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Epstein-Barr Virus Infection in Human T-cells

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Abstract The oncogenic potential of Epstein-Barr virus (EBV) for Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) has been extended to other neoplasias, such as Hodgkin's disease, gastric cancer, and peripheral T-cell tumor. The pattern of EBV gene expression observed in some EBV-associated T-cell tumors is similar to the pattern also seen in NPC and in EBV-positive cases of Hodgkin's disease. However, the inability to reproduce this latency II form of EBV infection *in vitro* has hampered study of the factors influencing EBV gene expression in different cellular environments. We have generated a recombinant EBV carrying a selectable marker (neomycin resistance gene) targeted to the viral thymidine kinase locus. Infection of the MT-2, a human T-cell line, with the recombinant virus and subsequent isolation of drug resistant clones resulted in the lines carrying EBV episomes. EBV-infected MT-2 cell clones expressed EBNA 1 and LMP 1 and very little of EBNA 2, showing the *Bam*HI F promoter-driven latency II form of infection, which is seen in non-B cell tumors. Thus, latency II type EBV infection could be reproduced *in vitro*. A very useful model system for establishing stable EBV infection of different cell lineages *in vitro* has been provided.

Key words : *Bam*HI F/Q promoter, CD21, Epstein-Barr virus, Recombinant virus, T-cell

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpes virus and more than 90% of the people in Japan have been infected with this virus at the age of 20. While primary infection of this virus in infancy appears to be asymptomatic, it causes infectious mononucleosis in early adult life. EBV has been also shown to be necessary to induce human cancer.

EBV has been conventionally thought to infect B lymphocytes and epithelial cells, since it was discovered in Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) 30 years ago (1, 2). However, subsequent studies linked EBV to post-transplant B-cell lymphoma, HIV-associated lymphoma, Hodgkin's disease (HD), and gastric carcinoma (3, 4, 5). Recently, the infection of T-cells and T-cell lymphomas by EBV in patients with chronic EBV infection has been demonstrated (6). The detailed clinicopathological, immunological, and virological aspects of EBV-containing T lymphoproliferative disorders have been increasingly clarified (7).

All the EBV-carrying cells regulate the expression of the viral genomes in non-lytically infected cells in different ways (Table 1) (8). Lymphoblastoid cell lines express six nuclear antigens (EBNAs 1 to 6) and three membrane antigens (LMPs 1, 2A and 2B). This full pattern of latent gene

Table 1. Promoter usage and EBV-gene expression in EBV-associated tumors

Latency	Type I	Type II	Type III
Antigen expression			
EBNA 1	+	+	+
EBNA 2, 3ABC, LP	−	−	+
LMP 1	−	+/−	+
LMP 2A	+/−	+/−	+
LMP 2B	−	+/−	+
BARF 0	+	+	+
EBERs	+	+	+
Promoter	F/Qp	F/Qp	Cp or Wp
Tumors	Burkitt's Lymphoma	Nasopharyngeal Carcinoma	Lymphoblastoid Cell Lines
	Gastric Carcinoma	Hodgkin's Disease	Lymphoma in Immuno-compromised Host
		T-cell Lymphoma	

expression was designated type III form of latency. The individual EBNA mRNAs are generated by differential splicing of long primary transcripts expressed from one of two promoters (Wp and Cp) located in the *Bam*HI W and adjacent *Bam*HI C regions of the EBV genome, respectively. The LMP transcripts are expressed from separate promoters in the *Bam*HI N region that are responsive to transactivation by EBNA 2. EBV-carrying BL cells express only EBNA 1. This type I form of latency is reflected at the mRNA level by a distinct pattern of virus gene transcription. Thus, type I latency does not use either Wp or Cp promoter, but a novel promoter located near the *Bam*HI F/Q boundary (F/Qp). In contrast to the well-characterized forms of latency I and III, latency II is characterized to express all the LMPs and F/Qp-driven EBNA 1. This latency II form of infection was recognized in non-B-cell tumors, such as NPC, T-cell tumors, and HD. However, there was no good *in vitro* model of latency II infection (9).

