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AN EFFICIENT METHOD FOR ROUTINE EPSTEIN-BARR VIRUS IMMORTALIZATION OF HUMAN B LYMPHOCYTES

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SUMMARY

A variety of methods exist for the immortalization of B lymphocytes by Epstein-Barr virus due to the simplicity of such techniques to establish cell lines with stable genomic DNA. Two different methods for immortalizing lymphoblastoid cell lines were compared for differences in techniques and materials, time between initiation and immortalization, and success rate of immortalization. An incubation period in Epstein-Barr virus and the use of conditioned media improved immortalization efficiency from 86 to 98% and decreased the time (usually weeks) from culture initiation to cryopreservation. The resulting cell bank was used to produce DNA for genetic studies focusing on the genes involved in non-insulin-dependent diabetes mellitus.

Key words: immortalization; B lymphocytes; Epstein-Barr virus; transformation; human cell lines.

INTRODUCTION

Several methods have been developed for the immortalization of B lymphocytes by Epstein-Barr virus (EBV) (7,21,24,31,37, 38,40). Immortalization is the process by which cells are changed by an exogenous agent—in this case a virus—which enables them to replicate and grow indefinitely in vitro (17,29). Sometimes this process is also called virus-induced transformation in which cells are altered in their morphology, proliferation, and genetic makeup (25).

Very little has been written on the methods for the establishment of lymphoblastoid cell lines (LCLs) in the past year or so, although the use of such methods is prevalent in many works. Most of these immortalization procedures have two major similarities: a) use of a separation medium to isolate mononuclear leukocytes from whole blood, a practice developed by Boyum (4,5); and b) use of EBV supernatants obtained from the B95–8 marmoset cell line (this cell line produces high titers of infectious EBV) (3,7,12,20,21,24,28, 31,32). Additional differences among methodologies have also been described. Some bypassed the separation step and instead used whole blood, either fresh or frozen (37,38) or previously cryopreserved non-immortalized cells (15,26,27). In addition, others have used gamma-irradiated fibroblast type cells as feeder layers to increase the efficiency of culture immortalization (7,24). To that end, mitogens such as phytohemagglutinin (PHA) (12,22), pokeweed mitogen (PWM) (3), and lipopolysaccharide (LPS, from *Escherichia coli*) (12) were also used to stimulate lymphocytes. Sometimes Cyclosporin A (CSA), a T-cell inhibitor, has been used (21, 24,26,38). Overall, methods have varied in media formulations, virus preparation, B cell separation, the use of feeder layers (7,24, 35) and inclusion of CSA during selection.

Successful immortalization has provided continuous cell lines which can be maintained indefinitely and used for studies about EBV (11,14,18,19,33), B lymphocytes (12,22,23,25,28,29, 30,39), antigen/antibody and protein production (6,8,9,13,41) as well as various genetic studies (1,2,10,16,36) that require large amounts of stable genomic DNA. Although these methods frequently result in transformation, the variability in success within a rapid time frame reduces confidence in use of method selection when there are large numbers of pedigreed donors (e.g., for genetic studies), relatively small numbers of collected cells, and the need to bank large numbers of cells as quickly as possible. Because of these requirements and the precious nature of these resources, we undertook this study to provide a routine method to facilitate EBV-induced B lymphocyte immortalization. Two methods were compared: method 1 is a general composite of standard methods, and method 2, in which a composite method includes supplementation of the culture growth medium with conditioned medium from an EBV-transformed B cell line.

MATERIALS AND METHODS

Reagents. The sources of reagents and materials used in this work were: filter cap 25-cm^2 and 75-cm^2 tissue culture flasks from Corning, Corning, NY; tryptone, yeast extract, and fluid thioglycollate medium from DIFCO, Detroit, MI; RPMI 1640, gentamicin, 100 m*M* L-glutamine, Histopaque 1077, dimethylsulfoxide and mouse IgG_1 from Sigma, St. Louis, MO; Fungizone from Irvine Scientific, Santa Ana, CA; cyclosporin A from Sandimmune, Sandoz, East Hanover, NJ; 100× GlutaMAX-1, 10× Hanks' balanced salt solution, and penicillin-streptomycin (5000 U/ml) from

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GIBCO Laboratories, Grand Island, NY; 10× trypsin-EDTA from JRH Biosciences, Lenexa, KS; fetal bovine serum (FBS) from Hyclone Laboratories Inc., Logan, UT; sodium chloride from Baker Company Inc., Standford, ME; monoclonal antibodies to EBV nuclear antigen (EBNA-2), latent membrane protein (LMP-1), biotinylated anti-mouse IgG (gamma chain specific), and fluorescein-streptavidin from Vector Laboratories, Inc., Burlingame, CA.

