Epstein–Barr virus-induced transformation of B cells for the diagnosis of genetic metabolic disorders – enumerative conditions for cryopreservation

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Abstract. Epstein–Barr virus (EBV) infection *in vitro* causes transformation of B cells and generates B lymphoblastoid cell lines (LCLs). These LCLs have been widely used for the diagnostic of several genetic metabolic disorders. However, up to now, efficiency of LCL generation has been based on misleading subjective analysis. In this study, quantitative analyses have been performed to indicate efficiency of B-cell transformation to measuring human lysosomal acid hydrolases associated with: GM1-gangliosidosis type I, Gaucher disease and mucopolysaccharidosis type I. Peripheral blood mononuclear cells were isolated from 13 subjects, and LCLs were produced by culturing them with EBV for 12 days. Activities of the enzymes β -galactosidase, β -glucosidase and α -iduronidase were measured before and after cryopreservation in liquid nitrogen for 30 days. Efficiency of the B-cell transformation was screened every 4 days by the enumeration of cell proliferation, cell counts and changes in granularity estimated by flow cytometry. We observed the generation of 13 LCLs. Cell transformation was confirmed by the gradual increase of cellular clusters, cell size and granularity. In addition, we determined that the activity of the enzymes mentioned above did not change following cryopreservation. These data suggest that our enumerative approach for screening of EBV–LCLs is efficient for the enzymatic determination of human lysosomal acid hydrolases and may thus replace misleading subjective analyses.

INTRODUCTION

In vitro infection of B cells with Epstein–Barr virus (EBV) results in the development of infected blasts into lymphoblastoid cell lines (LCLs) in which continuous growth is driven by viral gene products. These cells are apparently equivalent to those produced *in vivo* by initial

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infection, and much of our knowledge of EBV biology comes from studies on these cells (Cohen 2000). LCLs have been used as models in various biological and medical studies (Minami *et al.* 1977) as they can be easily established and continue proliferating for many generations.

During B-cell transformation, a series of morphological changes takes place including the formation of cytoplasmic vesicles enriched with lysosomes (Maret *et al.* 1985). For this reason, several studies have used EBV as tool for evaluating the presence of human lysosomal acid hydrolases (Negre *et al.* 1986). LCLs have several advantages over skin fibroblast lines; the material is more readily obtainable, it exhibits far less cytogenetic variation, cell proliferation is more rapid and the cells have a longer lifespan, there is more massive production of cellular material, and the cells are easier to store frozen, to be followed by re-establishment in culture (Minami *et al.* 1978). These advantages render a faster and reliable diagnosis using this system (Dreyfus *et al.* 1980; Neitzel 1986). Although LCLs can easily be obtained, the efficiency of their transformation is based, at the moment, on potentially misleading subjective analyses (Maret *et al.* 1987; Oh *et al.* 2003).

In this study, quantitative analyses have been performed to control for efficiency of B-cell transformation and to measure human lysosomal acid hydrolases associated with GM1-gangliosidosis type I, Gaucher disease and mucopolysaccharidosis type I. The efficiency of B-cell transformation was overseen every 4 days by enumerating cell proliferation, performing cell counts and noting changes in cellular granularity estimated by flow cytometry. The activities of the enzymes β -galactosidase, β -glucosidase and α -iduronidase were measured before and after cryopreservation.

MATERIALS AND METHODS

Collection of peripheral blood and isolation of mononuclear cells

Twenty millilitres of peripheral blood was collected from 13 healthy young adults (25.07 ± 5.0 year; 20–40 years) by venepuncture in the morning (between 9:00 and 10:00 h), and samples were stored in lithium–heparin tubes prior to analysis. The study protocol was approved by both scientific and ethics committees (Pontifical Catholic University of Rio Grande do Sul, PUCRS, Porto Alegre, Brazil), and written informed consent was obtained from all subjects. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque (Sigma, St. Louis, MO, USA) gradient (900 *g*, 30 min). Cells were counted microscopically (100 $\times$), and viability was always found to exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma).