In vitro host range restriction of EBV is partly due to the level of expression of a cell surface protein CD21, which is also the receptor for the C3d component of complement (10). EBV infection of B lymphocytes starts from its binding with cell surface molecule CD21 through viral envelope protein, gp350/220 (11). Although high-level expression of CD21 could be observed on human T-or erythroleukemic cells and human epithelial cells, EBV infection was aborted after the step of adsorption (12).

We have reported the generation of a system, which allows production of large amount of recombinant EBV and reconstitution of *in vitro* model of EBV infection of human T-cells (9). An EBV recombinant in which viral thymidine kinase encoding BXLF-1 gene was replaced with a selectable marker (neomycin resistance gene) was infected with a human T-lymphotropic virus type I-infected T-cell line, MT-2. Subsequent isolation of drug resistant clones resulted in the lines carrying EBV episomes and expressing the latency II form of EBV infection. Interestingly, EBV infection had no effect on the growth or phenotypic characteristics of the MT-2 cells.

EBV Infected T-Cell Lymphomas Observed *In Vivo*

A wide range of T-cell malignancies have been examined for EBV status by detection methods of varying sensitivity, mostly by EBER *in situ* hybridization. Three distinct categories of T-cell tumor are considered to be linked with the virus (13).

1) Hemophagocytic Syndrome-Associated T-Cell Lymphocytosis/Lymphoma

This entity covers a spectrum of diseases in the context of a primary EBV infection, which includes virus associated hemophagocytic syndrome-like disease, fatal form

of infectious mononucleosis, and malignant histiocytosis. EBV-positive T-cell proliferations are ranging from polyclonal lymphocytosis to monoclonal lymphoma. The T-cell subset involved can be variable. The release of cytokines, particularly tumor necrosis factor-α (TNFα) and interferon-γ from the infected and activated T-cells has been suggested to play a role in disease pathogenesis.

2) Nasal T-Cell Lymphoma

This group of disorders are labelled with extranodal T-cell lymphoma of angiocentric immunoproliferative lesions in the nasal cavity and are common in Southeast Asia. This entity has been previously designated polymorphic reticulosis, lethal midline granuloma, or lymphomatoid granulomatosis and causes progressive erosion of bone tissue. Once disseminated, it usually runs an aggressive course. EBV has been consistently present in all tumor cells. The tumor cells do not demonstrate clonal rearrangement of T-cell receptor genes, but display any one of T-cell phenotypes. However, the expression of natural killer (NK) cell antigen, CD56 and the lack of expression of CD3 and CD4/CD8 markers may suggest a linkage of the neoplastic cells to NK cells (14). Selective expression of F/Qp-initiated EBNA 1 transcripts and the LMP mRNAs like Latency II pattern has been detected.

3) Peripheral T-Cell Lymphomas of Angioimmunoblastic Lymphadenopathy (AILD)/ Pleomorphic Types

Lymphomas with AILD-like appearance and pleomorphic medium/large cell lymphomas have been identified with an EBV association, which may range from a benign hyperimmune process to neoplastic disease. EBV infection could be identified by EBER *in situ* hybridization, but EBER-positive cells constituted only a fraction (5 to 50%) of the total malignant population. The viral gene expression pattern shows EBNA 2-negative/ LMP 1-positive type II pattern of latency. The facts that LMP 1 expression is restricted to the EBER-positive cells and EBV-positive fraction of the malignant cell population is substantially increased on the progression of tumors suggest that the virus can confer a growth advantage.

Several human T-cell lines of leukemic origin express CD21 molecule and are capable of binding EBV, although this dose not always lead to viral penetration and active infection (12). However, once it happens to gain access to this lineage *in vivo*, EBV has oncogenic potential for T-cells. Such access probably occurs only rarely. In this regard, T-cells infiltrating sites of virus replication *in vivo* might be expected to be at particular risk for EBV infection and subsequent malignant transformation.

Clonal Propagation of EBV Recombinants

We have generated a system for producing large quantities of different EBV recombinants without having to select on the basis of transformation competence (15). Akata cell line is derived from an EBV-positive BL of a Japanese patient and posesses around 20 copies of EBV plasmid per cell. This Akata line expresses surface Ig of the G (k) class (16). The Akata cell line was subcloned to isolate 100% EBV-positive Akata clones and completely EBV-negative Akata clones (17).

