Indian Journal of Clinical Biochemistry, 2004, 19 (2) 95-99

ENUMERATION OF LYMPHOCYTE SUBSETS USING FLOW CYTOMETRY: EFFECT OF STORAGE BEFORE AND AFTER STAINING IN A DEVELOPING COUNTRY SETTING

Sanju Jalla*, Sunil Sazawal*, Saikat Deb***, Robert E. Black**, Satya Narayan Das****, Archana Sarkar***, Maharaj K. Bhan*****

**Center for Cancer Research, John Hopkins University School of Medicine, Baltimore MD USA.*
***Blumenberg School of Public Health, Johns Hopkins University, Baltimore, MD USA*
****Center for Micronutrient Research, Annamalai University, Chidambaram, Tamil Nadu,*
*****Department of Biotechnology, All India Institute of Medical Sciences, New Delhi.*
******Department of Pediatrics, All India Institute of Medical Sciences, New Delhi*

ABSTRACT

Lymphocyte subset estimations by flow cytometry in population-based studies require transportation of samples from the field site to the laboratory. As samples arrive late in the day they have to wait overnight before being processed. The effect of two possible approaches, sample storage for 24 h before staining and immediate staining with analysis after 24 h and 48 h were evaluated. Two sets of experiments were performed with EDTA (ethylenediamine tetra-acetate) anticoagulated peripheral blood. In the first experiment, after collection, each sample was divided into two portions. One portion was stained at the time of blood collection and the other 24 h later after keeping it at room temperature (38-45^{0}C). In the second experiment, blood samples were stained within 1-2 h. Each sample was analyzed immediately upon completion of staining process and subsequently after 24 h and 48 h of storage at 4^{0}C. Results suggest that blood collected in EDTA can be processed using whole blood lysis method, after storage at room temperature (38-45^{0}C) for 24 h with some but not significant alteration in T-cell subsets. Storage at 4^{0}C after staining for 24 h results in a lesser and insignificant loss of cells or alteration of T-cell subsets and may be the method of choice.

KEY WORDS

Lymphocyte storage, flow cytometry, leukocyte phenotypes.

INTRODUCTION

The analysis of peripheral blood for lymphocyte subsets is used to assess immunological status in a wide variety of clinical conditions (1). Lymphocyte subset analysis is important in the diagnosis and management of autoimmune diseases, viral infections, leukemia, renal transplant and acquired immune deficiency syndrome (2). The development of monoclonal antibodies to lymphocyte cell surface antigens or receptors has revolutionized T and B cell typing. Combined with flowcytometry, it is now possible to accurately and rapidly analyze T and B-lymphocytes for presence of surface antigens or receptors (3, 4, 5). The introduction of multiple conjugated antibodies makes it possible to examine a number of different subsets simultaneously (6, 7). These improvements have greatly expanded the capacity of flowcytometry and have made it very sensitive (8).

Currently, lymphocyte subset determination techniques are not available in many clinical laboratories, necessitating transportation of specimens to specialized reference laboratories. Even when the test is performed in-house, limited access to the equipment among multiple users may warrant delay in analysis of the specimens. Similarly, in community-based studies, as specimens are brought from the field site to the laboratory late in the day, being processed the same day is often not feasible. It is thus necessary to determine the effect of storage on lymphocyte subset

Author for correspondence :

Dr. Sunil Sazawal
Center For Micronutrient Research
New Delhi Field Office, M-30, Left Basement
Lajpat Nagar - II, New Delhi - 24, INDIA
email: ssazawal@jhsph.edu

to determine the effect of storage on lymphocyte subset estimations in order to define the limits of acceptable delays and plan laboratory procedures for such studies.

A number of biological and technical factors are known to affect the analysis of lymphocyte subsets. The technical variables include the anticoagulant into which the specimen is drawn (9), the time and temperature at which the specimen is held before staining with monoclonal antibody (10, 11).

