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REPORT

# Development of a robust method for establishing B cell lines using Epstein-Barr Virus

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**Abstract** B lymphoblastoid cell lines (B-LCLs) are generally established from B lymphocytes by infection with Epstein-Barr virus (EBV). As their genomic structure is stable in culture, B-LCLs are a valuable resource for many types of analysis. The efficiency of establishing B-LCLs from freshly obtained blood samples from healthy individuals is almost 100 %; however, for blood samples stored inappropriately after collection or held in long-term storage as peripheral blood mononuclear cells (PBMCs) in liquid nitrogen, the efficiency of B-LCL establishment can be considerably lower. To date, we have established more than 550 B-LCLs from 685 PBMC samples that have been stored in liquid nitrogen for over 20 yr. The PBMCs were prepared from blood samples donated by individuals belonging to native minority ethnic groups in outlying regions of South America and elsewhere. The establishment of B-LCLs from this material is difficult, and failure results in the waste of valuable and rare samples. We sought to improve our success rate for establishing B-LCLs from these difficult and irreplaceable samples by a detailed examination of each step of the process. The analysis showed that two parameters were particularly critical to the success rate: the density of the PBMCs plated after EBV infection and the EBV titer. These observations shed light on cases where establishment of B-LCLs was hard due to the small number of PBMCs or damage to the cells.

**Keywords** South America · Ethnic group · B cell line · EBV · Genome analysis

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Blood cells are a valuable resource for genome research as they can be obtained without risk to the donor and because intact genomic DNA can easily be obtained from them. However, they have the drawback that the quantity of DNA that can be obtained from a standard blood sample is limited. This problem can be avoided through establishment of cultured cell lines from the blood samples, which allows repeated preparation of genomic DNAs. Currently, establishment of B cell lines by transformation with the Epstein-Barr virus (EBV), creating cell lines usually designated B-LCLs, is a favored approach (Nilsson 1979). Although genomic mutations can accumulate in cultured cells, analyses have indicated that the genomes of B-LCLs are relatively stable (Nilsson 1992; Lalle et al. 1995; Okubo et al. 2001; Simon-Sanchez et al. 2007; Herbeck et al. 2009; Danjoh et al. 2011). For this reason, B-LCLs have been adopted as the material of choice for many genome analysis projects, such as the International Histocompatibility Working Group (http://ihwg.org/index.html), the International HapMap Project (http://hapmap.ncbi.nlm.nih. gov/), the Human Genome Diversity Project (http://www.cephb.fr/en/hgdp/table.php), the genotyping of blood cell alloantigens (Kroll et al. 2001), the detection of copy number variation (Redon et al. 2006), population genetics (Li et al. 2008), and the 1000 Genomes Project (1000 Genomes Project Consortium 2010).

The Sonoda-Tajima Cell Collection is a collection of cryopreserved peripheral blood mononuclear cells (PBMCs) derived from blood samples donated by volunteers from many indigenous populations living in isolated parts of the world. The PBMCs have been cryopreserved in liquid nitrogen for over 20 yr (Danjoh et al. 2011; Sonoda et al. 2011). As some of these populations no longer exist, the collection is a unique repository of the human genome. In total, the collection contains samples from more than 3,500 individuals. Each PBMC sample is composed of approximately $1 \times 10^7$ cells that are divided among

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several cryotubes (approximately $1\times10^6$ cells/tube) before cryopreservation.

In order to make the Sonoda-Tajima Cell Collection available for research without depleting it, we decided to establish B-LCLs. However, the success rate of B-LCL establishment was much lower for these PBMCs than is routinely achieved with fresh samples. For the latter, B-LCLs can be established from almost 100 % of PBMC samples (without cryopreservation) from healthy individuals. By comparison, the success rate for cells of the Sonoda-Tajima Cell Collection can be low for samples with small numbers of PBMCs and also for those containing PBMCs that were prepared and/or preserved under sub-optimal conditions because of the unavailability of electricity at the sampling location.

For samples from which B-LCLs cannot be established, then the stored PBMCs risk being exhausted. In order to enable repeated use of the precious samples without exhausting them, we decided to examine ways of improving the success rate of establishing B-LCLs from the PBMCs of the Sonoda-Tajima Cell Collection. During the remaking of the Sonoda-Tajima Cell Collection, we have encountered many problems, including reduced success rates for establishing B-LCLs and instability of some of the established cell lines. The PBMCs of the Sonoda-Tajima Cell Collection are not all in a good state because of the difficult conditions under which they were collected as described above. Although the methodology for establishing B-LCLs was developed at the end of 1970s, surprisingly little attention has been given over the years to identifying the factors that determine the success rate of generating cell lines. It was therefore essential that the methodology used for establishing B-LCLs from these was improved in order to create a resource that would not be rapidly depleted. To achieve this aim, we examined each step of the method to identify ways of improving the success rate.

One of the first aspects of the process that we examined was the survival rate of PBMCs after thawing. We found that rapid thawing of the cells and keeping them at around 4 °C improved the rate of survival (data not shown); expeditious processing of the cells after thawing also aided survival. Next, we examined the importance of various factors, namely, cell density, co-culture with feeder cells, EBV titer, and different immunosuppressive agents on the rate of success for producing B-LCLs.

When we initiated production of B-LCLs from the Sonoda-Tajima Cell Collection, EBV-infected PBMCs were simply inoculated into two wells of a 24-well plate (surface area of each well 2.0 $cm^2$) without regard to cell density. The method of Danjoh et al. (2011) was used here to establish B-LCLs. In brief, EBV solution was added to the PBMC culture for 2 h at 37 °C. The cells were then washed once with RPMI1640 supplemented with 20 % fetal bovine serum (FBS), then resuspended in RPMI1640 supplemented with 20 % FBS and 0.1 μg/ml cyclosporin A (CsA, trade name Sandimmune; Novartis Pharma, Basel, Switzerland), inoculated into a multi-well plate at various cell densities and cultured at 37 °C under 5 % $CO_2$. Twice per week, half of the culture medium was replaced with fresh medium containing CsA. The cultures were maintained as scaled up two- to four-fold dilutions until efficient proliferation of B-LCLs could be confirmed after a couple of months. At the initial scaling up, the culture was divided into three batches that were maintained independently. After establishment of B-LCLs, the three batches were checked with polymorphic short tandem repeats (STRs) originally developed as forensic markers, using an Amp FLSTR Identifiler kit (Applied Biosystems, Foster City, California) to confirm that they were derived from the same person and that they did not have the same STR pattern as other cell lines in the RIKEN Cell Bank, in order to eliminate the possibilities of misidentification or cross-contamination.

