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Establishment of Stably EBV-Transformed Cell Lines from Residual Clinical Blood Samples for Use in Performance Evaluation and Quality Assurance in Molecular Genetic Testing

Susan H. Bernacki,* Ana K. Stankovic,† Laurina O. Williams,† Jeanne C. Beck,‡ James E. Herndon,§ Karen Snow-Bailey,¶ Thomas W. Prior,‖ Karla J. Matteson,** Linda M. Wasserman,†† Eugene C. Cole,‡‡ and Timothy T. Stenzel*

From the Molecular Diagnostics Laboratory, Department of Pathology, and the Comprehensive Cancer Center,§ Duke University Medical Center, Durham, North Carolina; the Public Health Practice Program Office,† Centers for Disease Control and Prevention, Atlanta, Georgia; Coriell Cell Repositories,‡ Coriell Institute for Medical Research, Camden, New Jersey; the Molecular Genetics Laboratory,¶ Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota; the Department of Pathology,‖ Ohio State University Hospital, Columbus, Ohio; the Department of Medical Genetics and the Department of Pathology,** University of Tennessee Medical Center, Knoxville, Tennessee; the Department of Medicine,†† University of California San Diego, La Jolla, California; and the Department of Health Science,‡‡ Brigham Young University, Provo, Utah*

Positive control materials for clinical molecular genetic testing applications are currently in critically short supply or non-existent for many genetically based diseases of public health importance. Here we demonstrate that anonymous, residual, clinical blood samples are potential sources of viable lymphocytes for establishing Epstein-Barr virus (EBV)-transformed blood lymphocyte cell lines. We attempted to transform 34 residual blood samples, and analyzed transformation success with respect to sample age, anticoagulant, storage temperature, volume, hemolysis, and patient age and sex. In univariate analysis, sample age was significantly associated with transformation success ($P = 0.002$). The success rate was 67% (6 of 9) for samples 1 to 7 days old, 38% (3 of 8) for samples 8 to 14 days old and 0% for samples 15 to 21 (0 of 11) days old. When we controlled for sample age in multivariate logistic regression, anticoagulant and storage temperature approached significance ($P = 0.070$ and 0.087, respectively; samples in acid citrate dextrose (ACD) and refrigerated samples were more likely to transform). Based on these findings, we suggest that samples collected in either ACD or ethylene diamine tetraacetic acid, and up to 14 days old (refrigerated) or 7 days old (stored ambient), are reasonable candidates for EBV transformation. The transformation rate for samples that met these criteria was 63% (10 of 16). Implementation of this process could help alleviate the shortage of positive control materials for clinical molecular genetic testing. ***(J Mol Diagn 2003, 5:227–230)***

The deficiency of positive control material for molecular genetic testing has been identified as "the issue of utmost urgency" for clinical genetic testing.[1,2] Molecular genetic testing is becoming an increasingly important component of routine health care as the genetic foundations of a growing number of diseases are revealed. The availability of positive control material for performance evaluation and quality assurance (PE/QA) of existing tests, and for developing and validating new tests, is critical to the genetic testing community.

Reliable, high-quality control material for genetic testing can be provided by stably transformed B-lymphocyte cell lines derived from blood from subjects with genetic disease mutations. Lymphoblastoid cell lines are used extensively by proficiency testing groups, and many cell lines of this type are available in repositories such as the National Institute of General Medical Sciences (NIGMS) collection, maintained by the non-profit Coriell Institute for Medical Research (Coriell; http://cimr.umdnj.edu). However, control materials for many mutations of public

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Timothy T. Stenzel's current affiliation is Vysis, Inc., Abbott Laboratories, Downers Grove, IL.

Address reprint requests to Dr. Timothy T. Stenzel, Vysis, Inc., an Abbott Laboratories Company, 3100 Woodcreek Drive, Downers Grove, IL 60615-5400. E-mail: timothy.stenzel@vysis.com.

health importance can be difficult to obtain. For example, in 2001, the American College of Medical Genetics recommended a core panel of 25 mutations to be used in general population screening for cystic fibrosis,[3] yet positive genetic material for several of these mutations is not routinely available through public sources. Current and future genetic testing needs could be met in part by developing a standardized process to create control cell lines with mutations of interest.

