original research report

Validation of a modified cryopreservation method for leukemic blasts for flow cytometry assessment

Huda Alsayed, Tarek Owaidah, Faisal Al Rawas

From the Department of Diagnostic Hematology, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia

Correspondence and reprints: Tarek Owaidah, MD · Head, Diagnostic Hematology · King Faisal Specialist Hospital and Research Centre · PO Box 3354, Riyadh 11211, Saudi Arabia · T: +966-1-442-4328 F: +966-1-442-4280 · towaidah@kfshrc.edu.sa · Accepted for publication March 2008

Hematol Oncol Stem Cel Ther 2008; 1(2): 94-97

BACKGROUND: Cryopreservation, a common method for storing human cells, has advantages when cells are used in retrospective studies of selected cell populations. Frozen lymphocytes can be used for tissue typing, for monitoring cell-mediated immunity, and for various immunological tests. Our report describes an efficient, simple and inexpensive method for cryopreservation of human acute leukemia cells.

METHODS: Leukemia cells from 20 newly diagnosed cases were frozen at -80ºC after cryopreservation with 5% dimethysulfoxide and then assayed by flow cytometry for antigen expression determined by monoclonal antibodies at different time intervals.

RESULTS: All cases had viability above 75% at presentation. After 4 weeks, 91% of pre-B ALL, 88% of T-ALL, 100% of AML, and 100% of biphenotypic aliquots had viability over 75%. Viability continued to be reliably above 75% at 6 weeks from cryopreservation.

CONCLUSION: We confirm that the method does not significantly alter the viability of cells and it preserved the antigenic expression of leukemia cells.

Cryopreservation is a method commonly used to store human blood that also has numerous other advantages. The cryopreservation of human cells for long-term storage has a vital advantage, especially in a setting of bone marrow transplantation and blood transfusion. Frozen lymphocytes can be used for tissue typing, for monitoring cell-mediated immunity, and for various immunological tests.[1-4] Studies requiring a lymphocyte-mediated response for sequential analysis during the course of the disease require the use of cryopreserved lymphocytes.[5] Immunophenotyping is crucial for diagnosis and risk stratification of acute leukemia. Although many studies have addressed the cryopreservation of lymphocytes and its value in immunological studies,[6-10] not many have addressed the use of cryopreservation for different blasts cells, with the impact of cryopreservation on blast viability and the expression of various antigens.[10,11] We report a modified cryopreservation method for long-term storage of leukemic blast cells with a study of the effect of this cryopreservation on viability and antigen expression.

METHODS

A total of 20 newly diagnosed cases of acute leukemia were collected from January to April 2007 of which 9 cases were acute pre-B lymphoblastic leukemia (Pre-B-ALL), 6 cases were acute myeloblastic leukemia (AML), 4 cases were T-cell acute lymphoblastic leukemia (T-ALL) and 1 case was acute biphenotypic leukemia (ABL). Residual anticoagulated bone marrow or peripheral blood were frozen and processed as below.

Freezing Methodology. Residual samples from immunophenotyping were washed twice using cold Hanks Balanced Salt Solution HBSS (SAFC Biosciences, Lenexa, Kansas, USA) after which the cell count was adjusted to 5-12×10^9/L. A 10% dimethyl sulfoxide (DMSO) freezing solution was prepared by mixing fresh and cold 10 mL of HBSS with 6 mL of fetal calf serum and 1.8 mL of DMSO. The freezing media was then placed on ice. Six aliquots were labeled week 1 through week 6 using cryogenic vials. Equal volumes of blood sample and chilled freezing medium (500 µL blood+500 µL freezing medium) was added to each of the vials. This gave a final concentration of 5% of the