During the initial process for establishing B-LCLs, it became clear that there was a correlation between the density of the PBMCs used to initiate the culture and the likelihood of success in establishing B-LCLs. We therefore investigated this relationship using PBMCs obtained from a Japanese healthy volunteer. In this experiment, $5\times10^4$ cells were inoculated into plates with different numbers of wells to give cell densities of $1.96\times10^5$ cells/$cm^2$ in a 96-well plate, $0.66\times10^5$ cells/$cm^2$ in a 48-well plate, and $0.25\times10^5$ cells/$cm^2$ in a 24-well plate (Table 1). We found that the rate of establishment of B-LCLs was positively correlated with cell density (Table 1).

Next, we examined the effect of cell density on establishing B-LCLs from the Sonoda-Tajima Cell Collection samples collected from South America; a detailed description of this collection was provided by Danjoh et al. (2011). After infection with EBV, PBMCs were inoculated into differently sized multiwell plates depending on cell density: If the cell number was lower than $1\times10^6$, then a 48-well plate (0.75 $cm^2$ for each well) was used; for a cell number between 1 and $2\times10^6$, a 24-well plate was used; and, if the cell number exceeded $2\times10^6$, then two wells of a 24-well plate were used. We did not use 96-well plates in order to reduce the risk of contamination; the wells of these plates are too small and densely packed for long-term culture with frequent changes of medium and for handling a large number of samples in parallel as in this experiment.

Analysis of the results obtained from the 685 samples treated with EBV showed that B-LCLs were established at a significantly reduced rate ($p<0.0001$) in cultures initiated at lower cell densities (Table 2); the Sonoda-Tajima Cell Collection samples were analyzed by a contingency table analysis with Fisher's exact test and a chi-square test using StatView ver. 5.0 software (SAS, Cary, North Carolina). B-LCLs were established in approximately 84 %

**Table 1.** Cell density and success rate for establishment of B-LCLs

| Cell density ($\times 10^5$ cells/cm$^2$) | Number of wells | Success | Failure | Success rate (%) | | | |
|---|---|---|---|---|---|---|---|
| 1.96 | 20 | 13 | 7 | 65.00 | * | * | * |
| 0.66 | 60 | 14 | 46 | 23.33 | | | |
| 0.25 | 40 | 2 | 38 | 5.00 | | | |

PBMCs were from a healthy Japanese volunteer

*$p<0.05$

of the cultures initiated at a PBMC density in the range 2.5–$4.9\times10^5$ cells/cm$^2$ (Table 2). By comparison, we found that PBMCs prepared from fresh blood and stored in good condition in liquid nitrogen always produced B-LCLs in cultures initiated at a cell density greater than $2\times10^5$ cells/cm$^2$ (data not shown). In order to make further comparisons of our data from cultures using fresh PBMCs (Table 1) with those from Sonoda-Tajima Cell Collection, we sub-divided the data from cultures initiated at cell densities in the 1.0–$2.4\times10^5$ cells/cm$^2$ and 0.1–$0.9\times10^5$ cells/cm$^2$ (Table 2, numbers in parentheses), although these sub-divisions were not used for the statistical analysis. At a cell density of $2\times10^5$ cells/cm$^2$, the success rate for the PBMCs from the healthy Japanese volunteer was 65 % (Table 1) but 46.51 % for Sonoda-Tajima Cell Collection PBMCs at a cell density 1.5–$2.4\times10^5$ cells/cm$^2$ (Table 2). These results suggest that the PBMCs in the latter suffered damage during the field work to collect the blood samples.

To further analyze our results, we examined the possible influence of the date and location of collection of Sonoda-Tajima Cell Collection samples and also of differences between EBV batches as several lots were used during establishment of B-LCLs (this aspect is described in detail later). Even if samples were collected at the same place and at the same time, they were sorted into different groups if EBV lots with different titers were used to stimulate B-LCLs. The following criteria were used to identify categorized groups suitable for statistical analysis: a minimum of 15 samples, and containing several samples with a cell density below $2.5\times10^5$ cells/cm$^2$ (see Table 2). As described above, the statistical analysis was carried out within individual groups. Although the success rate for establishment of B-LCLs at each cell density varied among groups, it was evident that the success rate depended on cell density in all groups except those obtained in Colombia in August 1992 and Chile in December 1992 (Table 2). The absence of statistical significance in the data from the latter group might be due to the small sample size.

It has been reported that the use of Raji cells or allogeneic PBMCs as feeder cells improves the efficiency of establishing B-LCLs (Hakoda et al. 1996; Yang et al. 2004). In general, the success rate for establishing B-LCLs approaches 100 % when a sufficient number (over $5.0\times10^5$ cells/cm$^2$) of freshly prepared PBMCs from healthy individuals is used. However, for the Sonoda-Tajima Cell Collection, the success rate decreased when the numbers of PBMCs were low (less than $2.5\times10^5$ cells/cm$^2$). We therefore decided to investigate whether the success rate was improved by choice of feeder cell when using small numbers of PBMCs.

In addition to Raji cells, OP9 and 10T1/2 cells were used as feeder cells because they are known to support the growth of hematopoietic stem cells. As B-LCLs are thought to secrete an as-yet unidentified growth factor(s) that promotes cell growth in a paracrine manner, we also investigated the B-LCL cell lines KOIA-LCL and JTK-LCL as feeder cells. All five cell lines were obtained from the Cell Engineering Division of RIKEN BioResource Center (RIKEN Cell Bank, Tsukuba, Ibaraki, Japan) (http://www.brc.riken.jp/lab/cell/english/). Each feeder cell line was $\gamma$-irradiated at a dose that halted the growth of the cells but did not affect their survival (data not shown); they were added to the indicated number of wells (Table 3) of the culture plate 1 d before inoculation with EBV-infected PBMCs (a feeder cell density of $1.33\times10^6$ cells/cm$^2$ per well for a 48-well plate and of $0.5\times10^6$ cells/cm$^2$ for a 24-well plate). Our investigation of the influence of feeder cells was restricted to cultures initiated with fewer than $1\times10^5$ PBMCs/cm$^2$ , which have a low success rate, and did not examine cultures with over $2\times10^5$ cells/cm$^2$ that gave success rates over 50 % (Tables 1 and 2).