An EBV recombinant with a selectable marker at the viral thymidine kinase (TK) gene, which homologue of herpes simplex virus type 1 is nonessential for infection and replication, has been generated (Fig. 1) (9). The neomycin resistant (neo^r) gene was inserted into the TK-encoding BXLF-1 site of an EBV plasmid of Akata cells by the homologous recombination technique. An EBV recombinant clone has been isolated through EBV-negative Akata cell clone, then induced in large amount by cross linking cell surface IgG (15) (Fig. 2).

EBV Infection of T-Cell Line

Cell-free EBV was obtained by centrifuging induced cultures and filtrating the supernatants through a 0.45 μm filter. Viral supernatants in growth medium was infected with EBV-negative MT-2 cells (18). After 90 min, cells were pelleted, resuspended in fresh medium, and incubated for 2 days. Cells were, then plated in 96-well, flat-bottom plates with complete medium containing 0.5 mg/ml of G418. Cells were fed every 5 days until colonies were emerged. EBV-infection of MT-2 cells was examined by the expression

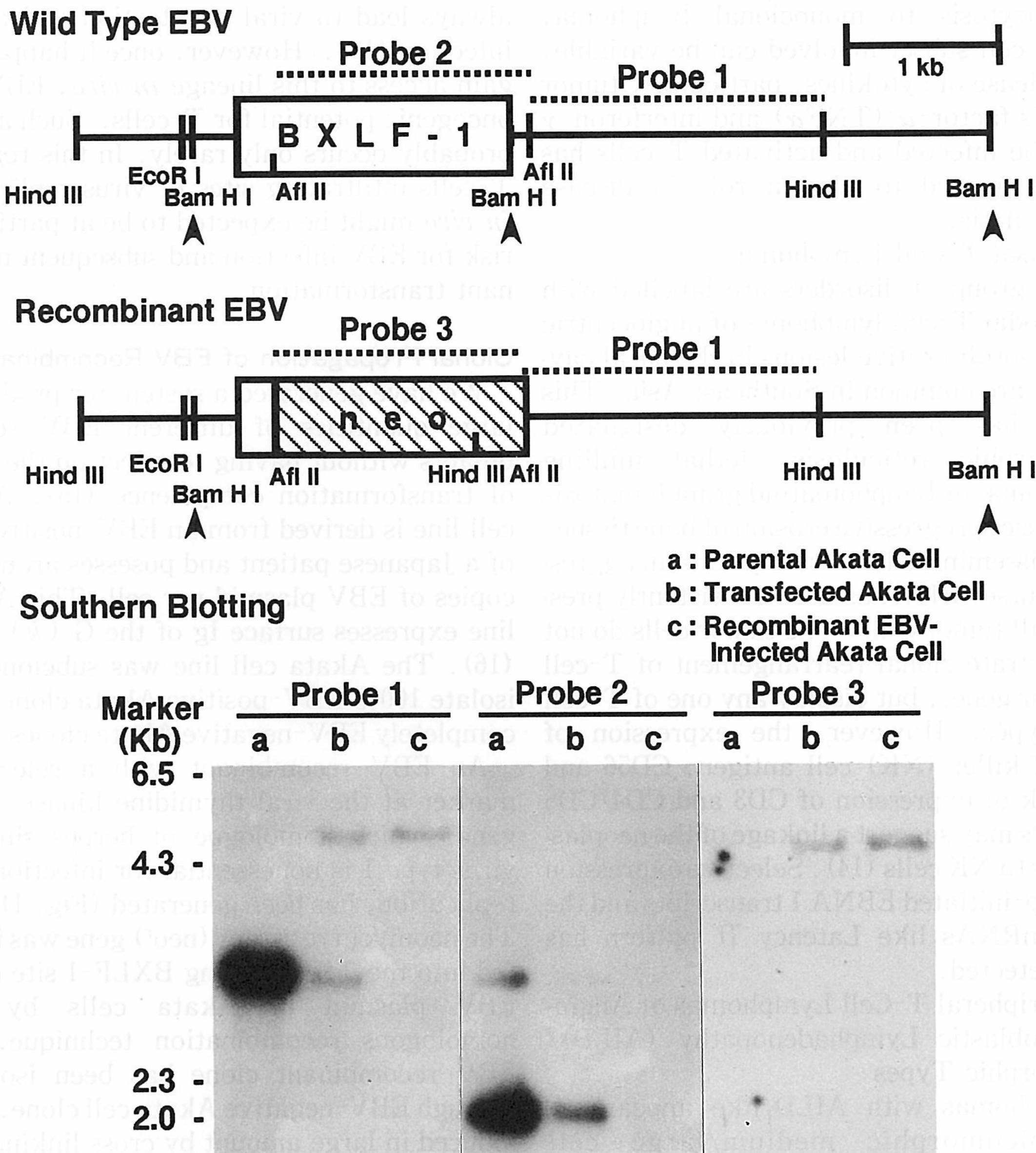