Media. RPMI 1640 was used as the wash medium to rinse the cells and as the base medium for cell culture. RPMI complete medium was RPMI 1640 supplemented with 20% FBS, 2 m*M*/ml GlutaMAX-1, 50 μg/ml gentamicin, 2.5 μg/ml Fungizone, and 0.2 μg/ml CSA. Addition of penicillin-streptomycin antibiotics was optional. RPMI complete conditioned medium consisted of conditioned media harvested from the H01006 cell line [a line maintained at the Center for Human Cell Biotechnology (CHCB) and developed by Dr. Richard D. Henkel, Southwest Foundation for Biomedical Research (SFBR), San Antonio, Texas] mixed 1:1 with RPMI complete medium with extra GlutaMAX-1 and CSA added (enough to supplement the harvested spent conditioned media). Histopaque was passed through a 0.22-μm filter and sterility checked before use. Luria broth (LB) consisted of 5 g tryptone, 2.5 g yeast extract, and 5 g sodium chloride (NaCl) dissolved in 500 ml sterile water and autoclaved before use. Fluid thioglycollate (TG) medium was made according to instructions from DIFCO.

Sterility measures. All media were tested for microbial contamination using LB and TG media. EBV stocks and H01006 media supernatants were also separately incubated in 25-cm^2 flasks at 37° C and 5% CO_2 to check for the presence of cells and to avoid cross-contamination of lymphocyte cell lines. Mycoplasma testing of cell lines was done periodically using Gen-Probe (Fisher Scientific, Pittsburgh, PA) with negative results.

Safety considerations. Because human blood is capable of carrying a variety of pathogens, all operations were performed at biosafety level 2 in a vertical laminar flow safety cabinet. Latex gloves and lab coats were worn at all times, and all samples were centrifuged in Aerosolve canisters (Beckman, Palo Alto, California). All contaminated materials were autoclaved before disposal.

Maintenance of H01006 cell line and harvest of conditioned media. The H01006 cell line, originally developed by Dr. Henkel of the Department of Genetics, SFBR, San Antonio, Texas, was maintained with media harvests at the CHCB, University of Texas Health Science Center at San Antonio (UTHSCSA), TX. H01006 is an EBV-immortalized human B lymphocyte line that is free of bacterial, mycoplasmal, and viral contaminants. Used as the source of conditioned medium in the immortalization of human B lymphocytes, the H01006 cells were grown as suspension cultures to a density of about 5×10^6 cells/ml in 75-cm^2 tissue culture flasks containing 30 to 40 ml of RPMI complete medium. Harvesting was done weekly by removing all spent media except for 10 ml from each flask. The flasks were then subcultured at a ratio of 1:2 to 1:4 and fresh complete RPMI culture medium added. Extra cells were either cryopreserved or discarded. The spent medium was kept at 4° C until 300 to 500 ml was accumulated and then the whole batch was filtered 3 times through 0.22-μm filters. Each batch was sterility checked, and separate aliquots were incubated at 37° C in 5% CO_2 to further ensure that no cells were present.

