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Use of EBV-Based Vector/HVJ-Liposome Complex Vector for Targeted Gene Therapy of EBV-Associated Neoplasms

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Targeted suicide gene therapy for Epstein-Barr virus (EBV)-associated neoplasms was attempted by using EBV-based plasmid vectors coupled with hemagglutinating virus of Japan (HVJ)-liposome *in vitro*. Expression of EBV nuclear antigen (EBNA)1 is a common feature of the neoplasms associated with EBV. When various leukemic cell lines were transduced with a vector carrying a marker gene and EBV replication origin of plasmid (oriP), the marker gene product was exclusively detected in cells expressing EBNA1. Transduction of herpes simplex virus (HSV)-1 thymidine kinase (Tk) gene resulted in a marked reduction in viable cell number by ganciclovir (GCV) specifically in EBNA1 positive cells. The results demonstrate that this virus-free system may be applicable to gene therapy of EBV-associated neoplasms. © 1997 Academic Press

Numbers of protocols have been proposed for gene therapy of neoplasms. Although majority of these systems/methods utilize recombinant viruses as gene transfer vehicles, they inherently carry potential risks of generating wild-type recombinants and/or being recognized by host immune system. In contrast, non-viral plasmid vectors have advantages in safety and various methods have been devised to deliver plasmid vectors to target cells. These include electroporation (1), gene gun (2), cationic liposome (3) and HVJ-liposome (4, 5). But the issue has been that the gene transduction efficacies by non-viral vectors have been generally poor.

We have previously reported that extremely efficient transfection can be succeeded by using EBV-based vector (1, 6, 7), a plasmid vector carrying EBNA1 gene and oriP region from EBV genome (8, 9). EBNA1 facilitates the retention, nuclear localization, transcription and replication of the plasmid DNA through the binding to oriP and makes this vector highly efficient (8-10). No infectious virus particle can be produced by using the vector (10).

In order to apply the EBV-based vector to gene therapy, we have recently combined the EBV-based vector with HVJ-liposome system (7). By using this complex vector, we could transduce exogenous genes into various cells including human primary fibroblasts and bone marrow cells.

In this study, we have applied this EBV-based vector/HVJ-liposome complex system to targeted gene therapy of EBV-associated neoplasms, which commonly express endogenous EBNA1 (11, 12). When a plasmid vector carrying oriP but EBNA1 was transduced into a cell, constitutive expression of cellular EBNA1 is a prerequisite for strong expression of transduced genes (1). Thus, we hypothesized that the targeted expression of exogenous gene could be possible in EBNA1-positive cells. Here, we transduced HSV-1 Tk gene into EBNA1-positive or negative cell lines and analyzed the sensitivities of the transduced cells to GCV, as a model of non-viral, specific gene therapy for neoplasms associated with EBV.

MATERIALS AND METHODS

Plasmid vectors. Plasmid vectors were constructed as previously described (1, 6). pOTkA is composed of HSV-1 Tk gene located between Tk promotor and polyA signal (BamH I-BamH I 3.4 kb fragment from pHSV-106 (Gibco-BRL, Gaitherburg, MD)(Fig. 1A, upper right panel)), EBV oriP (Sal I-BamH I fragment from pSOP.CD8α (1)), ampicillin resistant gene and the replication origin for E. coli (Fig. 1A, upper left panel). pOTkB is essentially the same as pOTkA except for the direction of HSV-1 Tk gene expression unit relative to oriP (Fig. 1A, upper middle panel). pNS.CD8α carries murine CD8α gene cDNA under the control of SRα promoter (ClaI-ClaI 0.9

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kb fragment from pcDL-SR α (13)), the neomycin resistant gene, and oriP (Fig. 1A, lower left panel). p(−)NS.CD8α was constructed by deletion of oriP from pNS.CD8α (Fig. 1A, lower right panel).

Cell lines. K562, a human erythroleukemia cell line, and Raji, an EBV-positive Burkitt's lymphoma cell line, were maintained in RPMI1640 medium (Gibco-BRL, MD) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids (Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 % FCS (Equitech-Bio, Ingram, TX) (complete medium). KE is a K562-derived transfectant constitutively expressing EBNA1 (1).