Fig. 1. Generation of recombinant EBV with a selectable marker. (Upper panel) Schematic representation of plasmid constructs used to insert *neo*^{r} gene into the EBV genome. Map of the BXLF-1 region is shown at the top. The pXneo plasmid was derived by inserting an SV40 early promoter-driven *neo*^{r} gene into the BXLF-1 open reading frame in an EBV (Akata) *Eco*RI/*Hind*III DNA clone. When the pXneo plasmid had recombined into the BXLF-1 site, a novel 5.3 Kb *Bam*HI fragment should appear due to a deletion of *Bam*HI site on the right (the bottom line). (Lower panel) Southern blot analysis of targeted Akata cell clones. Cellular DNAs were digested with *Bam*HI, blotted, and hybridized with the probe 1, probe 2, or probe 3. Probe 2 did not hybridize with recombinant virus, whereas probe 3 did not hybridized with wild virus.

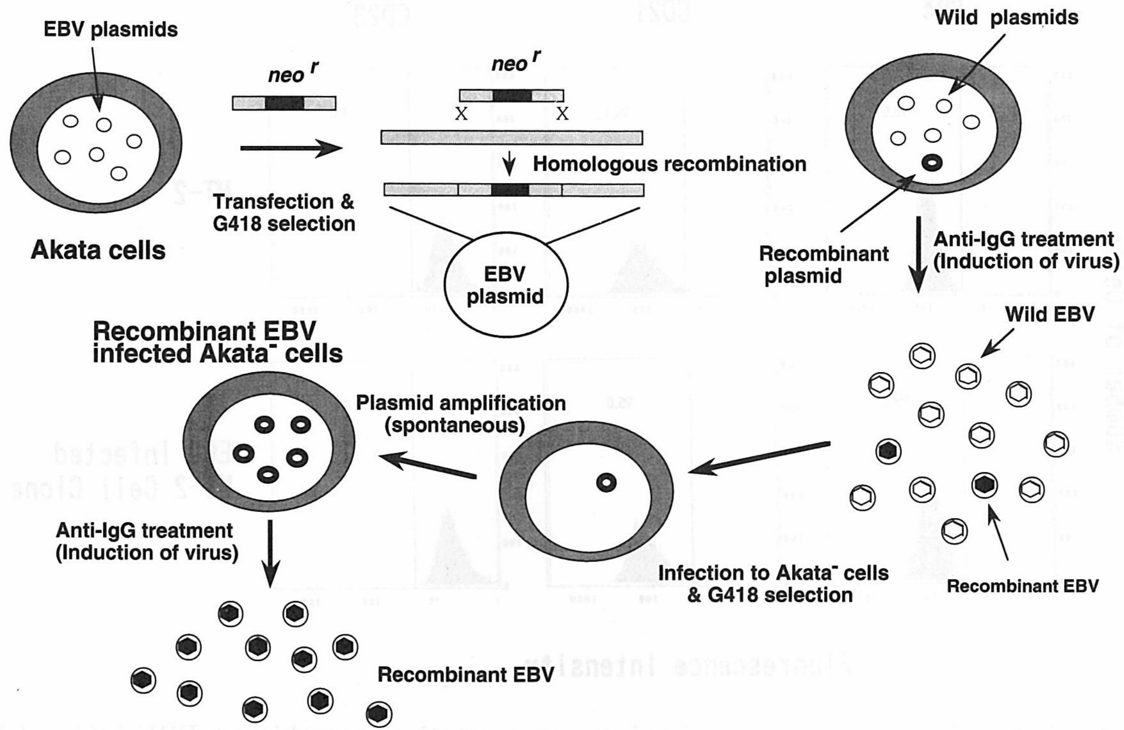

Fig. 2. Schematic representation of the recombinant EBV generating system. Clonal propagation of EBV recombinants could be performed by cross linking cell surface Ig G by anti-Ig antibody. *neo*^{r} : neomycin resistant gene.

