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Efficient Gene Transduction by Epstein–Barr-Virus-Based Vectors Coupled with Cationic Liposome and HVJ-Liposome

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We show here a novel non-viral strategy to transduce human cells by using an EBV-based vector system. The EBV-based vectors, the plasmid vectors carrying EBV oriP (origin for plasmid replication) and EBNA (EBV nuclear antigen) 1 gene from EBV genome, were combined with 2 gene delivery systems, i.e., cationic liposome and HVJ-liposome. By both methods, EBV-based vectors could be more efficiently transfected into HeLa cells than non-EBV, conventional plasmid vectors. When human primary fibroblasts were transfected, EBV-based vectors coupled with cationic liposome but HVJ-liposome resulted in successful gene transduction, while human bone marrow cells were transduced with both HVJ-liposome- and cationic liposome-EBV vectors. These results suggest the potential applications of the EBV-based vector system for gene therapy.

Various gene transfer vectors as well as delivery methods have been devised to transfer genes into human cells for the sake of the gene therapy. Among them, viral vectors have greatly contributed to many basic and clinical studies on gene therapy, but they inherently carry potential risks of generating wild-type recombinants and/or being recognized by host immune system (1). On the other hand, non-viral vectors may be safe, but the problem has been that the transduction and expression efficiency was generally poor.

The EBV-based vector is a plasmid vector carrying the EBNA1 gene and oriP region from EBV as *trans* and *cis* elements for DNA replication, respectively (2, 3). No infectious virus particles can be produced by using these vectors. Through the binding to oriP, EBNA1 facilitates the retention, nuclear localization, and replication of the plasmid DNA (4-7), giving the EBV vector favorable features of artificial chromosomes.

We have previously estimated the transfection efficiency of the EBV-based vectors into various human lymphoid cell lines (8, 9). Flowcytometric and limiting dilution analyses revealed that the transfection efficiencies were 500 to 1,000-fold higher than that of conventional, non-EBV plasmid vectors. These results suggested that EBV vector system could offer powerful non-viral strategies to transfer genes into various human primary cells. To examine the possible application of this vector system to gene therapy, we transfected primary fibroblasts from skin and bone marrow cells, by combining the vector with 2 gene delivery systems, namely, cationic liposome (10, 11) and HVJ-liposome (originally devised by Kaneda et al., Osaka University (12)) methods.

MATERIALS AND METHODS

Plasmids vectors. The plasmid vectors were constructed as described previously (8, 9). The pSES.β (Fig. 1A, left) and pSES.CD8α (Fig. 1A, right) are composed of (i) the E. coli β-gal gene (pSES.β) or murine CD8α cDNA (pSES.CD8α) located between SRα promoter (ClaI-ClaI 0.9 kb fragment from pcDL-SRα (13)) and the SV40 polyA additional signal, (ii) EBV oriP (derived from p220.2)(3), (iii) EBV EBNA-1 gene (derived from p220.2) under the control of SRα promoter, and (iv) the ampicillin resistant gene and the replication origin for E. coli. Another plasmid, pS.β (Fig. 1A, middle), was constructed from pSES.β by deleting EBNA1 and oriP.

Cells. HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM)(Gibco-BRL, Grand Island, NY) supplemented with 10 % FCS (Equitech-Bio, Ingram, TX). HLE, a cell line constitutively

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Abbreviations: β-gal, β-galactosidase; EBNA, EBV nuclear antigen; EBV, Epstein–Barr virus.

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expressing EBNA-1, was established by transfecting HeLa cells with p220.2 (3), a plasmid carrying the EBNA-1 and hygromycin B resistant genes, followed by selection with Hygromycin B. Primary human fibroblasts were established by culturing skin fragments, which were obtained as operation specimens from abdomen of a patient with colon cancer. The human bone marrow cells were obtained as biopsy specimens from iliac bone of a patient with lymphoma. In both cases, informed consent was obtained from the patients.