Reverse transcriptase-polymerse chain reaction (RT-PCR) analysis. The RT-PCR was carried out as described previously (6, 14). Briefly, RNA was extracted from cells by using ISOGEN (Nippon Gene, Tokyo, Japan). After reverse transcription, the cDNA was subjected to PCR amplification using synthetic primers for EBNA1 (5′-AGCGATAGAGCAGGGCCCCGCAGAT-3′ and 5′-CAAAACCTCAGCAAATATATGAGTT-3′ for sense and antisense primers, respectively (15)), EBV-encoded small RNA (EBER) (5′-GTGGTCCGCATGTTTTGATC-3′ and 5′-GCAACGGCTGTCCTGTTTGA-3′ for sense and antisense primers, respectively (16)) or β-actin gene (5′-GTGCTATCCCTGTACGCCTC-3′ and 5′-GTTGTAGCTTCCTGGCGGTG-3′ for sense and antisense primers, respectively (7)). The PCR products were electrophorased through 2% agarose gel and stained by ethidium bromide.

Electroporation. Electroporation was carried out as described previously (1, 6). Briefly, 5×10^{6} cells were mixed with 20 μg of plasmid DNA, transferred into a cuvette (the distance between the electrodes was 0.4cm) (Bio-Rad Laboratories, Richmond, CA), and pulsed using a Gene Pulser II apparatus (Bio-Rad Laboratories) set at 370 V/500 μF. The cells were cultured at 37°C in 5% CO_2/95% humidified air, and 3 days later, cells were subjected to an Alamar Blue assay or subsequent culture with 50 μM of GCV (F.Hoffmann-La Roche Ltd., Basel, Switzerland).

HVJ-liposome. The HVJ-liposome was prepared as described (4, 7). Briefly, dried lipid mixture composed of 1 mg of 3β-[N-(dimethylaminoethane) carbamoyl] cholesterol (Sigma), 4 mg of cholesterol (Nacalai Tesque, Kyoto, Japan) and 8mg of L-α-phosphatidylcholine

