

Characterization of Epstein-Barr Virus-Infected Mantle Cell Lymphoma Lines

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It has been reported that Epstein-Barr virus (EBV) resides in resting B cells *in vivo*. However, an ideal *in vitro* system for studying EBV latent infection *in vivo* has not yet been established. In this study, a mantle cell lymphoma line, SP53, was successfully infected with a recombinant EBV containing a neomycin-resistant gene. The EBV-carrying SP53 cells were obtained by selection using G418. They expressed EBER-1, EBNA1, and LMP1; this expression pattern of the EBV genes was similar to that in a lymphoblastoid cell line (LCL). However, proliferation assay showed that the EBV-carrying SP53 cells have a doubling time of 73 h, compared with 57 h of SP53 cells. Transplantation of 10⁸ SP53 cells to nude mice formed tumors in 4 of 10 mice inoculated, but the EBV-carrying SP53 cells did not. Unexpectedly, EBV infection reduced the proliferation and tumorigenicity of SP53 cells. However, the EBV-carrying SP53 cells showed higher resistance to apoptosis induced by serum starvation than did the SP53 cells. The inhibition of proliferation and the resistance to apoptosis induced in SP53 cells by EBV infection indicate that this cell line might to some extent provide a model of *in vivo* EBV reservoir cells.

Key words: Epstein-Barr virus, mantle cell lymphoma, latent infection, *in vivo* reservoir, SP53 line

Epstein-Barr virus (EBV) can immortalize B lymphocytes *in vitro*. EBV-infected B cells grow as lymphoblastoid cell lines (LCL) which express 6 nuclear proteins (EBNA 1-6) from C promoter, 3 plasma membrane proteins (LMP-1, -2a and -2b), and 2 small

nuclear RNAs, (EBER1 and 2) (1). The EBV-encoded proteins interact with cellular proteins in a complex manner which is only partially understood. EBV infection makes resting B cells express B-cell activation markers, such as CD23 and CD30. In contrast to LCL, only EBNA1 from Q promoter and EBERs are expressed in Burkitt's lymphoma *in vivo* and in its cell lines that retain the original phenotype (2). The latent infection of a Burkitt's lymphoma type has been rarely established *in vitro* infection. However, the outgrowth of LCLs might be a kind of *in vitro* artifact which does not exist in healthy individuals (3). EBV harbors in the resting B cells of healthy individuals (4). Immunosurveillance does not allow the growth of LCL *in vivo* (2). Even in immunosuppressed patients, EBV-carrying cells in the peripheral blood are resting memory B cells, not LCL-like B cells (5). EBV infection of Burkitt's lymphoma type is not found in healthy individuals, but is present exclusively in lymphoid malignancies. The detailed mechanism that regulates the virus-induced activation, immortalization, and silent carrier-state of B cells remains to be clarified. Each LCL has an essentially similar phenotype, while each B cell in a primary EBV-infected culture behaves in a different manner from each other (2). Some freshly-EBV-infected B cells die quickly after infection, some grow as lymphoblasts, and some remain in a resting state until they receive additional stimulation. What keeps EBV-carrying B cells in a resting state as EBV reservoir cells *in vivo* remains to be determined.

B-cell chronic lymphocytic leukemia (B-CLL), a CD5⁺ B-cell lymphoid malignancy, represents an accumulation of a single mature B-cell clone in the blood (6). Follicular mantle zone B cells may be the closest normal

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counterpart of B-CLL cells. The majority of CLL cells are readily infected with EBV and express all the virally-encoded nuclear proteins, but are refractory to entering the cell cycle (7-9). EBV-carrying B-CLL cells have weak allostimulatory capacity (7, 10). Thus, EBV infection of B-CLL cells has been studied as an experimental model of B-cell subsets which do not outgrow into LCL after EBV infection and have a greater chance to be free from immunosurveillance. However, B-CLL cells are prone to apoptotic death after EBV infection perhaps due to cell cycle block (9). Thus, B-CLL cells pose certain difficulties due to the problems involved in studying cells or non-proliferating cells in a heterogeneous population.

