EBV immortalization of human B lymphocytes separated from small volumes of cryo-preserved whole blood

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EBV immortalized B lymphocyte cell lines have been extensively used as a source of biological material for functional and molecular studies and represent a potentially limitless source of genomic DNA. Current technologies for EBV transformation are costly and use relatively large volumes of peripheral blood. Alternative methods were examined to determine whether smaller volumes of cryo-preserved whole blood could be subsequently transformed and which could provide a more cost-effective strategy for large population-based studies such as UK Biobank. A successful method was established where viable B cells were positively selected from 0.5 ml cryo-preserved whole blood samples. These were EBV transformed in microtitre plates and subsequently expanded in culture. A pilot study within UK Biobank was performed, which confirmed its potential usefulness for this study.

Keywords EBV, cell lines, Biobanks, genetic epidemiology

Introduction

Since it was recognized that the Epstein Barr Virus could be used in vitro, to infect human B lymphocytes and produce immortalized polyclonal B cell lines in culture,¹ this technique has been used with great effect to facilitate immunological and molecular studies. This procedure was originally used extensively by the Histocompatibility research community to generate reference HLA typed cells for use in functional and serological studies.²⁻⁴ Subsequently, EBV cell lines have also been used widely in molecular and genetic research including applications such as transcriptomic and exposure sensitivity studies.⁵⁻⁶ A major application of EBV immortalization has been to establish cell line resources, which can potentially provide limitless amounts of genomic DNA. Even though more recent molecular techniques have been introduced to amplify the ‘whole genome’ of individuals,⁷⁻⁹ such techniques have certain limitations,¹⁰ and EBV immortalization remains the ‘Gold Standard’ for long-term provision of high molecular weight DNA.

Consequently, banking of EBV cell lines has been of great importance in providing reference material for rare genetic diseases, and the management of large amounts of DNA for the genetic analysis of complex conditions in population and family-based disease collections.¹¹,¹² Thus, EBV transformation has been key to the long-term management of important disease collections and progress in genetic epidemiology. A number of major facilities currently establish and manage extensive collections of cell lines (www.ecacc.org.uk; www.alspac.bris.ac.uk; http://cimr.umdNJ.edu; www.lgc promochem-atcc.com;/www.rutgers.edu) for the international research community.

The technical procedures used to establish EBV transformed B cell lines have not changed substantially over the last 25 years. Free EBV particles are produced by maintaining an EBV infected marmoset cell line (e.g. B95.8), which is overgrown and subsequently lysed. Human lymphocyte cultures are inoculated with free virus that gains entry into B lymphocytes via their CD21 (CR2) cell surface molecules (receptor for
complement C3 fragments). As the virus becomes integrated into the B cell, cytotoxic T lymphocytes can be generated, which subsequently kill the infected B cells, leading to transformation failure. A range of techniques have been developed to avoid this.\textsuperscript{13–17}

These include removal of T lymphocytes following resetting with sheep red blood cells or immune suppression of T cells using cyclosporin A. An alternative strategy has been to incubate lymphocyte cultures with T cell mitogens such as phytohaemagglutinin (PHA), which encourages T cells to rapidly transform into blast cells and die before cytotoxic T cells can be generated.

Most current techniques are based on variations of these procedures and use separated lymphocytes from 10 ml of citrated or EDTA blood samples. These are either transformed as fresh cells or subsequently processed following cryo-preservation with 10\% DMSO and storage in liquid nitrogen. These procedures are both expensive and time consuming and require relatively large volumes of blood (10–20 ml blood). For EBV immortalization to be considered as a feasible procedure for large population studies such as UK Biobank (\url{www.ukbiobank.ac.uk}), a more cost-effective strategy based on small blood volumes is required. We have explored whether cryo-preservation of 0.5 ml anticoagulated whole blood samples could be subsequently EBV immortalized using positive B cell selection.

**Methods**

**Blood samples**

Ten millilitre ACD blood samples were provided by 10 healthy volunteers. The samples were maintained at room temperature until being mixed with an equal volume of RPMI containing 20\% DMSO at 4°C (final DMSO concentration 10\%). One millilitre aliquots of diluted whole blood were pipetted into Nunc cryo-tubes and placed into a programmable cell freezer (Planer Products, UK) held at 4°C. The aliquots were frozen using a standard programme (a reduction of 1°C per minute to a temperature of −30°C and reduction of 10°C per minute to a temperature of −100°C). On completion of the programme the cryo-tubes were transferred to storage in the vapour phase of a liquid nitrogen refrigerator and maintained until required.

**Sample thawing**

Frozen ampoules were removed from nitrogen storage and thawed by being partially immersed in tepid water (37°C). On thawing, the contents were transferred by pipette into sterile centrifuge tubes and were diluted gradually with cold hepes buffered RPMI. Tubes were centrifuged at 3000 rpm for 10 min at room temperature and the process repeated. Any large particulate debris and clumps were removed by pipette.