Maintenance and harvest of B95-8 cell line. The B95-8 cell line was obtained from the American Type Culture Collection, Rockville, MD (ATCC 1612-CRL). Flasks were seeded at approximately 1×10^6 cells/ml in RPMI complete medium and handled weekly if needed. To passage or subculture, all medium was collected from the flask (which contains the floating cell population) and placed into a 50-ml centrifuge tube. The attached cells were removed using 0.1% trypsin, 0.04% EDTA solution and placed in the tube. The suspension was centrifuged at 500 ×*g*, 23° C, for 10 min. The cell pellet was subcultured at a split ratio of 1:5 to 1:10 and the resulting flasks incubated at 37° C, 5% CO_2. Extra cells were either cryopreserved or discarded. To produce EBV stock, dense cultures of B95-8 cells were incubated for 8 days with no medium added. The supernatant was collected and spun down at 500 ×*g*, 23° C, for 10 min, and the cell pellet discarded. After the supernatant was passed through three 0.22-μm filters, each batch was sterility checked with LB and TG media and a separate aliquot incubated at 37° C and 5% CO_2 to detect for the presence of cells. Each EBV batch was aliquoted out and stored at 4° C with an expiration date of 1 to 2 mo. The efficacy of the B95-8 cell supernatant-derived viral stocks was tested for immortalizing of peripheral blood lymphocytes (PBLs) from control individuals before use (procedure described below).

Cryopreservation, thawing, and cell viability. Cell suspensions were spun down into pellets at 500 ×*g*, 23° C, for 10 min, resuspended in 1-ml aliquots of freezing medium [RPMI complete medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO)] and dispensed into 1.8 ml Nunc cryovials. The vials were labeled and put into Nalgene Cryo 1 freezing containers (cat. no. 5100-0001) and placed into a −86° C freezer overnight. The next day the vials were transferred into either liquid nitrogen or appropriate inventory boxes at −86° C. Upon thawing, cryovials were removed from liquid nitrogen or −86° C and immersed in 37° C water bath. After thawing, cells were washed with 10 to 20 ml RPMI 1640 to remove all DMSO before being placed in culture. Random cell line samples were assayed for viability at 24 to 48 h post seeding, using standard trypan blue exclusion assays.

Blood collection and lymphocyte separation, method 1. This method involved isolating the lymphocytes from only 10 ml of whole blood and immortalizing them in EBV-containing culture supernatants and RPMI complete medium. Samples were processed as soon as possible after receiving them. Ten milliliters of blood were diluted 1:1 with 37° C RPMI 1640 supplemented with glutamine, gentamicin and Fungizone (concentrations described above). Four milliliters of diluted blood were layered over 3 ml Histopaque, room temperature, in 15-ml centrifuge tubes (a total of 5 tubes per blood sample) and spun at 400 ×*g* for 30 min at 23° C. The clear top plasma layer was discarded. The opaque lymphocyte layers were collected from the five tubes and pooled into a 50-ml centrifuge tube. The cells were washed twice by repeat centrifugation rinses in the supplemented RPMI 1640 medium mentioned above.

Immortalization of lymphocytes, method 1. The resulting cell pellet was resuspended in 1 ml EBV supernatant and 3 ml of RPMI complete medium, then transferred to a 25-cm^2 tissue culture flask. Unlike method 2, there was no EBV incubation step, no use of conditioned medium, no cryopreservation of non-immortalized cells, and no use of filter-cap flasks. Cells were observed weekly for signs of transformation and RPMI complete medium added if required. When the cells reached a density of about 4 to 5×10^5 per ml, they were transferred into a 75-cm^2 flask. Cells were counted and frozen at a density of 5×10^6 cells per cryovial.

Blood collection and lymphocyte separation, method 2. For each selected family member, 20 ml of peripheral blood were collected in 10-ml Vacutainer tubes containing heparin. All reagents to be used in the lymphocyte isolation were warmed to 37° C. The blood was pooled in a 50-ml centrifuge tube, diluted with RPMI 1640 up to 35 to 40 ml, underlayered with 10 ml Histopaque 1077, and spun at 400 ×*g* for 20 to 30 min at 23° C. The clear top plasma layer was discarded. The opaque lymphocyte layer along with the Histopaque was collected down to the erythrocyte pellet and transferred to another 50-ml centrifuge tube. The cells were washed 3 times by repeat centrifugation rinses in RPMI 1640 and 60% of the pellet was cryopreserved in RPMI + 20% FBS and 10% DMSO. (These vials of non-immortalized cells, approximately 5×10^6 per vial, were cryopreserved as a backup for each donor.)