Residual, clinical blood samples hold promise as a sustainable, economical source of B-lymphocytes for the development of lymphoblastoid cell lines with important mutations. The use of residual samples avoids the need to draw additional blood, which can be problematic, particularly when pediatric or incapacitated patients are involved. Also, residual samples can be made completely anonymous, so Institutional Review Board (IRB) procedures are simplified. Because of the numerous samples processed by many clinical testing laboratories, it may be possible to obtain less common mutations, which are especially valuable.

In this study, we investigated the feasibility of developing Epstein-Barr virus (EBV)-transformed B-lymphocyte cell lines from residual, anonymous clinical blood samples that had been determined in the course of standard clinical testing to contain mutations of public health importance. Blood lymphocytes transform readily, and numerous EBV transformation protocols have been developed.[4–8] Coriell has developed protocols for EBV transformation of both fresh and cryopreserved blood lymphocytes that have success rates of 94% and 90%, respectively. These protocols use two 10-ml Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) of blood with acid citrate dextrose (ACD) as the anticoagulant, and the samples are maintained at ambient temperature during transit and processed within 6 days of collection. The transformation success rate approaches 100% for any given sample if additional transformation attempts are made using lymphocytes frozen and stored at the time of initial sample processing.[9]

Most EBV transformation protocols use freshly drawn blood or freshly frozen blood or lymphocytes. Residual clinical blood samples, however, may be 2 to 3 weeks old by the time a mutation is identified, and clinical samples vary with respect to blood volume, anticoagulant, storage temperature, and other factors that could affect transformability. A high transformation success rate was observed at Coriell for samples up 6 days old,[9] however, and we thought it likely that some residual clinical blood samples would also transform successfully. We wished to determine the transformation success rate for residual clinical samples, and also to identify the sample variables that were important predictors of transformation success.

Materials and Methods

To obtain blood samples for this study, we contacted clinical genetic testing facilities and requested contributions of residual clinical samples with specified sample characteristics. All laboratories that participated in this study obtained prior IRB approval as appropriate for their institutions. All patient identifiers were removed from the samples to make them completely anonymous, and, as an additional precaution to protect patient confidentiality, the identity of the contributing laboratory was not recorded.

We solicited blood samples drawn in either ACD or ethylene diamine tetraacetic acid (EDTA), in age ranges of 1 to 7, 8 to 14, and 15 to 21 days from venipuncture to initiation of transformation. The submitting laboratories provided information on age of the sample, anticoagulant used, storage temperature, sample volume, patient age and patient sex. We also recorded whether the sample hemolyzed before transformation was initiated. Although all of the samples had been originally submitted for clinical testing for a mutation or disease of interest, testing results were not always available at the time samples were submitted to this project; therefore some samples could have been negative.

EBV transformation was attempted on 34 samples. EBV shed by a marmoset cell line (GM07404; NIGMS Human Genetic Cell Repository) was used as the transforming agent as previously described,[9] with modifications as necessary to accommodate variations in blood sample volume. Briefly, blood was diluted 1:1 with RPMI 1640, layered onto a Histopaque-1077 Hybrimax gradient (Sigma-Aldrich, St. Louis, MO) and centrifuged. The lymphocyte layer was harvested and resuspended in culture medium containing EBV and the mitogen phytohemagglutinin (PHA; Sigma-Aldrich). In cases where small sample volume or hemolysis of the sample made purifying the lymphocytes impractical, the sample was mixed directly with culture medium containing EBV and PHA. Medium was replenished twice weekly, and the cultures were monitored for signs of transformation (increased cell growth, cell aggregates, or clumping), which were generally apparent within 3 to 8 weeks in successful cultures. The culture medium was antibiotic-free to increase the likelihood that contamination would be readily detected. Transformed cultures were grown to approximately 1×10^8 cells, then cryopreserved in heat-sealed borosilicate glass ampules (20 1-ml ampules of five million viable cells each), and stored in liquid nitrogen (liquid phase). "Successful" transformations were free from bacterial, fungal, and mycoplasmal contamination and were viable after cryopreservation, as evidenced by a doubling of the cell number within 4 days of recovery. Samples that did not show obvious signs of transformation were discarded after an average of 167 ± 6 (SEM) days in culture.