Mantle cell lymphoma (MCL) is another CD5⁺ B-cell non-Hodgkin's lymphoma that has a strong association with t (11;14) chromosomal translocation (11). Overexpression of cyclin D1 due to this translocation is considered responsible for lymphomagenesis (12). Several cell lines, which retained the MCL phenotype and characteristic translocation, have been established from MCL (13-15). An earlier attempt to infect MCL line cells with EBV were unsuccessful although they express CD21, an EBV receptor (13). We postulated that this is because EBV infection, by means of some unknown mechanism, inhibits proliferation of the infected MCL cells, which are then extinguished. In the present study, we infected a MCL line, SP53, with recombinant EBV containing a neomycin-resistant gene which allowed elimination of the EBV-negative cells by selection under the existence of G418 (16). EBV-positive MCL lines were then readily established. On the other hand, an EBV-carrying MCL line, SP50B, was established independently from SP53 from the same patient (14, 17). This cell line had the typical LCL phenotype and had the same chromosomal translocation as SP53. We report here the influence of EBV infection on SP53 cells and compare the EBV-carrying SP53 cells with the SP50B cells.

Materials and Methods

Cells. Three mantle cell lymphoma cell lines (SP-49, SP-53 and SP50B) were cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS). To induce apoptosis, the cells were incubated in RPMI1640 supplemented with 1% FCS for 0 h, 24 h, 48 h, and 72 h. The cells autosmeared onto glass slides were fixed in 1:1 acetone/methanol at -20 °C for immunostaining, or were fixed in 10% formalin-0.1 M phosphate buffer (pH

7.4) overnight at 4 °C for *in situ* hybridization.

EBV infection. The cell lines were infected with EBV from the culture supernatant of the B95-8 cells or from anti-IgG-treated Akata cells carrying recombinant EBV that contained the neomycin-resistant gene at the BXLF1 site of EBV DNA (Akata-EBV). After infection, the cells were selected by 200-300 µg/ml of G418 (Sigma Chemical Co., St Louis, MO, USA). Peripheral blood mononuclear cells were prepared from healthy individuals by centrifugation on a Ficoll-Hypaque gradient. The whole mononuclear cells were treated with the virus for 1 h and cultured in RPMI1640 supplemented with 20% FCS and 500 ng/ml of cyclosporine A.

Immunostaining and flow cytometric analysis. EBNA was examined on acetone/methanol-fixed cells by anticomplement immunofluorescence (ACIF) using EBV-seropositive human serum (18). EBNA2 and LMP1 were detected by mouse monoclonal antibodies (mAbs), PE2 and CS1-4, respectively. Immunofluorescence was captured using a CCD camera (C4880, Hamamatsu Photonix Ltd, Hamamatsu, Japan). To detect Ki67 antigen in nuclei, cells were fixed in 400 µl of 2% paraformaldehyde solution (Sigma Chemical Co.) on ice for 10 min and followed by treatment with 1.0% solution of saponin (Sigma Chemical Co.) in phosphate buffered saline (PBS) for 5 min (19). For detection of apoptosis, the cells were incubated in 10 µl each of FITC-conjugated Annexin V (10 µg/ml, Bender Med-Systems Diagnostics GmbH, Vienna, Austria), and propidium iodide (50 µg/ml in PBS) on ice for 10 min in the dark. Fluorescence was measured immediately by flow cytometry using FACScan (Beckton Dickinson, San Jose, CA, USA). Annexin V⁺PI⁻ cells in early apoptosis were quantified separately from Annexin V⁺PI⁺ cells in late apoptosis and necrosis after serum starvation. Immunophenotypes of the cells were also analyzed by flow cytometry. The antisera used are listed in Table 1.

In situ hybridization. EBER1 *in situ* hybridization was performed by applying 0.5 ng/µl of FITC-labeled anti-sense EBER1 oligonucleotide probe as described previously (20).

Western blot analysis. Western blotting was performed as reported previously (21). Briefly, the lysate of 1 × 10⁶ cells of each cell line were size-fractionated by SDS-10% polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Ltd, Bedford, MA, USA). After blocking with 5% non-fat dry milk in PBS, the mem-

brane was incubated with anti-EBNA2 mAb, PE2, and then with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin. The antigens were visualized using the enhanced chemiluminescence Western blotting system (Amersham Japan Co., Tokyo, Japan) according to the manufacturer's instructions.

