Original Article

Application of Epstein–Barr Virus for Optimization of Immortalized B-lymphocyte Production as a Positive Control in Genetic Studies

Behnaz Tousizadeh[1,2], Sharareh Moghim[3], Ahmad Reza Salehi Chaleshtori[4], Maryam Ghanbarian[5], Mina Mirian[6], Mansoor Salehi[1,4,6], Sepideh Tousizadeh[7], Fatemeh Zaboli[1]

From the [1]Department of Microbiology, Faculty of Basic Sciences, Ayatollah Amoli Azad University, Amol, [2]Pediatric Inherited Disease Research Center, Departments of [3]Microbiology and [6]Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, [4]Medical Genetics Center of Genome, [5]Department of Microbiology, Faculty of Basic Science, Islamic Azad University, Falavarjan Branch, Isfahan, [7]Department of Health, School of Health, Shahrekord University of Medical Sciences, Shahrekord, Iran

Address for correspondence:
Dr. Mansoor Salehi, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: m_salehi@med.mui.ac.ir

Abstract

Background: Infection of B-cells with Epstein–Barr virus (EBV) leads to more and subsequent immortalization. This is considered as the method of choice for generating lymphoblastoid cell lines (LCLs). Producing LCLs, although very useful but is very time consuming and troublesome, drives the requirement for quicker and more reliable methods for EBV-driven B-cell transformation. **Materials and Methods:** After successfully production of LCLs, different parameters including temperature, serum concentration, type of culture medium, and CO_2 concentration were evaluated on EBV-transformed B-cells. In this study, we were able to produce LCLs and optimize condition. **Results:** The best condition for generating LCLs was 37°C, 5% CO_2, 20% fasting blood sugar, and RPMI 1640. The study results were to establish a reliable method for producing LCLs that can be used to produce immortalized B-cells from almost any sources. **Conclusion:** This can help with tumorgenecity studies, as well as producing control material for rare genetic disorders and so on. The aim of this study was to determine optimized condition for reliable and reproducible LCLs from different sources.

Keywords: *B95 cell, Epstein–Barr virus, immortalization, lymphoblastoids cell line, optimization*

Introduction

Since 1968, it is recognized that the Epstein–Barr virus (EBV) could be used *in vitro*, to infect human B-lymphocytes and produce lymphoblastoid cell lines (LCLs) *in vitro*. EBV [Figure 1], a virus of herpes family, also called human herpes virus 4, which is one of the most important viruses in human being.[1] LCLs have been used for simplification of immunological and molecular studies, effectively. Banking of EBV cell lines also holds great potential for providing reference material for rare genetic disorders diagnosis. This virus was first discovered by Sir Michael Anthony Epstein and Yvonne MR. Barr in Burkitt lymphoma cell line mononucleosis and was then linked to the infectious mononucleosis disease by the Histocompatibility Research Community. LCLs are used to produce reference HLA typed cells for use in functional and serological studies, extensively.[2] LCLs were also used in the management of large amounts of DNA for genetic analysis of complex conditions in population and family disease collections.[3]

It has been reported that the produced EBVs by B95 cell line are biologically and antigenically indistinguishable from other isolates of EBV.[4] Also, it has been the prototype for previous studies of EBV DNA. B95 do not secrete immunoglobulin in response to B-cell differentiation factor containing supernatants.[5] B95 releases with high titers of transformation activity and is widely used as a model in cancer research, as well as virology.[5]

The triggering of B-lymphocytes to clonal expansion and terminal differentiation is punctuated by a series of cell cycle arrest points which require precise biochemical signals in order to be traversed.[6] Primarily, antigen presentation in the physiologically appropriate issue detected through appropriate way.[7] In addition, it is used for management of large amounts of DNA for genetic analysis of complex conditions in population and family disease. Thus, EBV transformation has been critical for long-term management of significant disease and progression in genetic epidemiology. Nowadays, some major facilities established and managed extensive collections of cell lines.[4,8] If EBV-mediated transformation be successful, cell lines can be observed by sophisticated unarmed eye

