

Short Communication

Whole Blood Cryopreservation in Epidemiological Studies

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Abstract

Standardized and cost-effective biological sample collection, processing, and storage procedures are needed in large-scale epidemiological studies to provide material for testing a broad range of etiological hypotheses. One component of sample collection in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial involves shipment of blood in acid-citrate-dextrose anticoagulant to a central processing laboratory, where 10% DMSO is added, and whole blood aliquots are cryopreserved. A single technician is able to routinely process 50–60 samples/day. Tests conducted to evaluate potential uses of cryopreserved whole blood showed successful EBV transformation (>90%, up to 20 months of storage). In addition, lymphocytes maintained good viability and stable T-cell:B-cell ratios, and T cells maintained the capacity to proliferate in response to solid phase anti-CD3/CD28 plus interleukin 2. Whole blood cryopreservation is a cost-effective approach to large-scale storage of viable cells in epidemiological studies.

Introduction

Nonviable cellular DNA sources (*e.g.*, frozen leukocytes and buccal cells) are adequate for most current genotyping needs in cancer epidemiology. Viable blood cells are a replenishable source of DNA and could potentially be used for cytogenetic and functional studies; however, the large sample numbers involved in epidemiological cohort studies often make it impractical to use standard lymphocyte separation techniques for long-term storage of viable cells. We applied a simple whole blood cryopreservation protocol to address this need in the PLCO² Screening Trial (1, 2). In the present study we show that B-cell transformation by EBV, lymphocyte viability, and T-cell proliferation in response to solid phase anti-CD3/anti-CD28 plus IL-2 are maintained over time in cryopreserved whole blood.

Materials and Methods

We evaluated whole blood cryopreservation among screening arm participants in the PLCO Screening Trial (1, 2). At 10 screening centers, blood is collected in 2 × 10-ml acid-citrate-dextrose Vacutainer tubes (Becton Dickinson, Inc., Rutherford, NJ) and shipped ambient overnight to a single facility for processing. Upon receipt, blood is mixed, and sterile DMSO (Sigma, St. Louis, MO) is added to a final concentration of 10%. The samples are aliquoted to cryovials, cryopreserved using a controlled rate freezer (Cryomech, Cryomech, and stored in a vapor phase liquid nitrogen freezer. Sample processing is batched to limit the time between addition of DMSO and initiation of freeze (35 min). A single technician can aliquot 10–15 samples within the allotted time, depending on speed and experience. Pilot studies showed that lymphocytes from blood collected in acid-citrate-dextrose maintained viability equal or superior to other anticoagulants, especially at time points beyond 48 h, and that cryopreserved samples yielded viable lymphocytes.

Flow Cytometry. Cryopreserved whole blood was thawed at room temperature, washed with RPMI 1640 plus 5 mM EDTA (Life Technologies, Inc.), and diluted in RPMI 1640 supplemented with 10% FCS. Viability was determined using CD45 FITC to identify leukocytes (Becton Dickinson) and PI to identify nonviable cells. A second tube was incubated with a three-color combination of antibodies to CD45, CD3, and CD19 to distinguish T and B lymphocyte subsets. Cells were analyzed on a Coulter XL flow cytometer. PI uptake by CD45 bright, low side scatter cells was used to identify nonviable lymphocytes, and back-gating confirmed reduced forward light scatter of these cells compared with CD45+/PI– cells. CD45 and light scatter were used to identify lymphocytes in the second tube. The percentage of T and B lymphocytes was determined and used to calculate the T-cell:B-cell ratio of all lymphocytes and those with light scatter consistent with viable lymphocytes.

T-Cell Proliferation. Anti-CD3 (at 1.0 μg/ml) and anti-CD28 (at 0.1 μg/ml; BD Biosciences Pharmingen) were used to precoat wells of 96-well Costar high absorbance ELISA plate(s) in a volume of 0.2 ml. Unbound antibody was removed by washing with sterile PBS. Blood samples were thawed as described above, resuspended in RPMI 1640 containing 10% FCS to 1 × 10⁶ cells/ml, and then plated into replicate uncoated and antibody-coated wells. Complete media were added to control wells, and media supplemented with 1000 IU/ml recombinant IL-2 were added to antibody-coated wells to a final volume of 0.2 ml. Culture plates were incubated at 37°C for 5 days, pulsed with 1 μCi of [³H]thymidine for 18–24 h, harvested using a Tomtec 96-well plate harvester, and counted on a beta counter (Wallac Microbeta). A stimulation index was calculated by dividing the average count of wells containing anti-CD3/CD28 by the average count of the media control wells.

