High-potentiality preliminary selection criteria and transformation time-dependent factors analysis for establishing Epstein–Barr virus transformed human lymphoblastoid cell lines

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Abstract. Infection of freshly isolated and cryopreserved lymphocytes with Epstein–Barr virus (EBV) leads to the establishment of human B lymphoblastoid cell lines (LCLs). Techniques for optimal infection of the lymphocytes are vital for the establishment of a human biobank. The present study found that more than half (58–86%) of such established LCLs had transport times of less than 48 h, cell densities exceeding 10^6 cells/ml and cell viabilities greater than 90%. After EBV infection, 3306 freshly isolated lymphocytes required 30.0 ± 0.1 days to become LCLs. Conversely, 1210 cryopreserved lymphocytes required 36.2 ± 0.4 days. Cell density and viability of the culture affected transformation time in freshly isolated lymphocytes. On the other hand, blood transport time, cryopreservation time and initial cell viability were major factors in cryopreserved specimens. These results contribute to general information concerning the establishment of a human biobank for EBV infected cells.

INTRODUCTION

Providing adequate amounts of DNA for genetic research is an important advantage of using cell lines. Peripheral blood, which is easy to access, is the primary source of human blood cells, including B lymphocytes. These can be transformed by the use of Epstein–Barr virus (EBV) infection, a currently available technique for B-lymphocyte immortalization (Borrebaeck & Ohlin 1996; Raff *et al.* 1996; Biddison 1998). Once permanent lymphoblastoid cell lines (LCLs) have been established, each of them represents a unique genetic information carrier and can provide unlimited quantities of DNA for downstream applications or research. This is of immense

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value to human clinical and experimental genetic studies (Heidemarie 1986). Several international cell banks, such as the Bioresources Collection and Research Center (BCRC), European Collection of Animal Cell Cultures (ECACC), American Type Culture Collection (ATCC) and Coriell Institute for Medical Research, have employed EBV transformation to establish human B lymphoblast cultures. To date, several studies (Heidemarie 1986; Fiebig *et al.* 1997; Tremblay & Khandjian 1998; Kleeberger *et al.* 1999; Beck *et al.* 2001; Cheng *et al.* 2001; Hayes *et al.* 2002; Oh *et al.* 2003) have focused on techniques for EBV transformation. However, the preliminary criteria for isolation of lymphocytes and the factors that might influence the required transformation times were not addressed in these studies. Several sources of information have documented EBV-induced changes in signal transduction pathways (Sinclair & Farrell 1995), membrane receptors of B cells, binding of EBV (Kilger *et al.* 1998; Yang *et al.* 2000; Sarrias *et al.* 2001) and B-cell gene expression (Carter *et al.* 2002), but these data do not concern the establishment of a human B-lymphocyte bank. It is therefore necessary to explore easily manipulable parameters to determine whether they could be employed as selection criteria for B-cell isolation for the establishment of EBV-transformed LCLs. The advantages of preliminary selection of isolated cells help lessen labour and material expense. This type of work has been rarely reported in previous studies on EBV transformation (Heidemarie 1986; Fiebig *et al.* 1997; Tremblay & Khandjian 1998; Kleeberger *et al.* 1999; Beck *et al.* 2001; Cheng *et al.* 2001; Hayes *et al.* 2002; Oh *et al.* 2003). The BCRC has been the core facility of the Animal Cell Bank of the National Health Research Institute (NHRI) in Taiwan since 1996. In addition to collecting cell lines, BCRC has established, since 2001, standardized protocols and quality control systems for EBV transformation to immortalize human B lymphocytes. Owing to a well-established depository system and experience in cell management and EBV transformation, the BCRC has helped establish the Taiwan Han Chinese Cell and DNA bank, which was initiated by the Institute of Biomedical Sciences (IBMS), Academia Sinica, Taiwan in 2003 (Pan *et al.* 2004). To date, more than 2000 human B-lymphocyte cell lines have been established. Based on our experience with the immortalization of B lymphocytes, we have found that: (1) the preliminary selection criteria for isolated lymphocytes for EBV transformation have been proposed. (2) The transformation time required for EBV transformation is dependent on whether the lymphocyte preparation used was cryopreserved or freshly isolated. The present study has shown that the immortalization period for cryopreserved lymphocytes is about 6 days longer than that required by freshly isolated cells. (3) The viability of isolated lymphocytes, whether cryopreserved or freshly isolated, when used for immortalization is a major factor affecting the required transformation time as transformation time for lymphocytes decreases with an increase in cell viability.

