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Efficient Generation of BLCL Expressing Foreign Antigen as Antigen-presenting Cells with Recombinant Retroviruses

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Epstein-Barr Virus (EBV)-transformed lymphoblastoid B cell lines, BLCL, which expresse antigens, are potential antigen-presenting cells (APCs) for the induction of CTL *in vitro*. However, transfection of BLCLs with subsequent selection by antibiotics is notoriously difficult because plating efficiencies of BLCLs are reported to be 1% or less. To generate stable transfectants of BLCLs, we produced high titers of retroviruses encoding pp65 antigen of human cytomegalovirus as foreign antigens and transduced them to BLCLs. The pp65 gene was cloned into the retroviral vector pLXSN. The recombinant retroviral vector was transfected to ecotropic packaging cell line, GP&E86, and this polyclonal recombinant retrovirus was transduced to PA317 that is amphotropic packaging cell line. The titers of cloned PA317 amphotropic retroviruses ranged from 5 to 7×10^6 colony forming units (CFU) per ml (CFU/ml). We performed three rounds of consecutive transductions to BLCLs in order to improve the cloning efficiencies. The expression of recombinant HCMV-pp65 antigen was more than 20% after the final transduction. The third-transduced BLCLs were easily selected in optimal concentration of G418. BLCLs expressing foreign antigens could be used as target cells for CTL assay and/or as APCs for induction of *in vitro* CTL responses specific for viral and tumor antigens.

Key words: EBV-transformed lymphoblastoid B cell lines (BLCL), antigen-presenting cells (APCs), Recombinant retrovirus, Cytotoxic T lymphocytes (CTL)

Epstein-Barr virus (EBV) is a potent inducer of polyclonal B lymphocyte proliferation and is a tool for the establishment of human B lymphoblastoid cell lines (BLCL) (1-3). In studies on the human immune system, BLCL are convenient sources of antibody-producing cells and of antigen-presenting cells, as they can be readily established from each individual. Moreover, BLCLs frequently express high constitutive levels of MHC class I and class II antigens (4, 5), and of molecules involved in second signals required for T cell activation such as B7 molecules (6). These obvious advantages of BLCL are counteracted to some extent by poor tissue culture performance resulting mainly in poor cloning efficiencies (7, 8). Because plating efficiencies of BLCL are reported to be 1% or less, transfection of BLCLs with subsequent selection by antibiotics is notoriously difficult.

In the classic model of antigen presentation to CTLs, foreign antigens must be synthesized in the cytoplasm of the infected cell and processed by a cellular protease. In order to study the CTL response to individual viral antigens, it is usually necessary to express the viral gene of interest within the target cell itself. A number of vectors have been used for this purpose. We hypothesized that the retroviral transduction for the expression of a foreign antigen might have important advantages over other methods of antigen delivery to BLCLs. Retroviral gene transfer is presently one of the most powerful techniques for introducing stably heritable genetic material into mammalian cells (9, 10). Retroviral vectors seem to offer unique advantages for studies of gene expression since they can be used to introduce an intact single copy of a gene into most mammalian cell types *in vitro* and somatic and germ cells *in vivo*. Their ability to deliver an unrearranged, single-copy gene into a broad range of human somatic cells in primary culture makes them well suited for the purpose.

In this study, we successfully produced BLCLs expressing HCMV-pp65 antigen by transduction of recombinant retrovirus. EBV-transformed BLCLs grow well and can be easily maintained *in vitro*. This paper provides a strategy useful for analysis of human T cell response to viruses and tumors.

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Materials and Methods

Generation of EBV-transformed B cell lines

BLCLs were prepared from donor peripheral blood mononuclear cells (PBMC). These were plated at 2×10^6 cells per well in flat-bottomed 24-well plates in supplemented RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) and 1 μg/ml of cyclosporin A, with 1 ml supernatant derived from cultures of B95-8, a marmoset B cell line transformed by human type 1 EBV. Once B95-8-infected BLCLs were established, they were expended into 75 cm^2 flasks for long-time culture and aliquots were frozen. Human leukocyte antigen (HLA) typing of BLCL lines was performed using the amplification refractory mutation system (ARMS)-PCR for HLA class I and by PCR-sequence-specific oligonucleotide probes (SSOP) method for HLA class II (11, 12).