Although it has been reported elsewhere that Raji cells can act as feeder cells, in the present study, we found a significant reduction in the establishment of B-LCLs at both cell densities tested in cultures using Raji cells compared with those with no feeder cells (Table 3). By contrast, KOIA cells gave a significant increase in the success rate at both cell densities tested; JTK had a significant effect at the lower but not the higher cell density (Table 3). Neither OP9 nor

**Table 2.** Cell density and success rate for establishment of B-LCLs from the Sonoda-Tajima Cell Collection

| Cell density ($x10^5$ cells/$cm^2$) | Number of samples | Success | Failure | Success rate (%) | Number of samples | Success | Failure | Success rate (%) | Number of samples | Success | Failure | Success rate (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Argentine (1997 Oct); EBV lot# T1, T2 | | | | Chile (1991 Jan); EBV lot# J3 | | | | Colombia (1991 Aug); EBV lot# J2, T1, T2 | | | |
| 10< | 6 | 6 | 0 | 100.0 | 8 | 8 | 0 | 100.0 | 5 | 4 | 1 | 80.0 |
| 7.5-9.9 | 5 | 5 | 0 | 100.0 | 15 | 15 | 0 | 100.0 | 5 | 5 | 0 | 100.0 |
| 5.0-7.4 | 12 | 12 | 0 | 100.0 | 24 | 21 | 3 | 87.5 | 9 | 7 | 2 | 77.8 |
| 2.5-4.9 | 0 | 0 | 0 | - | 15 | 13 | 2 | 86.7 | 6 | 4 | 2 | 66.7 |
| 1.0-2.4 | 2 | 1 | 1 | 50.0 | 10 | 4 | 6 | 40.0 | 4 | 1 | 3 | 25.0 |
| 0.1-0.9 | 0 | 0 | 0 | - | 7 | 1 | 6 | 14.3 | 0 | 0 | 0 | - |
| <0.1 | 3 | 0 | 3 | 0.0 | 3 | 0 | 3 | 0.0 | 4 | 0 | 4 | 0.0 |
| | Colombia (1991 Aug); EBV lot# J3 | | | | Colombia (1992 Aug); EBV Lot# J3[a] | | | | Ecuador (1999 Oct); EBV Lot# J3, J4 | | | |
| 10< | 0 | 0 | 0 | - | 1 | 1 | 0 | 100.0 | 8 | 8 | 0 | 100.0 |
| 7.5-9.9 | 4 | 2 | 2 | 50.0 | 6 | 2 | 4 | 33.3 | 9 | 9 | 0 | 100.0 |
| 5.0-7.4 | 5 | 5 | 0 | 100.0 | 5 | 2 | 3 | 40.0 | 16 | 16 | 0 | 100.0 |
| 2.5-4.9 | 9 | 7 | 2 | 77.8 | 8 | 4 | 4 | 50.0 | 9 | 8 | 1 | 88.9 |
| 1.0-2.4 | 8 | 0 | 8 | 0.0 | 1 | 0 | 1 | 0.0 | 8 | 1 | 7 | 12.5 |
| 0.1-0.9 | 5 | 0 | 5 | 0.0 | 2 | 0 | 2 | 0.0 | 5 | 0 | 5 | 0.0 |
| <0.1 | 0 | 0 | 0 | - | 0 | 0 | 0 | - | 1 | 0 | 1 | 0.0 |
| | Peru (1997 May); EBV Lot# T3 | | | | Venezuela (1993 Jan); EBV Lot# J2, T2 | | | | Venezuela (1993 Jan); EBV Lot# J3 | | | |
| 10< | 2 | 2 | 0 | 100.0 | 4 | 4 | 0 | 100.0 | 0 | 0 | 0 | - |
| 7.5-9.9 | 2 | 2 | 0 | 100.0 | 6 | 6 | 0 | 100.0 | 4 | 4 | 0 | 100.0 |
| 5.0-7.4 | 6 | 6 | 0 | 100.0 | 12 | 12 | 0 | 100.0 | 5 | 5 | 0 | 100.0 |
| 2.5-4.9 | 7 | 6 | 1 | 85.7 | 7 | 6 | 1 | 85.7 | 1 | 1 | 0 | 100.0 |
| 1.0-2.4 | 4 | 3 | 1 | 75.0 | 4 | 3 | 1 | 75.0 | 3 | 0 | 3 | 0.0 |
| 0.1-0.9 | 2 | 0 | 2 | 0.0 | 7 | 2 | 5 | 28.6 | 3 | 0 | 3 | 0.0 |
| <0.1 | 1 | 0 | 1 | 0.0 | 0 | 0 | 0 | - | 0 | 0 | 0 | - |
| | Chile (1991 Dec); EBV lot# T1 | | | | | | | | Sonoda-Tajima Cell Collection All samples | | | |
| 10< | 2 | 2 | 0 | 100.0 | | | | | 86 | 84 | 2 | 97.7 |
| 7.5-9.9 | 3 | 3 | 0 | 100.0 | | | | | 133 | 125 | 8 | 94.0 |
| 5.0-7.4 | 9 | 9 | 0 | 100.0 | | | | | 220 | 209 | 11 | 95.0 |
| 2.5-4.9 | 6 | 6 | 0 | 100.0 | | | | | 122 | 102 | 20 | 83.6 |
| 1.0-2.4 | 3 | 2 | 1 | 66.7 | | | | | 63 | 24 | 39 | 38.1 |
| (1.5-2.4) | - | - | - | - | | | | | 43 | 20 | 23 | 46.5 |
| (1.0-1.4) | - | - | - | - | | | | | 20 | 4 | 16 | 20.0 |
| 0.1-0.9 | 3 | 3 | 0 | 100.0 | | | | | 52 | 11 | 41 | 21.2 |
| (0.5-0.9) | - | - | - | - | | | | | 29 | 8 | 21 | 27.6 |
| (0.1-0.4) | - | - | - | - | | | | | 23 | 3 | 20 | 13.0 |
| <0.1 | 0 | 0 | 0 | - | | | | | 9 | 0 | 9 | 0.0 |

[a]Statistical significance was not observed between cell density and success rate
*p<0.05 by Fisher's exact probability test