MATERIALS AND METHODS

Blood collection

In the Taiwan Han Chinese Cell and DNA bank project, the IBMS collected selected blood samples from all over Taiwan and sent these to the BCRC for B-cell immortalization. Approximately 5–10 ml of blood was collected from each donor and placed in 10-ml tubes, each containing citrate-phosphate-dextrose-adenine (CPDA). The blood samples were then sent to the BCRC by overnight courier service. The delivery temperature was maintained at 4 °C. When the blood samples arrived, they were immediately processed for EBV transformation.

Lymphocyte isolation

Blood was diluted with an equal volume of phosphate-buffered saline (PBS). Six millilitres of PBS-diluted blood was then carefully layered onto 4.5 ml of Ficoll plaque for density gradient centrifugation at 620 g for 30 min. After centrifugation, the interface band, which is enriched with lymphocytes, was transferred and rinsed with wash medium (Roswell Park Memorial Institute (RPMI) 1640 with 2% (v/v) foetal bovine serum). The cell pellet was then re-suspended in 1 ml of freezing medium (RPMI1640 with 20% (v/v) foetal bovine serum and 7% dimethyl sulfoxide) for cryopreservation or in 1 ml of culture medium for EBV transformation. For cryopreservation, the lymphocytes were transferred to a cryotube and were then placed in a programmable freezer (Planer, Kryo 10 Series III; Planer Biomed, Sunbury-on-Thames, UK) while the temperature was lowered at 1–5 °C/min to –100 °C. The cryopreserved ampoule was then transferred to liquid nitrogen for storage until EBV transformation was performed.

EBV transformation

Transformation of EBV can be initiated with cryopreserved lymphocytes or with freshly isolated ones. After centrifugation at 270 g for 10 min, the cell pellet is infected with 1 ml EBV at 37 °C for 1 h. The sample was then centrifuged at 270 g for 10 min to remove the EBV. Procedures using phytohemagglutinin (PHA) utilized in EBV transformation are common to a standard EBV transformation protocol (Borrebaeck & Ohlin 1996), which has been applied to establish LCLs (Penno *et al.* 1993; Beck *et al.* 2001). The infected lymphocytes were re-suspended in 5 ml of transformation medium composed of RPMI1640 with 20% (v/v) foetal bovine serum and 1% PHA. The lymphocyte suspension was then added to a 25-cm² cell culture flask, which is incubated at 37 °C in a 5% CO₂ atmosphere. The culture medium was renewed twice weekly. During the incubation period, signs of immortalization, such as an exponential increase in cell number, clumping of cells and production of a yellowish (acidic) medium, were noted.

The use of cryopreserved lymphocytes was similar to that previously described except for rapid thawing in a 37 °C water bath and recovery of cryopreserved lymphocytes as described in succeeding discussions. After thawing, the cryopreserved lymphocyte solutions were diluted with an equal volume of 5% human albumin and 10 U/ml heparin in PBS by centrifugation for 10 min at 270 g. The cell pellets were re-suspended in 5 ml of culture medium and were then incubated overnight at 37 °C in a 5% CO₂ atmosphere to recover the lymphocytes.

Preparation of EBV

The source of our EBV was the B95-8 EBV-transformed marmoset cell line (BCRC 60199). B95-8 cells were maintained in Iscove's modified Dulbecco's media (IMDM) with 10% foetal bovine serum and were incubated at 37 °C in a 5% CO₂ atmosphere. Before harvesting the EBV, the required density of the B95-8 cell culture was $(1-2) \times 10^6$ viable cells/ml; incubation was then necessary for 10–12 days without the medium being renewed. At this point, the medium, now enriched with EBV, was collected and centrifuged at 270 g for 10 min. The supernatant was sterilized by 0.2-µm filtration, and the filtered supernatant was split into aliquots and stored at –80 °C.

