MODIFICATIONS IN FLOW CYTOMETRIC ESTIMATION OF T CELL SUBSETS AND B CELLS IN PERIPHERAL BLOOD TO REDUCE THE COST OF INVESTIGATION

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ABSTRACT

The development of monoclonal antibodies combined with flow cytometry has revolutionized the analysis of lymphocyte subsets. These newer methods using the Q-prep leucocyte preparation system require only 1-2 ml of blood as compared to 10 ml required traditionally. One of the main impediments in the use of this superior technology in Indian laboratories has been the high cost of reagents. This study evaluated methods to reduce the cost of assays. In the first experiment from 26 healthy subjects, 2ml venous blood samples in EDTA (ethylenediamine tetra-acetate) were obtained. Each sample was divided into two equal portions, one portion was stained using diluted monoclonal antibody, whereas the other portion was stained using standard concentrations of antibodies. In the second experiment, blood samples from 12 subjects were again divided into 2 portions; one portion of each pair was processed using commercial Q-prep reagents while the other portion was processed using our own reagents. In the first experiment, which evaluated use of a diluted antibody against the standard recommended concentrations, a 5-tube panel that estimated CD3, CD4, CD8, CD20 was used. In the second experiment CD3, CD4 and CD8 were estimated. The total cost per sample for a 5-panel estimation was however reduced from \$39.11 to \$1.10.

Given the proven advantages of using a whole blood stain-lyse method for T cell subset estimations, its use should be encouraged in developing country settings. With the suggested methods the whole blood Q-prep could be performed at appreciably reduced costs, without loss in precision.

KEY WORDS :

Flow cytometry, lymphocyte subsets, cost reduction, immunity

INTRODUCTION

The study of sub populations of T cells in peripheral blood by immuno phenotyping is helpful in evaluation of various malignancies of the hematological system, as well as in assessment of cellular immune functions. With a number of monoclonal antibodies directed against cell surface antigens of human lymphocyte subsets becoming available, quantitative immunofluorescence

analysis using commercial monoclonal antibodies and flow cytometry techniques are increasingly used in a variety of settings for immunological research and clinical diagnostic investigations. These methods have the advantage of being considerably less cumbersome and more accurate than previously used methods (1,2). The recent development of Q-prep leucocyte preparation system (Q-prep EPICS, Coulter Diagnostics, Hialeah, FL, USA), which uses 1-2 ml of whole blood (compared to 10 ml necessary for previous methods), has made the technique more feasible, especially evaluations in children.

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The main impediment for employment of this technology in developing country settings is the cost of reagents. Even in Institutions like All India Institute of Medical Sciences, where flow-cytometers are available, most investigators cannot afford the cost of lymphocyte subset estimation, using monoclonal antibodies for staining and Q-prep leucocyte preparation system for processing the sample.

We have standardized assays using diluted monoclonal antibodies and self-prepared Q-prep reagents that can appreciably reduce the cost of the estimations. In this paper, we evaluate these methods against the standard concentration of antibodies and use of commercial Q-prep reagents.

MATERIALS AND METHODS

Experimental design

We performed two sets of experiments. In the first experiment, 2ml venous blood samples in EDTA (ethylenediamine tetra-acetate) were obtained from 26 apparently healthy students and technical staff from April-July 1994. We collected blood from 2 subjects every day at 10 a.m. and processed it within 1 hour of blood drawing. Each sample was divided into two equal portions and processed in parallel using two procedures. One portion was stained using diluted monoclonal antibody (Table 1), whereas the other portion was stained using the standard concentration (20 μ l / 100 μ l of whole blood) of monoclonal antibody. In the second experiment, we collected blood from 12 subjects. Again, each blood sample was divided into 2 portions. Staining and processing was similar (using diluted monoclonals), but one portion of each pair was processed using commercial Q-prep reagents, while the other was processed using our own reagents.

Sample staining

Samples were processed using whole blood lysis method (3). 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 the greatest that could be used with optimal results during pretesting.