EBV Transformation. Cryopreserved blood was thawed and manipulated under sterile conditions. After washing, the cells were resuspended in 1.0 ml of Iscove's medium containing 20% fetal bovine serum for determination of cell counts and

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² The abbreviations used are: PLCO, Prostate, Lung, Colorectal and Ovarian Cancer; PI, propidium iodide; IL, interleukin.

Table 1 Selected characteristics of study subjects

Characteristic	Cytologic studies	EBV transformation
N of subjects	109	60
Age (mean) (yrs)	64.8	65.0
Gender (% male)	53.2	75.0
Days in transit (mean)	1.0	1.1

viabilities. Leukocytes were seeded at 5×10^5 viable cells in a 25-cm² flask containing 1.5 ml of EBV stock (ATCC.VR-1492) and irradiated feeder cells (ATCC.X-55, irradiated MRC-5), previously seeded at approximately 4×10^5 cells/flask. The EBV-infected cultures were incubated undisturbed for 10 days and then observed for the appearance of cell clusters indicative of transformation. Cultures were observed regularly and supplemented as needed with complete medium (3). If transformation was not noted within 1 month of initiation, additional EBV stock was added. EBV-transformed cells were cryopreserved at $5\text{--}15 \times 10^6$ viable cells/ml in cryovials.

Study Populations. Through December 2000, blood samples from 29,999 subjects had been processed by the central laboratory. Samples were received within 48 h of blood draw for 95.5% of these subjects, and all bloods were cryopreserved. For flow cytometry and T-cell proliferation investigations, we studied 109 subjects (Table 1) with whole blood samples frozen either for 1 day ($n = 20$), 24–30 days (~1 month; $n = 19$ due to loss of one sample because of technical error), 218–232 days (~7 months; $n = 20$), 588–596 days (~18 months; $n = 20$), or 976–1021 days (~31 months; $n = 30$). The number of samples in the >30 month group was increased to 30 to provide a better statistical evaluation of the group most likely to have degradation of sample quality. The 109 samples evaluated in this study represent 0.36% of total cryopreserved samples. Samples were selected for study based on numbers of blood vials stored, and an effort was made to select samples received for processing on multiple dates within the specified date range. For comparison, we studied lymphocytes separated on Ficoll-Hypaque from fresh unfrozen blood (fresh sample; $n = 20$) from the same subjects who were included in the 1-day frozen blood group. For EBV transformation, we studied 60 subjects, with cryopreserved sample up to 20 months (Table 1).

Statistics. Comparison between results for the same subjects measured at two points in time were conducted using Student's *t* test for matched pairs. Correlations were assessed using Pearson's correlation coefficient. Changes in blood parameters per month were calculated by linear regression analysis. Comparisons were considered significant when $P < 0.05$ with two-sided tests.

Results

As measured by PI staining, >94% of lymphocytes separated by Ficoll-Hypaque from fresh whole blood samples receipted from the screening centers were viable (Fig. 1a; mean = 98.4%). Lymphocyte viability was significantly reduced among the same subjects (paired *t* test, $P < 0.001$) after samples were frozen for 1 day, ranging from 65.8% to 93.3% (mean = 85.2%). Restricted to frozen samples, however, no further differences in viability were observed for samples stored up to 31 months (Pearson correlation, $P = 0.74$), with minimum viability of about 72% among the 89 subjects tested.

No differences were observed between freshly isolated lymphocytes (separated by Ficoll-Hypaque) and samples of whole blood frozen for 1 day, with respect to T cells as a

percentage of total lymphocytes (Fig. 1b; paired *t* test, $P = 0.51$) or T-cell:B-cell ratio (Fig. 1c; paired *t* test, $P = 0.14$). Restricted to frozen samples, however, there were modest increases with increasing storage time in T cells as a percentage of total lymphocytes ($P = 0.03$) and in T-cell:B-cell ratios ($P = 0.07$). Although changes over time are relatively small, the highest level of change is in samples frozen for the longest period of time (about 31 months). Solid phase (anti-CD3/CD28 + IL-2) lymphocyte stimulation gave better results in samples frozen for 1 day than in fresh samples (Fig. 1d; paired *t* test, $P = 0.008$) when measured as stimulation index (mean, fresh samples = 504.6; mean, frozen samples = 769.7). However, this difference was primarily due to lower background counts in the media control wells in the frozen samples.

The lymphocyte stimulation index decreased moderately with increasing time in the frozen state ($P = 0.07$). For samples frozen 1 month or more, the changes per month in the respective parameters were as follows: percentage of viable lymphocytes (-0.01 per month; $P = 0.74$); T cells as a percentage of total lymphocytes (0.25 per month; $P = 0.009$); T-cell:B-cell ratio (0.06 per month; $P = 0.04$); and lymphocyte proliferation stimulation index (1.8 per month; $P = 0.56$).