Statistical analysis

All values are presented as the mean $\pm$ SE (*n*). In the present study, Cox proportional hazards regression model was applied to investigate the associations between transformation time and four covariates, namely, blood transport time, cryopreservation time, cell density and cell viability. The transformation time is defined as the period from initiation of EBV infection to occurrence of immortalization. Four covariates were considered for fitting the Cox model (Kalbfleisch & Prentice 2002), selected for inclusion in a proportional hazard model using stepwise selection

methods from SAS software packages. Entry and removal *P* values were set at 0.05. For each significant covariate, one-way analysis was conducted to compare the difference among subgroups of the covariates. Because the null hypothesis of the normality test indicates that there is significant departure from normality, a non-parametric procedure, the Kruskal–Wallis test, was implemented (see Hollander & Wolfe 1999). In addition to this, the differences in blood transport time, cell density, cell viability and transformation time required by freshly isolated and cryopreserved lymphocytes were determined by employing the Mann–Whitney test.

RESULTS

Efficiency of EBV transformation

With freshly isolated lymphocytes as the starting material for EBV transformation, 3306 cell lines were established from a set of 3516 freshly isolated lymphocytes with a success rate of 94.0%. In addition, 1210 cell lines were established from a set of 1419 cryopreserved lymphocytes with a success rate of 85.2%.

Preliminary criteria for performing EBV transformation

The 3306 transformed cell lines initiated from the freshly isolated lymphocytes were divided into groups as indicated in Table 1. In Table 1, the frequency (relative percentage) of each group's different parameter levels, such as cell density, cell viability and blood transportation time, were determined. Total percentages of groups with cell densities exceeding 10^6 cells/ml,

Table 1. Relationships between initial status of freshly isolated lymphocytes (cryopreservation time = 0) and corresponding related percentages in 3306 transformed lymphocyte cell lines

Initial status of freshly isolated lymphocytes			Related percentage in transformed cell lines (%)
Cell viability (%)	Cell density ($\times 10^6$ cells/ml)	Blood transport time (h)	
> 90	> 1	0–24	37.4168
		24–48	48.6993
		48–72	6.68481
		> 72	2.57109
> 90	< 1	0–24	0.0605
		24–48	0.15124
		48–72	0
		> 72	0
< 90	> 1	0–24	1.5729
		24–48	2.08712
		48–72	0.36298
		> 72	0.30248
> 90	< 1	0–24	0
		24–48	0
		48–72	0.09075
		> 72	0

Note. Initial freshly isolated lymphocytes were divided into groups by combination of cell viability, cell density and blood transport time. Related percentages of each group were indicated.

Table 2. Relationships between initial status of cryopreserved lymphocytes (cryopreserved time $\neq 0$) and corresponding related percentages in 1210 transformed lymphocyte cell lines

Initial status of cryopreserved lymphocytes			Related percentage in transformed cell lines (%)
Cell viability (%)	Cell density ($\times 10^6$ cells/ml)	Blood transport time (h)	
> 90	> 1	0–24	32.3967
		24–48	25.7025
		48–72	1.98347
		> 72	0.7438
> 90	< 1	0–24	0.41322
		24–48	0.66116
		48–72	0
		> 72	0.24793
< 90	> 1	0–24	11.90082
		24–48	18.34709
		48–72	3.96694
		> 72	1.15701
> 90	< 1	0–24	0.41322
		24–48	0.33057
		48–72	0.16529
		> 72	0.08264

Note. Initial cryopreserved lymphocytes were divided into groups by combinations of cell viability, cell density and blood transport time. Related percentages of each group were indicated.

cell viabilities of 90–100% and blood transportation times of less than 48 h accounted for 86% of the total. Transformation efficiencies of special sets and non-special sets of freshly isolated lymphocytes were both 94.0%. With regard to the 1210 transformed cell lines initiated from cryopreserved lymphocytes, more than half (58%) were also found to be in the previously mentioned groups (Table 2). Unlike freshly isolated lymphocytes, transformation efficiencies of the special sets and the non-special sets of cryopreserved lymphocytes were 90.7% and 78.7%, respectively. This indicates that blood samples collected within 48 h, with isolated cell densities exceeding 10^6 cells/ml and with 90–100% cell viability, could be criteria for EBV transformation.