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after several weeks before microscopic clusters of cells can be demonstrated, even.[4,9]

In this study, we developed an optimized method including temperature, CO_2, quantity of fasting blood sugar (FBS), and culture media to produce lymphoblastoids from B-cells repetitively.[9,10] Moreover, we generated several human LCLs using this optimized protocol.[11] The EBV was concentrated to obtain excessive load of virus to estimate the number of inflexible cells. The B-lymphocytes were purified to allow direct deduction of the number of inflexible and growing cells after infection.[12] Benefits of human immortal blood lymphocytes is that it provides a permanent storage source of the desired genetic materials,[13] preventing the repetitive sampling. The produced lymphoblastoids has been used as control in many laboratories to prevent repetitive blood sampling.[14]

Materials and Methods

Blood cultures

The marmoset lymphoblastic cell line B95.8 was used as the potential source of free EBV particles. The cell line was grown in RPMI complete medium supplemented with 10% fetal calf serum (FCS) and cultured in flasks at 37°C in a 5% CO_2. The cultures were allowed to overgrow and a final medium change of RPMI with 2% FCS was used to encourage virus production. 10 ml of donor's blood was transferred into a heparinized syringe or blood tube, then it was diluted with 20 ml phosphate-buffered saline (PBS) in room temperature in a 50 ml conical tube. Thereafter, 15 ml of Ficoll-Hypaque lymphocyte separation medium were added and reserved at room temperature for 30 min. After centrifugation, the buffy coat was removed and the volume was increased to 50 ml associated with PBS, then it was spin at ×600 g at room temperature for 10 min. Subsequently, cells were washed using 50 ml PBS and spin at ×600 g at room temperature for 10 min 1 more time and then washed cells were added to 1 ml of complete RPMI. Also we were used cyclosporine A to prevent growth of T-cells. The cells were added to a 25 cm^2 tissue culture flask containing complete RPMI and cultured to obtain 2×10^6/ml cells.[15]

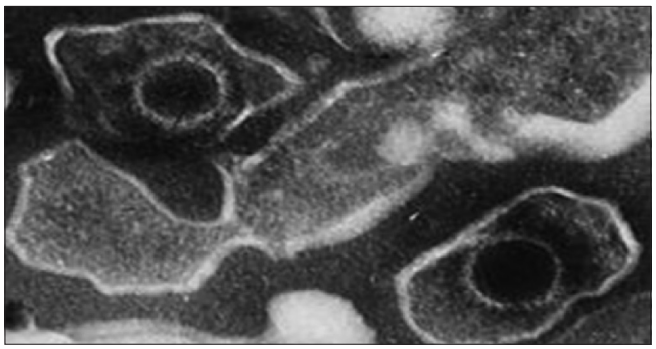

Figure 1: Epstein–Barr virus: Once the virus initiates lytic infection, the virus brought under control, it can persists in the individuals B-cells for the rest of life finally

Production and Infection with Epstein–Barr virus

0.5 ml of product of B95 cells were added into two wells of a 24-well plate. In the next step, any of 1 ml lymphocyte suspension, higher than 10^6 cell/ml in a complete culture medium, were added. Then 100 µl of cyclosporin A (CSA) stock, diluted 1:50 in RPMI, were added. Finally 100 µl of penicillin/streptomycin diluted 1:50 in RPMI were added. The final volume in each well was 1.6 ml. All the wells were marked properly, covered and placed in a humidified 5% CO_2 incubator at 37°C. The transformed cells were assessed under the microscope after approximately 48 h [Figure 2]. These cells were bigger and their fibroblastic morphology was transformed to lymphocytes.[15]

Cells were grown for 5 or 7 days and when the medium turns yellow, cells were collected and poured off into two 25 cm^2 flask.[16] Then 2 ml of complete medium were added to each flask, and put the flasks, with the slightly opened cap, into the CO_2 incubator. In the next step, 2–2.5 ml of complete medium was added to the flasks up to 10 ml volume in a 6 or 7 days period of time.