Transfection by cationic liposome. For cationic liposome-mediated transfection, HLE, HeLa, and fibroblasts were seeded into wells of a 6-well plate (Beckton-Dickinson Laboware, Franklin Lakes, NJ) and allowed to proliferate until they were 70-80 % confluent. Shortly before the transfection, the culture medium was replaced with 1 ml of fresh medium. Plasmid DNA (3 μg) in 20 mM HEPES, 150 mM NaCl, pH 7.4, was mixed with 15 μg of 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propylamid (DOSPER; Boehringer Mannheim, GmbH, Germany) to a final volume of 100 μl. After incubation at room temperature for 15 min, 60 μl of the plasmid-cationic liposome complex was added to the culture, which was then incubated at 37 °C in 5 % CO_2/95 % humidified air. Six hours later, 1 ml of fresh medium was added, and 24 hrs after the transfection, culture medium was replaced with fresh medium. After 3 days of cultivation, cells were subjected to the X-gal staining.

Transfection by HVJ-liposome. The HVJ-liposome was prepared as described (12, 14). Briefly, dried lipid mixture composed of 2 mg of 3β-[N-(dimethylaminoethane)carbamoyl]cholesterol (Sigma), 8 mg of cholesterol (Nakarai Tesque, Kyoto, Japan) and 166 μg of L-α-phosphatidylcholine (Nakarai Tesque) was mixed with 200 μg of plasmid DNA in 200 μl of BSS (140 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6). The mixture was then shaken vigorously to form liposome. HVJ, propagated in the chorioallantoic fluid of embryonated eggs, were purified by differential centrifugation and then inactivated by UV irradiation. The liposome suspension and 30,000 HAU of inactivated HVJ were mixed and incubated on ice for 10 min, followed by further incubation at 37 °C for 60 min. After removal of free HVJ by ultra centrifugation, the HVJ-liposome complex (approximately 1 ml) was harvested and added to 2×10^6 bone marrow cells in 1 ml of Iscove's modified Dulbecco's medium (IMDM)(Gibco-BRL, Grand Island, NY) supplemented with 10 % FCS, 10 μg/ml of interleukin (IL)-3 (Gibco-BRL), 10 U/ml of IL-6 (Gibco-BRL) and 10 U/ml of stem cell factor (SCF)(Pepro Tech Inc., Rocky Hill, NJ). After 2 days of cultivation, the medium was replaced with fresh one, and on day 4 posttransfection, cells were harvested for flowcytometric analysis.

Flowcytometric analysis. Immunofluorescence and FACS analysis were carried out as described previously (15, 16), using phycoerythrin (PE)-conjugated anti-murine CD8α antibody (Caltag, San Francisco, CA) and PE-conjugated control antibody. Analysis was carried out by a FACScan (Becton-Dickinson, Mountain View, CA).

X-gal staining. The cells were fixed with 1% glutaraldehyde/PBS for 10 min, washed 3 times with PBS, and then incubated in X-gal staining solution (0.05 % (v/v) 5-bromo-4-chloro-3-indolyl-β-D-