Comparison between EBV transformation initiated with freshly isolated and cryopreserved lymphocytes

In the present study, both freshly isolated and cryopreserved lymphocytes were used as starting materials for EBV transformation. Before EBV transformation was carried out, the transportation times, cell densities and viabilities of freshly isolated and cryopreserved lymphocytes were determined (Table 3). Significant differences between freshly isolated and cryopreserved lymphocytes were found in blood transport time ($P = 0.0001$), cell density ($P = 0.0000$) and cell viability ($P = 0.0000$). Because cryopreservation time in freshly isolated lymphocytes is 0 day, it was reasonable to conclude that cryopreservation time was different between freshly isolated and cryopreserved groups. At the end of EBV transformation, when the B lymphocytes were immortalized, total transformation times required by freshly isolated and cryopreserved lymphocytes were 30.0 ± 0.1 ($n = 3306$) and 36.2 ± 0.4 ($n = 1210$) days, respectively. There was a significant difference ($P = 0.0000$) in transformation times required by the two preparations of lymphocytes.

Table 3. Status of freshly isolated and cryopreserved lymphocytes for EBV transformation

Parameters	Lymphocytes	
	Freshly isolated	Cryopreserved
Blood transport time (h)	34.1 ± 0.2 (3516)	32.6 ± 0.4 (1419)*
Cryopreserved time (days)	0.0 ± 0.0 (3516)	313.3 ± 3.6 (1419)*
Cell density (×10 ⁶ cells/ml)	10.1 ± 0.0 (3516)	7.8 ± 0.1 (1419)*
Cell viability (%)	95.8 ± 0.0 (3516)	89.7 ± 0.2 (1419)*

Note. Mean ± SE (*N*) is indicated. The Mann–Whitney test was conducted to evaluate significance of parameters between freshly isolated and cryopreserved groups. Since the cryopreserved time in freshly isolated lymphocytes is 0 day, it was reasonable to conclude that the cryopreserved time was different between freshly isolated and cryopreserved groups. Asterisks indicate significant differences between freshly isolated groups and cryopreserved groups ($P < 0.05$).

Table 4. Test of significance for main effect on required transformation time for freshly isolated lymphocytes

Parameters	Chi-square score	<i>P</i> value
Blood transport time	0.0213	0.8839
Cell density	5.3536	0.0207
Cell viability	61.3286	< 0.0001

Note. Cox model analysis was conducted to evaluate the significance of factors on required transformation times. The entry and removal *P*-values were set at 0.05.

Major factors associated with transformation times required for freshly isolated lymphocytes

When fresh isolated lymphocytes were used as the starting material, cell density ($P = 0.0207$) and viability ($P < 0.0001$) were both highly significant whereas the transport time was not ($P = 0.8337$) (Table 4).

Effects of major factors on transformation times required for freshly isolated lymphocytes

Freshly isolated lymphocytes were distinguished by different levels of cell density: < 10⁶, 10⁶–10⁷ and > 10⁷ cells/ml. As shown in Table 5, the transformation times decreased with increasing cell density. The transformation times of groups with different cell densities were significantly dif-

Table 5. Test of significance for cell density on required transformation time for freshly isolated lymphocytes

Cell density (cells/ml)	Transformation time (day)
< 10 ⁶	41.7 ± 7.9 (9)*
10 ⁶ –10 ⁷	30.5 ± 0.2 (1887)†
> 10 ⁷	29.3 ± 0.2 (1410)†

Note. Mean ± SE (*N*) is indicated. The Kruskal–Wallis test was conducted amongst groups of different cell densities. *† Different symbols indicate that there were significant differences ($P < 0.05$) in transformation time among different levels of cell density.