In the first experiment, which evaluated use of the diluted antibody against standard recommended concentrations, a 5-tube panel of antibodies was selected for processing each sample to estimate lymphocyte subsets (CD3, CD4, CD8, CD20). The panel selected consisted of simultaneous labelling of two different molecules on one cell with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). In first tube (leucogate reagent for electronic gating), FITC(CD45) + PE(CD14) was used, the second tube had unstained cells as negative control, third had (helper/inducer T cells) FITC (CD3) + PE(CD4), fourth had (cytotoxic/suppressor T cells) FITC(CD3) + PE(CD8) and the last tube had (B cells) FITC (CD20). We used Becton Dickinson immunocytometry monoclonal antibodies. For each sample two pairs of 5 tubes each were prepared. In the 5 tube panel using standard concentration of monoclonals, 10 μ l of each of the two monoclonals (20 μ l/tube) were added. For the 5 tube panel using diluted monoclonals, 20 μ l each of CD45 and CD3, 10 μ l each of CD14, CD4 and CD8 and 5 μ l of CD20 were used. After adding 100 μ l of blood to the monoclonal antibody combination, each tube was gently vortexed for 15 seconds. The tubes were then incubated for 20-25 minutes in a dark space at room temperature. After incubation, the erythrocytes were lysed by an automated lysing instrument (the Q-prep EPICS work station, Coulter cytometry Hiatch FL), which performs a three step procedure of preparing lymphocytes for analysis. Q-prep leucocyte 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 leucocyte preparation, reduces non specific binding without need for centrifugation and cell loss associated with it. The process essentially consists of 3 steps, each step lasting 35 seconds.

The standard kit consists of 3 reagents labelled A, B, and C. In the first step, reagent A is mixed with the whole blood sample, as a result red cell lysis takes place while maintaining cellular integrity and morphology of leucocyte. In the second step, reagent B is mixed with lysed sample which buffers the solution and stops lysis process. In the last step, reagent C is mixed with the buffered solution which fixes the cells. For this experiment, we used the standard Q-prep reagents (Coulter cytometry Hiatch FL). The samples after this step were stored at 4°C until they were analysed. The

analysis was performed the same day. The technician performing the analysis was masked to the identity of tubes as regards the use of diluted or undiluted antibodies.

Assessment of reagents for the Q-prep

The second experiment evaluated our own verses commercial Q-prep reagents. A staining procedure similar to the first experiment was used except that the panel consisted of 3 tubes; the first tube was a negative control, the second had FITC (CD3) + PE(CD4) and the third had FITC (CD3) + PE (CD8). In this experiment, the diluted antibodies as described in first experiment were used, 20 µl of CD3 and 10µl of CD4 or CD8 were added to the respective tubes. Six tubes (2 pairs of 3 tube panels) were set up for each sample. One tube of each pair was processed using the commercial Q-prep, whereas the other tube of each pair was processed using own reagents. Three reagents were prepared in our laboratory, reagent A (70 ml) - contained formic acid (1.2 ml/l), reagent B (32 ml) contained sodium carbonate (6.6 g/l), sodium chloride (14.5 g/l), sodium sulphate (31.3 g/l), and reagent C (14ml) contained paraformaldehyde (10.0 g/l). These reagents were then respectively filtered through 0.2-micron millipore filter and stored at room temperature. The reagents were freshly prepared every three days. The self-prepared reagents were poured into empty vials of the commercial reagents and these were then used in the Q-prep station in similar fashion as the commercial reagents. The technician was masked to the identity of the reagents. At the time of switching the reagents, 3 blanks were run to clean the tubing.

Analysis of samples

Samples were analysed using Becton-Dickinson flowcytometer calibrated with CALIBRITE beads. The Becton-Dickinson Lysis II programme 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. The first tube of the five tube panel served the purpose. This gate included at least 95% lymphocytes and less than 5% non lymphocytes (granulocytes, monocytes and debris). Quadrant markers were set using negative controls (unstained cells) to define the limits of combined effect of auto fluorescence and non-specific staining. For single color analysis, the

FL1-PMT level was set using FITC-stained negative isotype matched control which was not PI-stained in order to define the limits of non-specific staining for FITC-stained samples. The FL2-PMT level was set using a positively stained PI control (dead cells), which was not FITC-stained. Finally, the instrument was compensated using two separate positive controls for each color. This ensured that there was no spectral overlap between FITC and PI in the samples and that PI-stained (dead or dying)

FITC⁺ cells in the second quadrant, could be distinguished from viable (PI-negative FITC⁺ cell) in the fourth quadrant. Having established the readiness of the instrument, the sample was run with the positive control (CD45 and CD14 combination) first, followed by the negative control and patient sample. For each sample 10,000 gated events were analysed per sample. The senior technician operating the flowcytometer was blinded to the antibody strength or Q-prep reagent type for a given sample.

Statistical methods

Means and standard deviations for percentages of each of the subsets were estimated. These were compared using paired 't-test'. For non-normally distributed variables, log transformations were applied. The difference between estimates of each sample with and without dilution or with two sets of reagents was estimated. Mean and standard deviations of these paired differences were calculated for each of the subsets. Further results using diluted and undiluted monoclonals were evaluated by estimating a paired correlation coefficient. Statistical analysis was performed using SPSSPC ver 6.1.