EBV stock preparation

The virus stock was obtained from an EBV-transformed B95-8 marmoset cell line (Laboratório de Imunologia, INCOR, University of São Paulo, Brazil). The cells were grown in complete RPMI-1640 medium, and cultures were maintained for 18–20 days. After centrifugation for 10 min at 850 *g*, the supernatant was filtered through a 0.22- μ m membrane (Millipore, Bedford, MA, USA) and stored at -40°C until use. Cells were washed (400 *g*, 10 min) on day 7 and were frozen in liquid nitrogen prior to use.

Transformation of lymphocytes

PBMCs were cultured to a final concentration of 9×10^6 cells/ml in plastic flasks (25 cm², Corning, New York, NY, USA) containing 3 ml of complete medium (RPMI-1640 medium supplemented with gentamicin 0.5%, fungizone 0.1%, glutamine 1%, Hepes 1%, and fetal calf serum 10%; all from Sigma). One millilitre of the supernatant derived from the EBV stock preparation and 3 μ l

of cyclosporin A (2 µg/ml; Sandimmun, Sandoz, East Hanover, NJ, USA) were added to the PBMCs. Transformation by EBV occurs with greater efficiency if the immune T cells are either physically removed from culture or are functionally inactivated (for example with cyclosporin A) (Tosato *et al.* 1982). Cells were cultured for 12 days at 37 °C in 5% CO₂ in accordance with previous studies (Oh *et al.* 2003). LCLs were subcultured every 4 days.

Evaluation of cellular transformation

Cell growth was checked every 4 days by evaluation of number of proliferative clusters observed by optical microscopy (Olympus, AX70TRF, Tokyo, Japan). The following scale was employed: number of clusters/image area. Each photograph was scanned and processed through imaging analysis (IMAGE-PRO PLUS® 4.5.1, MediaCybernetics, Silver Spring, MD, USA). Perimeters of clusters were manually selected and areas within were automatically calculated by the software. A minimum of 16 clusters was measured each day. Data are presented as mean µm² ± SE.

Flow cytometric analyses

Cells were washed twice in phosphate buffered saline (PBS) with 2% FCS and 0.01% sodium azide then resuspended in PBS. Aliquots of the cell preparations were added to each test tube and were promptly analysed using a FACScalibur (BD Pharmingen, San Diego, USA). A minimum of 20 000 events, gated by cell size (FSC) and granularity (SSC), were live-analysed using the CELLQUEST software (BD Pharmingen, San Jose, CA, USA).

Cell proliferation/viability assay

Proliferative responses were determined every 4 days by colourimetric assay (Mosmann 1983). In the last 4 h of culture, 100 µL of the supernatant was gently discarded and 30 µL of freshly prepared MTT (3-(4,5-dimethyl 2-thiazolyl) 2,5 diphenyl-2H-tetrazolium, Sigma) solution (5 mg/ml in RPMI-1640) was added to each well. The cell cultures were incubated for 4 h at 37 °C in 5% CO₂ atmosphere. After complete removal of the supernatant, 100 µL of dimethyl sulfoxide (Sigma) was added to each well. Optical density (OD) of preparations was determined using Bio-Rad ELISA plate reader (Biorad Benchmark, Tokyo, Japan) at a wavelength of 570 and 630 nm. Proliferation/viability was expressed as OD of stimulated – OD of non-stimulated cultures.

Collection of LCLs and freezing

LCLs were collected on day 12 of cell culture by washing twice in PBS (400 g, 10 min). Cryopreservation was performed according to previous studies (Tremblay & Khandjian 1998). Briefly, 1 ml of freezing medium (FCS 90% and DMSO 10%) was added, and LCLs were gradually frozen in appropriate containers with isopropyl alcohol (Nalge Nunc Int., Rochester, NY, USA). Cells were stored frozen at –80 °C for 24 h and then deep frozen in liquid nitrogen.