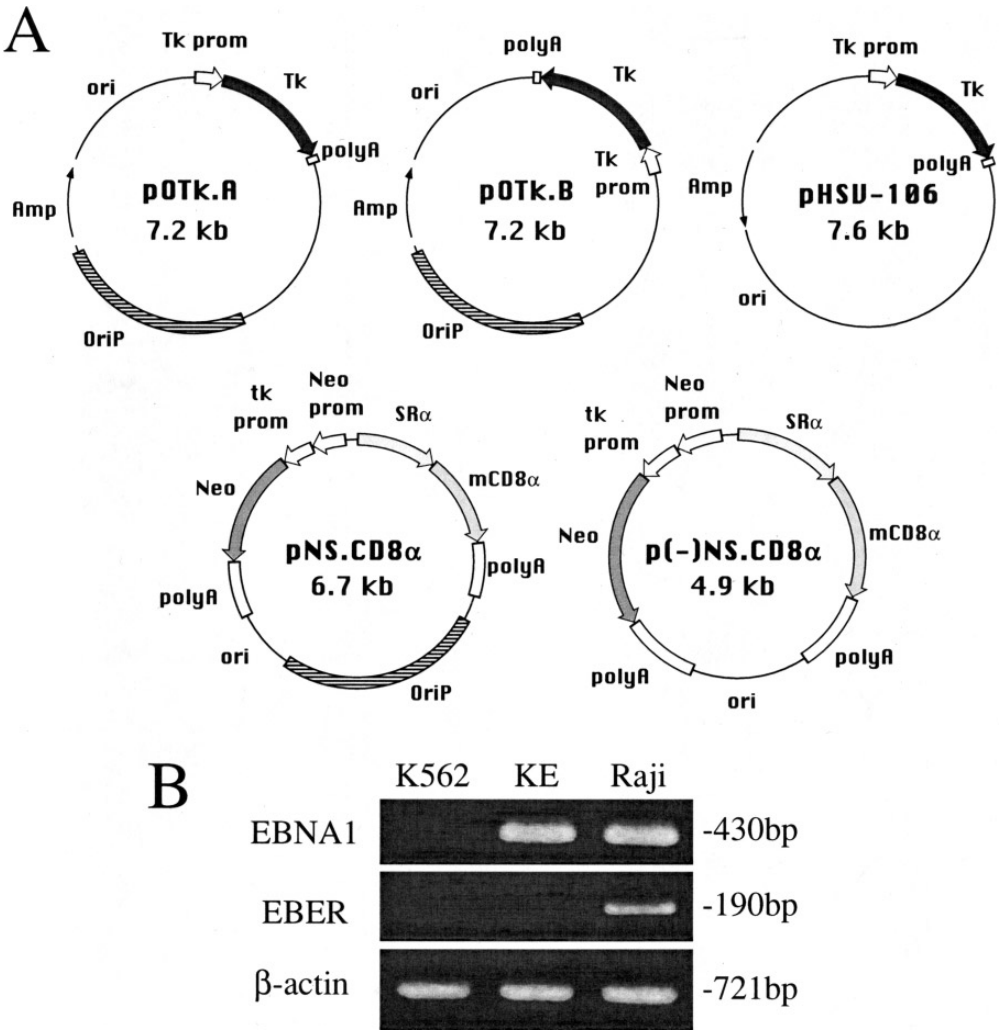

FIG. 1. *A*, plasmids used in this study. Maps of pOTkA, pOTkB, pHSV-106, pNS.CD8α and p(−)NS.CD8α are shown. Prom: promoter; polyA: poly A additional signal. *B*, expression of EBV-specific RNAs in leukemia cells. RNA from K562, KE or Raji cells were subjected to RT-PCR using primers for EBNA1 gene, EBER or β-actin gene as indicated. The PCR products were electrophorased through 2% agarose gel and stained by ethidium bromide. The 430-, 190- and 721-base pair (bp) bands represent the specific RT-PCR products from EBNA1 mRNA, EBER and β-actin mRNA, respectively.

(Nacalai 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 ultracentrifugation, the HVJ-liposome complex (approximately 1 ml) was harvested, and 60 μl of the complex was added to 2×10^6 cells in 1 ml of complete medium. On day 3 posttransfection, cells were subjected to a flowcytometric analysis or an Alamar Blue assay.

Flowcytometric analysis. Immunofluorescence and flowcytometric analyses were carried out as described previously, using PE-conjugated anti-murine CD8α antibody (Caltag, San Francisco, CA) and phycoerythrosin (PE)-conjugated control antibody. A FACScan (Becton-Dickinson, Mountain View, CA) flowcytometry was used.

Alamar blue assay. Susceptibility of cells to GCV was examined by an Alamar Blue assay (14). Briefly, quadruplicate aliquots of cells were seeded in 96-well flat-bottom microtiter plates (FALCON, Lincoln Park, NJ) (1×10^4 cells in 200 μl of complete medium per well) and cultured in the presence or absence of various concentrations of GCV. After incubation for 72 hours at 37°C in 5% CO_2/95% humidified air, Alamar Blue (Alamar Biosciences Inc., Sacramento, CA) was added according to the manufacturer's protocol. The cells were further cultured for 4 hours, and the optical density (OD) of each well was measured with a microplate reader (International Reagents Corporation, Kobe, Japan), at test and reference wave lengths of 570 and 600 nm, respectively. Percentages of viable cells were calculated according to the following formula: % Viable cells = (OD570-OD600) of GCV-treated cells/(OD570-OD600) of untreated cells.

RESULTS

RT-PCR analysis was carried out to examine whether Raji, KE and K562 cells express EBNA1 mRNA and EBER. Raji, an EBV-positive Burkitt's lymphoma cell line, expressed both of the EBV-specific RNA (Fig. 1B). This is compatible with earlier reports showing that EBV-associated neoplasms commonly express these RNAs (17, 18). Although K562 expressed neither EBNA1 mRNA nor EBER, KE cells were positive for EBNA1 mRNA.

