The Egyptian Journal of Medical Human Genetics 19 (2018) 221–223

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Contents lists available at ScienceDirect

The Egyptian Journal of Medical Human Genetics

journal homepage: www.sciencedirect.com

Original article

A modified protocol for highly efficient EBV-mediated immortalization of human B lymphocytes from small volumes of peripheral blood serum

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ARTICLE INFO

Article history:
Received 18 September 2017
Accepted 8 October 2017
Available online 6 December 2017

Keywords:
Immortalization
B lymphocytes
Epstein-Barr virus
Human cell culture

ABSTRACT

Background: Many human molecular and genetic studies require the use of a renewable biological material. Although primary fibroblast cell lines can be used for this purpose, there are disadvantages associated with human biopsies including the limited number of cell divisions. Peripheral blood has the advantage of being easier to obtain but also the drawback that blood cells produce only short-term cultures. Epstein-Barr virus is capable of transforming human B lymphocytes into indefinitely proliferating cells that can be maintained in tissue culture. Here, we report a convenient method of B-lymphocyte immortalization using small volumes of freshly collected human blood serum saturated with nucleated blood cells.

Aim of the study: The aim of the present study is modification and improvement of the protocol for highly efficient immortalization of human B lymphocytes from small volume of blood samples.

Material and methods: Cell line B95-8 was used as Epstein-Barr virus source for viral stock preparation. Immortalizing medium contains RPMI-1640, viral stock and additives. No feeder layer was used.

Results: As result we present high efficient method for B lymphocytes immortalization with start blood volume less than 5 ml.

Conclusion: The method is applicable for immortalization of B lymphocytes from small blood samples and is essential for studies involving children or patients when large blood volume sampling is impossible.

1. Introduction

Human blood cell lines are powerful tools for a variety of *in vitro* functional and molecular studies and an inexhaustible source of high molecular weight DNA for genetic disease collections. Cellular immortalization is defined as the phenotypic transformation of a normal cell with a limited life span to a malignant one. The immortalization of mammalian cells can be induced by chemical carcinogens, transfection of oncogenic viruses, or telomerase reactivation [1,2]. Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and is etiologically associated with Burkitt's lymphoma and nasopharyngeal carcinoma [3]. Over the past 40 years, cell line supernatants (e.g., from the marmoset B-lymphoblastoid cell line B95-8) were commonly used to confirm the neoplastic potential of EBV infection by virus-mediated immortalization of cultured B-lymphocytes from human blood [4–8]. Traditionally, these techniques require approximately 50 ml peripheral blood for one sample, special kits or media for B-cell isolation, and the use of feeder cell layers [9]. This is quite laborious and inapplicable for the immortalization of B-cells derived from small volumes. Research requiring cell immortalization from small sample sizes, for example blood collected from hyperactive patients or children, often lack the material volume necessary for immortalization by standard approaches. The aim of the present study is modification and improvement of the protocol for highly efficient immortalization of human B lymphocytes from small volume of blood samples, without feeder cells layer and virus activating agents.

2. Ethics

Patient involvement in this study was strictly designed in accordance with international standards, which include the awareness of the subject, his or her consent to participate in the study in its

Abbreviations: EBV, Epstein-Barr virus; BSC, biosafety cabinet; BSL-2, biosafety level 2; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide.

Peer review under responsibility of Ain Shams University.

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https://doi.org/10.1016/j.ejmhg.2017.10.002
1110-8630/

entirety, and the guarantee of confidentiality. All studies conformed to ethical standards developed in accordance with the Helsinki Declaration of the World Medical Association, as amended in 2000. In addition, these studies were supervised by the Institutional Review Board.

3. Materials and methods

3.1. Safety

All experiments with human samples, primate samples, and virus stock must be performed in a Class 2 BSC in a BSL-2 laboratory, by properly trained laboratory personnel wearing appropriate personal protective equipment including a lab coat, gloves and safety glasses.

3.2. Viral stock preparation

B95-8 marmoset suspension cell culture from IMCB SB RAS cell repository was tested for mycoplasma by PCR screening as previously described [10]. Cells were cultured in RPMI 1640 medium (Gibco, USA) with 20% of Fetal Bovine Serum (Gibco), 4 mM L-glutamine (twice the normal culture concentration) (Gibco) and 50 μg/ml gentamicin sulfate (KRKA, Slovenia). After 3 days of incubation, supernatant was collected without mixing the cells and passed through a 0.22 μm membrane filter (Corning, USA) to remove living marmoset cells.

3.3. Immortalization and freezing medium

Cells were immortalized in a medium containing 50% culture medium, 50% viral stock, 3% Phytohaemagglutinin-M (Gibco) and 0.04 mg/ml Cyclosporin A (Goldbio, USA). Immortalized cells were frozen in 90% Fetal Bovine Serum (Gibco) with 10% DMSO (Sigma, USA).

3.4. Samples

Blood samples were collected in Novosibirsk State Regional Clinical Diagnostic Center of Ministry of Health of Russian Federation according to ethical standards. Informed consent was obtained from every involved individual.