Immortalization of lymphocytes, method 2. The remaining 40% of the pellet obtained from the Histopaque isolation was resuspended in 1 ml of sterile cell-free EBV supernatant obtained from the culture of B95-8 cells. The cell suspension was placed in a filter-cap 25-cm^2 tissue culture flask and incubated 1 to 3 h in an upright position at 37° C and 5% CO_2. After the incubation 3 ml of RPMI complete conditioned medium were added to the flask, the incubation continued in a prone position with the cell suspension evenly covering the bottom of the flask. The cells were observed every week under an inverted microscope for signs of immortalization (increased cell growth, cell aggregates or clumping, and lymphocyte blastogenesis) and to add fresh RPMI complete conditioned medium to prevent the flasks from drying out. The first passage or subcultivation of the culture usually took place 2 to 4 wk after initiation. Parallel cultures from each family member were incubated and maintained separately as an extra precaution against loss. For each immortalized cell line 10 vials (1 vial equivalent to $^1/_2$ to 1 dense 75-cm^2 flask, i.e. 12×10^6 to 24×10^6 cells) were cryopreserved as a stock in liquid nitrogen or at −86° C.

Occasionally those cultures that were initial slow growers or doing poorly were treated again with EBV, which sometimes aided in the immortalization process.

Detection of EBNA-2 and LMP-1 antigens in EBV immortalized cells.

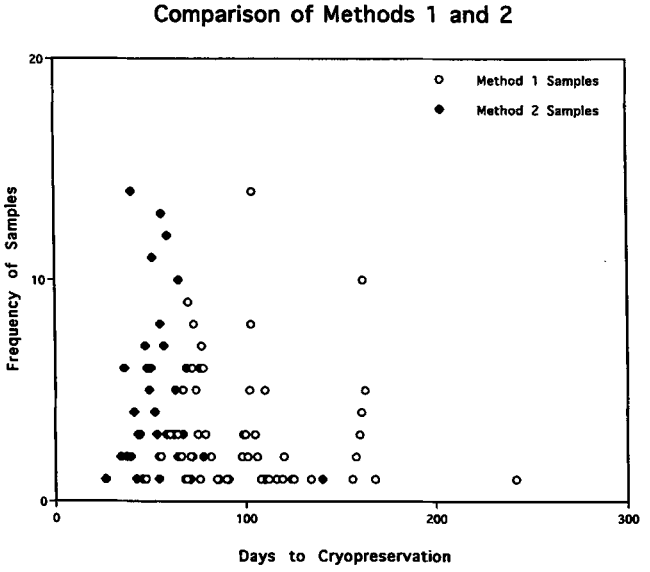

FIG. 1. Number of days to cryopreservation for methods 1 and 2, according to sample frequency.

Cell lines immortalized with EBV from the B95–8 cell line were selected at random and fixed in suspension with cold acetone: methanol (1:1) and stored at 4° C until stained. Two positive control cells [human cord lymphocytes (HUCL) immortalized with EBV and RAJI] and one negative cell line (MOLT-4) were also tested. Fixed cells were placed on 5-mm wells of 14-well slides and allowed to air dry. Each cell line was tested with monoclonal antibody (MAb) to EBNA-2, MAb to LMP-1, and mouse IgG_1. Antibody controls included incubating with primary or secondary antibodies alone to ensure no evidence for nonspecific reactivity. Cells were incubated with diluted MAb (1:5) at 37° C for 1 h, washed, and subsequently allowed to react with biotinylated anti-mouse IgG (1:20) for 30 min, followed by fluorescein-streptavidin (1:50). Slides were mounted with glycerol:phosphate buffered saline (9:1, pH 8.5) and viewed with a 50× water immersion objective of a Zeiss epifluorescence microscope.

RESULTS

Immortalization efficiency. Over a period of a year and a half 370 cell lines were successfully established out of 405. Under method 1, 233 samples were processed with 32 unsuccessful due to problems with cell yields or contamination (although high, only an 86% success rate was achieved). In contrast, of the 172 samples processed by method 2, only 4 samples were unsuccessful (i.e. there was a 98% success rate). Those that did not grow had very low initial numbers of viable white blood cells in the samples. The chi-square test (15.902 at $P = 0.0001$) and G-statistic (18.523 at $P = 0.0001$) indicated that the success rates for the two methods in establishment of cell lines were indeed significantly different.