We next estimated the efficiency of transduction/expression into the leukemic cell lines by the EBV/HVJ liposome. When KE cells were transduced with pNS.CD8α/HVJ liposome, 72 % of the cells expressed murine CD8α on day 3 posttransfection, as revealed by a flowcytometric analysis (Fig. 2B). The expression of murine CD8α by KE cells transduced with p(−)NS.CD8α, which does not carry oriP, was hardly detectable (Fig. 2C). Similar results were obtained from experiments using Raji (data not shown). In contrast, K562 cells, which had been transduced and analyzed as above, expressed the marker gene product only marginally (Fig. 2A). These results are compatible with our previous study (1) indicating the requirement of EBNA1 for the strong expression of oriP-bearing plasmid vector.

To examine the possible application of the EBV-based vector system to selective killing of EBV-associated malignant cells, we transduced pOTkA and pOTkB, both of which carry HSV-1 Tk gene expression unit and EBV oriP, into the leukemic cell lines and analyzed their susceptibilities to GCV.

Fig. 3 shows the results of a series of experiments,

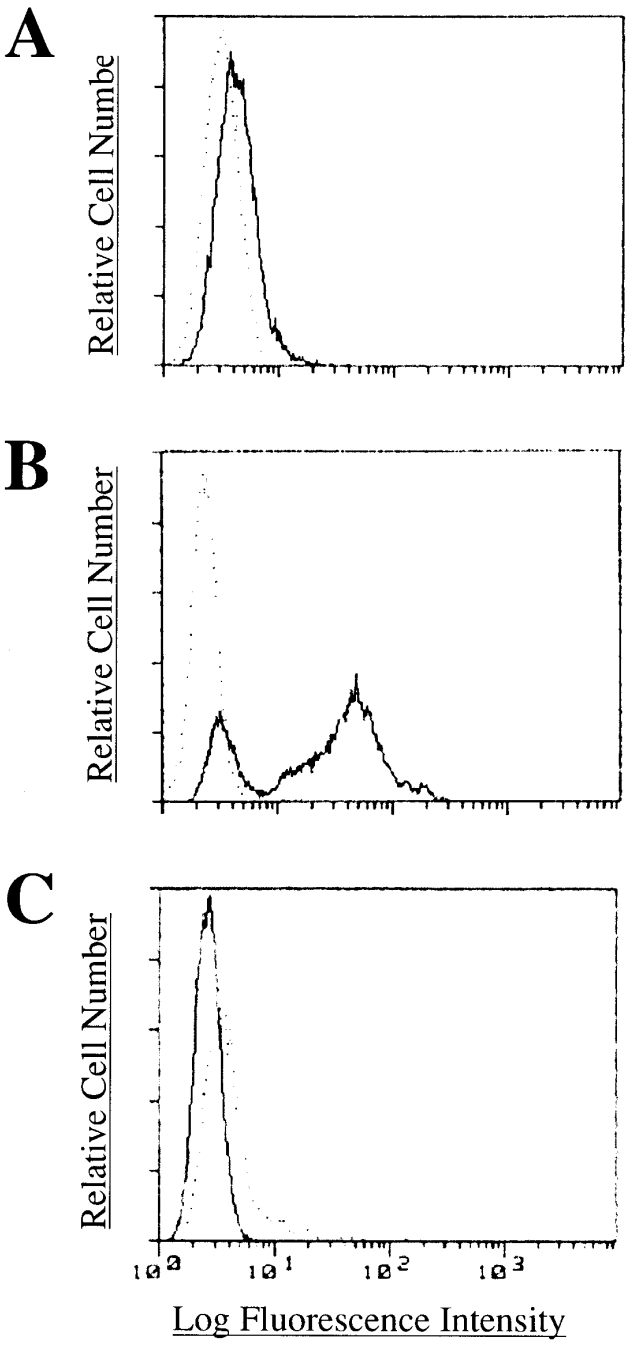

FIG. 2. Flowcytometric analysis of K562 and KE cells transduced with EBV-based vector/HVJ-liposome complex vectors. K562 (*A*) or KE (*B* and *C*) cells were transduced with pNS.CD8α (*A*, *B*) or p(−)NS.CD8α (*C*) coupled with HVJ-liposome, and after 3 days of cultivation, stained with PE-anti-murine CD8α and analyzed by FACScan.