**Development of protocol**

A range of experiments were performed to assess cell viability of lymphocytes and the relative proportions of T and B cells in the thawed samples of whole blood. The former was assessed using a colorimetric assay and incubation with the tetrazolium salt WST-1 (Roche Applied Science). Samples were assayed at a range of dilutions and measured on an ELISA reader. These were compared to a standard curve prepared using fresh lymphocytes, separated using density centrifugation. T and B cell differential counts were assessed using standard FACS analysis procedures.

Further experiments were performed to establish conditions for effective positive selection of B lymphocytes using the Milteny autoMACSTM cell separation station (Milteny Biotec). This is a bench-top computer-controlled magnetic cell sorter designed for sterile cell separation from whole blood. The following protocol was finally used.

Following thawing and washing of whole blood aliquots as described earlier, cell pellets were incubated with 35 μl of Milteny’s-MAC beads conjugated with anti-CD19 antibody. After thorough gentle mixing, the samples were incubated at 4–8°C for 15 min. After incubation 5 ml of cold running buffer were added and gently mixed. The tubes were then centrifuged at 2000 rpm for 10 min and the supernatant carefully removed leaving 2 mm of liquid above the pellet. A further 1 ml of running buffer was added and gently mixed. A pre filter was placed onto a fresh 15 ml centrifuge tube and wetted with 1 ml of running buffer. The samples were then allowed to pass through the wet pre-filters.

The autoMACSTM was maintained within the sterile environment of a class II safety cabinet and working areas of the device were swabbed with 70\% ethanol. New separation columns were installed and purged to manufacturer’s instructions. Prior to cell sorting, new 50 ml tubes were placed underneath uptake and elution ports and a purge ‘clean’ programme performed.

Cell samples were placed under the uptake port and separated using the programme ‘positive selection’ D2. B cell fractions were collected at the positive 2 port and the debris/other cell fractions at the negative port. Prior to processing further samples, the uptake and positive 2 ports were again swabbed with ethanol and a rinse programme performed with 50 ml tubes on all ports. A number of experiments were performed to assess the viability and purity of the B cells produced by this procedure. Viability was visually assessed by phase contrast microscopy and B cell purity by FACS analysis using CD19 and CD3 antibodies.

**Production of EBV**

The marmoset lymphoblastoid cell line B95.8 (kindly provided by Dr B. Bolton—ECACC, UK) was used as a source of free EBV particles. The cell line was grown in RPMI complete medium supplemented with 10\%
foetal calf serum (FCS) and cultured in flasks at 37°C in a 5% CO₂ humidified atmosphere. The cultures were allowed to overgrow and a final medium change of RPMI with 2% FCS was used to encourage virus production. Cultures were harvested and freeze-thawed at −20°C twice to lyse cells. Cultures were transferred to sterile 50ml centrifuge tubes and spun at 2000 rpm for 10 min. Supernatants were removed and put through 0.5μm Millipore filters. Filtered supernatant was aliquotted into 1ml volumes in Nunc cryotubes and stored at −80°C until required.

B cell fractions produced by the autoMACSTM were centrifuged at 2000 rpm for 10 min. Supernatants were carefully aspirated and 200μl of EBV particles added. After careful mixing, 100μl volumes were transferred in duplicate into round-bottomed well culture plates. Culture plates were placed in an incubator at 37°C in a 5% CO₂ atmosphere for 2 h. A further volume of 100μl of culture medium (RPMI + 20% FCS) was then added to each well. All samples were routinely checked microscopically for signs of microbiological contamination and cell line transformation. As cultures transformed, they were gradually increased in volume before transfer to culture flasks.

**UK Biobank pilot study**

A pilot study was performed to determine whether ACD whole (unfractionated) blood samples collected to the UK Biobank protocol and cryo-preserved in liquid nitrogen can be effectively used as a source for subsequent EBV immortalization of B cell lines. A further objective was to assess whether this could also be achieved for blood samples that had been maintained for 24 h at room temperature prior to cryo-preservation, thought to reflect the situation with UK Biobank more accurately.

Eight millilitre blood samples were collected from volunteers into ACD vacutainer tubes and transferred to CIGMR’s laboratory. Initial blood samples from 40 different volunteers were collected and delivered in four batches of 10 samples. These samples were divided and cryo-preserved at two different time points; on the day of delivery and the following day. Cryo-preservation was performed as described earlier.

The samples frozen after 24 h standing at room temperature were thawed and processed using the autoMACSTM and the protocol described earlier. EBV transformation was performed on these samples using the method previously described.

**Results**

**Viability of cells in cryo-preserved whole blood**

After thawing and washing whole blood sample, considerable haemolysis and clumping of cells was observed. Clumps appeared to consist largely of agglutinated platelets and dead neutrophils. An estimation of lymphocyte viability compared with a calibration curve of fresh viable cells revealed an average of 200,000 viable cells per millilitre of thawed blood. FACS analysis revealed a range of B cells in the thawed samples, but in all samples there were >10% B lymphocytes present.