Table 6. Test of significance for cell viability on required transformation time for freshly isolated lymphocytes

Cell viability (%)	Transformation time (day)
70–80	40.3 ± 8.3 (6)*
80–90	33.0 ± 0.9 (140)†
90–100	29.8 ± 0.1 (3160)†

Note. Mean ± SE (*N*) is indicated. The Kruskal–Wallis test was conducted amongst groups with different cell survival rates. *† Different symbols indicate that there were significant differences ($P < 0.05$) in transformation time among different levels of cell viability.

ferent ($P = 0.0000$). The transformation times required for groups with cell density exceeding 10^6 cells/ml decreased to 75% of that in the group with cell density of less than 10^6 cells/ml. With regard to the effect of cell viability on the transformation time, significant difference in transformation time was found amongst groups with different cell viabilities ($P = 0.0000$). As shown in Table 6, freshly isolated lymphocytes with 90–100% cell viability required shorter transformation times than did the other two groups.

Major factors associated with transformation times required for cryopreserved lymphocytes

When EBV transformation was initiated with cryopreserved lymphocytes, blood transport time, cryopreservation time, cell viability and cell density were all considered respective to their influence on transformation times. Consequently, it was found that blood transport time ($P = 0.0001$), cryopreservation time ($P < 0.0001$) and cell viability ($P < 0.0001$) influenced the required transformation time; however, cell density ($P = 0.4210$) did not (Table 7).

Effects of major factors on transformation times required for cryopreserved lymphocytes

Significant difference in transformation time was found amongst groups with different blood transportation time ($P = 0.0000$). Once the blood transportation time had exceeded 48 h, at least 40 days were required for transformation; i.e. transformation only took 35–36 days when blood transportation time was less than 48 h (Table 8). Significantly different times for transformation ($P = 0.0000$) were required for groups with different levels of cryopreservation time. Increase in cryopreservation time led to an increase in transformation time, as indicated in Table 9. However, groups with cryopreservation times exceeding 270 days required less transformation time than the other three groups. Meanwhile, it should be noted that cell viability of these two groups was significantly higher than that of the other three groups (Table 9). With regard to the effect

Table 7. Test of significance for main effects on required transformation times for cryopreserved lymphocytes

Parameters	Chi-square score	<i>P</i> value
Blood transport time	14.8056	0.0001
Cryopreserved time	213.1299	< 0.0001
Cell density	0.6476	0.4210
Cell viability	63.8281	< 0.0001

Note. Cox model analysis was conducted to evaluate the significance of factors on required transformation time. The entry and removal *P* values were set at 0.05.

Table 8. Test of significance for blood transport time on required transformation time for cryopreserved lymphocytes

Blood transport time (h)	Transformation time (day)
0–24	36.0 ± 0.8 (547)*
24–48	35.1 ± 0.5 (561)†
48–72	40.8 ± 1.9 (74)‡
> 72	49.1 ± 4.8 (28)‡

Note. Mean ± SE (*N*) is indicated. The Kruskal–Wallis test was conducted amongst groups with different blood transport times. *,†,‡ Different symbols indicate that there were significant differences ($P < 0.05$) in transformation time among different levels in blood transport time.

Table 9. Cell viability and required transformation times for cryopreserved lymphocytes with different cryopreserved times

Cryopreserved time (day)	Cell viability (%)	Transformation time (day)
0–90	82.2 ± 1.2 (66)*	40.7 ± 1.2 (66)*
90–180	86.4 ± 0.8 (155)†	42.4 ± 0.9 (155)*
180–270	87.9 ± 0.5 (258)†	47.2 ± 1.6 (258)†
270–360	93.2 ± 1.1 (27)‡	30.0 ± 0.9 (27)‡
> 360	92.6 ± 0.2 (704)‡	30.6 ± 0.4 (704)§

Note. Mean ± SE (*N*) is indicated. The Kruskal–Wallis test was conducted amongst groups with different cryopreserved times. *,†,‡,§ Different symbols indicate that there were significant differences ($P < 0.05$) in cell viability or transformation time among different levels in cryopreserved time.