**Table 3.** Effect of feeder cells on B-LCL establishment

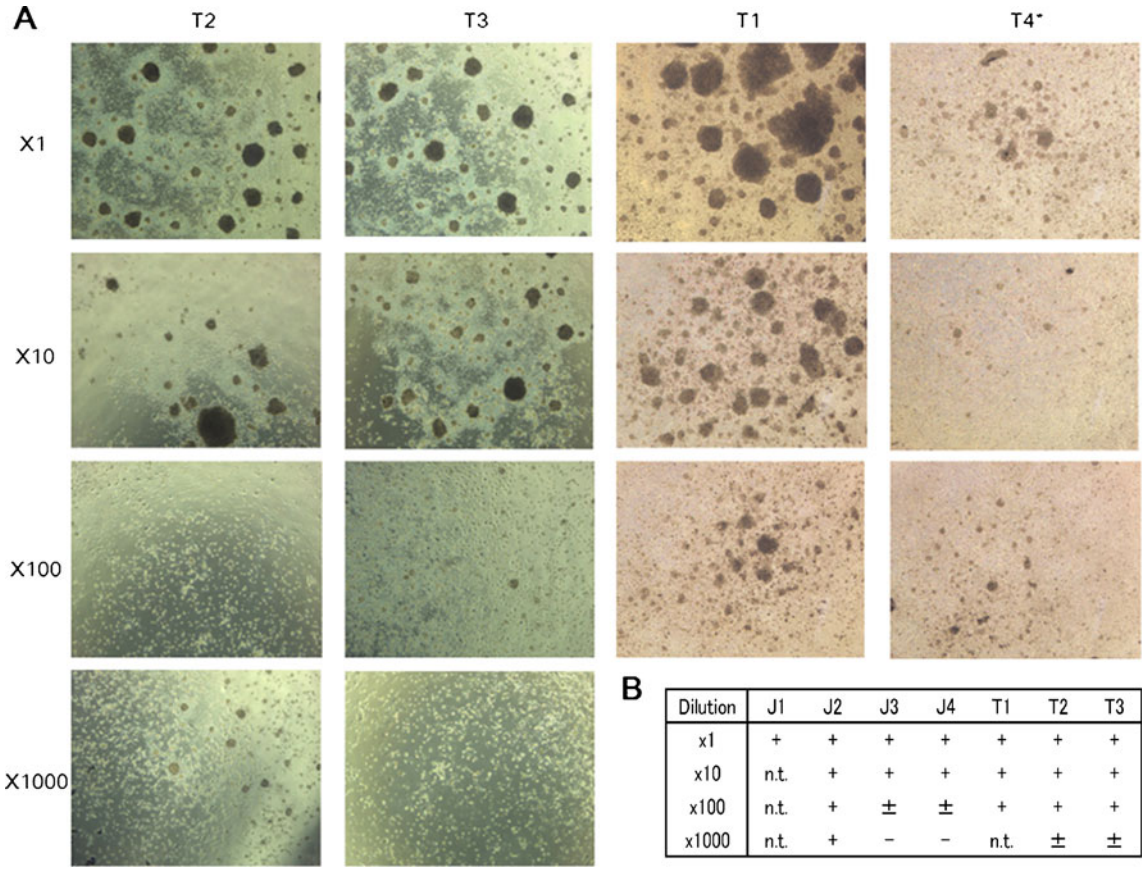
| B-LCL density ($x10^5$ cells/$cm^2$) | Cell lines used as feeder | Number of wells | Success | Failure | Success rate (%) |
|---|---|---|---|---|---|
| 0.67[a] | no feeder | 50 | 20 | 30 | 40.00 |
| | Raji | 20 | 3 | 17 | 15.00 |
| | KOIA | 30 | 22 | 8 | 73.33 |
| | JTK | 30 | 17 | 13 | 56.67 |
| 0.25[b] | no feeder | 50 | 15 | 35 | 30.00 |
| | Raji | 20 | 0 | 20 | 0.00 |
| | KOIA | 30 | 19 | 11 | 63.33 |
| | JTK | 30 | 20 | 10 | 66.67 |

[a]$5x10^4$ cells were inoculated in each well of a 48-well plate
[b]$5x10^4$ cells were inoculated in each well of a 24-well plate
*p<0.05 by Fisher's exact probability test

10T1/2 had any effect on the rate of success for establishing B-LCLs (data not shown).

Most researchers prepare their own EBV solutions for use in establishing B-LCLs. As there are no good methods to quantify the titer of EBV, the quality of the prepared EBV solution is checked pragmatically by testing its ability to establish a B-LCL following infection of cultured PBMCs. The latter approach was the one initially adopted for establishing B-LCLs from the Sonoda-Tajima Cell Collection. However, while using some batches of EBV solution prepared from independent cultures of the B95-8 cell line, we gained the impression that the success rate for B-LCL establishment varied despite the fact that each EBV lot could establish B-LCLs from PBMCs prepared from healthy volunteers under regular laboratory conditions. We therefore investigated the influence of the EBV preparation on the efficiency of establishing B-LCLs.

The method of Danjoh et al. (2011) was used here to prepare EBV solutions. In brief, B95-8 cells were cultured in RPMI1640 (Gibco, Carlsbad, CA) supplemented with 10 % FBS. The culture supernatant was collected, filtered to remove any remaining cells, and then stored at −80 °C. Seven different preparations of EBV solutions have been used to establish B-LCLs from the Sonoda-Tajima Cell Collection; four of these were derived from the B95-8 cell line obtained from the Japanese Collection of Research Bioresources (JCRB Cell Bank, Osaka, Japan; J1-J4 in Fig. 1), the other three were from the Cell Resource Center for Biomedical Research, Tohoku University (CRCBR Cell Bank, Sendai, Miyagi, Japan; T1-T3 in Fig. 1). Each EBV preparation was diluted in a ten-fold series, and these diluted preparations were used to infect PBMCs obtained from a healthy Japanese volunteer; the various dilutions were assessed for their abilities to induce formation of B-LCLs (see examples in Fig. 1). B-LCLs could be established using the 1,000-fold dilution of EBV lot #T2, whereas only small B-LCL clusters were present using the 100-fold dilution of lot #T3. The effectiveness of lot #T1 was not checked at the 1,000-fold dilution, however, many large B-LCL clusters were observed at the 100-fold dilution. Although some




| Dilution | J1 | J2 | J3 | J4 | T1 | T2 | T3 |
|---|---|---|---|---|---|---|---|
| x1 | + | + | + | + | + | + | + |
| x10 | n.t. | + | + | + | + | + | + |
| x100 | n.t. | + | ± | ± | + | + | + |
| x1000 | n.t. | + | − | − | n.t. | ± | ± |

**Figure 1.** Titration of EBV preparations. (*a*) EBV titration experiment. EBV-infected PBMCs from a healthy Japanese volunteer were inoculated into two wells of a multiwell plate at a cell density of approximately $1\times10^6$ cells/cm$^2$ for each EBV dilution. The images shown are of cultures at 31 d after infection with the EBV preparation. The *left numerical characters* indicate the extent of serial dilution of each EBV solution. T2, T3, T1, and T4 indicate EBV solutions prepared from independent cultures. *Asterisk*, not suitable for use in establishment of B-LCLs from the Sonoda-Tajima Cell Collection PBMCs because of a low EBV titer. (*b*) Success or failure of different concentrations of EBV preparations for producing B-LCLs. +, B-LCLs could be established; − B-LCLs were not established; *nt* not tested.

