Short Communication

Successful Transformation of Cryopreserved Lymphocytes: A Resource for Epidemiological Studies[1]

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The Cooperative Family Registry for Breast Cancer Studies (CFRBCS) is an international multisite cooperative consortium of investigators who collaborate to ascertain families at risk for breast cancer. Sufficient blood was collected from each individual participating in the CFRBCS to permit the isolation of lymphocytes, distribution into four aliquots, and cryopreservation for future transformation. To determine the success rate for transformation of these cryopreserved lymphocytes and whether processing and cryopreservation of lymphocytes affected the length of time required to achieve a successful transformation, a study was initiated with cryopreserved samples from CFRBCS participants. To date, using EBV transformation protocols, noncontaminated, viable cell lines have been successfully established from lymphocytes cryopreserved from 124–678 days (mean ± SE = 418 ± 6 days). Cryopreserved lymphocytes from these CFRBCS participants were successfully transformed within an average of 40.2 ± 0.8 days (mean ± SE). The time required for successful transformation for freshly isolated lymphocytes from the same study population was 35.2 ± 0.3 days. Although the success rate for transformation was slightly less for the cryopreserved sample set (90% *versus* 94% for freshly isolated lymphocytes), the availability of multiple aliquots of cryopreserved lymphocytes from each registry participant for additional transformation attempts virtually guarantees the establishment of a cell line. Cryopreservation of isolated lymphocytes for future transformation represents an economical alternative to the establishment of cell lines at the time of venipuncture for epidemiological studies such as the CFRBCS that may require an unlimited source of genetic material for future studies.

Introduction

The CFRBCS[3] is an international multisite cooperative consortium of investigators who collaborate to ascertain families at risk for breast cancer; to collect pedigree information, epidemiological and clinical data, and biological specimens from these individuals; and to provide these resources to the research community. Sufficient blood is collected from each individual participating in the family registry to permit the isolation of lymphocytes, which are divided among four aliquots, cryopreserved with 6% DMSO, and stored in liquid nitrogen. These cryopreserved lymphocytes may be used for nucleic acid preparation and/or the establishment of a cell line. To date, more than 15,000 blood samples have been obtained from CFRBCS participants.

In anticipating the resources required for gene discovery and characterization, it seemed prudent to have available a source for production of unlimited quantities of DNA. Establishment of a cell line would provide such a resource. In addition to providing an unlimited supply of DNA, the establishment of cell lines would eliminate the need to resample individuals, an important consideration because some individuals may no longer be available or may refuse to be resampled. Therefore, to enhance the CFRBCS resource, transformation of cryopreserved lymphocytes from participants was attempted to determine the efficiency of transforming these cryopreserved lymphocytes and whether the processing and cryopreservation protocols affected the length of time required to achieve a successful transformation. Although previous studies had shown that cryopreserved lymphocytes could serve as a source for the establishment of cell lines (1–5), little detail was provided on the time to achieve a successful transformation with respect to the length of time between venipuncture and cryopreservation of lymphocytes, the length of cryopreservation time, the age of the individual at sampling, or the disease status of the donor subject.

To date, cell lines have been successfully established from cryopreserved lymphocytes using EBV transformation protocols. The methods presented in this article describe a highly efficient system for the cryopreservation of lymphocytes in a large-scale production laboratory such as might be necessary for large epidemiological studies.

Materials and Methods

Informed Consent for CFRBCS Participants. An informed consent document approved by institutional review boards at the Northern California Cancer Center and the Coriell Institute for Medical Research (Coriell) was given to each CFRBCS participant during a personal interview. After obtaining informed consent, approximately 30 ml of blood from each CFRBCS participant recruited through the Northern California Cancer Center were collected in three 10-ml Vacutainer tubes, each containing 1.5 ml of the anticoagulant acid citrate dextrose (Becton Dickinson, Inc. Rutherford, NJ). Blood samples were shipped to the Coriell by overnight courier service and processed within 1–6 days after venipuncture.