After a week, when the medium turns yellow, cells were harvested by pouring off 8 ml medium of both flasks into one 75 cm^2 flask and then it was filled with up to 25 ml medium. The 75 cm^2 flask reloaded with 3 ml of complete medium and return to the incubator again. After 5–7 days, the 75 cm^2 flask was filled with 15 ml of fresh complete medium.

After approximately 60 days, it should be of a concentration of nearly 10^6 cell/ml, If the culture was grown properly. The number of clots, the cloudy, and the color of the medium [Figure 3] indicated that it was the right time. 20 ml of culture was centrifuged and the supernatant was removed in order to freeze in liquid nitrogen. These processes were repeated until 10^7 cells/ml were acquired.[16]

Finally, the followings were optimized to determine the best conditions for production of lymphoblastoids:

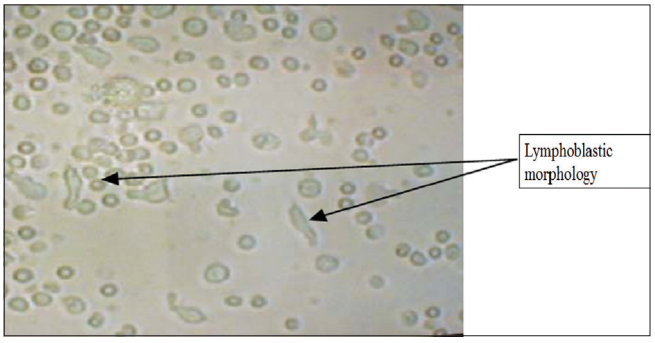

Figure 2: Lymphoblastic morphology[5]

Different temperatures including 36°C, 36.5°C, 37°C, 38°C, and 39°C were used in culturing the produced lymphoblastoids. Also lymphoblastoids were grown in different percentages of CO_2 including 5%, 10%, 15%, and 20% and lymphoblastoids were grown in different medium cultures, including DMEM, RPMI 1640. Likewise, lymphoblastoids were grown in different percentages of FBS including 10%, 15%, 20%, and 25%.[17]

To confirm LCL, we performed polymerase chain reaction (PCR) amplification using normal and mutant primers for some of our patients.

Results

In this study, after 4 weeks, we were able to generate lymphoblastic cell line. Different temperatures were used for culturing lymphoblastoids ranging between 36°C and 39°C. The best temperature was 37–37.5°C ($P < 0.05$). The number of cells produced in each temperature is summarized in Table 1 and image was prepared from lymphoblastoid cells [Figure 4].

Different culture mediums including RPMI and DMEM were applied for culturing the lymphoblastoids. The RPMI was recognized as the best medium. The number of cells produced in each culture medium is summarized in Table 2.

Different percentages of CO_2 including 5%, 7%, 10%, and 15% were used for culturing lymphoblastoids that the first one recognized as the finest one. The number of produced cells is summarized in Table 3.

Different percentages of FBS such as 10%, 15%, 20%, and 25%, were applied for culturing lymphoblastoids and the FBS concentration of 20% was determined as the most appropriate concentration. The number of produced cells are summarized in Table 4.

Table 1: Number of cells produced in different temperature

Temperature	Conditions	Number of cells
36°C	CO_2 5%, FBS 10%	500,000
36.5°C	CO_2 5%, FBS 10%	800,000
37°C	CO_2 5%, FBS 10%	900,000
38°C	CO_2 5%, FBS 10%	950,000 until 48 h, after 5 days decreased to 400,000
39°C	CO_2 5%, FBS 10%	300,000

FBS: Fasting blood sugar

Table 2: Number of cells produced in different culture medium

Culture medium	Number of cells	Condition
DMEM	900,000-950,000	37°C, CO_2 5%, FBS 10%
RPMI 1640	1,000,000-1,300,000	37°C, CO_2 5%, FBS 10%

FBS: Fasting blood sugar

PCR amplification procedure was performed and products were electrophoresed on an agarose 2% gel electrophoresis. The mutants have two bands, including PCR products with mutant primers and a positive internal control near the 550 bp. Moreover, the normal ones have a single band for PCR amplification including internal control band. Data were shown in Figure 5 and Table 5.