B-LCL clusters were induced using the 100-fold dilution of lot #T4, overall, this lot only achieved slow establishment of B-LCLs compared with other EBV lots (Fig. 1*a*, x1 and x10 EBV dilution) and also generated many dead cells. Therefore, we concluded that lot #T4 was unsuitable for use in establishment of B-LCLs from the Sonoda-Tajima Cell Collection PBMCs. A summary of the results of the titration experiment for seven EBV lots is shown in Fig. 1*b*. Our results clearly showed that the virus titers varied considerably between EBV preparations. Notably, all seven virus lots shown in Fig. 1*b* could establish B-LCLs without any problems from PBMCs obtained from healthy volunteers and prepared under controlled conditions.

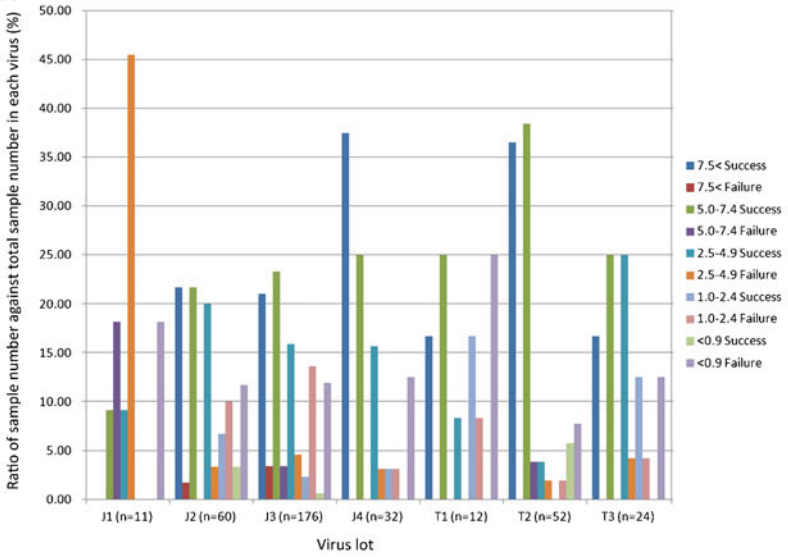
After confirming that each virus lot had a different virus titer, we expected that the EBV lots with higher virus titers would be more efficient at establishing B-LCLs than those with lower virus titers. As the PBMCs of some of the Sonoda-Tajima Cell Collection were so seriously damaged that all the cells were dead upon thawing, we decided to use two group settings to determine whether virus titer affected the success rate for establishment of B-LCLs: One group contained all 685 EBV-treated samples; the second group omitted problematic samples and only contained the remainder of the EBV-treated samples. As it is essential that the groups have statistically homogeneous sample variance in order to apply a contingency table analysis, we first carried out a Bartlett's test to check that cell density variance was homogeneous across the virus lots using the sample numbers ($n$) and standard deviations in the tables in Fig. 2. With the exceptions of some of the selected cultures using T2 and all Sonoda-Tajima Cell Collection samples using J1, the analysis indicated that the other virus preparations showed homogeneity of variance for cell density (data not shown). The T2 and J1 data were therefore excluded from the respective contingency table analysis of the relationship between virus lot and ability to establish B-LCLs. This analysis identified a statistically significant relationship between virus lot and ability to establish B-LCLs ($p<0.0001$). The influence of PBMC density on success rate is illustrated in Fig. 2 by plotting the ratio of sample number in each category of cell density against total number of samples within each virus lot. Interestingly, the J2, J3, T1, and T2 lots of virus produced B-LCLs in a few cultures initiated with cell densities below $1.0\times10^5$ cells/cm$^2$ (Fig. 2, rows labeled "<0.9"). This observation suggests that EBV lots with higher virus titers can establish B-LCLs at low PBMC density with a higher rate of success than those with lower virus titers. However, the small sample sizes precluded testing this apparent difference for statistical significance. By comparison, some of the B-LCLs established using J3 or J4 (which had comparatively low virus titers among the seven EBV lots analyzed here, Fig. 1*b*) showed premature growth arrest at approximately passage 15 (data not shown).

Although it has been reported that use of allogeneic PBMCs or Raji cells as feeder cells (Hakoda et al. 1996; Yang et al. 2004), or addition of supernatants from cell line cultures (Mevissen et al. 1993), can increase the rate of success, we found here that cell density was the most critical parameter with regard to improving the success rate for establishing B-LCLs. We also identified significant variation between virus lots with respect to their abilities to establish | B-LCLs. Indeed, the virus titer in some lots was sufficiently high that they continued to function even at a 1,000-fold dilution (Fig. 1). Some of the virus lots with a lower titer produced unstable B-LCLs that showed growth arrest shortly after expansion. Our analysis, therefore, indicates that the EBV solution is a critical factor for improving the success rate of establishing B-LCLs and for the stable growth of the B-LCLs.