Table 1. Antigen expression in 20 cases of acute leukemia

Type of leukemia	Number of cases	Frequency of antigen expression at presentation		Frequency of antigen expression at 4 weeks	Frequency of antigen expression at 6 weeks
Pre-B ALL	9	CD10	9/9	9/9	9/9
		CD19	9/9	9/9	9/9
		CD79a	5/9	5/5	5/5
		CD34	8/9	8/8	8/8
		TdT	9/9	9/9	9/9
		HLA-DR	9/9	9/9	9/9
		CD45	7/9	6/9	6/9
AML	6	CD7	2/6	2/2	2/2
		CD13	5/6	5/5	5/5
		CD33	6/6	6/6	6/6
		CD117	5/6	5/5	5/5
		CD34	2/6	2/2	2/2
		MPO	4/6	3/6	3/6
		HLA-DR	6/6	6/6	6/6
		CD45	6/6	6/6	6/6
T-ALL	4	CD2	4/4	4/4	4/4
		CD3	4/4	4/4	4/4
		CD5	4/4	4/4	4/4
		CD7	4/4	4/4	4/4
		TdT	3/4	3/4	3/4
		CD45	4/4	4/4	4/4
ABL	1	CD7	1/1	1/1	1/1
		CD19	1/1	1/1	1/1
		CD33	1/1	1/1	1/1
		CD34	1/1	1/1	1/1
		CD79a	1/1	1/1	1/1
		CD117	1/1	1/1	1/1
		TdT	1/1	0/1	0/1
		HLA-DR	1/1	1/1	1/1
		CD45	1/1	1/1	1/1

freezing media. The vials were mixed and quickly placed in the –70°C freezer. After every week, frozen samples were thawed immediately and rapidly at 37°C and then washed twice using cold HBSS and filtered. Frozen cells then were stained with different monoclonal antibodies.

Immunophenotyping methodology. The following monoclonal antibodies were used: T-cell markers CD1a-PE (coulter), CD2-PE (BD), CD3-FITC (B.D), CD4-APC(BD), CD8-PE(BD), CD7-FITC (BD), CD5-FITC(BD), TCR((-FITC(BD), TCR((-PE(BD) where as B-cell markers included CD10-FITC(Coulter), CD19-APC(BD), CD20-PE(BD), CD22-APC(BD), Kappa-FITC(BD), Lambda-PE(BD), IgG-FITC(Biosource), IgM-FITC(Biosource). The myelomonocytic markers

used were CD14-FITC (BD), CD13-PE (BD), CD33-PE (BD), CD64-FITC (BD), CD65-FITC (BD), CD117-PE (BD), CD15-FITC (BD), and CD11b-PE (BD). Monoclonal antibodies against cytoplasmic and nuclear antigens were used MPO-FITC (Coulter), TdT-FITC "polyclonal" (Coulter), CD79a-PE (Coulter), CD3-FITC (Coulter). Additional monoclonal antibodies used were CD45-PerCP (BD), HLA-DR-FITC (BD), CD34-APC (BD), NG2-PE (Coulter), CD11c-PE (BD), CD9-FITC (BD), CD56-APC (BD), CD61-FITC (BD) to identify megakaryocytes, CD235a-FITC (Coulter) and CD71-FITC (BD) to identify erythroid precursors. Cell viability was measured by staining the cells using 7-amino actinomycin D (7-AAD) dye.

After the second wash the cells were filtered and the count adjusted to 0.5-1×10^9/L of which a 50 µL was placed in each tube and incubated with the appropriate combination of monoclonal antibodies for 15-20 minutes in the dark (room temperature). Once the incubation period was finished the cells were washed one more time as above. The cells were then resuspended in 0.5 mL of 1% paraformaldehyde and stored in the refrigerator till time of analysis.

For staining of the cytoplasmic and nuclear antigens the IntraPrep Permeabilization Reagent kit (Beckman Coulter, Fullerton, CA, USA) was used, strictly following the recommendations of the manufacturer. Data acquisition was performed using a FACSCalibur system equipped with two lasers and Cell Quest Pro software (Becton Dickinson, San Juan, CA, USA) where a minimum of 10 000 events/tube were acquired. Instruments were daily calibrated using CALIBRITE beads (Becton Dickinson, San Juan, CA, USA) and two levels (normal and low) of controls using CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19 run daily to ensure consistency in the functions of the instrument. Data analysis was performed using Cell Quest Pro software and the blast cells were gated on using CD45 vs. SSC technique. The following criteria were used to score the blast population: negative <20% and positive: >20% of the gated blasts.

