Cryopreservation of Whole Blood Samples Collected in the Field for a Large Epidemiologic Study

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Abstract

Cryopreserved lymphocytes can be used to measure various cellular functions and are an excellent source of DNA. However, functional studies of lymphocytes have been largely limited to specimens collected in laboratory settings because of the perception that specialized equipment and handling are needed to successfully cryopreserve biospecimens. In this study, we have developed a protocol to successfully cryopreserve blood samples collected in the field as part of a pilot study of Cancer Prevention Study-3. Blood was collected in sodium heparin–containing vacutainers at six outdoor events, transported via courier to one of four different processing labs for cryopreservation, and stored in the vapor phase of liquid nitrogen. After 2 to 6 weeks of storage, the effectiveness of the protocol was evaluated by testing 30 samples for their viability, lymphocyte yield, and ability to be transformed by EBV. Although lymphocyte recovery varied considerably, all samples yielded at least $2 \times 10^9$ cells with at least 86% of the cells being viable. All samples were successfully transformed by EBV and yielded immortalized cell lines within 15 days of treatment with the virus. These findings indicate that whole blood samples collected in the field can be successfully cryopreserved and that the normal variation in sample handling expected in a large epidemiologic study does not compromise the quality of the cryopreserved specimens. (Cancer Epidemiol Biomarkers Prev 2007;16(10):2160–3)

Introduction

Cryopreserved lymphocytes are an extremely valuable resource for epidemiologic studies, both because they can be used to measure a variety of cellular functions and biomarkers and, after transformation, they provide a virtually infinite source of DNA. Successful cryopreservation requires the addition of a cryoprotective agent such as DMSO to the blood followed by freezing at a controlled rate to a temperature less than $-50^\circ\text{C}$ (1) and subsequent long-term storage at a temperature below $-130^\circ\text{C}$. Typically, cryopreservation is done under very controlled conditions in a laboratory equipped with either a controlled-rate freezer or commercially available sample containers that control the rate of freezing. This requirement for defined conditions and specialized equipment has inhibited the collection of blood for this purpose in epidemiologic studies wherein biospecimens are obtained in the field and multiple processing laboratories are used for cryopreservation. Collection of these samples has been further discouraged by our limited understanding of factors that affect the viability of the cryopreserved lymphocytes when biospecimens are collected in the field. These include variable transport temperatures, times between collection and processing and between freezing and transfer to long-term storage in liquid nitrogen, and potential variation in the final concentration of cryopreservative in each sample.

Successful cryopreservation of isolated lymphocytes (2–5) or whole blood samples (6, 7) has been reported by epidemiologic studies in which the samples were handled by a limited number of laboratories, ranging from one (2, 4–7) to four (3). The major focus of most of these studies (2, 4, 6, 7) was to establish that the cryopreserved samples were similar to fresh samples in several important characteristics including viability and transformability. Other variables investigated were the effect of extended storage in liquid nitrogen on transformation by EBV (2, 4, 7) and the influence of delayed blood processing on cell viability (4, 5). Transformation by EBV was consistently found to be unaffected by the length of time samples were stored in liquid nitrogen (2, 4, 7). However, delayed processing was found by Kristal et al. (5), but not by Beck et al. (4), to greatly reduce cell viability. An important difference between the two studies that maybe responsible for this difference is the additive in which the blood was collected, which was EDTA in one case (5) and acid-citrate-dextrose (ACD) in the other (4). The influence of different transport temperatures or time between cryopreservation and transfer to liquid nitrogen for long-term storage has not been investigated.
In this study, we developed and tested a protocol to cryopreserve whole blood samples collected in the field without using specialized equipment. Blood was collected from participants in the Cancer Prevention Study-3, a prospective cohort study being conducted by the American Cancer Society. Cancer Prevention Study-3 will enroll 500,000 individuals through Relay For Life fund-raising events that will be held throughout the country over the next 5 years to examine the role of lifestyle and molecular factors in cancer incidence. To assess the effectiveness of our cryopreservation protocol, we determined the viability and transformability by EBV of 30 blood samples collected at six Relay For Life sites that were processed by four different laboratories.