Discussion

The method was described in this study can produce LCL from donor peripheral blood cells with rapid immortalization. These developments make the described

Figure 3: Clots of transformed cells

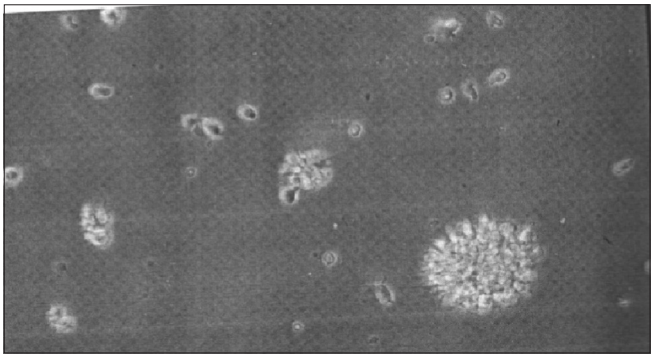

Figure 4: Lymphoblastoid cell line

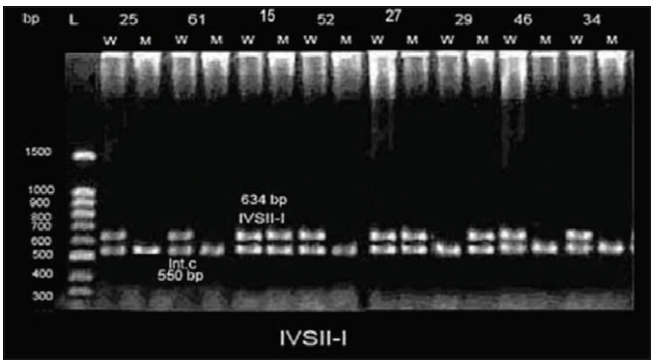

Figure 5: W: Wild; representing normal primers, M: Mutant; representing mutant primers, L: Ladder. Internal control size is equal to 550 bp size. The sample number 29 is homozygous for mutation, because the mutant primers have a band and normal primers has no band. The samples number 15 and 27 are heterozygous for mutation, because normal primers and mutant primers have bands

Table 3: Number of produced cells in different percentages of CO_2

Percentage of CO_2	Number of cells	Condition
5	1,000,000-1200,000	37°C, CO_2 5%, FBS 10%
7	900,000-1000,000	37°C, CO_2 7%, FBS 10%
10	500,000-250,000	37°C, CO_2 10%, FBS 10%
15	10,000-5000	37°C, CO_2 15%, FBS 10%

FBS: Fasting blood sugar

Table 4: Number of cells produced in different percentage of FBS

Percentage of FBS	Number of cells	Condition
10	1000,000	37°C, CO_2 5%, FBS 10%
15	1000,000-1200,000	37°C, CO_2 5%, FBS 15%
20	1200,000-1500,000	37°C, CO_2 5%, FBS 20%
25	1500,000	37°C, CO_2 5%, FBS 25%

FBS: Fasting blood sugar

Table 5: Sequence of applied primers

Primer	Sequence	Primer type
Normal primer	5'AAGAAAACATCAAGGGT CCCATAGACTGAC3'	Forward
	5'ACCTCACCCTGTGGAGCCAC3'	Reverse
Mutant primer	5'AAGAAAACATCAAGGGT CCCATAGACTGAT3'	Forward
	5'ACCTCACCCTGTGGAGCCACATA3'	Reverse

method more effective, resulting in rapid expansion of cells for subsequent experiments[18] which is not accessible in Iran currently. In this study, EBV used for lymphoblast immortalization. EBV were grown in B95.8 that do not require interleukin-2 (IL-2) for their growth, but addition of IL-2 may stimulate growth of these cells.[18] Okano *et al.* used H-*ras* and c-*myc* oncogenes to immortalize lymph node lymphocytes. The cell lines were growing for 3 months after establishment, but cells needed IL-2 for growth stimulation. In this study, we used B95.8 for generation of high titer of EBV. In another study, FK506 (AG scientific) utilized for cell suspension from 2.6 nM to a final concentration of 20 nM.[19] Using FK506, high titers of infectious virus is achievable and promote proliferation of EBV-infected B-cells from peripheral blood cells, but these cells needed more FCS and long time for generating.[19] We used CSA to prevent growth of T-cells. An alternative strategy has been to incubate lymphocyte cultures with T-cell mitogens such as phytohaemagglutinin, which encourages T-cells to rapidly transform into blast cells and die before cytotoxic T-cells can be generated, but in presence of CSA, T-cells were killed rapidly.[1]