RESULTS

Table 1 shows frequency of antigen expression for each leukemia at different time intervals. While all cases had viability over 75% at presentation, 33/36 (91%) of pre-B ALL, 14/16 (88%) of T-ALL, 24/24 (100%) AML and 4/4 (100%) biphenotypic leukemia aliquots, respectively, had viability over 75% by 4 weeks. The viability continued to be reliably above 75% when aliquots were tested at 6 weeks from cryopreservation. The flow cytometry analysis of the six aliquots for each case demonstrated good preservation of various antigen expression compared to the original fresh samples results with no major change.

DISCUSSION

Preservation of human cells such as blood and bone marrow cells has become increasingly important in both basic research and clinical medicine. Cryopreserved cells proved to be excellent controls for determining the day-to-day variability and for selecting optimum conditions for laboratory tests in the clinical immunology laboratory.[4,10] Cryopreservation of human mononuclear cells has been used for quality control materials in clinical immunology.[12] In multicenter studies, the precision and accuracy of complex immunologic assays could be greatly improved if specimens obtained at multiple sites could be analyzed in a single, highly skilled laboratory, which requires adequate preservation of the cells. Riemann et al had demonstrated in multicenter AIDS clinical trials the feasibility of cryopreserving peripheral blood mononuclear cells for immunophenotyping and functional testing.[13] Although it had been shown in a number of studies that cryopreservation has some deleterious effect on the viability of cells and antigen expression[14,15] that we did not have in the method we are presenting here. A number of methods were introduced for cryopreservation, with different effects on cell function and antigen preservation.[5,13-18] Use of DMSO is considered the method of choice as it is easy, rapid and effective. The effect of various concentrations of DMSO, the composition of the medium, and other parameters were investigated in several trials to work out optimal conditions for freezing human lymphocytes. Although the ideal concentration of DMSO has been extensively studied, concentrations as low as 5% have resulted in a good recovery of cryopreserved lymphocytes.[5] However, this was complemented with gradual freezing at 2°C per minute.

In this study, we validated a simple method that uses a concentration of 10% DMSO when mixed with freezing media and added to an equal volume of blood that can be frozen immediately. In a retrospective analysis of specimens obtained at presentation of acute leukemia and cryopreserved by this method, we found that the viability had been preserved in more than 88% of the cells with variability between the types of leukemia. The myeloid leukemia had better viability than the lymphoid leukemia. Both B and T lymphoid cells had shown some degree of decrease in viability with no significant change in antigen expression. These results are somewhat comparable to that reported by Thiry et al for B-cell chronic lymphocytic leukemia[19] and Romue et al for lymphocyte

subset.[20]

Immunophenotyping of acute leukemias is the cornerstone of diagnosis and very important in risk stratification and follow-up post therapy. In many situations the leukemia diagnosis needs to be done in a remote center, which necessities transfer of samples.

This project was aimed at determining the applicability of using cryopreservation in diagnosis of acute leukemia with the intention to use cryopreserved leukemic blasts for further studies, especially minimal residual disease detection. It will also provide a good tool for quality control for leukemia studies.

REFERENCES

1. Bohm F, and Rychlikova: The blastogenic reaction in mixed cultures of lymphocytes preserved by freezing to -79ºC with DMSO.Folia Biol 1967;13:204.

2. Chess L, Bock G.N, Mardiney R: Restoration of the reactivity of frozen stores human lymphocytes in the mixed lymphocyte reaction and in response to specific antigens. Transplantatio. 1972;14:728.

3. Nathan P: Freeze-thaw-refreeze cycle to prepare lymphocytes for HLA antibody detection or tissue typing. Cryobiology 1974;11:305.

4. Sleasman J.W, Leon B.H, Aleixo L.F, Rojas M, Goodenow M.M:Immunomagnetic selection of purified monocyte and lymphocyte populations from peripheral blood mononuclear cells following cryopreservation. J Clin Diagn Lab Immunol 1997;4(6): 653-658.