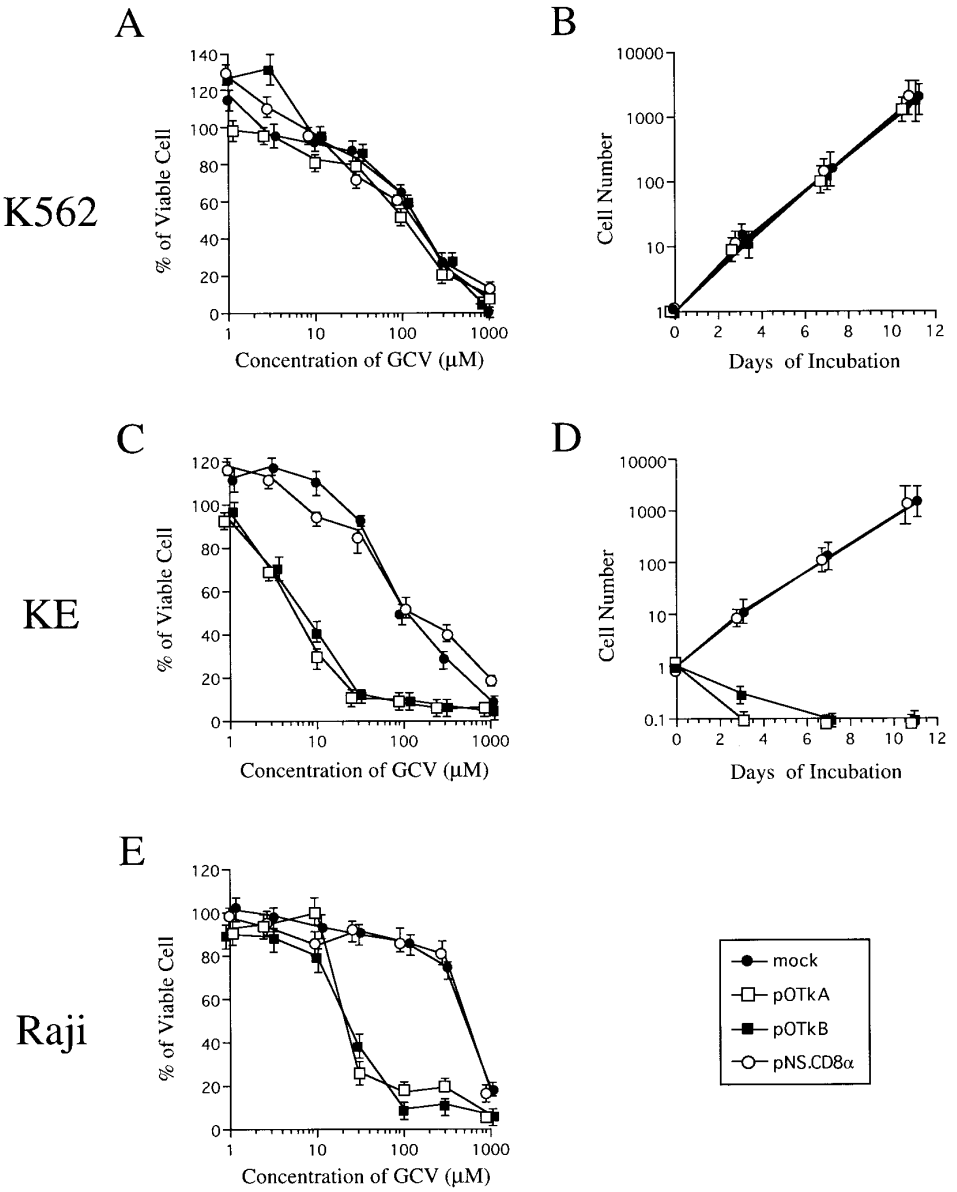

FIG. 3. Susceptibilities to GCV of cells transfected with EBV-based vectors by electroporation. K562 (*A* and *B*), KE (*C* and *D*) and Raji (*E*) cells were transfected with the indicated plasmids, and 3 days later, subjected to an Alamar Blue assay (*A*,*C* and *E*) or an proliferation assay (*B* and *D*). *A*,*C* and *E*, 10^4 Cells were seeded and their viabilities were measured after 72 hours of incubation with or without various concentrations of GCV. Percent of viable cells (y-axis) are plotted against GCV concentrations (x-axis). Each point represents the mean ± SEM of quadruplicates. *B* and *D*, 10^6 cells were cultured with 50 μM of GCV and viable cell numbers were counted on day 3, 7 and 11. Each point represents the mean ± SEM of quadruplicates.