Southern blot analysis. The extracted DNA (5 μ g) was digested with BamHI or with BamHI and BglII (Bethesda Research Laboratories, Rockville, MD, USA). After being transferred onto nylon membranes, the digested DNA was hybridized with BamHI-W fragments of EBV DNA. The hybridized probe was detected using Gene Images TM (Amersham).

Proliferation assay. G418 was washed out from the culture one day before the experiment. The population-doubling time and saturation cell density were determined by seeding 4×10^5 viable cells into the wells of 12-well culture plates (Nippon Becton Dickinson Co., Tokyo, Japan) and counting the number of cells in each well every day for 8 days.

Table 1 The antibodies used in the study

Antibodies	Clone	Isotype	Source
CD3	UCHT1	Mouse IgG1	BD
CD5	UCHT2	Mouse IgG1	BD
CD10	SS2/26	Mouse IgG1	Dako
CD15	C3D-1	Mouse IgM	Dako
CD19	HD37	Mouse IgG1	Dako
CD20	B-LY1	Mouse IgG1	Dako
CD21	IF8	Mouse IgG1	Dako
CD23	MHM6	Mouse IgG1	Dako
CD25	ACT-1	Mouse IgG1	Dako
CD30	Ber-H2	Mouse IgG1	Dako
CD38	HIT2	Mouse IgG1	BD
CD39	TU66	Mouse IgG2b	BD
CD40	5C3	Mouse IgG1	BD
CD80	BB1	Mouse IgM	BD
CD86	FUN-1	Mouse IgG1	BD
BCL-2	124	Mouse IgG1	Dako
CyclinD1	P2D11F11	Mouse IgG1	Novo
EBNA2	PE2	Mouse IgG1	Dako
LMP-1	CSI-4	Mouse	Novo
Ki67		Rabbit	Dako
Human IgA		Rabbit	Dako
Human IgD		Rabbit	Dako
Human IgG		Rabbit	Dako
Human IgM		Rabbit	Dako

BD, Becton Dickison, San Jose, CA, USA; Dako, Dako, Glostrup, Denmark; Novo, Novocastra, London, UK.

Tumorigenicity in mice. Tumorigenicity was tested by inoculating 1.0×10^7 or 1.0×10^8 cells subcutaneously into 5-week-old female nude mice (BALB/c AnN cri-nu from Charles River Japan, Inc, Yokohama, Japan) which were subsequently observed for tumor formation for 16 weeks.

Results

EBV infection of mantle cell lymphoma lines. A few percent of the SP53 cells infected with B95-8 EBV were positive for EBNA2 in the first week after the infection. However, EBNA2-positive cells became undetectable in the 3rd week (data not shown). In contrast, in Akata-EBV-infected SP49 and SP53 cultures incubated in 100–200 μ g of G418, the proportion of EBNA2-positive cells gradually increased and reached almost 100% at the 3rd week (Fig. 1F). They were also positive for ACIF staining with the serum of an EBV-seropositive individual (Fig. 1H). About one-third of the cells showed cytoplasmic and membranous staining for LMP1 (Fig. 1J). About 80% of the cells were positive for EBER1- *in situ* hybridization (Fig. 1L). These results indicated that EBV-carrying SP53 has an LCL-like EBV gene expression pattern with the full spectrum of latent EBV genes. Western blot analysis also confirmed EBNA2 expression (Fig. 2). Existence of the EBV genome in the neomycin-resistant cells was confirmed by Southern hybridization using an EBV BamH-W probe (Fig. 3). Because the growth of EBV-carrying SP49 cells was quite slow, the Akata-EBV-carrying SP53 line was subjected to the subsequent study.

