanded B blasts switch to resting cells. This happens twice: after immunoglobulin rearrangement in virgin B cells and after clonal expansion, when the memory cells are generated. The methylation status of EBV-DNA in both BL and CLL cells may reflect the large-scale closing-down of viral and cellular genomes at the time of this transition.

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