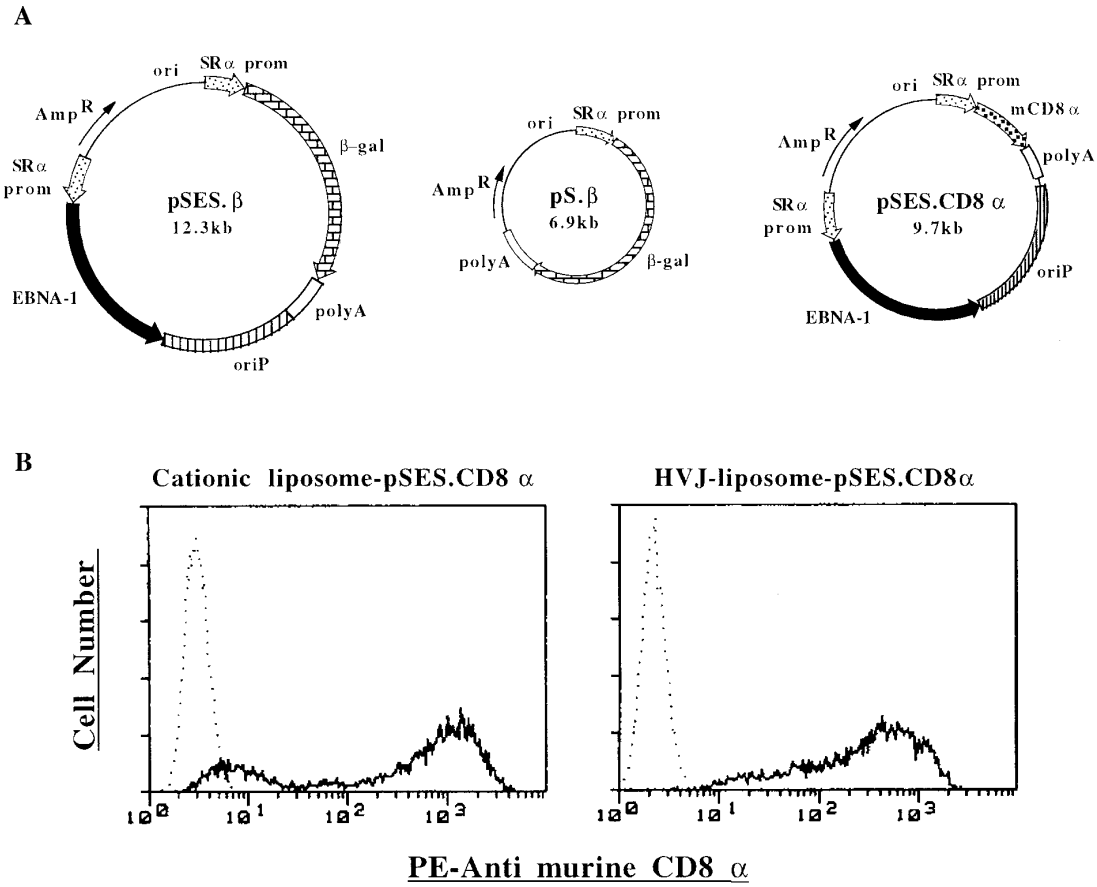

FIG. 1. (A), Plasmids used in this study. Maps of pSES.β (left), pS.β (middle) and pSES.CD8α (right) are shown. Prom: promoter; polyA: SV40 polyA additional signal. (B), FACS analysis of HLE cells transduced with EBV-based vectors. HLE cells were transfected with 60 μl of cationic liposome-pSES.CD8α (left) or 60 μl of HVJ-liposome-pSES.CD8α (right), and analyzed 3 days later.