To establish B-LCLs from Sonoda-Tajima Cell Collection PBMCs, we adopted the common and popular method of using cyclosporin A (CsA) as an immunosuppressive agent. We also tested the effect of another immunosuppressive agent, FK506 (trade name Prograf, Astellas Pharma Inc., Tokyo, Japan). FK506 is an inhibitor of calcineurin and suppresses cytotoxic T lymphocytes in the same manner as CsA (Yoshimura et al. 1989a, b; reviewed by Schreiber and Crabtree 1992), therefore, in theory, it could be used instead of CsA in the establishment of B-LCLs. First, we carried out a titration experiment to compare the effects of CsA and FK506 on the sensitivity of PBMCs to EBV. We found that CsA and each of the tested concentrations of FK506 gave similar EBV titers (Fig. 3*a*). Thus, the concentration of FK506 did not influence the sensitivity of PBMCs to EBV; however, the number and the sizes of B-LCL clusters at all concentrations of FK506 in the undiluted EBV solution were larger than those with CsA (Fig. 3*b*). Small dead cell clusters were observed with diluted EBV solutions (data not shown). Next, we compared the effects of the two agents on the rates of successful establishment of B-LCLs using different densities of PBMCs. Mononuclear blood cells from a Japanese adult volunteer and from neonatal cord blood (CB) were used for this experiment. Various densities of PBMCs or CBMCs were inoculated into ten wells of multi-well plates after EBV infection, and the frequency of establishment of B-LCLs was determined at 23 d after inoculation (Fig. 3*c*). Statistical analyses of the results indicated that there was no significant difference between CsA and FK506 at establishing B-LCLS at each seeding cell density. In contrast, however, comparison within both samples showed a significant difference in success rates at different cell densities for CsA but not for FK506: $1.96\times10^5$ cells/cm$^2$ versus $0.25\times10^5$ (Fig. 3*c*). These results suggest that FK506 did not affect the sensitivity of the cells to EBV but maintained a high promotive effect on cells entering the growth cycle. The presence of small clusters of dead cells in cultures with FK506 at the highest EBV

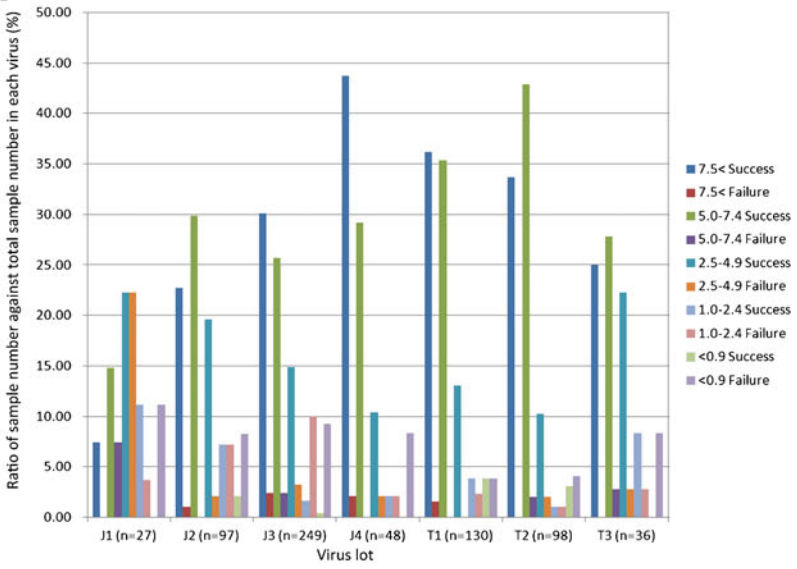
**Figure 2.** Relationship of virus lot and cell density to successful establishment of B-LCLs. (*a*) Results from the selected samples in Table 2. (*b*) Results from all Sonoda-Tajima Cell Collection samples. The *bar graph* indicates the percentage of samples in each category against the total sample number in each virus lot. The tables below provide the actual numbers in each category. The tables also show the average cell density and standard deviation in each virus lot; these data were used for the Bartlett's test.

A




| Cell density ($\times 10^5$ cells/cm$^2$) | | J1 (n=11) | J2 (n=60) | J3 (n=176) | J4 (n=32) | T1 (n=38) | T2 (n=52) | T3 (n=24) |
|---|---|---|---|---|---|---|---|---|
| 7.5< | Success | 0 | 13 | 37 | 12 | 7 | 19 | 4 |
| | Failure | 0 | 1 | 6 | 0 | 0 | 0 | 0 |
| 5.0–7.4 | Success | 1 | 13 | 41 | 8 | 12 | 20 | 6 |
| | Failure | 2 | 0 | 6 | 0 | 0 | 2 | 0 |
| 2.5–4.9 | Success | 1 | 12 | 28 | 5 | 7 | 2 | 6 |
| | Failure | 5 | 2 | 8 | 1 | 0 | 1 | 1 |
| 1.0–2.4 | Success | 0 | 4 | 4 | 1 | 4 | 0 | 3 |
| | Failure | 0 | 6 | 24 | 1 | 2 | 1 | 1 |
| <0.9 | Success | 0 | 2 | 1 | 0 | 3 | 3 | 0 |
| | Failure | 2 | 7 | 21 | 4 | 3 | 4 | 3 |
| Average of cell density ($\times 10^5$ cells/cm$^2$) | | 4.10 | 4.80 | 5.00 | 6.00 | 4.40 | 6.90 | 4.50 |
| SD of cell density (x105 cells/cm2) | | 2.20 | 3.80 | 3.20 | 3.40 | 4.50 | 4.90 | 3.10 |

B

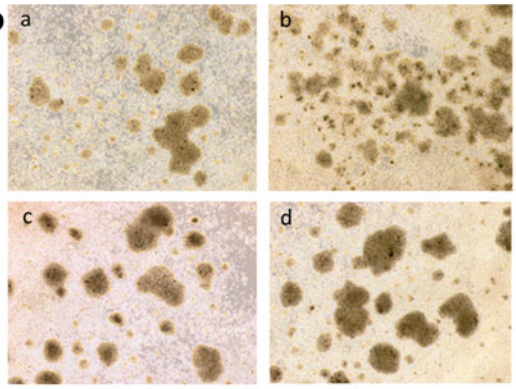



| Cell density ($\times 10^5$ cells/cm$^2$) | | J1 (n=27) | J2 (n=97) | J3 (n=249) | J4 (n=48) | T1 (n=130) | T2 (n=98) | T3 (n=36) |
|---|---|---|---|---|---|---|---|---|
| 7.5< | Success | 2 | 22 | 75 | 21 | 47 | 33 | 9 |
| | Failure | 0 | 1 | 6 | 1 | 2 | 0 | 0 |
| 5.0–7.4 | Success | 4 | 29 | 64 | 14 | 46 | 42 | 10 |
| | Failure | 2 | 0 | 6 | 0 | 0 | 2 | 1 |
| 2.5–4.9 | Success | 6 | 19 | 37 | 5 | 17 | 10 | 8 |
| | Failure | 6 | 2 | 8 | 1 | 0 | 2 | 1 |
| 1.0–2.4 | Success | 3 | 7 | 4 | 1 | 5 | 1 | 3 |
| | Failure | 1 | 7 | 25 | 1 | 3 | 1 | 1 |
| <0.9 | Success | 0 | 2 | 1 | 0 | 5 | 3 | 0 |
| | Failure | 3 | 8 | 23 | 4 | 5 | 4 | 3 |
| Average of cell density ($\times 10^5$ cells/cm$^2$) | | 4.10 | 5.20 | 5.90 | 6.70 | 6.60 | 6.80 | 5.20 |
| SD of cell density ($\times 10^5$ cells/cm$^2$) | | 2.60 | 3.40 | 3.80 | 3.10 | 3.70 | 3.90 | 2.90 |