Isolation, Cryopreservation, and Storage of Lymphocytes. Upon receipt at Coriell, the blood from one Vacutainer was

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[3] The abbreviations used are: CFRBCS, Cooperative Family Registry for Breast Cancer Studies; PHA, phytohemagglutinin.

diluted with an equal volume of RPMI 1640 with 0.02 M HEPES, layered onto a Histopaque-1077 HybriMax (Sigma-Aldrich, St. Louis, MO) gradient, and centrifuged for 30 min at 400 × *g* at 18–20°C. The lymphocyte layer was harvested and washed twice in RPMI 1640 [BioWhittaker (Walkersville, MD) or Sigma-Aldrich] with 0.02 M HEPES. The pellet was resuspended in 4 ml of freeze medium [RPMI 1640 with HEPES and 30% (v/v) heat-inactivated fetal bovine serum (Intergen, Purchase, NY or Sigma-Aldrich) and 6% DMSO (J. T. Baker Chemical Co., Phillipsburg, NJ)] and distributed among four glass ampules (Wheaton Science Products, Millville, NJ). Because cells were not counted before aliquotting, each ampule contained a variable number of cells isolated from approximately 2–2.5 ml of blood (between 2–8 × 10^6 white cells). Ampules were sealed and placed in a programmable freezer (Gordinier Electronics, Roseville, MI), and the temperature was lowered 1–5 degree(s)/min to −80°C. The cryopreserved ampules were transferred to liquid nitrogen for storage.

Establishment of Lymphoblastoid Cell Lines. Cell lines from CFRBCS participants were established from freshly isolated lymphocytes or from cryopreserved lymphocytes using standard EBV transformation protocols that include cell separation by gradient centrifugation and lymphocyte growth enhancement by the mitogen PHA. Each data set contains a unique group of participants. Cell lines were established and grown in the absence of antibiotics.

To initiate transformation with cryopreserved lymphocytes, the ampule of cells was thawed quickly in a 37°C water bath, the cells were washed twice with cell culture medium [RPMI 1640 with 0.2 mM L-glutamine (BioWhittaker or Sigma-Aldrich) and 20% (v/v) heat-inactivated fetal bovine serum] by centrifuging for 10 min at 10°C at 60–100 × *g*. The cell pellet was resuspended in 2 ml of cell culture medium and added to a 25-cm^2 cell culture flask [Corning (Corning, NY) or Nunc (Naperville, IL)] containing 1 ml of EBV stock prepared as described below and 0.3 ml of PHA (Sigma-Aldrich). Cells were incubated at 37°C in 5% carbon dioxide. When there were at least 3 × 10^6 total viable cells, the flask was subcultured.

When freshly isolated lymphocytes were the starting material, the washed cell pellet from the Histopaque-1077 HybriMax gradient from one 10-ml tube of blood was resuspended in 8 ml of cell culture medium and transferred to a 25-cm^2 cell culture flask containing 1 ml of EBV stock and 1 ml of PHA. Transformed cells were cryopreserved as described above when sufficient cells were obtained to produce 20 one-ml ampules of 5 × 10^6 viable cells/ml.

Maintenance and Harvest of B95-8 Cell Line (GM07404). The source of the EBV was that shed by a transformed marmoset cell line held in the National Institute for General Medical Sciences Human Genetic Cell Repository collection (GM07404). The marmoset cells were grown loosely attached in RPMI 1640 with 0.2 mM L-glutamine and 10% uninactivated fetal bovine serum. When the culture reached approximately 1 × 10^6 viable cells/ml, the cells were harvested, and the viral stocks were prepared.

Cell Culture Quality Control. For a transformation to be considered successful, the cell line must be free of contamination and be viable after cryopreservation. To be considered viable, a cell line must double its cell number within 4 days of recovery from liquid nitrogen, and the final yield can be no lower than 8 × 10^6 total viable cells/10 ml medium. Cells and culture medium were tested for sterility with trypticase soy broth, Sabouraud's dextrose broth, tryptose phosphate broth and on blood agar plates and incubated at 30°C and 37°C for a minimum of 2 weeks. Mycoplasma contamination was assessed using a PCR-based method developed at Coriell (6).

Population Characteristics. An analysis of the data for cell cultures revealed that for those established from freshly isolated lymphocytes (421), approximately half of the participants were affected with breast cancer [211 (7 males/204 females)], and the remainder were unaffected [210 (59 males/151 females)]. All of the 326 (8 males/318 females) individuals from whom a viable culture was established from cryopreserved lymphocytes were affected with breast cancer.

Statistical Analyses. Data are presented as the mean ± SE in the tables and text. Comparisons between groups were conducted using Student's *t* test and were considered significant when $P < 0.05$.