The phenotype of SP53 cells did not change after EBV infection. The phenotypic features of non-infected SP53 and EBV-carrying SP53 cells and SP50B cells are summarized in Table 2, and representative results of FACS analysis are shown in Fig. 4. SP53 cells expressed CD5, CD19, CD20, CD21, IgD, IgM, and cyclinD1, and were negative for CD10 and some activation markers, CD23 and CD30, which were consistent with the MCL phenotype. Some other activation markers, CD38, CD80, and CD86, were positive. EBV infection did not induce the expression of CD23 and CD30 in SP53 cells, while they were induced in freshly EBV-infected PBL. The expression intensity of the other markers remained unchanged after EBV infection except for CD38, whose expression was augmented. Forward scatter showed that EBV-negative

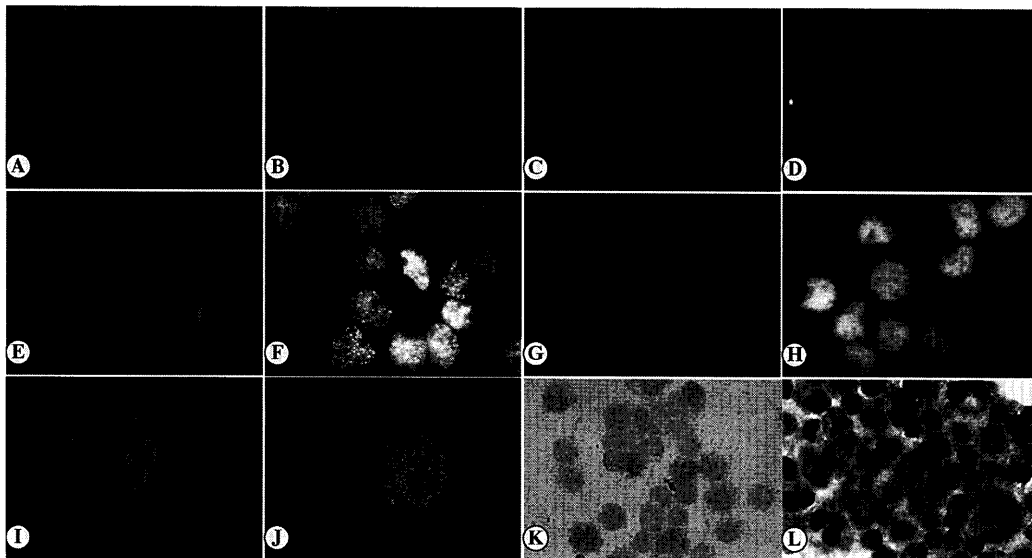


Fig. 1 Expression of Epstein-Barr Virus (EBV) genes in EBV-carrying SP53 cells. EBNA2 is not detected in SP53 cells by immunofluorescent staining using anti-EBNA2 mAb (PE-2) (A, Hoechst; B, PE-2, FITC), but is detected in Akata-EBV-infected SP53 cells (E, Hoechst; F, PE-2 FITC). Anti-complement immunofluorescence (ACIF) with anti-EBV-positive human serum demonstrates EBNA2 in Akata-EBV-infected SP53 cells (G, Hoechst; H, ACIF FITC), but not in noninfected SP53 cells (C, Hoechst; D, ACIF, FITC). LMPI is detected as numerous dots in the cytoplasm of Akata-EBV-infected SP53 cells (I, Hoechst; J, CSI-4, FITC). EBNA2 is detected in the nuclei of Akata-EBV-infected SP53 cells (L), but not in SP53 cells (K).

A-D, K, SP53; E-J, L, EBV-carrying SP53.

A-H, K and L, Original magnification, $\times 400$; I and J, Original magnification, $\times 1000$.

SP53 and EBV-carrying SP53 cells had a similar cell size. These observations indicated that EBV does not cause the phenotypic change after infection as it does in PBL-B cells. SP50B cells had the typical LCL-like phenotype expressing CD23, CD30, and CD40.

The proliferating activity of EBV-infected SP53 cells was lower than that of EBV non-infected SP53 cells. The EBV-carrying SP53 cells proliferated slower than did the original SP53 cells. The doubling time of EBV-negative SP53 cells was 57 h, while that of EBV-carrying SP53 cells was 73 h. The saturation density was higher in SP53 cells [$(2.00 \pm 0.11) \times 10^6$] than in EBV-infected SP53 cells [$(1.10 \pm 0.15) \times 10^6$]. The doubling time of SP50B cells was 34 h, and the saturation density was 3.5×10^6 (Fig. 5). FACS analysis showed that Ki67-expressing cells were more numerous in SP53 cells than in EBV-carrying SP53 cells. The EBV-carrying SP53 cells showed lower proliferation activity than did the original SP53 cells.