Assessment of lysosomal acid hydrolases

Analyses of human lysosomal acid hydrolases were performed on the 12th day of culture and 30 days following freezing of LCLs. Activity of the enzyme β-glycosidase was evaluated according to a previous method (Peters *et al.* 1976) that used 4-methylumbeliferil-β-D-glucosidase (Sigma) as synthetic substrate together with sodium taurocolate (Sigma) in standard conditions (pH 5.5 and 37 °C). The activities of the enzymes β-galactosidase (Minami *et al.* 1978) and α-iduronidase (Hopwood *et al.* 1979) were estimated by the incubation with 4-methylumbeliferil-β-D-galactoside (Sigma) as substrate in standard conditions (4.0 pH and 2.8 pH, respectively, at 37 °C). Fluorescence was evaluated by spectrophotometry (450 and 365 nm). Data are expressed as nmol/h/mg of protein (Lowry *et al.* 1951).

Figure 1. LCL proliferation. Proliferation/viability was estimated in a colourimetric assay by addition of MTT salt in the last 4 h of culture. Data presented as mean OD $\pm$ SE.

Statistical analysis

All variables were tested for normality of distribution by means of the Kolmogorov–Smirnov test. Data are reported as mean $\pm$ SE. One-way ANOVA was used to compare groups, followed by Tukey and Duncan post hoc tests when necessary. The analysis was performed using the statistical software package SPSS 11.5 (SPSS Inc., Chicago, IL, USA), and the level of significance was set at $P < 0.05$.

RESULTS

Generation of LCLs

PBMCs were initially cultured with the EBV-transformed B95-8 marmoset cell line for 20 days and cell proliferation was assayed every 4 days (Fig. 1). We observed a significant drop in cell proliferation on the 12th day of culture, $P < 0.0001$. This reduction can be explained in that only 8% of PBMCs are B cells committed to EBV-induced transformation. Paralleled to cell proliferation, PBMC counts were also reduced during the culture period with stabilization following day 8 (Fig. 2). Thus, day 12 was chosen as the ideal time for cryopreservation of LCLs.

Evaluation of cellular transformation

Imaging analysis permitted us to observe time-related morphological changes as well as to quantify a significant increase in cell clusters (Fig. 3a–d), $P < 0.001$. Cluster areas increased in area from 2000 to 37 000 μm^2 during the first 12 days of culture (Fig. 3d). Furthermore, cells had become more granular indicating EBV-induced cellular transformation. This cogent morphological change was also observed by flow cytometry (Fig. 4). Figure 4(a–c) shows a time-dependent switch from R1 to R2 gate, suggesting a significant increase in cellular granularity (an SSC parameter) – quantification of this is shown in Fig. 4d. In addition, we also observed a considerable switch in the FSC parameter, indicating an increase in cellular size.

Efficiency of transformation

During the first 12 days of culture, 13 LCLs were produced, indicating 100% success of EBV-induced transformation.

Figure 2. Enumeration of LCLs. This figure shows the absolute cell counts obtained by manual counting on a hemacytometer. Data shown as mean counts $\pm$ SE.

Figure 3. Visualization of transforming B lymphocytes infected with EBV. PBMCs were incubated with EBV for 4, 8 and 12 days (a–c) and cultures were photographed with the aid of an inverted microscope (100 $\times$). The number of cell clusters indicates the success of B-cell transformation (a–d).

Evaluation of lysosomal acid hydrolases

Activities of the enzymes β -galactosidase, β -glucosidase and α -iduronidase were measured on day 12 and on the following 30 days after cryopreservation. As shown in Table 1, activities of lysosomal acid hydrolases did not change following freezing in liquid nitrogen – $t = 0.644$, $P > 0.527$; $t = 0.103$, $P > 0.918$; $t = 1.014$, $P > 0.324$; β -galactosidase, β -glucosidase and α -iduronidase, respectively.