Time between initiation and cryopreservation. Signs of transformation (cell aggregates, increased rate of growth, etc.) were usually present within 1 to 3 wk as confirmed by the literature (7,21,24,25,37). The number of days between culture initiation and cryopreservation were plotted for method 1 and method 2 according to frequency (Fig. 1). The average length of time for method 1 was 99 ± 35 days and for method 2 was 57 ± 14 days. Bartlett's test indicated that the bell widths of the distribution curves of both methods were unequal, which invalidated any results obtained using the analysis of variance (ANOVA), *t* test, or other parametric methods. This was confirmed by Snedecor's approximate *F* statistic for unequal variances which was unable to compensate for the differences in the two distributions. Because the distributions for both methods were definitely not normal, the nonparametric Mann-Whitney U test was used to show that methods 1 and 2 were significantly different in the time between culture initiation and cryopreservation (U_s = 27 796 at $P = 0.0001$)(34).

Thawing efficiency. During the course of building and maintaining the cell bank, over 100 cell lines were removed from cryopreservation to amplify stocks or reinitiate cultures with 100% efficiency, demonstrating that the methods used to cryopreserve and store cells were effective in ensuring cell viability. This was verified by standard trypan blue dye exclusion studies of random samples where more than 90% of the cells were viable.

Evidence of immortalization by EBV. Of the 11 selected cell lines assayed for EBNA-2 and LMP-1 expression, all were positive, indicating that immortalization was EBV specific. Antibody and non-EBV containing cell line controls were negative for expression.

DISCUSSION

Of the two methods described in this paper, method 2 was superior in culture success, immortalization efficiency, and length of time to cryopreservation. Although differences between the lengths of time to cryopreservation might be partly due to differences in FBS lots and in virus batches as reported by others (38), the large numbers of samples and patient sources clearly demonstrated the superiority of method 2.

Method 1 was less successful overall due to several factors: a) occasional culture regression due to the presence of T cells or too few lymphocytes, b) contamination was sometimes a problem, and c) the length of time required to immortalize cell cultures and expand them to large enough numbers for cryopreservation was twice as long. A new isolated laboratory under GLP standards, stricter sterility checks enforced on all medias and reagents, use of filter-cap tissue culture flasks, and the use of conditioned media contributed to the improved success rate for transformations.

Although the success rate of method 2 compares favorably with those observed by other researchers (7,15,24,26,37,38), it has several advantages over other published methods. One of the primary advantages of method 2 was the use of conditioned medium harvested from the H01006 cell line, replacing the need for feeder layers. Although it seems that the use of feeder layers may enhance immortalization efficiency (7,24,35), such a procedure is not practical when samples are received at a high rate (i.e. up to 84/mo.) and when manpower and space are limited. Conditioned medium eliminated the need to grow up, maintain, and irradiate flasks of feeder cells while still providing growth and other factors for newly initiated cultures. This medium also aided in removing cells from cryogenic storage by increasing cell recovery and viable yield. With this system in place a staff of two can initiate, grow up, and cryopreserve an immortalized cell line in 4 to 6 wk (for a total of 9 to 10 vials at 2×10^7 to 5×10^7 cells per vial).

Possible areas of improvement and enhanced cost-effectiveness of method 2 include: a) reduction of serum concentration in the medium after the cells are immortalized and growing quickly; b) discontinued use of CSA after immortalization because it is not required for T-cell inhibition after the B cells are transformed (21), and c) assessment of a synergistic contribution of certain compounds to further increase the efficiency and shorten the time for immortalization (12,21,22).

In summary, the procedure described as method 2 in this paper is an excellent, routine way of establishing high numbers of continuous EBV-immortalized B-cell lines which can be grown to large cell numbers in a relatively short time. Thus, banks of cells can be readied as stocks of genetic reference material from the precious cells obtained from individuals and pedigreed study populations. These cellular tools subsequently allow genetic analyses of diseases such as diabetes.

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