Results and Discussion

Successful cell lines were established from 10 of the 34 submitted samples. Time from initiation of transformation to the growth of 1×10^8 cells ranged from 41 to 83 days, with an average of 52.5 ± 3.9 (standard error of the mean, SEM) days. This is longer than the historical average at Coriell of 35.2 ± 0.3 (SEM, $n = 421$) for freshly collected samples in ACD.

Table 1. Transformation Success in Stored Samples from a Single Donor

Sample age	Successfully transformed?	
	ACD	EDTA
7 days	Yes	Yes
14 days	No	No
21 days	No	No

Statistical analysis was performed to determine which sample variables were reliable predictors of successful transformation. Six of the 34 submitted samples were excluded from the analysis, including one successful transformant. One of the excluded samples had been stored for 21 days at ambient temperature before transformation was attempted, and exhibited bacterial contamination shortly after culture with EBV was initiated. The culture was destroyed before the outcome was evident. The other five excluded samples were part of a set of six samples from a single donor, where blood drawn in both EDTA and ACD was aged for 7, 14, and 21 days (see Table 1). One randomly selected sample from this set was included in the statistical analysis. We assessed the relationships of the individual sample variables to transformation success using exact inference analyses, either Fisher's exact test, or the Cochran-Armitage exact test for trend as appropriate (Table 2). We found a statistically significant relationship between age of the sample and the chances of successful transformation ($P = 0.002$). Samples that were from 1 to 7 days old at the time of setup had a higher transformation rate (67%) than older samples (38% for 8 to 14 days; 0% for >14 days), suggesting a linear relationship between age of sample and success rate. Samples collected in ACD were somewhat more likely to transform than those collected in EDTA, ($P = 0.095$), and unhemolyzed samples appeared more likely to transform than hemolyzed ones ($P = 0.098$). Sample volume, sample storage temperature, and age and sex of the donor were not significantly related to transformation success by this analysis.

Table 2. Univariate Relationships between Sample Variables and Transform-ation Success. Nine Samples Transformed

Sample variable	No. of successful Transformations/No. of attempts (%)	*P*-Value
Age of sample (days from venipuncture to addition of EBV)		0.002*
1–7 days	6/9 (67%)	
8–14 days	3/8 (38%)	
>14 days	0/11 (0%)	
Anticoagulant		
EDTA	3/17 (18%)	0.095†
ACD	6/11 (55%)	
Storage temperature		
4°C	6/14 (43%)	0.420†
RT	3/14 (21%)	
Hemolysis		
No	8/18 (44%)	0.098†
Yes	1/10 (10%)	
Sex‡		
Male	5/15 (33%)	>0.999†
Female	4/12 (33%)	
Age of subject (yrs)‡		
<20	2/8 (25%)	>0.999*
20–49	4/10 (40%)	
50+	2/9 (22%)	
Sample volume (ml)		
<3	3/8 (38%)	0.794*
3–5.99	4/12 (33%)	
6+	2/8 (29%)	

*, *P*-value from the Cochran-Armitage exact test for trend (two-sided).
†, *P*-value from Fisher's exact test.
‡, The sex of one subject was unknown, as was the age of another subject.

We then examined the relationship between the strongest predictor of transformation success (sample age) and selected sample variables: anticoagulant, storage temperature, and hemolysis. Hemolysis was strongly related to sample age (Fisher's exact test, $P = 0.002$). Most hemolyzed samples were over 14 days old, and none was 1 to 7 days old. No relationship was found between sample age and anticoagulant ($P = 0.624$) or sample age and storage temperature ($P > 0.999$). However, in this sample set, storage temperature and anticoagulant ($P = 0.120$) may be related. This apparently spurious relationship may be due to a non-random distribution of some characteristics for samples submitted by particular laboratories. For example, one facility may have submitted many samples to the project, and all may have had the same anticoagulant and storage conditions. Because the origins of the samples were not recorded, this cannot be verified.