in which the EBV-based vectors were delivered by electroporation. In case of K562 cells, the susceptibilities of transfectants to GCV were virtually the same as that of mock transfected cells, regardless of the plasmids transferred (Fig. 3A). In a marked contrast, KE cells transfected with pOTkA or pOTkB were 10 times more susceptible to GCV than pNS.CD8α-transfected or mock transfected KE cells (Fig. 3C). Similar results were also obtained from Raji cells (Fig. 3E). Transfer of the plasmids carrying Tk gene but oriP did not apparently affect the susceptibility of these cell lines to GCV (Fig. 4).

K562 or KE cells transfected as above were cultured in the presence of 50 μM GCV and numbers of cells were counted 3, 7 or 11 days later. KE cells transfected with pOTkA or pOTkB drastically reduced in cell number within 7 days of the culture (Fig. 3D), while K562 cells transfected with any of the plasmids proliferated well (Fig. 3B).

We next transduced the leukemic cell lines with the

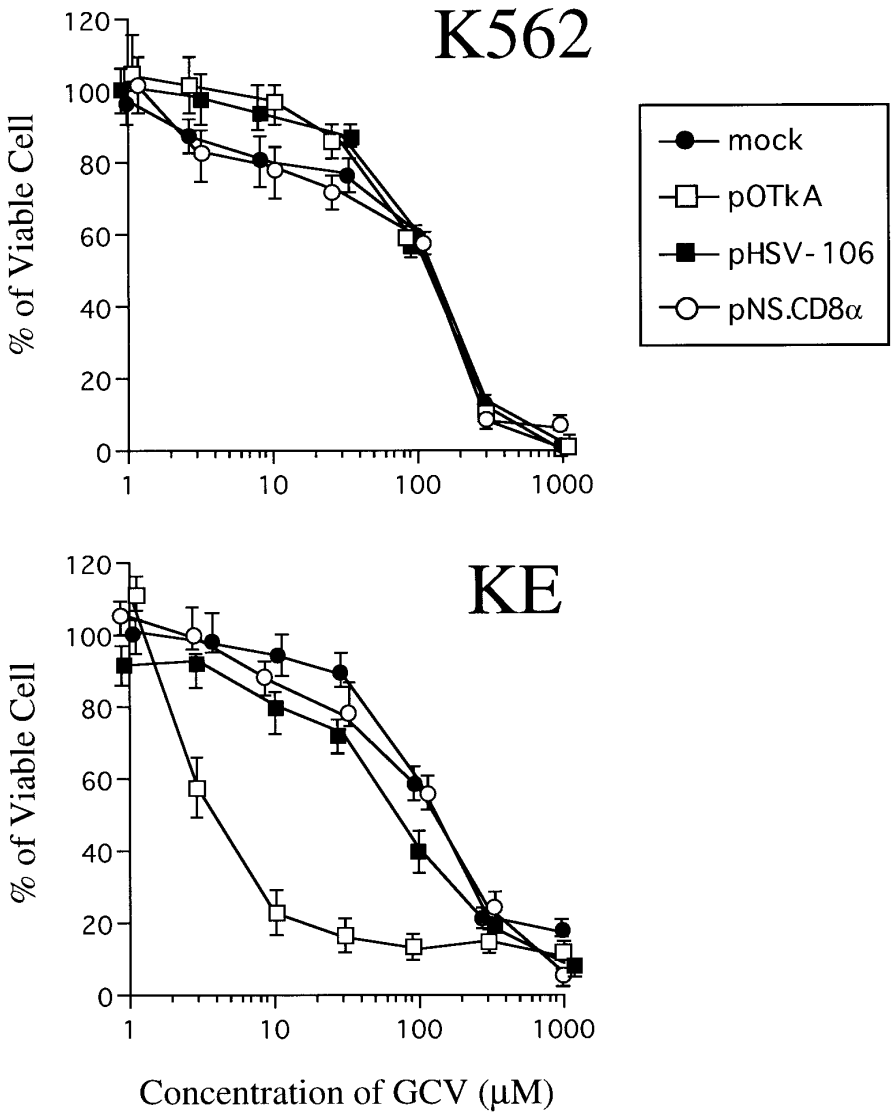

FIG. 4. Susceptibilities to GCV of cells transfected with EBV-based vectors by electroporation. K562 and KE cells were transfected with the indicated plasmids, and 3 days later, cells were subjected to an Alamar Blue. 10^4 Cells were seeded and their viabilities were measured after 72 hours of incubation with or without various concentrations of GCV. Percent of viable cells (y-axis) are plotted against GCV concentrations (x-axis). Each point represents the mean ± SEM of quadruplicates. Each point represents the mean ± SEM of quadruplicates.

EBV-based vector/HVJ-liposome, since unlike electroporation, this complex vector can be applicable to *in vivo* gene transfer (Fig.5). When KE cells were transduced with pOTkA/ or pOTkB/HVJ-liposome, the viabilities of the cells became less than 10 % after 3 days of incubation with 30 μM of GCV (Fig. 5B). This is not the case for mock transfected KE cells or KE cells transduced with pNS.CD8α/HVJ-liposome. Similar dose-response relationship was also obtained from Raji cells (Fig. 5C) but K562 cells (Fig. 5A). These results clearly indicate that the delivery of HSV-1 Tk gene by the EBV-based vector/HVJ-liposome system resulted in specific expression and function of the transgene in the EBNA1-positive leukemic cells.

DISCUSSION

In the present study, we have shown an *in vitro* model system for a targeted suicide gene therapy of neoplasms associated with EBV. EBV-based vector/HVJ-liposome complex vector system, which we have recently devised (7), was utilized.

Gene therapy for neoplasms comprises several different approaches, such as potentiating immunogenicity of tumor cells (19), supplementing anti-oncogenes (20) or transferring genes for prodrug metabolizing enzymes (21). The important features required for these systems are (i) to kill cancer cells effectively, and (ii) not to damage non-cancerous cells as possible. For these