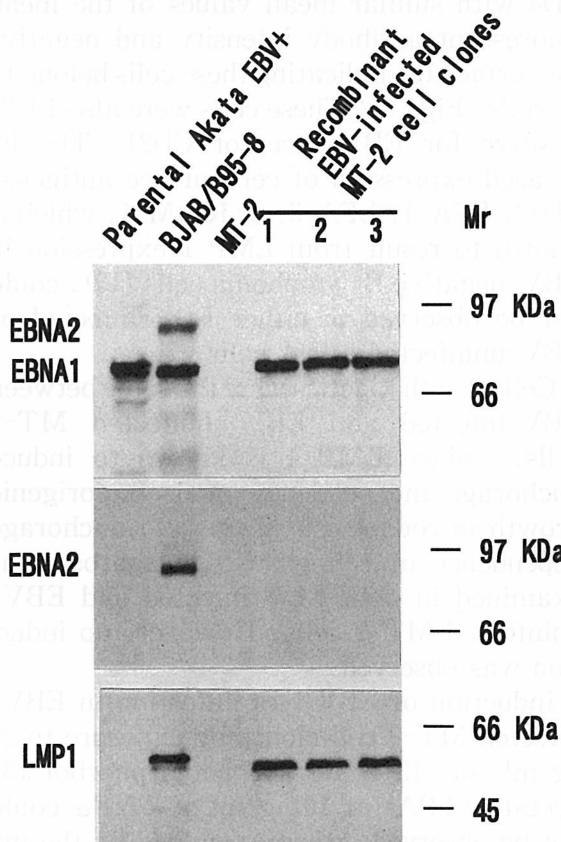

Fig. 3. Detection of EBV latent proteins in recombinant EBV-infected MT-2 cell clones by immunoblot analysis. For immunostaining, the top blot was treated with a standard EBNA-positive human serum and peroxidase-labeled protein A. The second blot was treated with an EBNA 2 monoclonal antibody (PE 2) and peroxidase-labeled anti-mouse Ig. The bottom blot was treated with an LMP 1 monoclonal antibody (CS1-4) and peroxidase-labeled anti-mouse Ig.