Production and titration of retrovirus

Plasmid expressing the proviral constructs were introduced into the murine ecotropic packaging cell line GP&E86 using a commercially available kit for Calcium-Phosphate transfection system (Gibco-BRL). After 48 hr, GP&E86 cells, transfected with pLXSN-pp65, were selected in 1 mg/ml of G418 (Invitrogen, San Diego, CA, USA) for 10 days. Supernatant from the resulting G418-resistant polyclonal population was used to transduce the murine amphotropic packaging cell line PA317. After a selection for 10 days in 1 mg/ml of G418, the transfected producer clones were isolated and titered on NIH-3T3 cells and used for transduction experiments.

Viral titer was determined as the average number of drug-resistant colony cells multiplied by a factor to account for magnification, plate size, and dilution of the infectious stock. NIH-3T3 cells were transduced by incubating a 60 mm plate containing 2×10^5 cells with 2 ml of a 10-fold diluted retroviral supernatant in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO, USA) for 4 hr. Subsequently, the medium was changed to 4 ml of fresh DMEM supplemented with 10% FBS. Two days post-transduction, the transduced cells were splited 1 : 20 into 1 mg/ml G418 selective medium. At day 3 of culture, the medium containing G418 was changed, and transformed colonies were counted 10 days post-transduction. In determining the G418-resistance titer, the number of colonies was divided by 4 to account for two cell doubling.

Transduction of Retrovirus and establishment of stable pp65-expressing BLCLs

The BLCLs were transduced by incubating on 24-well culture plates at a concentration of 1×10^6 cells/ml with viral supernatant diluted 1 : 1 in culture-medium containing 8 μg/ml polybrene for 4 hr. The medium was then removed and replaced by fresh normal medium. This procedure was carried out at 0, 1, and 3 days. Subsequently, the cells were analyzed or grown in culture-medium containing 0.8 mg/ml of G418. The BLCLs were selected for neomycin-resistance for 2 weeks in selective medium, and then maintained in culture-medium containing 0.5 mg/ml of G418. Bulk cultures of neomycin-resistant cells were used without further selection.

Indirect immunofluorescence assay

After the three-round transduction or selection, the cells were washed twice in phosphate-buffered saline (PBS). Approximately 2×10^5 cells are placed on each of 5 mm wells on preprinted slides, air-dried, and then fixed for 10 min in 4% paraformaldehyde (Sigma, St. Louis, MO, USA). Serial 2-fold dilutions of anti-pp65 antibodies (Fitzgerald, Concord, MA, USA) and negative control sera were added to each well. After incubation for 30 min on ice followed by thorough washing in PBS three times for 5 min, wells were similarly treated with fluorescein conjugated anti-mouse IgG. Slides were mounted in glycerol/PBS (1 : 1) and observed under a fluorescein microscope for the presence of nuclear and diffuse cytoplasmic fluorescence.

Results and Discussion

Retroviruses have been the most commonly employed vectors for the preparation of human gene therapy trials. For this purpose, retroviral stock of high titers are necessary for efficient introduction of recombinant genetic material into rare cells, and to be able to infect all cells in a large target population (10, 13). The success of gene therapy and marking protocols that utilize retrovirus vectors is dependent on a source of high-titer virus stocks. To isolate high-titer amphotropic packaging cell clones in this study, the supernatant obtained from a polyclonal population of neomycin-resistant ecotropic producer GP&E86 cells was used to transduce into the murine amphotropic packaging line PA317 cells. After the selection process, the subcloning was performed by limiting dilution. About 20 colonies were picked and expended. The resulting supernatant were then titered by G418 selection for the neomycin-resistant gene also contained in the retrovi-

Table 1. Titration of PA317 clone producing amphotropic recombinant retroviruses

CLONE	NO. of $G418^R$ colony[a]	Titer per milliliter[b]
1	144	7.20×10^6
2	103	5.15×10^6
3	128	6.40×10^6