Results

To determine the efficiency of transforming cryopreserved lymphocytes, the success rate for transformation was determined using cryopreserved lymphocytes from 363 CFRBCS participants and compared with that obtained using freshly isolated lymphocytes from 487 registry participants. From the freshly isolated lymphocytes, 460 noncontaminated, viable cell lines were obtained, a success rate of 94%. A total of 326 cell lines were established from the set of cryopreserved lymphocytes for a success rate of 90%.

Each of the success rates presented here is based on one attempt at transformation for each subject. When a second ampule of cryopreserved lymphocytes was recovered, and transformation was initiated in a subset of those that did not transform on the first attempt, a noncontaminated, viable cell line was obtained in all cases. Therefore, with the availability of multiple aliquots of isolated cryopreserved lymphocytes, cell lines may be established with nearly 100% efficiency, an observation noted previously (1, 2, 5).

Although noncontaminated, viable cultures were obtained for freshly isolated lymphocytes from 460 individuals, all data (*i.e.*, birthdate, disease status, and time in transit were available for only a subset of these (421 CFRBCS participants). Thus, in subsequent analyses, for freshly isolated lymphocytes, data from only 421 participants were used. For the cryopreserved lymphocytes, data were available for the cultures established from all 326 participants.

To address whether or not the time for establishment of a culture is affected by the length of time lymphocytes are cryopreserved before the initiation of transformation, we examined in detail the time for establishment of a lymphoblastoid culture and compared it with multiple variables that might affect the time to successful transformation. A summary of these variables for the two lymphocyte populations examined (*i.e.*, freshly isolated *versus* cryopreserved lymphocytes) appears in Table 1. For the freshly isolated lymphocytes, there was no difference in the mean time to successful transformation between affected (35.1 ± 0.5 days) and unaffected (35.3 ± 0.5) individuals and between males (34.8 ± 0.6) and females (35.8 ± 0.6). Furthermore, there was no difference in the time between venipuncture and the initiation of transformation, indicated as days in transit, between the two groups of participants (1.90 ± 0.08 days for the affected individuals compared with 2.00 ± 0.09 days for the unaffected individuals). Although there was a statistically significant difference in the mean age at sampling (53.2 ± 0.8 years for the 211 affected individuals compared with 55.4 ± 0.9 years for the 210 unaffected individuals; $P < 0.05$), an examination of the effect of age on time for transformation failed to indicate any relationship. There-

Table 1 Transformation of cryopreserved and freshly isolated lymphocytes by participant and sample processing characteristics

	Freshly isolated lymphocytes	Cryopreserved lymphocytes	*P*
Total no. of individuals	421	326	
Age of participant (yrs)	54.3 ± 0.6	52.6 ± 0.5	<0.025
Age range (yrs)	24–91	24–73	
Time in transit (days)	1.97 ± 0.06	1.79 ± 0.06	<0.05
Time in transit, range (days)	1–6	1–6	
Cryopreservation time before transformation (days)		418 ± 6	
Cryopreservation time before transformation, range (days)		124–678	
Time to successful transformation (days)	35.2 ± 0.3	40.2 ± 0.8	<0.0005
Time to successful transformation range (days)	25–91	28–200	