Sixty subject samples were evaluated for EBV transformation. The initial success rate was 80% with five of eight initial failures successful after a second attempt, bringing the overall success rate to 92.5%. Time for successful transformation ranged from 20 to 90 days (mean = 38.2 days; SD = 13.1 days). EBV transformation was tested for samples stored for up to 20 months, with excellent response through the time period investigated.

Discussion

This study shows that large numbers of blood samples from multiple study sites can be shipped to a central location and cryopreserved as whole blood, allowing for the subsequent recovery of viable lymphocytes. Others have demonstrated the successful cryopreservation of separated lymphocytes in an epidemiological setting (4, 5), although implementation in large-scale epidemiological studies remains challenging (6). EBV-transformation rates from whole blood, ranging from 83% by Penno *et al.* (7) to 92.5% in our study, compare well with EBV transformation rates from cryopreserved separated lymphocytes (7). Advantages of cryopreserving whole blood *versus* separated lymphocytes include increased total DNA available for direct extraction and the substantial cost savings associated with reduced materials and labor required for sample processing. We estimate that the lymphocyte separation approach would require five technicians to keep up with sample flow in the PLCO Screening Trial (50 to >100 samples/day), whereas whole blood cryopreservation only requires one to two technicians, using two Cryomed units.

We also demonstrated that T cells in cryopreserved whole blood could be stimulated to proliferate using solid phase anti-CD3/CD28 in samples stored for at least 31 months. Lymphocytes also responded to mitogenic signals such as phytohemagglutinin and pokeweed, but the response was less robust (data not shown), perhaps due to release of toxic products by dead cells or binding/inactivation of mitogens by dead cells and debris.

Cryopreserved whole blood showed decreased lymphocyte viability compared with freshly isolated mononuclear cells; however, some of the apparent decrease may result from increased difficulty in eliminating dead granulocytes with altered light scatter from the total lymphocyte gate in the frozen sample. Once samples were frozen, lymphocyte viability did not appear to further diminish with freeze duration (up to 31 months).