**Materials and Methods**

**Blood Collection.** Pilot enrollment for Cancer Prevention Study-3 was done at six Relay For Life events in Texas, Georgia, and California (two each) held in the spring of 2006. The setting for the blood draw included air-conditioned buildings (two sites), non–air-conditioned buildings (two sites), and outdoor tents with a comfortable temperature (one site) or an extremely warm temperature (one site). Enrollees were men and women between the ages of 30 and 65 years who had no previous history of cancer (except nonmelanoma skin cancer). Blood for cryopreservation was drawn via venipuncture by a licensed phlebotomist and collected in a 6-mL green-top vacutainer containing sodium heparin. The blood and anticoagulant were mixed by inverting the tube several times. Samples were routinely collected within 20 min and chilled before transport via courier to a nearby Quest Diagnostic Lab for processing within 14 h of collection. At the first enrollment event and for the first half of the second event, the blood was chilled by placing the samples in a cooler with frozen cold packs. For the second half of the samples at the second event and for all samples from the other events, the cold packs were replaced by an ice-water bath that covered at least the bottom portion of the vacutainers to chill the samples more effectively. The temperature of the samples during transport was monitored by electronic thermometers placed in the coolers.

**Blood Processing.** On arrival at the lab, the blood samples were transferred to a bench-top ice bath and remixed by inverting several times. Blood was then transferred from the vacutainers to prelabeled 1.8-mL cryovials containing 180-μL DMSO using a sterile plastic transfer pipette. The blood and cryopreservative were mixed by inverting the cryovials four to five times, after which they were placed in a Styrofoam tube rack that completely enclosed the samples and placed in an ultralow temperature freezer for slow freezing to approximately −80°C. The samples were handled in batches of about 50 to 80 tubes to maximize the efficiency of the blood processing. At each step, the samples were maintained on ice for the time required to complete the processing of the entire batch of blood. After at least 2 days at −80°C, the samples were transferred to cryovial boxes and shipped on dry ice to a biorepository, where they were transferred to liquid nitrogen vapor phase for long-term storage.

**WBC Separation and Immortalization.** WBC separation and lymphocyte immortalization with EBV was done at the American Type Culture Collection laboratory. Whole blood samples were shipped to the American Type Culture Collection on dry ice, thawed, and diluted with RPMI 1640. Fetal bovine serum was layered under the diluted sample in a sterile centrifuge tube, which was centrifuged at 200 × g for 10 min. The supernatant was removed and the pellet containing primarily WBC with some RBC was transferred to a culture flask and exposed to EBV. Samples were monitored for transformation starting at 8 to 10 days after initiation and then every 2 to 3 days afterwards, as described by Hayes et al. (7). Cell numbers were determined by counting with a hemacytometer and viability was assessed by erythrosin B staining.

**Data Analyses.** The influence of collection at six different sites, processing at four separate labs, variable transport times and temperatures, and different times between freezing and transfer to liquid nitrogen on cell viability and recovery was determined by averaging the results of three samples for each of 10 separate combinations of these variables (30 independent samples total). The results were expressed as the average ± SD.

**Results**

The scheme by which the blood samples were collected over a 3-month period at six different Relay For Life fund-raising events and processed at four different laboratories is diagrammed in Fig. 1. The average transport temperature and the time between blood draw and processing for three samples representing 10 combinations of these variables are shown in Table 1. Transport temperatures ranged from 37°C to 55°C and times to

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**Figure 1.** Blood collection and processing scheme. The three steps involved in the blood handling are indicated in the boxes and the variables at each step and during transport are indicated on the right.
Table 1. Influence of different processing variables on selected characteristics of the cryopreserved samples

<table>
<thead>
<tr>
<th>Condition</th>
<th>Lab</th>
<th>Transport temperature (°F)</th>
<th>Time to processing (h)</th>
<th>Time to liquid N₂ (h)</th>
<th>Viability (%)</th>
<th>Cell no. (%)</th>
<th>Time to transform (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>55</td>
<td>13.4</td>
<td>70.1</td>
<td>91.0 ± 2.0</td>
<td>56.2 ± 16.2</td>
<td>12.0 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>49</td>
<td>5.5</td>
<td>76.8 ± 1.3</td>
<td>89.7 ± 1.5</td>
<td>65.0 ± 14.9</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>39</td>
<td>8.2 ± 0.1</td>
<td>80.1 ± 3.1</td>
<td>91.3 ± 1.5</td>
<td>42.0 ± 15.7</td>
<td>11.0 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>37</td>
<td>3.9</td>
<td>84.4 ± 0.2</td>
<td>89.3 ± 1.5</td>
<td>80.0 ± 24.6</td>
<td>11.0 ± 1.7</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>37</td>
<td>6.1</td>
<td>84.7 ± 0.1</td>
<td>89.0 ± 1.7</td>
<td>32.2 ± 15.1</td>
<td>14.3 ± 2.3</td>
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<tr>
<td>6</td>
<td>A</td>
<td>40</td>
<td>8.5</td>
<td>74.3 ± 0.1</td>
<td>88.3 ± 1.5</td>
<td>100 ± 19.2</td>
<td>10.0</td>
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<tr>
<td>7</td>
<td>A</td>
<td>40</td>
<td>13.9</td>
<td>74.1</td>
<td>89.0 ± 2.9</td>
<td>40.7 ± 10.4</td>
<td>10.0</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>40</td>
<td>4.9</td>
<td>256.2</td>
<td>90.0 ± 2.0</td>
<td>40.3 ± 4.0</td>
<td>12.0 ± 1.7</td>
</tr>
<tr>
<td>9</td>
<td>D</td>
<td>39</td>
<td>4.9</td>
<td>62.7</td>
<td>91.3 ± 0.6</td>
<td>49.0 ± 10.1</td>
<td>9.7 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>D</td>
<td>39</td>
<td>8.3</td>
<td>62.8</td>
<td>87.3 ± 1.5</td>
<td>43.1 ± 23.1</td>
<td>11.0 ± 1.7</td>
</tr>
</tbody>
</table>