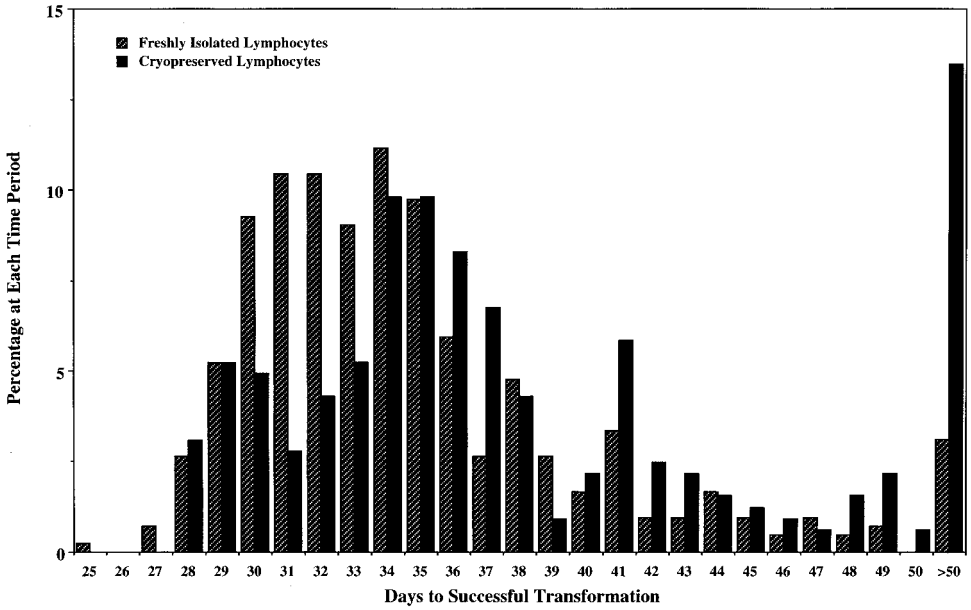

Fig. 1. Histogram illustrating the relationship between the time to successful transformation and the number of samples observed at each time period. For one set, freshly isolated lymphocytes were the starting material, and for the second set, cryopreserved lymphocytes were used to initiate transformation.

fore, the two data sets for the freshly isolated lymphocytes were combined for the analyses presented in Table 1.

The mean time for successful transformation with freshly isolated lymphocytes as the starting material was 35.2 ± 0.3 days, whereas that observed for cryopreserved lymphocytes was 40.2 ± 0.8 days ($P < 0.0005$). Fig. 1 depicts the relationship between the time to successful transformation and the number of samples observed at each time period for both data sets. Clearly, with freshly isolated lymphocytes as the starting material, the time from initiation of transformation to production of approximately 100 million cells is shorter than that observed with cryopreserved lymphocytes. Of note, a significant portion of the cryopreserved samples (13.5%) required more than 50 days for successful transformation when compared with the freshly isolated samples (3.1%). Interestingly, an examination of the cryopreservation time for those samples that transformed in less than 50 days (280) compared to the 46 samples that required more than 50 days for successful transformation indicated that the mean time each was cryopreserved was similar [418 ± 7 (<50 days) *versus* 418 ± 18 (>50 days)]. However, when the samples requiring more than 50 days for transformation are removed from the analyses, the time for transformation remains significantly greater for the cryopreserved lymphocytes [36.1 ± 0.3 days (cryopreserved lymphocytes) *versus* 34.3 ± 0.2 days (freshly isolated lymphocytes); $P < 0.0005$].

To determine whether variables other than cryopreservation had significantly increased the time to transformation, the time to successful transformation was examined as a function of the time between venipuncture and the initiation of transformation (in the case of freshly isolated lymphocytes) or cryopreservation (for cryopreserved lymphocytes). Due to the small number of samples processed on days 5 and 6 after venipuncture, the data for these two days have been combined. The data presented in Table 2 indicate that within each sample set (*i.e.*, for freshly isolated lymphocytes at each time point), there is no effect of time in transit on time for successful transformation. However, for freshly isolated compared with cryopreserved lymphocytes, the time to successful transformation is significantly increased by cryopreservation at each time point. Thus, the observed increase in time for transformation with cryopreserved lymphocytes is independent of the time between venipuncture and cryopreservation of the lymphocytes.

Although the mean ± SE number of days lymphocytes were cryopreserved before the initiation of transformation was 418 ± 6 days, the time ranged from 124–678 days. An examination of the relationship between the time required for successful transformation and the time lymphocytes are cryopreserved before the initiation of transformation suggests that the time required for successful transformation is independent of the time lymphocytes are cryopreserved before the initiation of transformation for samples that were cryopreserved for less

Table 2 Effect of time in transit on time for successful transformation

Days in transit	Freshly isolated lymphocytes		Cryopreserved lymphocytes		*P*
	No. of samples	Days to successful transformation	No. of samples	Days to successful transformation	
1	220	35.3 ± 0.5	186	39.7 ± 1.1	<0.0005
2	78	34.0 ± 0.5	64	39.6 ± 1.4	<0.0005
3	65	35.7 ± 0.7	43	40.5 ± 2.0	<0.01
4	35	35.8 ± 1.1	28	43.2 ± 2.6	<0.005
5 and 6	23	35.6 ± 1.7	5	48.2 ± 10.9	<0.025
Total	421	35.2 ± 0.3	326	40.2 ± 0.8	<0.0005

than 678 days (approximately 22 months). Whether or not this is true for longer periods of cryopreservation, (*e.g.*, 5–10 years) remains to be determined.