**a**

| EBV dilution | FK506 (ng/mL) | | | CsA (ng/mL) |
|---|---|---|---|---|
| | 1 | 10 | 100 | 100 |
| x1 | + | + | + | + |
| x10 | + | + | + | + |
| x100 | ± | ± | ± | ± |
| x1000 | − | − | − | − |

**b**



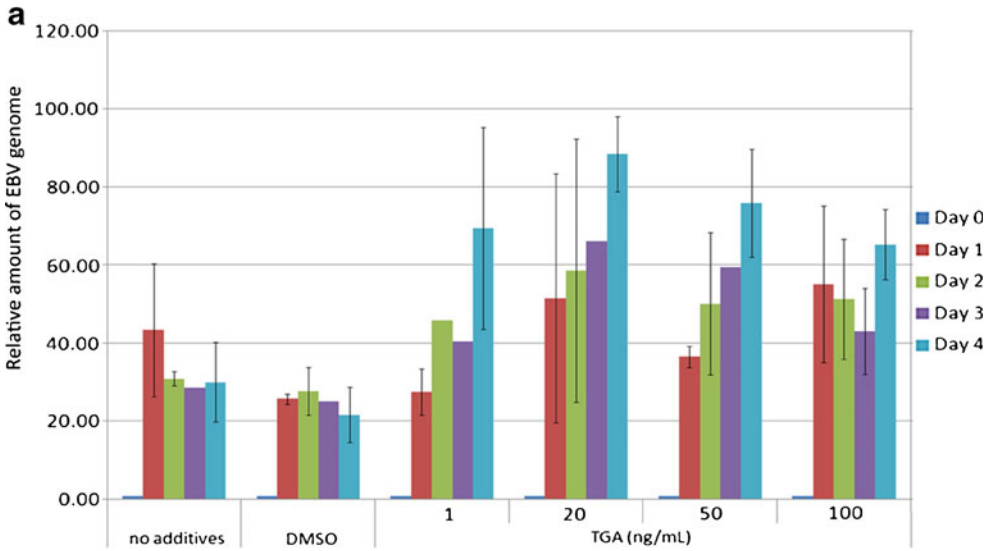

**c**

| Cell density ($\times 10^5$/cm$^2$) | Sample | Immunosuppressive agent | Number of wells | Success | Failure | Success rate (%) | |
|---|---|---|---|---|---|---|---|
| 1.96 | Adult | CsA | 10 | 9 | 1 | 90.00 | * |
| | | FK506 | 10 | 10 | 0 | 100.00 | |
| 0.66 | | CsA | 10 | 6 | 4 | 60.00 | |
| | | FK506 | 10 | 7 | 3 | 70.00 | |
| 0.25 | | CsA | 10 | 3 | 7 | 30.00 | |
| | | FK506 | 10 | 6 | 4 | 60.00 | |
| 1.96 | Cord blood | CsA | 10 | 9 | 1 | 90.00 | * |
| | | FK506 | 10 | 10 | 0 | 100.00 | |
| 0.66 | | CsA | 10 | 9 | 1 | 90.00 | |
| | | FK506 | 10 | 10 | 0 | 100.00 | |
| 0.25 | | CsA | 10 | 3 | 7 | 30.00 | |
| | | FK506 | 10 | 7 | 3 | 70.00 | |

**Figure 3.** Comparison of the immunosuppressive agents cyclosporin A (CsA) and FK506 on B-LCL establishment. (*a*) EBV titration. The experimental protocol was as for Fig. 1 except that various concentrations of FK506 were applied to some cultures instead of CsA. (*b*) The images show B-LCL establishment under various culture conditions: (*a*) CsA, (*b–d*) FK506 ((*b*) 1 ng/mL; (*c*) 10 ng/mL; (*d*) 100 ng/mL). (*c*) Success rates for B-LCL establishment using different cell densities from different individuals. 10 ng/mL of FK506 was used for this experiment. *Asterisk*, $p<0.05$ by contingency table analysis with Fisher's exact test and a chi-square test. As FK506 is insoluble in water, it was diluted in ethanol and added as a 1 % ethanol solution to the culture medium just before use.

dilutions may support this interpretation: PBMCs may have been infected by only a small number of EBV particles and forced into entering cell division by the presence of FK506, however, the cells may have been unable to continue growth and to stop after a few rounds of cell division. We conclude that use of FK506 might shorten the period available for establishment of B-LCLs.

To further refine the method for establishing B-LCLs, we tested the effect of the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (phorbol 12-myristate 13-acetate (PMA), Sigma-Aldrich Inc., Saint Louis, MO) on the activation of the EBV-producing cell line B95-8. PMA is structurally analogous to diacylglycerol and can activate protein kinase C and thereby induce a range of cellular responses. The B95-8 cell line has previously been reported to be activated by PMA (Callard et al. 1988; Taylor et al. 2011; Murata et al. 2012), and we first investigated EBV production in these cells after PMA treatment. B95-8 cells were cultured in the presence of various concentrations of PMA, and culture supernatants were collected to act as EBV preparations as described above. EBV genomic DNAs were extracted with the DNeasy Blood and Tissue kit (Qiagen, Dusseldorf, Germany). Known amounts of genomic DNA extracted from the EBV-free cell lines AZ521 and GSS were added to the EBV preparations following the addition of cell lysis buffer for extraction of EBV genomic DNAs. The relative amounts of EBV genomic DNAs were determined using a real-time PCR monitoring system. The beta-globin gene was used as the control as the EBV genome does not contain this gene, and any amplification product is therefore derived from the AZ521 and GSS genomes that were added ectopically. The *EBNA1* gene, which is specific for the EBV genome, was used as the indicator of the relative amount of EBV genomic DNA. The following primer sequences were used for the beta-globin gene: forward, 5'-ggttggccaatctactcccagg-3' and reverse, 5'-tggtctccttaaacctgtcttg-3'. The primers for *EBNA1* were: forward, 5'-ggcctaggagagaagggaga-3' and reverse, 5'-ctatgtcttggccctgatcc-3'. The amount of EBV genomic DNA released from B95-8 cells into the culture medium increased in a time-dependent manner after addition of PMA at concentrations up to 50 ng/mL (Fig. 4*a*). Addition of 100 ng/mL PMA did not alter the amount of EBV genomic DNA in the medium during the treatment period; however, dead cells were present in the 50 and 100 ng/mL PMA treatment cultures from 1 d after addition of PMA. The lack of effect in the 100 ng/mL PMA culture may therefore indicate a cytotoxic effect of PMA on B95-8 cells. In all experiments, the highest levels of EBV genomic DNA were recovered at 4 d after initiation of PMA treatment. We, therefore, used EBV