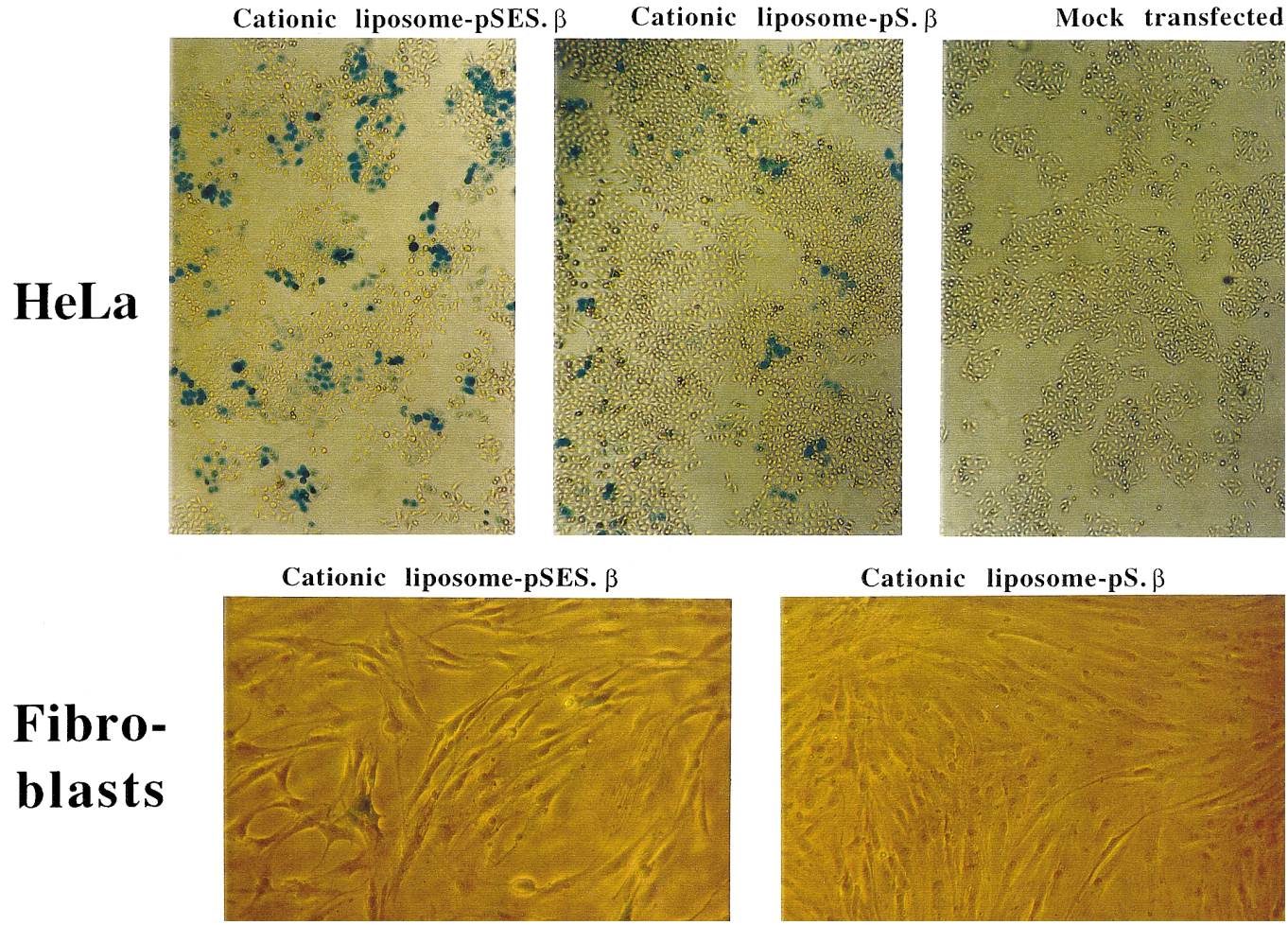

FIG. 2. X-gal staining of transduced cells. HeLa cells (upper panels)(original magnification, ×40), or primary human fibroblasts (lower panels)(original magnification, ×100) were transfected with 60 μl of cationic liposome-pSES.β, 60 μl of cationic liposome-pS.β, or mock transfected as indicated, and fixed and stained by X-gal on day 3 posttransfection.

galactoside (X-gal)(Nakarai Tesque), 1 mM $MgCl_2$, 150 mM $NaCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 0.1 % Triton X-100). After 12 hrs of incubation at 37 °C, the reaction was terminated by replacing the solution with 1 mM Na_2-EDTA/PBS.

RESULTS

To examine whether EBV-based vectors can be successfully combined with cationic liposome and HVJ-liposome methods, we first transfected HLE cells, the HeLa cell-derived transfectant constitutively expressing EBNA1, with pSES.CD8α encapsulated with these gene delivery vehicles. As shown in Fig. 1B, murine CD8α was expressed on 94 % and 100 % of the cells, by means of cationic liposome and HVJ-liposome, respectively. These efficiencies were comparable with that by electroporation (8, 9)

Then we transfected HeLa cells with pSES.β or pS.β by using the gene delivery methods. As shown in Fig. 2, 35 % of the cells transfected with cationic liposome-pSES.β expressed β-gal (Fig. 2, upper left panel), while only 3 % of the cells transfected with cationic liposome-pS.β were positive for the marker gene product (Fig. 2, upper center panel). The results indicated that the transfection efficiency by cationic liposome-EBV vector is actually higher than that by cationic liposome-non-EBV plasmid vector. Similar data were also obtained by HVJ-liposome-mediated transduction of pSES.β and pS.β (data not shown).