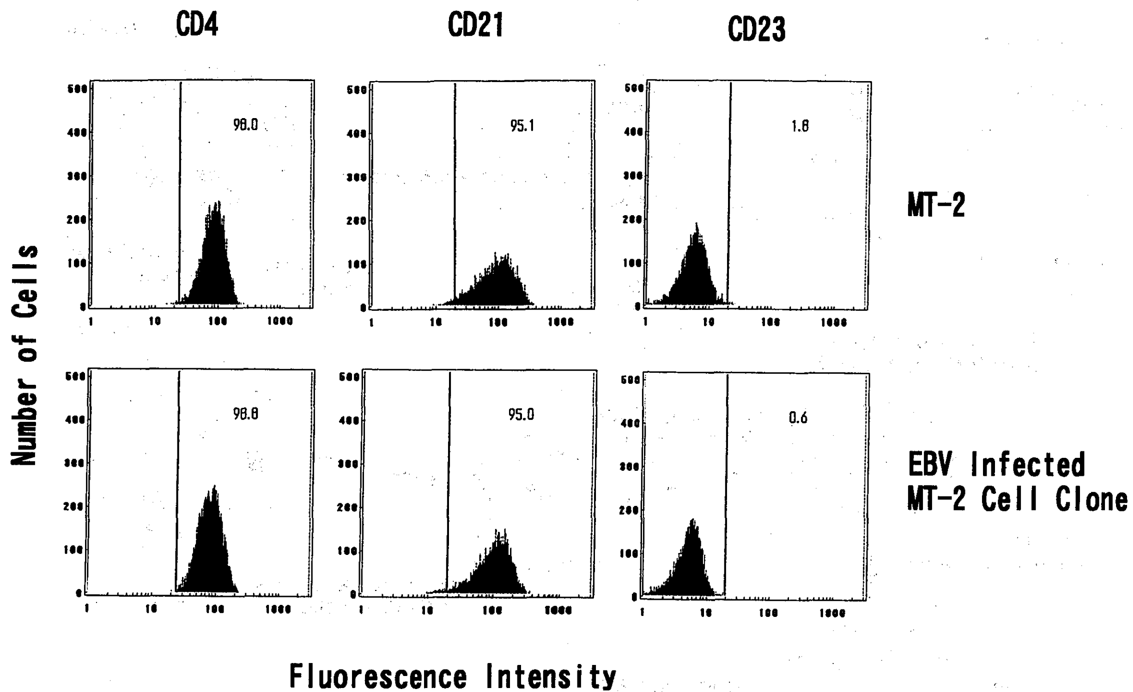

Fig. 4. Cell surface antigen expression in a representative recombinant-EBV infected MT-2 cell clone. Histograms are plotted as log fluorescence intensity (x-axis) vs. cell number (y-axis).

of EBNA 1 by indirect immunofuluorescent method. All of the three representative clones showed 100% positive for EBNA 1. EBV genes were also examined by Southern analysis and presence of 3 kb *Bam*HI W fragments evidenced that all the cell clones examined were infected with EBV. Infection of MT-2 with recombinant EBV was further confirmed by detecting PCR product of desired size using primer pair encompassing the recombination site. To examine the type of latency, expression of EBV latent proteins on EBV-positive MT-2 cells were examined by immunoblot analysis. The expression of both EBNA 1 and LMP 1, designated as type II latency (Fig. 3), could be observed (8). The state of EBV genome in infected MT-2 cell clones was examined by Gardella's gel analysis. Recombinant EBV genomes were maintained as episomes in MT-2 cells (data not shown).

Phenotypes of EBV-infected T cell lines

Cell surface antigen expression in EBV-infected and EBV-uninfected MT-2 cells was examined by the fluorescence activated cell sorting analysis. All of EBV-infected and EBV-uninfected MT-2 cells were positive for CD4 with similar mean values of the mean fluorescent antibody intensity and negative for surface Ig, indicating these cells belong to T-cells (Fig. 4). These cells were also 100% positive for EBV receptor CD21. The increased expression of cell surface antigens, CD23, LFA-1, LFA-3, or ICAM-1, which is known to result from LMP 1 expression in EBV-negative B-lymphoma cells (19), could not be observed in either EBV-infected or EBV-uninfected MT-2 cells.

Cell growth kinetics were the same between EBV-infected and EBV-uninfected MT-2 cells. Since LMP 1 is known to induce anchorage-independence and tumorigenic growth in rodent cell lines (20), anchorage dependence of cell growth in agarose was examined in both EBV-infected and EBV-uninfected MT-2 cells. However, no induction was observed.

Induction of EBV lytic infection in EBV-infected MT-2 cell clones by exposure to 20 ng/ml of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or 10 ng/ml of TNFα could not be observed, when examined by the im-

munoblotting method (data not shown). Moreover, EBV infection did not give any effect on HTLV-I production in MT-2 cells. Neither TPA or TNFα treatment affected the intensity of HTLV-I polypeptides (9).

Conclusion

Our recombinant virus producing system could prove extremely valuable in producing sufficient quantities of EBV recombinants for a variety of studies. This study also provides a very useful model system for establishing stable EBV infection of different cell lineages *in vitro*. A recombinant EBV carrying a selectable marker has the advantage to overcome the complicated factors in different cellular environments. However, the forms of EBV latency defined *in vitro* analysis of cell lines may not apply in the *in vivo* context.

Fujiwara et al. also reported the infection of the same MT-2 cell line with a recombinant EBV carrying the hygromycin resistance gene (21). Again, stable resistant lines expressed type II latency, but displayed a reduced growth rate as well as morphological changes. Though they did not show the site of the EBV gene disrupted by the insertion of drug resistant marker or biological function of recombinant EBV, together with our study, the type of the virus-infected cell may determine the form of EBV latency. These observations also evidenced that LMP 1 expression in MT-2 cell did not alter the expression of CD23, LFA-1, LFA-3, or ICAM-1 as well as the growth characteristics.

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