EBV-carrying SP53 cells were more resistant to apoptotic stimuli. Medium containing a very low percentage (1%) of FCS induced apoptotic cell

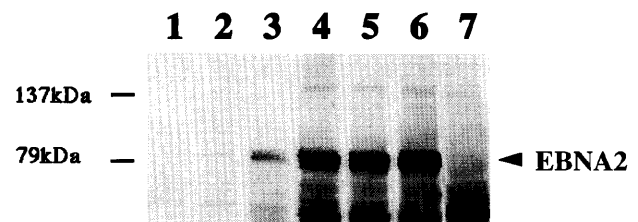


Fig. 2 Western blotting of EBNA2.

Lane 1, SP49; lane 2, Sp-53; lane 3, SP50B; lane 4, EBV-carrying SP49; lane 5, 6, EBV-carrying SP53; lane 7, Akata-EBV. Lanes 5 and 6 represent the different EBV-infected Sp53 cultures. Molecular size of EBNA 2 is indicated by the arrowhead.

death in SP53 cells during the first 8 h, with the number of dead cells increasing time-dependently, while EBV-carrying SP53 cells maintained their resistance to apoptotic cell death even after 72 h (Fig. 6).

Tumorigenicity of SP53 was suppressed by EBV infection. Subcutaneous inoculation of neither 10^7 EBV-negative nor EBV-carrying SP53 cells

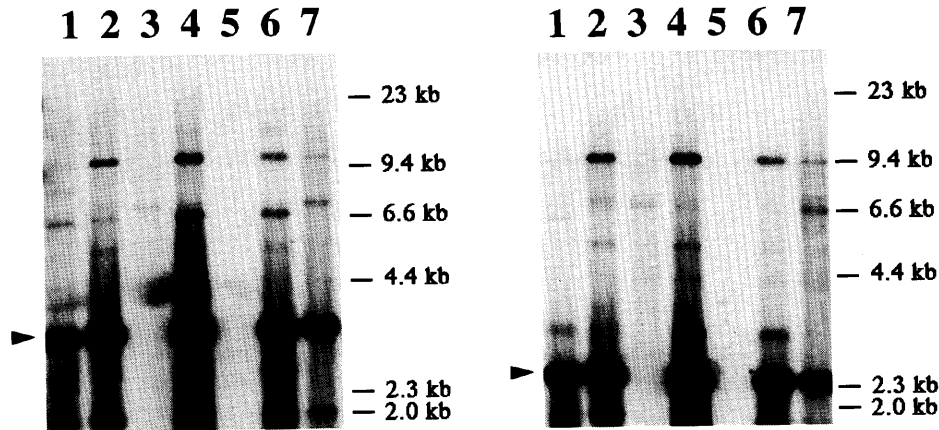


Fig. 3 Southern blot analysis of EBV DNA. *Bam*HIW fragment of EBV DNA (arrowheads) was detected. DNA was digested by *Bam*HI (a) and *Bam*HI and *Bgl*II (b).

Lane 1, SP50B; Lane 2, Akata-EBV; Lane 3, SP49; Lane 4, EBV-carrying SP49; Lane 5, Sp-53; Lanes 6, 7, EBV-carrying SP53. Lanes 6 and 7 represent the different EBV-infected SP53 cultures.