To examine whether primary cells can also be efficiently transduced by these systems, primary fibroblasts, derived from human skin, were transfected. When cationic liposome-pSES.β was transfected, 5 % of the cells were revealed to express β-gal on day 3 posttransfection, although the intensity of the expression is lower than that of HeLa cells (Fig. 2, lower left panel). In contrast, fibroblasts transfected with cationic liposome-pS.β failed to express the transgene at detectable level (Fig. 2, lower right panel), demonstrating the superiority of the EBV vector system in gene transduc-

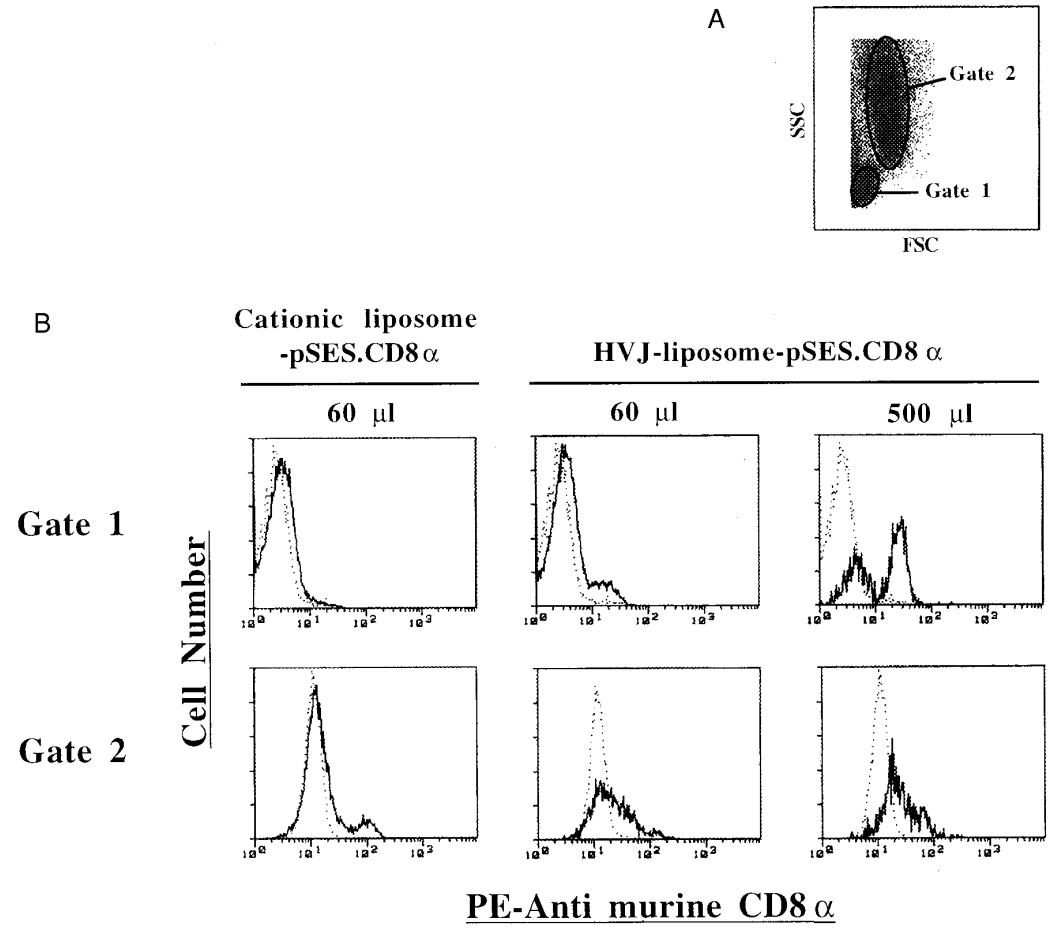

FIG. 3. Gene transduction into bone marrow cells. Human bone marrow cells were transfected with cationic liposome-pSES.CD8α (60 μl), or HVJ-liposome-pSES.CD8α (60 or 100 μl), and 4 days later, the cells were harvested and analyzed by FACS. A, Gates for lymphoid (Gate 1) or myeloid (Gate 2) cells are shown. FSC, forward scatter; SSC, side scatter. B, The CD8α expression on the transfected cells.

tion into primary fibroblasts. On the other hand, when pSES.β or pS.β was transfected into the primary fibroblasts by using the HVJ-liposome, expression of β-gal was very faint, if any (data not shown).

We next transduced human bone marrow cells, the potential target cells in gene therapy for hematopoietic disorders, with EBV-based vectors. Bone marrow cells from biopsy specimens were transfected with cationic liposome- or HVJ-liposome-encapsulated pSES.CD8α, and after 4 days of cultivation with IL-3, IL-6 and SCF, cells were stained with anti-murine CD8α antibody. As shown in Fig. 3B, FACS analysis indicated that the transfection with HVJ-liposome-pSES.CD8α resulted in the expression of the transgene product on the surface of both lymphoid and myeloid cells in a dose-dependent manner. When bone marrow cells were transfected with cationic liposome-pSES.CD8α, the lymphoid cells expressed murine CD8α only marginally, while 4 % of myeloid cells were positive for the marker gene product.

In contrast, the CD8α expression was almost undetectable on bone marrow cells which had been transfected with cationic liposome- or HVJ-liposome-pS.CD8α (a plasmid vector with SRα-murine CD8α but EBNA1 and oriP)(data not shown).

DISCUSSION

In the present study, we have shown that both human primary fibroblasts and bone marrow cells could be successfully transduced *in vitro* with the EBV-based vector system. As far as we know, this is the first report showing that primary human cells were transduced with EBV-based vectors.