We could not find any study evaluating the effect of storage on T-cell subset estimation in a developing country setting. Most previous reports evaluating storage effects are from industrialized countries where all the laboratories are air-conditioned and ambient temperature is controlled. The best-evaluated work from these studies suggested that temperature is the most significant factor in sample storage (16, 1). Further varying temperature may have differential effect on various subsets (8). The maximal temperature evaluated in their studies was 21°C. In contrast in developing country settings the ambient temperature in the field condition can be as high as 40°C. Further the laboratories are also not air-conditioned. In order to determine the best procedure for sample storage in field conditions, we evaluated effects of storage on lymphocyte subsets estimation at room temperature for 24 hours before staining and at 4°C after staining for 24 and 48 hours.

MATERIALS AND METHODS

Experimental design

Two sets of experiments were performed with EDTA (ethylenediamine tetra-acetate) anticoagulated peripheral blood collected on ten consecutive days between 9 a.m. to 10 a.m. In the first experiment, whole blood (2 ml) from 10 healthy individuals was collected on 4 different days. After collection, each sample was divided into two aliquots. One aliquot was processed at the time of blood collection (within 1-2 h), the other aliquot was processed 24 h later. Samples were held at room temperature (38-45°C). In the second experiment, blood samples were collected on 6 different days from 6 different individuals and processed within 1-2 h, each sample was analysed immediately upon completion of labeling process and then after 24 h and 48 h of storage at 4°C.

Monoclonal antibodies

We used monoclonal antibodies of Becton Dickinson Quanti BRITE products. These provide > 95% one PE molecule per antibody. The antibody preparation used was the standard preparation of Becton Dickinson.

The antibody preparation used clone SK3 for CD4 and CD8, clone MϕP9 for CD14, clone L27 for CD20, clone SK7 for CD3 and clone 2D1 for CD45.

For the reasons of lower cost and flexibility of varying the dilution of each antibody, we used single monoclonal antibody vials and then mixed the two in each tube of the panel. Before use, the monoclonal antibodies were diluted with phosphate buffer saline (PBS). The dilutions used were 1:5 for CD20, 1:20 for CD3, CD4 and CD8, 1:10 for CD45 and 1:2 for CD14. These dilution factors were found to be optimal during our standardization and pretesting (reported elsewhere). The antibodies used were of labelled type.

Sample processing

Specimens were processed using the whole blood lysis method (1). In both experiments, a 5-tube panel of antibodies was selected for processing each specimen to include major lymphocyte subsets. The panel selected consisted of simultaneous labeling of two different molecules on one cell with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). In the first tube (leucogate reagent for electronic gating), FITC (CD45) + PE (CD14) was used, in the second, unstained cells were used as negative control, in the third tube FITC (CD3)+PE (CD4) were used, in the fourth FITC (CD3)+PE (CD8) were used, and in the last tube FITC (CD20) was used. We used Becton-Dickinson Immunocytometry monoclonal antibodies, 20 µl each of CD45 and CD3, 10 µl each of CD14, CD4 and CD8 and 5 µl of CD20. After adding 100 µl of blood to the monoclonal antibody combination each tube was gently vortexed for 15 seconds. The tubes were then incubated for 20-25 minutes at room temperature in dark. After incubation, the erythrocytes were lysed by an automated lysing instrument (the Q-prep EPICS work station, Coulter cytometry Hialeah, FL) that performs a three step procedure of preparing lymphocytes for analysis. Q-prep leukocyte preparation system is an immunoprep reagent system which essentially lyses the red blood cells, maintains cellular integrity without distortion, minimizes debris or platelet adhesion to leucocytes preparation, reduces non specific binding without need for centrifugation and cell loss associated with it. For both experiments we used the standard Q-prep reagents (Coulter diagnostics, Hialeah, FL). The samples after this step were stored at 4°C until they were analysed.

Analysis of samples

The analysis was performed in the first experiment

experiment it was additionally repeated after 24 and 48 h. The samples were coded and were analysed using Becton Dickinson flowcytometer. Samples were analyzed using Becton-Dickinson flowcytometer calibrated with CALIBRITE beads. The Becton-Dickinson lysis II program was used for analysis; the leucogate (CD45+CD14) fluorescent information, with forward and side scatter, was used to set an electronic gate around the lymphoid population. These methods which are standard for flowcytometry have previously been described (19). The sample was run with the positive control (CD45 and CD14 combination) first, followed by the FITC-stained negative isotype matched control and sample. For each sample 10,000 gated events were analysed.