[a]1 : 20 split in 100 mm plates after 2 days post-transduction

[b]Viral titer was determined as the average number of drug-resistant colony cells multiplied by a factor to account for magnification, plate size, and dilution of the infectious stock. In determining the G418-resistance titer, the number of colonies was divided by 4 to account for two cell doublings.

ral vector. The titers of cloned PA317 amphotropic retroviruses ranged from 5 to 7×10^6 CFU/ml (Table 1) and the expression of HCMV-pp65 antigen was detected by indirect immunofluorescence. The titer of viruses varies widely and depends on a combination of factors. The vector, the inserted gene, the packaging line, and the method of harvesting all contribute to the final titer. Although titers from stable producers can be as high as 10^8 colony forming units (CFU) per ml, the titers of transient producted stocks from ecotropic line Ψ2, or amphotropic lines ΨCRIP and PA317 are generally lower, from 10^3 to 10^4 CFU/ml range (13). These stocks are conventionally prepared by transfection of retrovirus vector DNA or by infection of transiently produced virus stocks into a packaging cell line. However, only a small subset of packaging cell clones transduced in this manner actually produce high titers of retrovirus. It is therefore useful to screen several clones to identify the highest titer packaging cell line. Moreover, a clone is a more stable reagent than a pool of polyclonal producers.

In order to improve the transfection efficiencies of BLCLs, a number of vectors have been used. Many groups have used recombinant vaccinia virus (rVV) to express different viral antigens in BLCLs (14-16). However, rVV may not be the ideal candidate for antigen expression, because vaccinia itself is a potent immunogen and it is, therefore, difficult to properly interpret the results of studies using rVV in some vaccinia-immunized individuals. In addition, the vaccinia virus is lytic to cells. It is not possible to generate stable cell lines for the study of antigen processing or presentation using rVV to express individual viral proteins. Electroporation of BLCLs has also been used in the study of gene therapy for B-cell malignancies. However, it is necessary to use large numbers (up to 10^7) of cells to perform electroporation and the efficiency of gene transfer was only 5 to 10% in the case of B cell chronic lymphocyte leukemia (17). Curiel et al. described a high-efficiency gene transfer system to BLCLs based on receptor-mediated endocytosis using adenovirus as the DNA carrier (18, 19). Other methods of inducing foreign gene expression in BLCLs such as DEAE-dextran and lipofectin were not successful despite an optimization of the processing condition (20). We hypothesized that retroviral transduction as a technique for developing the expression of viral antigens might have important advantages over other methods of antigen delivery to BLCLs, and have tried to improve the cloning efficiencies of BLCLs using three consecutive transductions of high-titer retroviruses.

To investigate the retroviral transduction efficiencies of BLCLs, EBV-transformed BLCLs were first established from 5 healthy young adults, and transduced with recombinant retroviruses containing the HCMV-pp65 gene (MOI 1 : 2). The expression of HCMV-pp65 antigen was examined by Indirect immunofluorescence assay. The

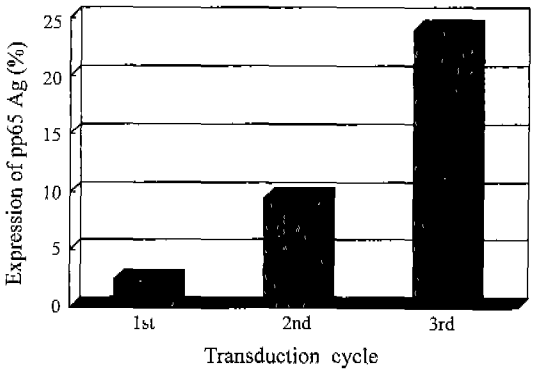

Fig. 1. Expression of the HCMV-pp65 antigen in EBV-transformed lymphoblastoid B cell lines (BLCL) after each transduction cycle. The percentage of positive cells was multiplied by the total number of cells on the plate in each cycle transduction 2 days post-transduction. The data are the mean of five experiments.