4. Results and discussion

EBV, also known as human herpesvirus 4, is one of the most common human viruses and the cause of infectious mononucleosis. Most people are infected with EBV at some point in their lives. Because EBV can sometimes cause malignant degeneration, tests using IgM (for recent infection) or IgG antibodies directed against the viral capsid antigen of EBV are often administered as a precondition for a person receiving permission to work.

To initiate the immortalization procedure reported here, we first thawed 5 million B95-8 marmoset cells infected with EBV in 10 ml culture medium, and 10–15 ml of fresh medium was added twice a week. Every three days, supernatant was collected without cell mixing and filtered for viral stock preparation. Note that it is important not to use inactivated bovine serum.

For immortalization, we used 3–5 ml blood samples collected in heparin test tubes (Vacutest, Kima, Italia). Blood must be at room temperature rather than chilled. One hour before culturing, it is necessary to mix and exfoliate the blood, but do not allow complete stratification into 3 layers (Fig. 1).

After this partial separation, we transferred the plasma containing nucleated cells into vertically oriented T25 flasks and added 9 ml of immortalization medium. One week later, we removed approximately half of this medium and replaced it with an equal volume of pre-warmed fresh growth medium containing 1–3% phytohaemagglutinin-M. Flasks should be incubated in an upright position with vented or loose caps at 37 °C in a CO_2 incubator. Important note: for the first four weeks, cells should be maintained by partially removing the growth medium without centrifugation based on its gradual acidification (i.e., when the color changes to yellow). The volume of medium in the flask can affect the growth of cells as the surface to air ratio is important in maintaining the proper pH of the medium. No more than 20 ml of medium should be used in a T25 flask. Cells are cultured in a medium containing virus and cyclosporine A for the first week; after this, growth medium should only be supplemented with 1% phytohaemagglutinin.

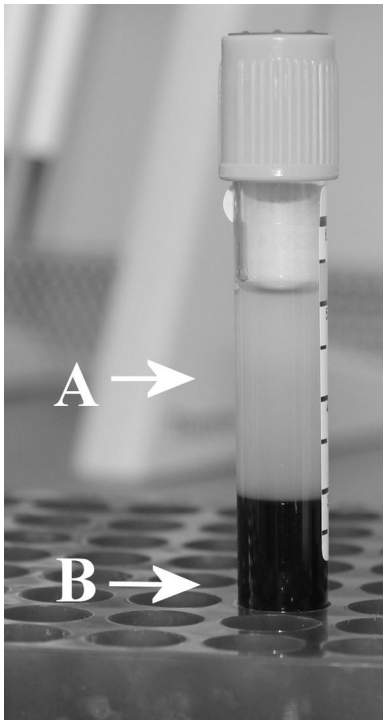

Fig. 1. Blood sample fractionation directly before plasma collection for immortalization. A. Blood plasma with nucleated cells. B. Erythrocyte layer. Important note: there is no ring of nucleated cells.

When the cells begin to form conglomerates and the number of cells doubles or triples twice a week, one can conclude that the culture is immortal. Establish the malignancy of cells as soon as possible, and then freeze the cells in a freezing medium using a −1 °C/min Slow Rate Freezing Container (Biocision). Vials containing immortalized cells should be stored at −70 °C and then transferred to a liquid nitrogen cell bank.

Traditionally, MRC-5 irradiated feeder cells are used during this process [11,6,9], which help to nourish the plasma cells during the transformation growth phase and possibly produce growth factors. In contrast, our method is feeder free. Plasma-containing cells can be used as a source of human serum and thus provide a number of advantages when grown in culture.

During the first week of incubation, Cyclosporin A suppresses the *in vitro* immune response of T-cells to the presence of EBV, but after 1 week this chemical must be removed. Generally, we see the steady formation of immortalized colonies by the third or fourth week. Freezing during these early stages allows the avoidance of cell subcloning.

Another advantage of our protocol is that it is not necessary to treat the B95-8 cell line with chemical agents (e.g., 12–0-tetra-de

canoylphorbol-13-acetat, phorbol 12-myristate-13-acetate or n-butyric acid) to induce viral production [12,13]. Perhaps this is because both cultured cells and virus are actively multiplying during the active growth phase. For small sample sizes, our method has the advantage that immortalization occurs in more than 90% of the time, which is essential when working with children or in other situations when one is unable to draw a large volume of blood.

5. Conclusion

Traditionally, immortalization require approximately 50 ml peripheral blood for one sample, special kits or media for B-cell isolation, and the use of feeder cell layers. This reproducible method is feeder free and activator of viral production free. It is easy to use and require small volumes of peripheral blood.

Acknowledgments

The study is supported by Russian Science Foundation Grant 15-15-10001. Author thank Alena S. Telepova at the Institute of Molecular and Cellular Biology SB RAS for help with photo preparations.

Conflict of interest

Author declares that there is no conflict of interest.

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