This study find an optimized method for generating LCLs that can be used to produce immortalized B-cells from almost any sources. This can help to studies around tumorigenicity, as well as producing control material for rare genetic disorders and so on. We used different temperatures, CO_2 and FBS concentrations, and different mediums for lymphoblastoid culturing. The best conditions for generating LCLs were 37°C, 5% CO_2, 20% FBS, and RPMI 1640.[11,20] These conditions were approved through our study and our results. More studies are required to improve these conditions.

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Conflicts of interest

There are no conflicts of interest.

References

1. Megyola C, Ye J, Bhaduri-McIntosh S. Identification of a sub-population of B cells that proliferates after infection with Epstein-Barr virus. Virol J 2011;8:84.
2. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-50.
3. Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. N Engl J Med 1984;310:1225-30.
4. Hui-Yuen J, McAllister S, Koganti S, Hill E, Bhaduri-McIntosh S. Establishment of Epstein-Barr virus growth-transformed lymphoblastoid cell lines. J Vis Exp 2011. pii: 3321.
5. Callard RE, Lau YL, Shields JG, Smith SH, Cairns J, Flores-Romo L, *et al.* The marmoset B-lymphoblastoid cell line (B95-8) produces and responds to B-cell growth and differentiation factors: Role of shed CD23 (sCD23). Immunology 1988;65:379-84.
6. Amoli MM, Carthy D, Platt H, Ollier WE. EBV Immortalization of human B lymphocytes separated from small volumes of cryo-preserved whole blood. Int J Epidemiol 2008;37 Suppl 1:i41-5.
7. Mosier DE, Subbarao B. Thymus-independent antigens: Complexity of B-lymphocyte activation revealed. Immunol Today 1982;3:217-22.
8. Miller G, Shope T, Lisco H, Stitt D, Lipman M. Epstein-Barr virus: Transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. Proc Natl Acad Sci U S A 1972;69:383-7.
9. Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, *et al.* An efficient method to make human monoclonal antibodies from memory B cells: Potent neutralization of SARS coronavirus. Nat Med 2004;10:871-5.
10. Yang ZY, Kong WP, Huang Y, Roberts A, Murphy BR, Subbarao K, *et al.* A DNA vaccine induces SARS coronavirus

neutralization and protective immunity in mice. Nature 2004;428:561-4.
11. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;298:2199-202.
12. Raab-Traub N, Dambaugh T, Kieff E. DNA of Epstein-Barr virus VIII: B95-8, the previous prototype, is an unusual deletion derivative. Cell 1980;22:257-67.
13. Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, *et al*. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 1991;83:757-66.
14. Ormerod MG. Flow Cytometry: A Practical Approach. Great Britain, Wiltshire: Oxford University Press; 2000.
15. Okano M, Thiele GM, Davis JR, Grierson HL, Purtilo DT. Epstein-Barr virus and human diseases: Recent advances in diagnosis. Clin Microbiol Rev 1988;1:300-12.
16. Beck JC, Beiswanger CM, John EM, Satariano E, West D. Successful transformation of cryopreserved lymphocytes: A resource for epidemiological studies. Cancer Epidemiol Biomarkers Prev 2001;10:551-4.
17. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med 2004;350:1328-37.
18. Gordon J, Guy G, Walker L. Autocrine models of B-lymphocyte growth. I. Role of cell contact and soluble factors in T-independent B-cell responses. Immunology 1985;56:329-35.
19. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757-68.
20. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, *et al*. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature 2007;448:470-3.