first and second transduction efficiencies of recombinant retroviruses to BLCL proved to be less than 3% and 11%, respectively. After three consecutive transductions, however, the expression of HCMV-pp65 exceeded 20% (Fig. 1). Therefore, to confirm the stable expression of the transduced gene, we cultured the first, second and third transduced BLCLs, without the addition of a selection drug for a period of one month. The ratio of BLCLs expressing HCMV-pp65 antigen increased slightly until 12 days after transduction, and it then decreased. In the case of the first and second transduced BLCLs, the expression ratio of HCMV-pp65 antigen was less than 3% after a month. However, the third transduced BLCLs expressed HCMV-pp65 antigen at approximately the 10% level after a month (Fig. 2).

To test whether the transduced BLCLs could efficiently grow in selective medium, we compared the cloning efficiency of transduced BLCLs from each transduction in

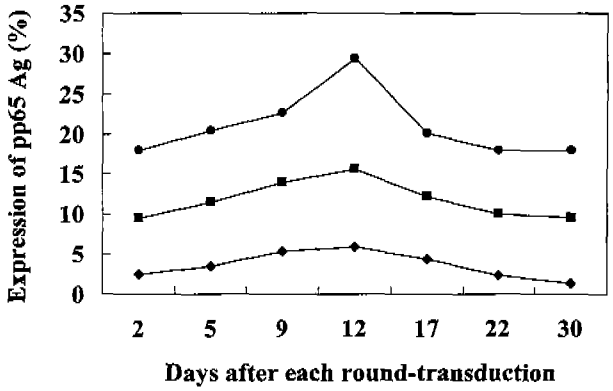

Fig. 2. Maintenance of HCMV-pp65 antigen expression in EBV-transformed Lymphoblastoid B cell lines (BLCL) after each round transduction. The percentage of positive cells was multiplied by the total number of cells on the plate in first-cycle (◆), second-cycle (■), and third-cycle (●) transduction. This data are representative of three experiments.

Table 2. Cloning efficiency of BLCLs transduced with retrovirus encoding HCMV-pp65 at 10% and 30% FBS[a]

Cells/well[b]	1st Transduction		2nd Transduction		3rd Transduction	
	10% FBS	30% FBS	10% FBS	30% FBS	10% FBS	30% FBS
3000	3	5	6	6	7	8
300	1	1	1	1	3	2
30	0	0	0	0	1	1

[a]BLCLs were grown in culture-medium containing 0.8 mg/ml of G418. After 2 weeks, number of wells containing viable growing cells by microscopic inspection.
[b]Twelve replicate wells/cell concentration.

Fig. 3. Immunofluorescence microscopy of expression of the major late HCMV matrix phosphoprotein, pp65 antigen. The EBV-transformed lymphoblastoid cell line (BLCL) was three-times transduced with retroviruses encoding HCMV-pp65. After a selection period, the cells were stained with anti-pp65 monoclonal antibody (manufactured at × 400).

10% and 30% FBS. The first and second transduced BLCLs could be cloned in cultures of 300 cells per well, whereas the neomycin-resistant viable colonies achieved 30 cells per well in the third transduced BLCLs. The serum concentration had no effect upon the growth of transduced BLCLs in selective medium (Table 2). The first and second transduced BLCLs were not grown in selective medium in bulk culture, whereas the final transduced BLCLs efficiently selected in G418 of optimal concentration. Following selection for a period of 2 weeks, the percentage of HCMV-pp65 viral antigen on neomycin-resistant BLCLs was determined to be more than 70% (Fig. 3). Only in the case of the third transduced BLCLs, the expression of transduced genes was maintained for a month and cloned in cultures of 30 cells per well. Finally, the eighteen stable BLCL transfectants were successfully established without any other feeder cells or cytokines. This data suggests that the stable transfectant of BLCLs could be reliably generated by three consecutive transductions with recombinant retroviruses. Our result implies that the transduced genes are accumulated in transduced BLCLs and the accumulation of genes might affect the selection of transduced BLCLs. We believe that the observations described in this paper will be useful to those investigators who need a convenient source of autologous antigen presenting cells for the study of human immune responses.

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