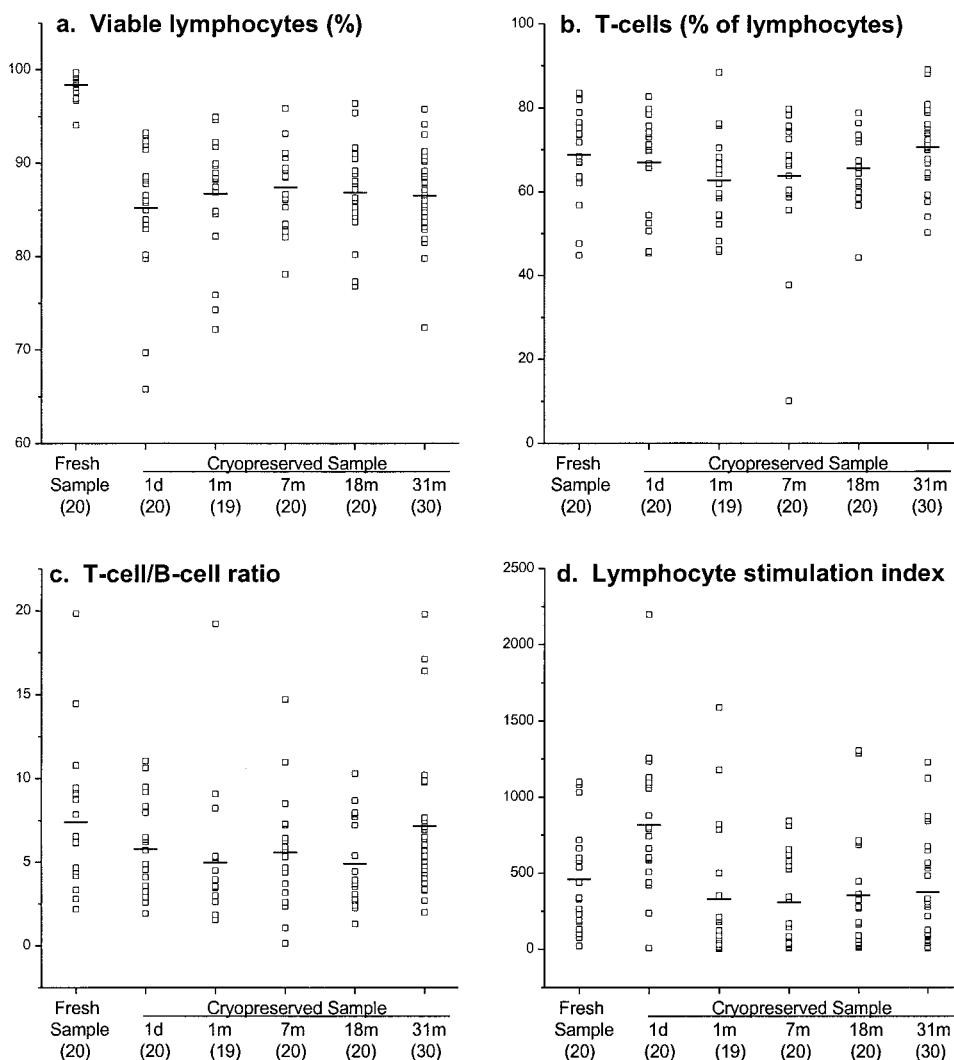


Fig. 1. Lymphocyte characteristics of fresh whole blood and cryopreserved samples held for 1 day (*d*) to 31 months (*m*). — indicates the mean of the distribution; number of subjects (*n*) is shown in parentheses.

Field collection and shipping of blood samples with centralized whole blood cryopreservation is a cost-efficient approach to large-scale blood storage, until samples of selected subjects can be earmarked for EBV transformation to lymphoblastoid cell lines, primarily serving as a DNA resource. In addition to providing a large-scale DNA resource, we are optimistic that other assays of interest to epidemiologists may be carried out with cryopreserved whole blood, although substantial protocol development and validation work will be needed to assess the suitability of this medium for each specific assay (8–10).

References

- Gohagan, J. K., Prorok, P. C., Hayes, R. B., Kramer, B. S., and PLCO Project Team. The Prostate, Lung, Colorectal and Ovarian (PLCO) Screening Trial of the National Cancer Institute: history, organization, and status. *Control. Clin. Trials*, 21: 251s–272s, 2000.
- Hayes, R. B., Reding, D., Kopp, W., Subar, A. F., Bhat, N., Rothman, N., Caporaso, N. E., Ziegler, R. G., Johnson, C. C., Weissfeld, J., Hoover, R. N., Hartge, P., Palace, C., and Gohagan, J. K. Etiologic and early marker studies in the Prostate, Lung, Colorectal and Ovarian (PLCO) Screening Trial. *Control. Clin. Trials*, 21: 349s–355s, 2000.
- Caputo, J., Thompson, A., McClintock, P., Reid, Y., and Hay, R. An effective method for establishing human B lymphoblastic cell lines using Epstein-Barr virus. *J. Tissue Culture Meth.*, 13: 39–44, 1991.
- Beck, J. C., Beiswanger, C. M., John, E. M., Satariano, E., and West, D. Successful transformation of cryopreserved lymphocytes: a resource for epidemiological studies. *Cancer Epidemiol. Biomark. Prev.*, 10: 551–554, 2001.
- Kleeberger, C. A., Lyles, R. H., Margolick, J. B., Rinaldo, C. R., Phair, J. P., and Giorgi, J. V. Viability and recovery of peripheral blood mononuclear cells cryopreserved for up to 12 years in a multicenter study. *Clin. Diag. Labor. Immunol.*, 6: 14–19, 1999.
- Pero, R. W., Olsson, A., Bryngelsson, C., Carlsson, S., Janzon, L., Berglund, G., and Elmstahl, S. Quality control program for storage of biologically banked blood specimens in the Malmo diet and cancer study. *Cancer Epidemiol. Biomark. Prev.*, 7: 803–808, 1998.
- Penno, M. B., Pedrotti-Kruger, M., and Ray, T. Cryopreservation of whole blood and isolated lymphocytes for B-cell immortalization. *J. Tissue Culture Meth.*, 15: 43–48, 1993.
- Fiebig, E. W., Johnson, D. K., Hirschhorn, D. F., Knape, C. C., Webster, H. K., Lowder, J., and Busch, M. P. Lymphocyte subset analysis on frozen whole blood. *Cytometry*, 29: 340–350, 1997.
- Cheng, L., Wang, L. E., Spitz, M. R., and Wei, Q. Cryopreserving whole blood for functional assays using viable lymphocytes in molecular epidemiology studies. *Cancer Lett.*, 166: 155–163, 2001.
- Hagmar, L., Bonassi, S., Stomberg, U., Brogger, A., Knudsen, L. E., Norppa, H., and Reuterwall, C. Chromosomal aberrations in lymphocytes predict human cancer: A report from the European study group on cytogenetic biomarkers and health (ESCH). *Cancer Res.*, 58: 4117–4121, 1998.