NOTE: Ten conditions (1-10) were selected that represent the range of transport variables that the samples were subjected to (N = 30 with 3 samples for each condition). The average (±SD) transport temperature, time to processing, time to storage in liquid nitrogen, cell viability, cell recovery, and time to transformation by EBV for each condition are shown. SD is not shown when the SD was zero.

Discussion

The blood samples used for this study were collected at six different sites, processed in four different laboratories, maintained at a range of temperatures for various times before addition of DMSO and slow freezing, and kept at approximately –80°C for different intervals before transfer to liquid nitrogen vapor phase for long-term storage. Although the range of variation was somewhat limited, we found that none of these variables affected the viability of the cryopreserved lymphocytes or their ability to be immortalized by EBV transformation. An additional factor that did not compromise lymphocyte viability was variation in the final concentration of DMSO that could be introduced when the blood was transferred into the cryovial containing the cryopreservation. The final volume of 1.8 mL in the cryovial was achieved by filling the vial with blood to a designated fill line; variability in this step caused the final volume to vary by ±0.2 mL and the DMSO concentration to range from 9% to 11%.

The high viability of the lymphocytes in our whole blood samples (87-91%) was similar (3, 7) or higher (2) than previously reported for either cryopreserved whole blood (7) or isolated lymphocytes (2, 3). Furthermore, the 100% success rate of immortalization by EBV transformation we achieved was higher and the time to transformation was shorter than for samples cryopreserved in previous epidemiologic studies (3, 7). Thus, our protocol was at least as effective as others previously tested.

The number of viable cells recovered from the samples varied by up to 3-fold. This variation was unlikely to be due to cell death because the viability was similar for all the samples. Possible explanations for the lower recovery from some of the samples include small differences in the volume of those samples and interindividual differences in the number of WBC in the blood. However, even with the variation in cell recovery, it is clear that all the samples had adequate numbers of lymphocytes for immortalization by EBV transformation.

Previous epidemiologic studies collecting samples for cryopreservation have used blood drawn into vacutainers containing various additives, including ACD (4, 7), EDTA (5), and heparin (2, 3, 6). Recently, attention has focused on the use of ACD because cryopreserved samples derived from blood collected in this additive have been shown to better tolerate delays in processing than those from EDTA-blood (4, 5) and ACD has been reported to maintain lymphocyte viability longer than heparin when blood is maintained at room temperature (4, 8). Despite this knowledge, we chose to collect the blood in sodium heparin-containing vacutainers for two reasons: First, the anticoagulant action of heparin is not lost during isolation of the lymphocytes as it is for ACD (because calcium is added back to the blood). Therefore, the problem of clot formation during the lymphocyte isolation, which can hamper the recovery of the WBC from the sample, can be avoided by using heparin. Second, the favorable effects of ACD on lymphocyte viability have only been noted when blood is maintained at ambient temperatures (8). Our samples were transported on ice rather than at ambient temperatures because this allowed us to better control this variable under the variety of weather conditions experienced during field collection of blood. Thus, the use of ACD as the blood additive was expected to provide no advantage over heparin. Our findings indicate that these conditions resulted in a high lymphocyte viability and immortalization success rate.

In addition to showing that our protocol allows for the successful collection and processing of cryopreserved blood samples, these findings also indicate where variation can be tolerated in the blood handling scheme...
without compromising the quality of the sample. Thus, a delay of up to almost 14 h between collection and processing of the blood and exposure to temperatures up to 55°C did not decrease the viability or the transformability of the samples. Extended storage at −80°C of up to 11 days after freezing but before transfer to liquid nitrogen storage and small variation in the final DMSO concentration also did not harm the samples. Our results showing that the normal variation in conditions that is inherent in the field collection of blood does not compromise the successful cryopreservation of blood samples should encourage the collection of these valuable samples by future epidemiologic studies.

References
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