Table 2 Immunophenotype of the cell lines

Antibodies	Cell lines			
	SP50B	SP53	SP53EBV-1	SP53EBV-2
CD3	-	-	-	-
CD5	-	+	-	-
CD10	-	-	-	-
CD15	-	-	-	-
CD19	++++	++++	++++	++++
CD20	++++	++++	++++	++++
CD21	+	+	ND	ND
CD23	++	-	-	-
CD25	-	-	-	-
CD30	++	-	-	-
CD38	++++	+	++++	++++
CD39	++++	+	+	+
CD40	++++	++	+	+
CD80	+	++	++	++
CD86	++++	++++	++++	++++
Human IgG	-	-	-	-
Human IgD	+	++	-	-
Human IgM	+++	+++	++	++
Human IgA	-	-	-	-
BCL-2	+	++	+	+
CyclinD1	++	++	ND	ND
Ki67	+++	+++	+	+

-, mean fluorescence intensity is less than 10; +, 10-49; ++, 50-99; +++, 100-149; +++++, ≥ 150.
 ND, not done.

induced tumor formation in nude mice. EBV-negative SP53 cells induced tumors in 4 of 10 nude mice inoculated with 10⁸ cells, while EBV-carrying SP53 cells did not (0/10) (*P* < 0.05). Thus, EBV infection decreased the tumorigenicity of SP53.

Discussion

It has been reported that SP53 cells were not converted into EBV-carrying cells by EBV infection (3). However, EBV-carrying SP53 cell line was readily established by the 3-week selection of Akata-EBV-infected cells in the G418-containing medium. The proliferation assay and the positive rate for Ki67 showed that EBV-carrying SP53 cells have lower proliferating activity. These results support our hypothesis that EBV cannot easily convert SP53 cells into EBV-carrying cells because EBV-carrying SP53 cells grow slower than do non-infected SP53 cells. EBV induced the proliferation of PBL-B cells, but did not augment the proliferation of SP53 cells.

EBV infection did not change the phenotype of SP53 cells to the LCL-like phenotype as shown by FACS analysis in spite of the full expression of latent EBV genes. EBV infection rendered SP53 cells less proliferative and less tumorigenic. It is known that EBV latent genes interact with a variety of cellular proteins. EBNA2 induces the transcription of LMP1, 2a and 2b, CD21, CD23, and c-fgr (1). LMP1 is a potent transforming

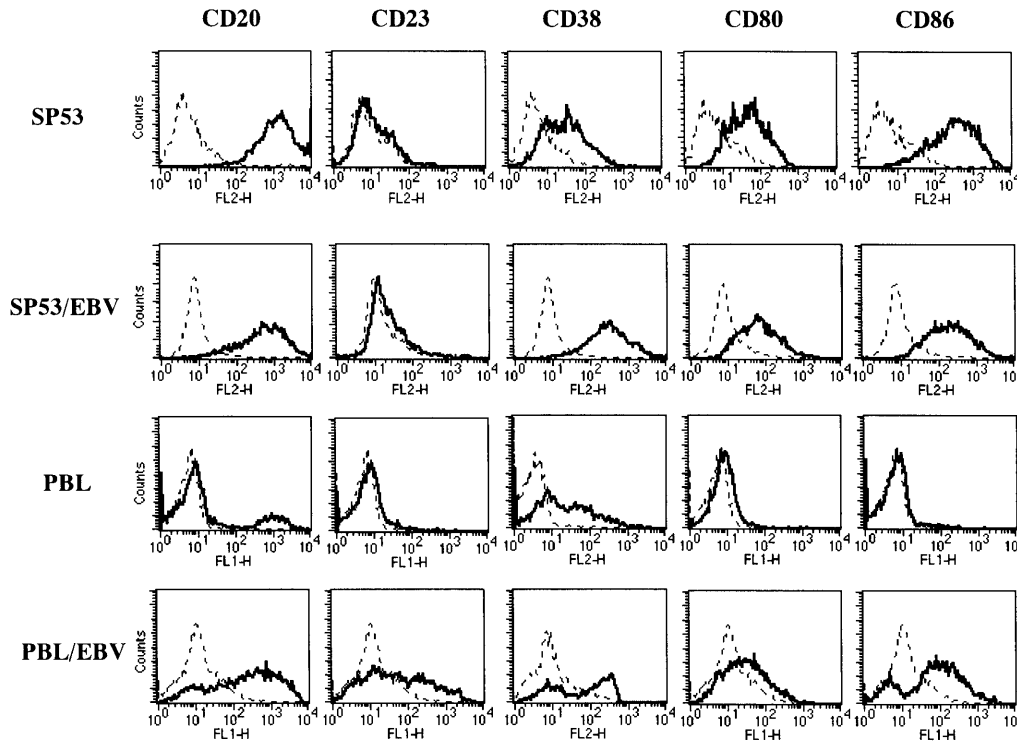


Fig. 4 Phenotypic analysis by flow cytometry. SP53/EBV, EBV-carrying SP53; PBL, peripheral blood lymphocytes; PBL/EBV, EBV-infected PBL 3 weeks after infection; broken line, reacted with control mouse IgG; solid line, reacted with examined antibodies.