Statistical methods

Means and standard deviations of the percentages of each of the subsets were estimated. These were compared using paired t-test. For non-normally distributed variables, log transformations were applied to the data. The differences between each pair of estimation on a sample at the beginning and end of a 24 h or 48 h interval for which effect of storage was assessed. Mean and standard deviations of these paired differences were calculated for each of the subsets.

RESULTS

We found that storage for 24 h before staining at room temperature (38-45^{0}C), resulted in small reduction in T. Cell subsets (2.3-2.9%), the B-cells (0.7%) and the CD4/CD8 ratio being least affected (0.05%) (Table 1) and the differences were not statistically significant. Storage after staining for 24 h and 48 h before analysis resulted in small, statistically non-significant reductions in T. Cell subsets (0.2-2.5%) and B-Cells (0.6%), as compared to samples processed within 1-2 h of staining. The differences were greater at 48 h than at 24h (Table II). Cell losses with storage for 24 h after staining resulted in values closer to the values obtained with storage for 1-2 h before staining, while losses were lesser than storage for 24 h before staining.

DISCUSSION

These results show that even during summer months in tropical setting the storage of a sample for 24 h at room temperature (38-45^{0}C) before staining gives acceptable results with insignificant losses of cell surface markers than estimation on fresh specimens, and storage at 4^{0}C for 24 h after staining is associated with even lower losses in cell markers. These data suggest that samples collected in field or distant locations can be analysed the next day, if they are stained in the evening of the day of collection.

We selected EDTA as an anticoagulant for blood collection as it allows both hematologic and flow cytometric estimation to be performed on the same sample. Commonly used lysing procedures as well as red cell lysis is optimized when EDTA is used as an anticoagulant as compared to ACD (acid citrate dextrose). Also EDTA has been shown to

Table I. Comparison of mean percentage values and mean of difference between paired samples of lymphocyte subpopulations stained at 1-2 hrs and 24 hrs interval after blood collection

Lymphocyte subpopulation (n=10)		Period of storage between collection and staining		Paired mean difference
		1-2 h	24 h	
Lymphocytes	(CD45$^+$, CD14)	95.3 ± 5.7*	88.0 ± 16.5	7.4 ± 11.9
T cell	(CD3$^+$)	64.4 ± 8.2	61.8 ± 17.8	2.9 ± 16.8
T helper Cells	(CD3$^+$, CD4$^+$)	36.6 ± 9.8	34.3 ± 16.2	2.3 ± 8.9
T suppressor cells	(CD3$^+$, CD8$^+$)	24.8 ± 6.3	22.1 ± 4.8	2.7 ± 7.7
Ratio *H:S*	(CD4/CD8)	1.5 ± 0.7	1.5 ± 0.7	0.05 ± 0.09
B cells	(CD20$^+$)	17.2 ± 12.7	16.4 ± 7.4	-0.7 ± 8.4

* Mean ± SD.
All comparisons were statistically Not Significant
H:S Helper : Suppressor Ratio

yield better results in withstanding storage for 24 h (15). The stability of lymphocyte subsets could be increased on storage by adding holding medium to the anticoagulated blood (13), but the disadvantage of adding holding medium to the blood is that the blood must be aliquoted into a separate tube and diluted with medium under sterile conditions. This may not always be practical (15). In our study we did not use a holding medium.