Table 10. Test of significance for cell survival rate on required transformation times for cryopreserved lymphocytes

Cell viability (%)	Transformation time (day)
< 70	43.4 ± 2.8 (45)*†
70–80	45.2 ± 2.3 (87)*
80–90	40.3 ± 1.1 (308)†
90–100	32.7 ± 0.5 (752)‡

Note. Mean ± SE (*N*) is indicated. The Kruskal–Wallis test was conducted amongst groups with different cell survival rates. *,†,‡ Different symbols indicate that there were significant differences ($P < 0.05$) in transformation time among different levels in cell viability.

of cell viability on transformation time required by cryopreserved lymphocytes, the significant difference in transformation time was found amongst groups with different cell viabilities ($P = 0.0000$). As shown in Table 10, the group with 90–100% cell viability required shorter transformation time than the other three groups.

DISCUSSION

Owing to ease of manipulation, lower labour and maintenance costs, exhibition of chromosomal stability, high resolution in chromosomal preparations and ideal resources for human genetic studies (Heidemarie 1986), EBV transformation has become a routine method for establishing LCLs. It is accepted that LCLs can provide unlimited quantities of DNA, which is helpful in

genetic research, human disease studies and new drug screening. Thus, a supply of genetic material and the exploration of target genes of diseases – two essential proponents for establishing a human genetic cell bank – have been linked by EBV transformation of LCLs. It should also note that the efficiency in time and materials is another necessary and important topic that should be considered when establishing genetic cell banks. Taken together, there are two ways to achieve the above aims. First, a selection principle for isolated B lymphocytes with high potentiality for EBV transformation should be established. Purified lymphocytes, even those maintained long-term in liquid nitrogen (Tremblay & Khandjian 1998), can be used as a source for establishing LCLs. However, the study of Tremblay & Khandjian (1998) did not provide data concerning transformation efficiency. Thus, establishing preliminary principles must allow estimates of efficiency of EBV transformation for the establishment of genetic banks.

Based on easily acquired parameters including blood transportation time, cell density, cell viability and cryopreservation time, the present study attempted to establish a preliminary selection for isolated B lymphocytes. As indicated in Tables 1 and 2, more than half of the LCLs were initiated from a special set of isolated lymphocytes with transport times of less than 48 h, cell densities exceeding 10^6 cells/ml and cell viabilities greater than 90%. Interestingly, the efficiency of EBV transformation in the special set and the non-special set of isolated lymphocytes vastly differed for cryopreserved lymphocytes as the starting material. The efficiency of the special set was about 1.2 times that of the non-special set. Additionally, when cryopreserved lymphocytes were divided into three groups according to the level of cryopreservation times (1–180, 180–360 and > 360 days), the efficiency in the special set was still around 1.1–1.2 times that of the non-special set (data not shown). With regard to freshly isolated lymphocytes, the efficiencies in the special set and non-special set were the same. We propose that this is because all the freshly isolated lymphocytes had similar physiological status for EBV transformation. These data not only show that the previously mentioned status of the lymphocytes (blood transportation times of less than 48 h, more than 10^6 cells/ml, and cell viabilities exceeding 90%) could be the preliminary selection criteria for EBV transformation performance of isolated B lymphocytes but they also imply that this criteria is helpful specially for cryopreserved lymphocytes as starting material. Molecular mechanisms beneath the proposed selection criteria might be associated with CD21 (the CR2 complement receptor), which has been demonstrated to mediate EBV binding to and infection of B lymphocytes (Martin *et al.* 1994). Expression of CD21 in the previously mentioned special set and non-special set of lymphocytes would be an interesting topic for exploration.