Sample age was strongly associated with transformation success, so we re-examined the effects of selected other variables using a logistic regression model that would assess the simultaneous effects of sample age, anticoagulant, and storage temperature. Hemolysis was not included in this multivariate analysis because the strong association with sample age would result in numerical instability in the estimation procedure. Due to the relatively small number of samples, and the association between anticoagulant and storage temperature, two separate models were developed, one including sample age and anticoagulant and one including sample age and storage temperature. The age of the sample was treated as a continuous variable. In both models, the age of the sample was found to be a significant predictor of successful transformation ($P = 0.017$ and $P = 0.011$). In the two separate models, after adjusting for sample age, the effect of both anticoagulant and storage temperature approached statistical significance ($P = 0.070$ and $P = 0.087$, respectively). Samples in EDTA and those stored at room temperature were less likely to transform.

The effect of sample age on transformation success is illustrated by the results of six separate transformation attempts using aged blood from a single donor (Table 1). Several tubes of blood from a single draw (both EDTA and ACD tubes) were stored at 4°C and transformation was initiated for blood with both anticoagulants at 7, 14, and 21 days. Both of the 7-day-old samples transformed successfully; however, none of the 14- or 21-day-old samples transformed.

Our results demonstrate that residual clinical blood samples are a potential source of B-lymphocytes for establishing stably EBV-transformed lymphocyte cell lines. Sample age was the strongest predictor of transformation success for a given sample, with success rates of 67%, 38% and 0% for samples aged 1 to 7, 8 to 14 and 15 to 21 days, respectively. When the age of the samples was included in a logistic regression model, the effects of anticoagulant and storage temperature approached statistical significance, with samples drawn in ACD and samples stored at 4°C more likely to transform. Although sample hemolysis was associated with a lower transformation rate, hemolyzed samples were also typically older samples, so it is not clear whether hemolysis itself affected transformation or was a confounder due to its association with sample age. One hemolyzed sample did transform successfully, indicating that hemolysis alone does not preclude successful transformation. Sample volume and the age and sex of the donor did not influence the likelihood of successful transformation.

Based on these findings, we suggest that the following criteria be used to identify residual blood samples that are good candidates for EBV transformation: samples should be no more than 14 days old (preferably no more than 7 days old) and should have been stored under ambient conditions or at 4°C for samples up to 7 days old and at 4°C only for samples 8 to 14 days old. Both ACD and EDTA appear to be acceptable as anticoagulants. Although samples in ACD were more likely to transform than those in EDTA, three of the ten successful cell lines were derived from samples in EDTA. We also recommend a minimum volume of 1.0 ml to facilitate sample handling. In this study, samples that conformed to these guidelines had a transformation success rate of 63% (10 of 16).

It is not known why younger samples are more likely to transform, although a likely possibility is that the number of viable B-lymphocytes decreases as the sample ages. Thus, the number of viable B-lymphocytes in a residual sample may be a useful predictor of transformation success. It is also not known if the increased time in culture needed to establish a transformed cell line from residual as opposed to freshly drawn blood samples is due to initially fewer viable B-lymphocytes, slower proliferation rates for the lymphocytes once transformed, or a combination of these or other factors.

It has been reported that although EBV-transformed lymphoblast cell lines are stable in culture, they are not truly immortal, and generally cease proliferating before 160 population doublings have been reached.[10,11] One-hundred sixty population doublings would, however, provide an extremely large quantity of genetic material. Appropriate archiving of seed stocks during early expansion of the cultures should obviate this potential limitation in the use of EBV-transformed cell lines as reliable sources of control materials.

In summary, we have shown that residual clinical blood samples can provide viable lymphocytes for the establishment of EBV-transformed cell lines with mutations of public health importance. We achieved a transformation success rate of 63% (10 of 16) for samples up to 14 days old. Successful samples up to 7 days old had been stored under ambient conditions or at 4°C; all successful samples 8 to 14 days old had been stored at 4°C only. The cell lines established in this study were all derived from residual samples no more than 14 days old, so this approach is likely to be most successful for mutations that can be detected clinically within this time frame. To maximize the utility of these types of cell lines as control materials for molecular genetic testing, work is in progress to verify the stability of cell line mutations in culture and to validate cell lines in various clinical testing settings.

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