The advantages of the EBV-based vectors are usually appreciated in stable rather than transient gene transduction. However, we have shown that even at a transient phase, EBV vectors give much higher expression of marker genes in cell lines (8, 9) as well as primary cells (the present study) than conventional plasmid vectors do. The high transient expression may results from the multiple functions of EBNA1, such as transfer

of the plasmid DNA into nucleus and transcriptional enhancement (4-7).

In the present study, the transfected fibroblasts and bone marrow cells did not proliferate significantly during the period between the transfection and harvest. During the period, the introduced EBV vectors may not have amplified, because the EBV plasmid replicates only once per cell cycle (17).

Interestingly, the cationic liposome but HVJ-liposome resulted in successful transduction into fibroblasts, whereas both HVJ-liposome and cationic liposome were effective in transfecting bone marrow cells. The results suggest that the gene delivery vehicles should be carefully chosen for each target cell type.

We have ever tried to transfect human peripheral blood stem cells by non-viral methods, and simply by electroporation, we succeeded in transducing the cells with the EBV-based vectors *in vitro* (Satoh et al., submitted for publication). Although electroporation seemed to be a good method for *ex vivo* transfer of plasmid vectors into floating cells like hematopoietic cells, combinations with other gene delivery systems are required for the EBV-based vectors to be applied to *in vivo* gene transfer. The present results suggest the availability of the cationic liposome and HVJ-liposome for this purpose, since both the former (18-20) and the latter (14, 21-26) methods have been shown to be effective in *in vivo* gene transfer.

The EBV is an oncogenic virus, whose oncogenicity is due to EBNA2, LMP(latent membrane protein)1 and other latent gene products. However, EBNA1 has been reported not to possess transformation activity (27-31). Since EBNA1 is the only viral protein produced in the EBV vector-transduced cells, the EBV-based vectors may not be oncogenic, although the safety must be demonstrated by *in vivo* experiments.

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