Secondly, an optimal EBV transformation protocol should be employed to save time and material. Based on the present protocol, freshly isolated lymphocytes require about 30 days for successful transformation, which is 5 days shorter than that in a previous study. Beck *et al.* (2001) provided no data about cell densities or viabilities of infection for further comparison. It is still important to note, however, that the efficiency in the present study (94.0%) was the same as that reported by Beck *et al.* (2001). It should also be noted that 10% PHA was used in their study whereas 1% PHA was used in the present study. Oh *et al.* (2003) employed secondary infection of EBV, an alternative method for achieving EBV transformation, to complete EBV transformation with an initial cell density of about 10^6 cells/ml, yet it took 36 ± 7 days to complete. In the present study, there were several freshly isolated lymphocyte cultures with mean cell densities also of about 10^6 cells/ml and with transformation times of about 30 days (data not shown). The required time for EBV transformation initiated with cryopreserved lymphocytes was around 36 days, which was less than the 40.2 ± 0.8 days reported by Beck *et al.* (2001) and 56 days reported by Tremblay & Khandjian (1998). It should be noted that according to comparison between the present data and those of Tremblay & Khandjian (1998), it was found that there was no significant difference in mean cell number of cryopreserved lymphocytes for infection

with EBV (data not shown). It should be emphasized here that protocols for EBV transformation in the mentioned studies (Tremblay & Khandjian 1998; Beck *et al.* 2001; Oh *et al.* 2003) and the present study all employ several similar signs for immortalization, namely, exponential increase in cell number, clumping of cells or production of a yellowish (acidic) medium to identify whether LCLs have established or not. Based on this qualitative examination, the required transformation time, which is the period between EBV infection and occurrence of immortalization signs, was determined. Based on data concerning comparison in transformation time, the present protocol is reliable, efficient and more economical to perform EBV transformation in terms of time than the other previously mentioned protocols, regardless of whether freshly isolated or cryopreserved lymphocytes are used.

During EBV transformation, the status of LCLs, their proliferation, the number of cell clumps or immunophenotyping could be examined by performing an assisting technique. Exploring whether or not the transformation time and efficiency of EBV transformation could be improved by employing assistant techniques during EBV transformation is an interesting topic, particularly for the establishment of a human B lymphoblast bank, which needs rapid methods for immortalization of B lymphocytes. It is accepted that growth of LCLs could be enhanced by mitogen stimulation such as with PHA (Penno *et al.* 1993; Borrebaeck & Ohlin 1996; Beck *et al.* 2001) and regression of LCLs, which may result from cytotoxic action of T cells, which could in turn be diminished by cyclosporin A (Tosato *et al.* 1982; Heidmarie 1986; Oh *et al.* 2003; Hur *et al.* 2005; Mello *et al.* 2006). These are helpful ways for establishing LCLs during EBV transformation. Oh *et al.* (2003) found that in the transformation efficiency of a group infected with tetradecanoyl phorbol acetate, the induced EBV was higher than that of their control group. Recently, Hur *et al.* (2005) found that numbers of cell clumps and expression of CD38 in LCLs could be enhanced by CD19 ligation. Mello *et al.* (2006) suggested that LCLs could be established with 100% efficiency and cryopreservation on day 12 after infection with EBV by performing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to examine cell proliferation during EBV transformation. This indicates that in the future, EBV transformation can be performed with more efficiency by combining further assistant techniques of cell proliferation, cell counting and cell granularity.

In parallel with performing EBV transformation using freshly isolated lymphocytes, cryopreserved lymphocytes are an alternative source that can be used to initiate EBV transformation for establishment of LCLs. Because of this, these two common preparations of lymphocytes were chosen in performing EBV transformation in the present study. It was found that using freshly isolated lymphocytes as the starting point saved around 6 days for completion of the transformation compared to using cryopreserved ones. In 1993, Penno *et al.* reported that they found no difference in transformation time between freshly isolated and cryopreserved lymphocytes. However, other studies (Tremblay & Khandjian 1998; Beck *et al.* 2001; Oh *et al.* 2003) indicated that transformation times for these preparations differed. The required transformation time for freshly isolated lymphocytes was 30.0 ± 0.1 days as determined in the present study, 35.2 ± 0.3 days in Beck *et al.* (2001) and 36 ± 7 days in Oh *et al.* (2003). Alternatively, cryopreserved lymphocytes required 36.2 ± 0.4 days in the present study, 40.2 ± 0.8 days in Beck *et al.* (2001) and 49–70 days in Tremblay & Khandjian (1998). An important question raised from this is: What causes the difference in the transformation time required for these two types of lymphocyte preparation? Based on influences of major factors in the required transformation time (see succeeding discussions), cell viability is a major factor in the transformation (Tables 4 and 7) in both lymphocyte preparations. In addition to this, Tables 6 and 10 also show that the transformation time significantly decreases with an increase in cell viability, regardless of whether freshly isolated or cryopreserved lymphocytes were used. Table 3, clearly indicates