b

| EBV Dilution | Day 0 | Day 1 | | | Day 4 | | | Day 4* | |
|---|---|---|---|---|---|---|---|---|---|
| | | - | D | 20 | - | D | 20 | - | 1 |
| x1 | − | ± | ± | + | + | + | + | + | + |
| x10 | − | − | − | ± | + | + | + | + | + |
| x100 | − | − | − | − | ± | ± | ± | + | + |
| x1000 | − | − | − | − | − | − | − | ± | ± |

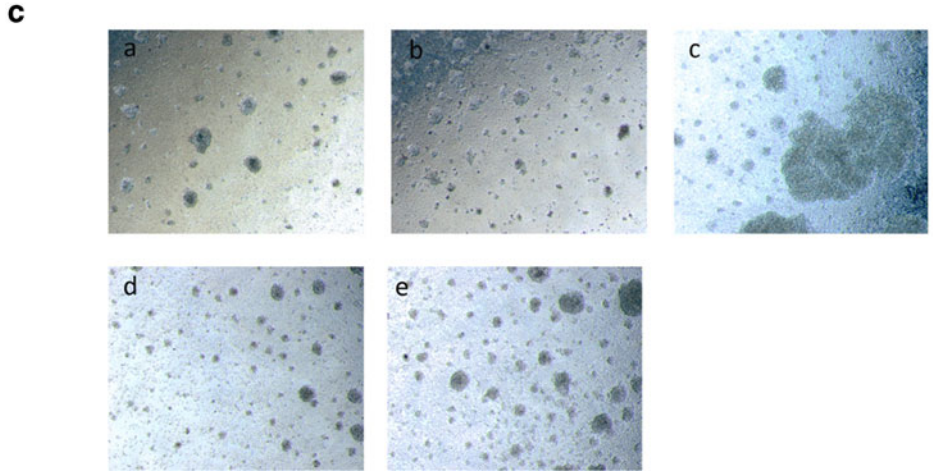



**Figure 4.** Production of EBV after activation of B95-8 cells with PMA. (*a*) Relative amounts of EBV genomic DNAs. *Vertical axis* indicates the ratio of amount of *EBNA1* against beta-globin gene. As PMA is insoluble in water, it was diluted in DMSO, and a 0.1 % DMSO solution was added to the culture medium just before use. (*b*) EBV titration. The experimental protocol to determine EBV titers was as described in Fig. 1. Two cord blood samples were used for this analysis. −, no additives; *D*, DMSO; *1*, 1 ng/mL PMA; *20*, 20 ng/mL PMA. The data shown for the Day 0, Day1 (−, D, 20) and Day 4 (−, D, 20) cultures were obtained from one sample, those for Day 4 (−, 1) cultures were from the second sample. (*c*) Selected images from cultures stimulated with undiluted EBV after PMA treatment for 4 d: (*a*) no additives; (*b*) DMSO; (*c*) 20 ng/mL PMA; (*d*) no additives; (*e*) 1 ng/mL PMA. (*a–c*) 15 d after EBV infection; (*d–e*) 13 d after EBV infection.

preparations at days 0, 1, and 4 for a titration experiment to determine if there were any differences in their ability to establish B-LCLs. EBV preparations treated with 50 or 100 ng/mL PMA were not used in the titration experiment because of their cytotoxic effect on B95-8 cells. After 1 d of PMA treatment, none of the EBV preparations had the ability to establish B-LCLs. In contrast, the 4-d treatment of the B95-8 cells yielded EBV preparations with sufficiently high virus titers to induce B-LCLs (Fig. 4*b*). Notably, EBV titer was very different between days 1 and 4 in the absence of additives, or with dimethyl sulfoxide (DMSO), in contrast to the similarity in the relative amounts of EBV genomic DNAs. Additionally, after treatment with 20 ng/mL PMA, the titer in the preparation from the 4-d treatment was more than ten times larger than that of the 1-d treatment group, although comparison of EBV genomic DNAs in the two preparations suggested only a twofold difference. Although the EBV titers from the cultures with no additives, or with DMSO, or in the 20 ng/mL PMA treatment group showed little difference, the PMA-treated EBV preparations generated big cell clusters (Fig. 4*c*). The EBV preparation from the culture in the 20 ng/mL PMA treatment group, however, had a cytotoxic effect

on PBMCs when used undiluted (data not shown). This effect was not evident in the EBV preparation in 1 ng/mL PMA-treated cultures (data not shown). These results suggest that B95-8 cells treated with PMA have enhanced EBV production; however, the genomic stability of B-LCLs established with a PMA-treated preparation needs to be monitored carefully as PMA is a strong tumor promoter.

Overall, the observations and conclusions described here are of relevance not only to the PBMC samples in the Sonoda-Tajima Cell Collection but also to other difficult blood samples. This is especially true of cases where collecting sufficient volume of blood is problematic, such as small children with thin blood vessels or patients whose blood cells have been decreased after therapeutic treatments with toxic compounds.

The Sonoda-Tajima Cell Collection is a very valuable cell collection obtained from various ethnic populations across the world, particularly from South America. As some of these populations in South America no longer exist, it will be impossible to prepare a similar collection in the future. Currently, more than 550 B-LCLs have been established from the 3,500 PBMC samples of the Sonoda-Tajima Cell Collection. All these B-LCLs are, or will shortly be, available from the cell bank held at the Cell Engineering Division of RIKEN BioResource Center in Japan (http://www.brc.riken.jp/lab/cell/english/). At the moment, approximately 150 B-LCLs are immediately available, whereas the others are now under preparation for distribution. In addition, we are willing to establish more B-LCLs on demand from scientists around the world. We believe that our development of this resource will contribute to various fields of science such as human genetics, human evolution, the history of human migrations across continents, and the pharmacokinetics of ethnic minority groups, among others.

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