Discussion

Preservation of sufficient genetic material for multiple analyses often requires the establishment of lymphoblastoid cell lines. Isolation and cryopreservation of lymphocytes for future transformation could represent a viable and economic alternative to this approach. Few studies have examined this rigorously. Although Tremblay and Khandjian (5) demonstrated that purified lymphocytes maintained in liquid nitrogen for several years (5.5–7.7 years) could be used for the establishment of lymphoblastoid cell lines, they transformed only 11 samples. These authors state that the time from initiation of transformation to banking averaged 8 weeks when cryopreserved lymphocytes were used as the starting material; no data on time to successful transformation were provided for freshly isolated lymphocytes. In the study of Penno *et al.* (4), there appeared to be no difference in the number of days required for successful transformation with either freshly isolated or cryopreserved lymphocytes. Both Louie and King (1) and Pressman and Rotter (2) examined larger sample sets; however, neither study assessed the effects of length of time between venipuncture and cryopreservation of lymphocytes, the length of cryopreservation time, the age of the individual at sampling, and the disease status on the time to achieve a successful transformation.

The data presented in this study confirm earlier observations that establishment of cell lines is possible from cryopreserved lymphocytes. Success rates for a single attempt at transformation compare favorably to the 90–95% success rate reported by others for freshly isolated lymphocytes (7) and to that of Penno *et al.* (4) for cryopreserved lymphocytes. It is somewhat higher than those reported by Reidy and Wheeler (3). Although the rate observed in this study is slightly less than those quoted in Louie and King [99% (1)] and Pressman and Rotter [97% (2)], it should be noted that in the previous studies (1, 2), penicillin and streptomycin were added to the medium in which the lymphocytes were cryopreserved and cultured. No antibiotics were included in any medium used in the current study. Furthermore, for a transformation to be considered successful, the lymphoblastoid cells must recover and proliferate after cryopreservation. This criterion is not mentioned in any of the previous studies (1–5). Interestingly, Reidy and Wheeler (3) noted a marked decrease in success rates as the length of time between venipuncture and cryopreservation of lymphocytes increased (from 2–4 days). This was not observed in the present study.

By controlling for such variables as disease state and examining age at sampling, time between venipuncture and cryopreservation, and length of cryopreservation time for their effects on time to successful transformation in a large number of samples, this study extends the initial observation of others (1–5) and provides information on the effect of cryopreservation on time from initiation of transformation to production of cell stock. The data suggest that the length of time lymphocytes are cryopreserved has little or no effect on time to successful transformation, an observation noted by Penno *et al.* (4). However, additional time in culture is required for cryopreserved lymphocytes for successful transformation when compared with that required for freshly isolated lymphocytes, an observation not supported by Penno *et al.* (4). Of note, in that study, the number of cells used for transformation of cryopreserved lymphocytes was five times greater than that used for initiation of transformation with freshly isolated cells. Because no cells were counted before the initiation of transformation in the present study, no distinction can be made between possible differences in cell numbers or differences in cell biology, *e.g.*, viability, as the cause for the increased time required for successful transformation between the freshly isolated and cryopreserved lymphocytes.

The results reported here were obtained in a laboratory where procedures have been streamlined for efficient production of 50–100 cultures/week. In such a setting, the use of feeder layers and conditioned medium is not feasible. The experience at Coriell has shown that dividing the lymphocytes among four aliquots without counting produces sufficient numbers of viable cells to permit transformation. The use of tissue culture flasks at the outset eliminates the need to transfer from 24-well tissue culture plates, thereby reducing bacterial, fungal, and cell-cell contamination. Adopting each of these procedures minimizes manipulation, increases success of transformation, and reduces labor. Thus, for large epidemiological studies, cryopreservation of isolated lymphocytes for future transformation represents an economic alternative to the establishment of cell lines at the time of venipuncture. Furthermore, the fact that the time required for successful transformation appears to be independent of the time between venipuncture and sample processing for up to 4–5 days for blood collected in acid citrate dextrose tubes validates the use of this methodology for samples collected at multiple centers and shipped to a central processing laboratory.

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