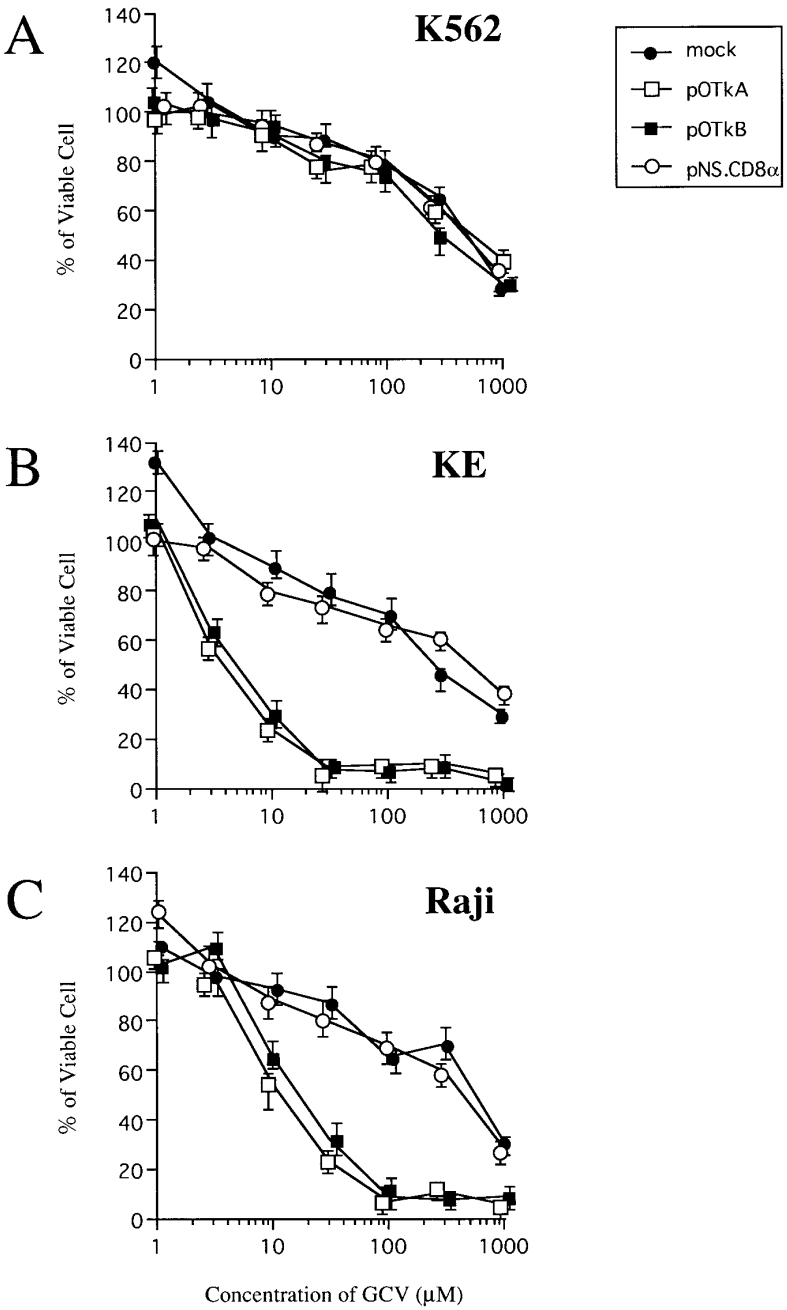

FIG. 5. Susceptibilities to GCV of cells transduced with EBV-based vector/HVJ-liposome. K562 (*A*), KE (*B*) and Raji (*C*) cells were transduced with the indicated plasmids coupled with HVJ-liposome, and 3 days later, subjected to an Alamar Blue assay. Ten thousand cells were seeded and their viabilities were measured after 72 hours of incubation with or without various concentrations of GCV. Percent of viable cells (y-axis) are plotted against GCV concentrations (x-axis). Each point represents the mean ± SEM of quadruplicates.

purposes, a number of strategies have been devised to allow specific expression of transduced genes in cancer cells, e.g. receptor-mediated targeting (22) and utilization of tumor specific promoter/enhancers (23).

Recently, it has been documented that EBV may be causally associated with not only Burkitt's lymphoma (24) and nasopharyngeal carcinoma (25) but also malignant lymphomas in immunodeficiency (26), Hodgkin's disease (27), thymic carcinoma (28) and gastric carcinoma (29). In the neoplastic cells, the EBV genome is present as episomes. Although some tumors fail to express EBNA2, latent membrane protein-1 and other latent gene products, EBNA1 is commonly expressed in all the EBV-associated cancer cells, because EBNA1 is essential for replication and maintenance of the viral episomes in the cancer cells (11, 12). In contrast, nor-