Miller and Levy (14) have shown that when blood is held in EDTA during storage, proportion of T cells increases while that of B cells decreases. These effects were most evident by 96 hours. In our study changes were evident by 24 hours, storing longer than 24 hours in the pretest did not yield satisfactory results. Ruiz *et al.* (18) also found the quality of counts remain high in the samples stored after preparation for 24 hours at 4°C, however, studies by Ekong *et al.* (8) have shown that samples for T cell measurements could be conveniently held at a temperature of around 17°C for up to 48 hrs with minimum changes in the level of expression of each subset. They, however, found loss of B cells at 21°C storage for any length of time. Paxton *et al.* (16), found effect of varying temperature to be a significant factor on 24 h storage with $CD19^+$ B-cells being most sensitive to temperature. Hensleigh *et al.* (13) reported that optimal storage condition without change was 22°C only when 50% RPMI 1640 was added to whole blood. In our experiment we did have air-conditioning in the laboratory that operated for 6-8 h, however the storage was done under normal room temperature but other than that this study was performed during April-July, which are the hottest months in Delhi.

Nicholson *et al.* (12) has shown that with whole blood stored at 22°C (room temperature) for 24 h there is no change in the relative levels of T and B cells when lysed whole blood was analyzed, but a significant rise in T cells and a fall in B cells was observed when lymphocytes were separated by density gradient. Llyod *et al.* (17) using whole blood method in sheep blood have shown that delaying staining to 48 hours did not have significant effect on CD4, CD8, CD5 and B cell markers. However, storage for more than 7 days was associated with significant decrease in percentage of cells expressing CD8, T1-9 and B cell markers.

In conclusion, our results show that under conditions, such as those in an Indian research laboratory, blood collected in EDTA can be processed using whole blood lysis method, after

Table II. Mean percentage values and mean of difference between paired samples of lymphocyte subpopulation when acquired at 1-2 h, 24 h and 48 h after staining

Lymphocyte subpopulation (n=6)	Duration of storage at +4°C before analysis				
	1-2 h (%)	24 h (%)	48 h (%)	Paired mean difference	
				0-24 hrs.	0-48 hrs.
Lymphocytes ($CD45^+$, $CD14^-$)	95.4 ± 4.1*	93.5 ± 8.0	90.0 ± 7.8	1.9 ± 6.2	5.4 ± 4.1
T cell ($CD3^+$)	66.5 ± 7.0	66.1 ± 7.7	60.3 ± 6.0	1.3 ± 2.2	5.9 ± 5.9
T helper cells ($CD3^+$, $CD4^+$)	36.9 ± 7.6	36.6 ± 8.3	32.0 ± 2.8	0.2 ± 1.4	4.7 ± 3.6
T suppressor cells ($CD3^+$, $CD8^+$)	26.9 ± 6.4	24.3 ± 4.9	24.4 ± 3.8	2.5 ± 2.3	2.6 ± 3.6
Ratio H:S (CD4/CD8)	1.4 ± 0.6	1.5 ± 0.7	1.5 ± 0.1	-0.1 ± 0.1	0.05 ± 0.3
B cell ($CD20^+$)	17.7 ± 10.2	17.2 ± 11.3	18.4 ± 11.3	0.6 ± 4.0	-0.7 ± 1.4

* Mean ± SD.
All comparisons were statistically Not Significant
H:S Helper : Suppressor Ratio

storage for 24 h at room temperature (38-45°C) with some but not significant loss in lymphocytes or T-cell subsets. Storage at 4°C after staining for 24 h results in a lesser loss of cells or T-cell subsets. These findings may help in reducing the effect of variations in ambient temperatures during storage in laboratories that do not have air-conditioning.

ACKNOWLEDGMENTS

The authors thankfully acknowledge Dr. I. Nath and Dr. Malavia for permitting them to use their flow cytometer facility, Financial support from: WHO-CDR, Thrasher Research Fund and the core support from ICMR is also acknowledged.