5. Simon J.D, Flinton L.J, Albala M.M: A simple method for the cryopreservation of human lymphocytes at -80º C. Transfusion 1977;17(1):23-28.

6. Venkataraman M.: Effect of cryopreservation on immune responses: VI.An inexpensive method for freezing human peripheral blood mononuclear cells. J Clin Lab Immunol 1992;37(3):133-143.

7. Ford CH, Newman CE, Carter AB.:The effect of cryopreservation of lymphocytes on E rosetting ability: a study in lung cancer patients and controls.J Immunol Methods. 1979;26(2):113-124.

8. Jennes W, Kestens L, Nixon D.F, Shacklett B.L: Enhanced ELISPOT detection of antigen-specific T cell responses from cryopreserved specimens with addition of both IL-7 and IL-15-the Amplispot assay. J Immunol.Methods 2002;99:270.

9. Weinberg A, Wohl D.A, Brown D.G, Pott G.B, Zhang L, Ray M.G, Horst V.D: Effect of cryopreservation on measurement of cytomegalovirus-specific cellular immune responses in HIV-infected patients.J.Acquir.Immune Defic.Syndr. 2000;25:109.

10. Mary L.D, Corazon D.R, Vivian G, Ling-Yu K, Jennie C.C.C, Kristina K.R, Timothy M.C, KIM Lyerly H, Sonny B, Smita A.G, Vernon C.M, Holden T.M: Maximizing the retention of antigen specific lymphocyte function after cryopreservation. J. Immunol. Methods 2006;308:13-18.

11. Costantini A, Mancini S, Giuliodoro S, Butini L, Regnery C.M, Silvestri G, Montroni M: Effects of cryopreservation on lymphocyte immunophenotype and function. J. Immunol. Methods 2006;278:145.

12. Douglas M.S, John R.O, Franco P, Annette M, Ronald B.H: Cryopreservation of human mononuclear cells for quality control in clinical immunology.I.Correlation in recovery of K-and NK-cell functions, surface markers, and morphology. Journal of clinical immunology 1982;2(3): 214-221.

13. Reimann KA, Chernoff M, Wilkening CL, Nickerson CE, Landay AL.Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type 1-infected donors: implications for multicenter clinical trials. The ACTG Immunology Advanced Technology Laboratories. Clin Diagn Lab Immunol. 2000 May;7(3):352-9.

14. Katkov II, Kim MS, Bajpai R, Altman YS, Mercola M, Loring JF, Terskikh AV, Snyder EY, Levine F. Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. Cryobiology. 2006 Oct;53(2):194-205.

15. Heng BC, Ye CP, Liu H, Toh WS, Rufaihah AJ, Yang Z, Bay BH, Ge Z, Ouyang HW, Lee EH, Cao T Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis. J Biomed Sci. 2006 May;13(3):433-45.

16. Stear M.J, Allen D, and Spooner R.L: A method for freezing sheep lymphocytes prior to cytotoxicity testing. Tissue Antigens 1982;19(2): 134-139.

17. Glassman A.B, Bennett C.E: Cryopreservation of human lymphocytes: a brief review and evaluation of an automated liquid nitrogen freezer. Transfusion 1979;19(2): 178-181.

18. Areman E.M, Simonis T.B, Carter C.S, Read E.J, Klein H.G: Bulk cryopreservation of lymphocytes in glycerol. Transfusion 1988;28(2):151-156.

19. Deneys V, Thiry V, Hougardy N, Mazzon AM, Leveugle P, De Bruyère M. Impact of cryopreservation on B cell chronic lymphocytic leukaemia phenotype. J Immunol Methods. 1999 Aug 31;228(1-2):13-21

20. Romeu MA, Mestre M, González L, Valls A, Verdaguer J, Corominas M, Bas J, Massip E, Buendia E. Lymphocyte immunophenotyping by flow cytometry in normal adults. Comparison of fresh whole blood lysis technique, Ficoll-Paque separation and cryopreservation. J Immunol Methods. 1992 Sep 18;154 (1):7-10.