DISCUSSION

LCLs have been used as models in various biological and medical studies as they can be easily established and continue proliferating for many generations. For instance, various groups have used EBV-induced LCLs for the assessment of lysosomal acid hydrolases (Minami *et al.* 1978; Dreyfus *et al.* 1980; Watanabe & Minami 1981; Levade *et al.* 1985; Maret *et al.* 1985; Negre

Figure 4. Flow cytometric analyses of LCLs. PBMCs were incubated with EBV for 4, 8 and 12 days and cultures were analysed by flow cytometry. Note the increase in granularity as shown by a switch from R1 to R2 gates (SSC parameter, Figures a–c). The mean percentage of R2 positive cells $\pm$ SE is also shown (Fig. d).

Table 1. Activities of lysosomal acid hydrolases in LCLs before and following cryopreservation. Data expressed as (nmol/h/mg of protein) $\pm$ SD

Enzymes	Before freezing	After freezing
β -galactosidase	81.2 $\pm$ 23.7	73.1 $\pm$ 29.9
β -glucosidase	21.1 $\pm$ 6.3	20.8 $\pm$ 8.0
α -iduronidase	28.3 $\pm$ 11.7	23.8 $\pm$ 7.5

et al. 1986). Although LCLs are easily obtained, efficiency of transformation currently is based on misleading subjective analyses (Maret *et al.* 1987; Oh *et al.* 2003). In addition, there is no information regarding the exact culture time for collecting or freezing LCLs. Considerable efforts have been made here to provide quantitative analyses that control the efficiency of B-cell transformation.

We observed that LCLs remained proliferating until day 12 of culture, showing a significant decline following this period. This reduction can be explained by the fact that only 8% of PBMCs are B cells that were committed to EBV-induced transformation. This infection is guided through binding of the viral glycoprotein gp350 with CD21 on B cells. Only B cells bound to the EBV will be proliferating and the remaining mononuclear cells perish. The phenomenon of

regression in EBV-transformed LCL may be a consequence of long-term T-cell mediated immunity to B cells presenting EBNA antigen (Neitzel 1986). In this study, however, cyclosporin A was added into cultures to suppress T cell responses *in vitro*. It should be taken into account that if culture is continued beyond day 20, LCLs are expected to increase later (data not shown). However, a shorter period of cell culture is warranted for diagnostic purposes. Flow cytometric analyses also confirmed a time-dependent increase in cellular granularity, a hallmark of EBV-induced transformation, which reached a plateau on the 12th day of culture. Taken together, these data suggest day 12 as ideal time for collecting and freezing LCLs from cultures.

Cell clusters were more evident after several days of culture, indicating the success of EBV infection. It should be kept in mind, however, that it is necessary to add a viral-induced activator in cell cultures in order to prevent EBV from entering at any latency phase (Bird *et al.* 1981). The activator employed here was cyclosporin A (CSA), an immunosuppressor agent used to prevent transplant rejection *in vivo*. In this study, CSA possibly had suppressed the *in vitro* T-cell response against EBV-induced infection of B cells. This response can also be dampened by vigorous shaking and the whole media changing, this is because degeneration of B cells in proliferation foci largely depends on the tight junction of B cells with suppressor or cytotoxic T cells (Oh *et al.* 2003).

In this study, activities of the lysosomal acid hydrolases β -galactosidase, β -glucosidase and α -iduronidase were measured before and following cryopreservation of LCLs. These enzymes were chosen because they are deficient in the most frequent genetic lysosomal storage diseases in southern Brazil: GM1-gangliosidosis type I, Gaucher disease and mucopolysaccharidosis type I (Coelho *et al.* 1997). Activities of these enzymes were found to be similar prior to and after cryopreservation of the LCLs. These data further indicate that long-term frozen LCLs can efficiently be used as diagnostic markers of genetic metabolic disorders. Determination of deficient lysosomal acid hydrolases can also be performed on skin fibroblasts. LCLs have several advantages over skin fibroblast lines, such as they are more readily obtainable, they have far less cytogenetic variation, more rapid cell proliferation combined with a long lifespan, they have more massive production of cellular material, and are easier to store in the frozen state followed by re-establishment in culture (Minami *et al.* 1978; Negre *et al.* 1986). These advantages render faster and reliable diagnosis for the diseases described (Dreyfus *et al.* 1980; Neitzel 1986).

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