REFERENCES

1. Ekong, T., Hill, A.M., Gomples, M., Brown, A. and Pinching, A.J. (1992) The effect of the temperature and duration of sample storage on the measurement of lymphocyte subpopulations from HIV-I-Positive and control subjects. J. Immunol. Methods 151 : 217-225.
2. Thornthwaite, J.T., Rosenthal, P.K., Vasquez, D.A. and Seckinger, D. (1984) The effects of anticoagulant and temperature on the measurement of helper and supresser cells. Diagn. Immunol. 2 : 167-174.
3. Reinherz, E.L., Kung, P.Z., Goldstein, G. and Schlossman, S.F. (1979) A monoclonal antibody with selective reactivity with functionally mature human thymocytes and all peripheral human T cells. J. Immunol. 123 : 1312-1317.
4. Thomas, Y., Sosman, J., Irigoyen, O., Friedman, S.M., Kung, P.C., Goldstein, G. and Chess, L. (1980) Expression of cell surface markers after human B lymphocyte activation. Immunol. 125 : 2402-2408.
5. Stashenko P., Nadler, L.M., Hardy, R. and Schlossman, S.F. (1981) Expression of cell surface markers after human B lymphocyte activation. Proc. Natl. Acd. Sci. 78 : 3848-3852.
6. Liu, C., Muirhead, K.A., George, S.P. and Landay, A.L. (1989) Flow cytometric monitoring of HIV-infected patients: Simultaneous enumeration of five lymphocyte subsets. Am. J. Clin. Pathol. 92 : 721-728.
7. Prince, H. and Jensen, E. (1991) Three colour cytofluorometric analysis of Cd8 cell substes in HIV-1 infection. J. Acquir. Immuno. Defic. Synd. 4 : 1227-1232.
8. Ekong, T., Hill, A.M., Clark, C., Alun, D. and Pinching, A.J.P. (1993) Technical influences on immunophenotyping by flow cytometry the effect of time and temperature of storage on the viability of lymphocyte subsets. J. Immunol. Methods. 164 : 263-273.
9. Shield, C.F., Martlett, P. and Smith, A. (1983) Stability of human lymphocyte differentiation antigens when stored at room temperature. J. Immunol. Methods. 62 : 347.
10. Bongers, V. and Bertrams, J. (1984) The influence of common variables on T cell subset analysis by monoclonal antibodies. J. Immunol. Methods. 67 (2) : 243-253.
11. Parker, J.W., Adelsberg, B., Azen. S.P., Boone, D., Fletcher, M.A., Gjerset, G.F., *et al.* (1990) Leucocyte immunophenotyping by flowcytometry in a multisite study: standardisation, quality control and normal values in the transfusion safety study. Clin. Immunal. Immunopathol. 55 : 187-220.
12. Nicholson, J.K.A., Jones, B.M. and Cross, D. (1984) Comparison of T and B cell analysis on fresh and aged blood. J. Immunol. Methods. 73 : 29-40.
13. Hensleigh, P.A., Waters, V.B. and Herzenberg, L.A. (1993) Human T lymphocyte differentiation antigens. Effects of blood sample storage of Leu antibody binding. Cytometry. 3 : 453-455.
14. Miller, C.H. and Levy, N.B. (1989) Effects of storage conditions on lymphocyte phenotypes from healthy and diseased persons. J. Clin. Lab. Anal. 3 : 296-300.
15. Nicholson, J.K.A. and Green, T.A. (1993) Selection of anticoagulants for lymphocyte immunophenotyping. J. Immunol. Methods. 165 : 31-35.
16. Paxton, H. and Bendele, T. (1993) Effect of Time, Temperature and Anticoagulant on Flow Cytometry and Hematological values. Annals of New York Academy of Sciences 677 : 440-443.
17. Lloyd, J.B., Gill, H.S. and Husband, A.J. (1995) The effect of storage on immuno-phenotyping of sheep peripheral blood lymphocytes by flow cytometry. Vet. Immunol. Immunopathol. 47 : 135-142.
18. Ruiz, A.R., Fernandez, I.P., Perea Baena, J.M., de La Torre, F.J. and Ramirez, G.R. (2000) Influence of sample storage time and temperature on lymphocyte subset count using a FACS count system. Haematologica. 85 : 550-551.
19. Jalla, S., Sazawal, S., Deb, S., Black, R.E. and Bhan, M.K. (2002) Modifications in flow cytometric estimation of T cells subsets and B cells in peripheral blood to reduce the cost of investigation. Indian Journal of Clinical Biochemistry. 17 (1) : 69-74.