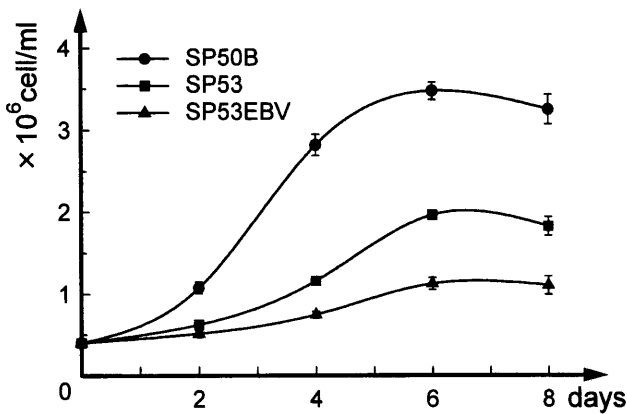


Fig. 5 Proliferation assay. Each value represents the average of triplicates \pm SD.

EBV gene which acts as a constitutively active CD40 (22), and is a key protein of EBV-induced B cell transformation. Transfection of LMP1 alone transforms B cells, and LMP1 works as a potent apoptosis inhibitor through activation of NF κ B (1). Thus, EBV-carrying SP53 cells' resistance to apoptotic stimuli may be due to the effect of LMP1 function. However, the reduced proliferation and tumorigenicity of EBV-carrying SP53 cells is inconsistent with the function of LMP1, a potent transforming viral oncogene. Perhaps some blockage might occur in the signal transduction pathway of LMP1 in the EBV-carrying SP53 cells. Recently, it has been reported that EBERs express anti-apoptotic activity (23). EBERs may also be responsible for the increased resistance to the apoptotic stimuli.

EBV is now considered to be reserved in resting B cells (2). EBV-reservoir B cells do not behave *in vivo* as LCLs do because the LCL causes severe immunological reactions (2). Only EBNA1 and EBERs are expressed in Burkitt-type latency. Since they are not immunoreactive,