mal cells rarely express EBNA1 (17, 18). By using EBV-based vector carrying oriP but EBNA1, we have obtained specific expression and function of the suicide gene in the EBNA1-positive leukemic cells.

Electroporation is a good method to transfer plasmid vectors into various types of cells *in vitro*. Indeed, we have recently succeeded in transfecting EBV-based vectors into human peripheral blood stem cells by electroporation. For *in vivo* gene transfer, however, plasmid vectors must be combined with other gene delivery systems. The HVJ-liposome, originally devised by Kaneda et al. (4), has been reported as an efficient non-viral method for *in vivo* gene transfer. The envelope proteins of inactivated HVJ mediate liposome-cell membrane fusion and facilitate cellular uptake of packaged plasmid DNA, bypassing endocytosis and lysosomal degeneration. We have reported that EBV-based vector/HVJ-liposome complex can successfully deliver marker genes into human primary fibroblasts as well as bone marrow cells (7). In the present study, HVJ-liposome was as effective as electroporation in killing EBNA-1 positive cells (Figs. 3 and 5). This complex vector may be applicable to *in vivo* use by local injection into solid tumors.

The strategy presented here seems to be safe and efficient because it is a virus-free system and the expression and function of the suicide gene may be restricted to the target cells. We are currently doing *in vivo* experiments with animal models.

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