that the cell viability of freshly isolated lymphocytes was significantly higher than that of cryo-preserved ones. Thus, it is reasonable to speculate that cell viability is an important factor that could explain why freshly isolated lymphocytes require shorter transformation times than cryo-preserved ones for successful transformation.

Differing techniques (Oh *et al.* 2003; Hur *et al.* 2005; Mello *et al.* 2006) or agents that can stimulate growth of LCLs (Penno *et al.* 1993; Borrebaeck & Ohlin 1996; Beck *et al.* 2001) or suppress cytotoxic action of T cell (Tosato *et al.* 1982; Heidemarie 1986; Oh *et al.* 2003; Hur *et al.* 2005; Mello *et al.* 2006) have been combined with the performance of EBV infection to raise transformation efficiency and decrease transformation time. However, the relationships between parameters concerning lymphocyte preparation and efficiency or the required time of EBV transformation are not well addressed. In the present study, several easily acquired parameters, blood transportation time, cryopreservation time, cell density and cell viability, associated with preparation of lymphocytes have been determined. The possible effects of these parameters on the transformation time required for the two types of lymphocyte preparation were evaluated. The present data show that major factors for EBV transformation initiated with freshly isolated lymphocytes were cell density and cell viability. As indicated in Tables 5 and 6, both these factors influenced the transformation time for freshly isolated lymphocytes in achieving successful transformation. Furthermore, an increase in cell density or cell viability was accompanied by a significant decrease in transformation time. Unexpectedly and interestingly, blood transportation time was not a major factor affecting transformation time for freshly isolated lymphocytes. Heidemarie (1986) reported that lymphocytes could still be prepared 5–7 days after blood sampling. Based on these data, one might suppose that an acceptable processing time for EBV transformation would be within 7 days once the blood was collected. In the present study, the mean of blood transportation time for freshly isolated lymphocyte was 34 h (1.4 days). This might explain why the blood transportation time was not a major factor. With regard to major factors in cryopreserved lymphocytes, Beck *et al.* (2001) described how cryopreserved lymphocytes require different times for successful transformation, even with similar cryopreservation periods. This indicates that parameters other than cryopreservation time influence the transformation time when cryopreserved lymphocytes are used as the starting material.

In the present study, blood transportation time, cryopreservation time and cell viability were demonstrated to be the three major factors. Based on the results shown in Table 8, collected blood samples with transport time in excess of 48 h were suggested on which to immediately perform EBV transformation in the interest of saving transformation time. Interestingly, cell density was not included in the major factors. Perhaps the recovered quality of lymphocytes was superior to the quantity of lymphocytes, the determination of which requires further study. Thus, it is worth noting that cell viability greater than 90% led to a significant decrease in the transformation time even when the cryopreservation time exceeded 360 days (Table 9). These data not only reveal that the effects of cryopreservation time and cell viability on transformation time differed, but also imply possible results from interactions among major factors on the required transformation time. Interactions of major factors on the required transformation time are complicated and cannot be ruled out.

In conclusion, the present study proposes a preliminary selection criterion of isolated B lymphocytes for EBV transformation. Additionally, transformation time-dependent factor analysis has not only shown the individual effects of each major factor on the required transformation time but has also provided data to demonstrate that major factors of freshly isolated lymphocytes were not the same as those of cryopreserved ones. It should be emphasized that further experiments are required to address the extent of the influence of each factor on the required transformation time. In the meantime, the present study also demonstrated that transformation time

required by freshly isolated lymphocytes for EBV transformation is significantly different from that required by cryopreserved ones.

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