**Viability and purity of autoMACSTM selected cells**

Using phase contrast microscopy, the viability of B cells positively selected using the autoMACSTM ranged between 70% and 75% for the samples processed. FACS analysis revealed a very high purity of the B cells selected by the autoMACSTM, with all >99% purity.

**EBV transformation**

All 10 samples processed following autoMACSTM B cell selection were successfully transformed into EBV cell lines and were eventually transferred into flasks and cryo-preserved. An average time of 5 weeks was taken for cell lines to become established in culture plates.

**Discussion**

The methodology developed and assessed clearly demonstrates that it is possible to cryo-preserve relatively small volumes of whole blood and subsequently generate EBV immortalized lymphoblastoid cell lines. Furthermore, we have been able to establish that such methodology would be appropriate for UK Biobank and they will be able to cryo-preserve whole blood samples confident with the knowledge that cell lines can be subsequently generated. These findings have since been replicated elsewhere, where similar levels of transformation success have been achieved with small volumes of cryo-preserved whole blood (Dr David Lewis, ECACC—personal communication).

The use of antibody conjugated magnetizable micro particles to produce a pure B lymphocyte population from cryo-preserved whole blood for EBV cell line transformation appears to be a procedure with great potential and worthy of further development. The B cell population produced is viable and free of T cells thus obviating the need for other steps.
to overcome cytotoxicity. The micro particles used are extremely small (50 nm) and although they remain attached to the cells following elution from the column, they quickly fall off the cells in culture and are biodegradable. They appear to have no detrimental effects on long-term culture of cells.

It is also apparent that long-term sterility of cultures using this method of separation is not a significant concern. The device used for separation has specially been designed for isolation of cells for long-term culture\textsuperscript{18} and has also been used for the separation of cells used for \textit{in vivo} therapeutic procedures. If used correctly in a biological safety cabinet there does not appear an issue regarding contamination of cultures.

It is likely that this methodology will be further developed and refined to produce a more automated and economic method for large-scale EBV cell line transformation from small volumes of cryo-preserved whole blood.

The UK Biobank samples tested presented their worst-case scenario i.e. a time delay of 24 h at room temperature before cryo-preservation. This again increases confidence that this represents a valid strategy for future generation of immortalized cells. This is not surprising as previous studies have revealed that residual blood samples can be left a considerable time period and still be successfully EBV transformed.\textsuperscript{19} A success rate of 67% has been given for samples up to a week old and 38% for samples up to 2-weeks old. Given the great expense involved in immortalizing cell lines from fresh cells or separating lymphocytes for cryo-preservation, a sensible strategy for large epidemiological studies would be to cryo-preserve small volumes of whole blood. Such samples could be selectively transformed in the future if specific needs arise, thus representing a cost-effective approach to future proofing studies.

The costs involved in cryo-preserving small whole blood samples are relatively low compared with EBV transformation. An alternative approach may be to cryo-preserve small volumes ofuffy coat rather than whole blood. Such processes can now be automated, covering unit costs further.

It is likely that successful EBV cell transformation can be achieved with even small blood samples if methods could be developed for blood collected from finger pricks, this could greatly facilitate paediatric and large-scale population-based studies.

The method of B cell transformation described here is relatively time consuming, but could potentially be automated. The reagent costs involved per separation are relatively low (<£5) and the major additional costs will be technical labour and consumables for expansion of cell cultures.

It is possible that even more cost effective methods for EBV transformation will be developed in the future. We have already established that just incubating thawed whole blood samples in round-bottomed culture plates at 37°C is sufficient to make B cells adhere and T cells can be removed by gentle aspiration. Subsequent addition of EBV was capable of successfully transforming 50% of the samples treated in this way. B cell purification using incubation on sterile nylon wool columns may also be an alternative method, which could be considered for automation. As methods are developed, which use fewer B cells it is likely that cell density will be the critical factor in determining success rates in EBV transformation. We and others have appreciated for some time that success rates are better using ‘V’ bottomed plates but these cannot be readily monitored by microscopy.

If new cost-effective and automated procedures can be introduced for EBV transformation and expansion it will make a major contribution to International Biobanking and genetic epidemiology. Although whole genome amplification of DNA samples has been of great benefit to research there are many applications for which it is not appropriate. The somatic mutation rate of DNA derived from EBV cell lines is low (0.3%)\textsuperscript{20} and somewhat counter intuitively it is now increasingly realized that gene expression in EBV cell lines is largely representative of a wide range of metabolic pathways specific for the individual from whom the cell line was generated.

In summary, there appears to still be a strong justification for potentially generating EBV cell lines in population studies and the long-term cryopreservation of small whole blood samples appears to be a valid strategy for fulfilling future needs.

\textbf{Conflict of interest:} None declared.

\textbf{References}