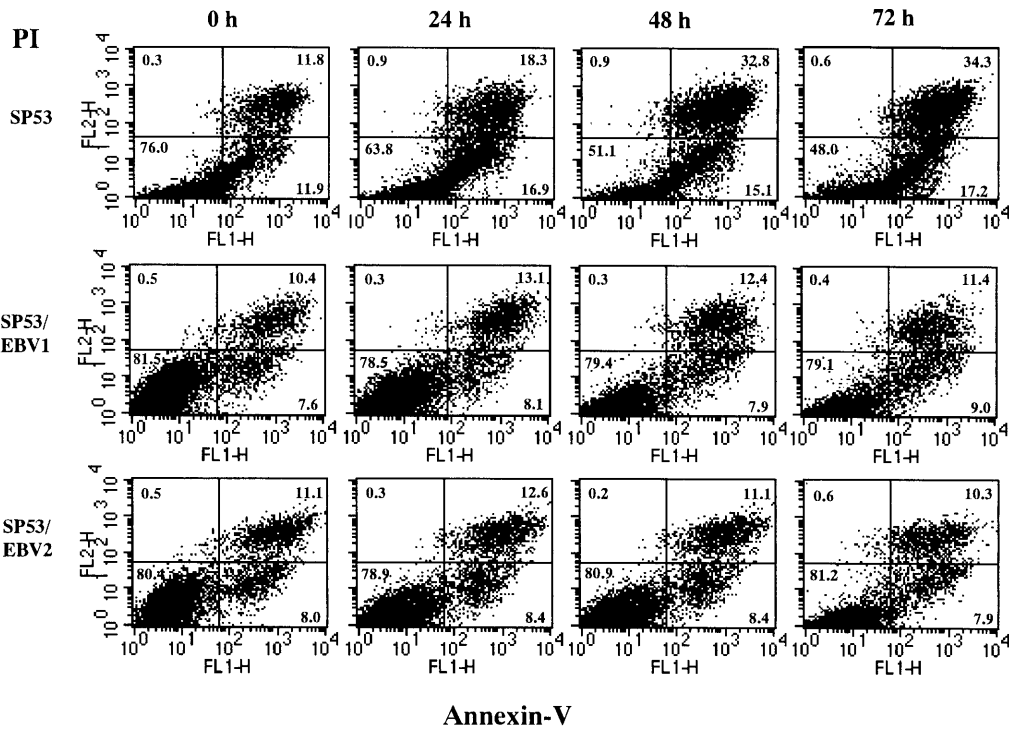


Fig. 6 Apoptosis after serum starvation. Cells were incubated in the culture medium with 1% fetal calf serum (FCS) and harvested after 0 h, 24 h, 48 h, and 72 h. The percentages of living cells (left lower corner), cells in early apoptosis (right lower corner), and cells in late apoptosis and dead cells (right upper corner) are provided in the figures. SP53/EBV-1 and SP53/EBV-2 represent the EBV-carrying SP53 established at different times.

it has been supposed that EBV in reservoir B cells shows Burkitt-type latency. However, whether or not such latency plays an important role in healthy individuals remained undetermined. Some freshly EBV-infected B-cells expressing EBNA2 have been seen to remain resting *in vitro* (9). Niedobitek *et al.* reported that non-proliferative EBV-infected cells showing EBNA2 expression were detected in the lymph nodes of patients with infectious mononucleosis (24). Non-proliferating EBV-infected B cells are an important form of EBV infection *in vivo*. Non-proliferating cells are supposed to be less immunoreactive even if the full spectrum of EBNA2 is expressed (7, 10). Non-proliferating cells in a fresh EBV-infected culture might be precursors of EBV reservoir cells.

Since EBV-carrying B cells *in vivo* are non-proliferating, it is interesting to investigate non-proliferating EBV-positive B cells. However, it is very difficult to analyze non-proliferating cells in a heterogene-

ous population of primary cultures. Since B-CLL comprises clonal cells, it has been used as an example of a pure subset of B cells which do not proliferate after EBV infection (7-9). However, some B-CLL cells have demonstrated the cell cycle block which might affect the interaction of EBV and B cells (9). SP53 cells showed no cell cycle block. The inhibition of proliferation and resistance to apoptosis induced in SP53 cells by EBV infection indicates that, to some extent, this cell line might be a model of an EBV reservoir cell *in vivo*.

Although SP53 cells expressed CD21, the EBV receptor, they have been previously reported to be unsusceptible to EBV infection. Our study showed that this is because SP53 cells proliferate more slowly after EBV infection. SP50B cells had a character quite different from the EBV-carrying SP53 cells although both came from the same patient with MCL and had the same chromosomal translocation, t(11;14). SP50B cells showed the LCL phenotype, expressing CD23, CD30,

and CD40, but not CD5. SP50B cells showed much higher rates of proliferation and tumorigenicity than did SP53 cells and EBV-carrying SP53 cells (data not shown). Therefore, some stimulatory condition other than expression of the full spectrum of EBV latent genes might be responsible for induction of the LCL phenotype and the outgrowth of SP50B cells. The LCL phenotype might be also induced in EBV-carrying SP53 cells by some additional stimulation. This hypothesis is in line with our previous report that some B cells can remain in a resting state after EBV infection but can be activated later (9). However, the precise mechanism that modulates the dormancy and activation of EBV-infected B cells remains to be elucidated.

Acknowledgments. We thank Ms. Sachiyo Onoda, Ms. Reiko Endo, Ms. Miyuki Shiotani, and Ms. Mutsumi Okabe for their technical help and Dr. Ichiro Kubonishi (Department of Internal Medicine, Kochi Medical School) for providing the mantle cell lymphoma cell lines (SP49, SP53 and SP50B).

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Received April 24, 2000; accepted June 2, 2000.