RESULTS

The mean paired difference between diluted and undiluted monoclonal varied between -1.7% for total lymphocytes to 1.4% for CD8 subset (Table 1). These results show that the values of lymphocyte subsets are not very different when the whole blood sample is stained with diluted monoclonal antibodies as compared to antibodies in standard concentration. The difference was the least for CD4/CD8 ratio. None of these differences were statistically or clinically significant. The correlation coefficient for paired comparisons between undiluted and diluted antibody was 0.71

for %CD3, 0.90 for %CD4, 0.77 for %CD8, 0.93 for CD4/CD8 ratio and 0.98 for %CD20.

The mean percentage values and differences between estimates of %CD3, %CD4, and %CD8 using our own reagents and commercial Q-prep reagents are presented in Table 2. There were no significant differences in the mean %CD3, %CD4 and %CD8.

DISCUSSION

This study indicates that, diluted antibody and self prepared reagents performed as well as standard concentrations of monoclonals and commercial Q-prep reagent for estimation of T-cell subsets. The use of proposed methods, however, result in a substantial cost saving for the given assays.

The development of monoclonal antibodies combined with flow cytometry has revolutionized the analysis of lymphocyte subsets (4). Traditional immunophenotyping methods using lymphocyte separation required 10 ml of blood. Techniques using whole blood refined in the last 10 years, require only 1-2 ml of blood (5). The whole blood lysis methods are now recommended because they result in a cell population similar to that found in vivo (6). The Q-prep leucocyte preparation system is considered better than Lyse-stain or Ficoll separation methods (7). Use of the blood lysis method further helps in elimination of cell losses induced during washing and also reduces processing time. The only impediment to the use of this superior technology in Indian laboratories has been the high cost of reagents. Use of methods reported in this paper should eliminate this impediment.

The use of the suggested dilutions would reduce the cost of monoclonals for analysing one blood sample, with a panel of 5 antibody

combinations, from \$14.11 to \$0.60. In addition, the \$25.00 cost for preparation of samples by Q-prep reagents, is reduced to about \$0.50 with the use of self prepared reagents. The total cost per sample for a 5-panel estimation would thus be \$1.10 instead of \$39.11. Although many laboratories do use monoclonal antibodies in dilution, we could not locate any published evaluation of these methods. Use of standardized validated procedures and publication of such results is important and could save other experienced investigators considerable time and expense and provide better compatibility of studies in different laboratories. It needs, however, to be emphasized that there may be variations in concentration of monoclonal antibody probes from kit batch to batch. Investigators need to confirm and work out the possible optimal dilutions maintaining the precision for each batch.

These results need to be further confirmed with very low lymphocyte counts such as may be found in patients with AIDS. Also we only used a 3-panel test for evaluation of reagents for the Q-prep and cannot comment about its performance for estimation of other sub-sets.

In conclusion, the preferred whole blood stain-lyse method for T-cell subset estimations should be encouraged in developing country settings, since estimations can be performed at an appreciably reduced cost, without loss of precision using the proposed methods.

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TABLE 1

Mean percentage values and mean of difference between paired samples of lymphocyte subpopulation : paired comparison of two concentrations of monoclonal antibodies.

Lymphocyte subpopulation	Values using standard concentration (n=26)	Dilution Factors	Values using diluted Monoclonal (%) (n=26)	Paired mean difference
Lymphocytes (CD45 ⁺ , CD14 ⁻)	92.6 ± 6.0	1:10	94.1 ± 6.2	1.7 ± 1.1
T cells (CD3 ⁺)	64.7 ± 6.8	1:20	66.1 ± 6.0	1.2 ± 1.4
T helper cells (CD3 ⁺ , CD4 ⁺)	35.6 ± 6.6	1:20	36.1 ± 8.0	0.5 ± 0.6
T suppressor cells (CD3 ⁺ , CD8 ⁺)	24.9 ± 5.2	1:20	23.5 ± 4.4	-1.4 ± 0.6
Ratio H:S (CD4/CD8)	1.4 ± 0.5	—	1.5 ± 0.5	0.08 ± 0.03
B cells (CD20 ⁺)	12.5 ± 9.6	1:5	11.8 ± 9.2	-0.78 ± 0.3

All comparisons were statistically Not Significant

TABLE 2

Mean percentage values of lymphocyte subpopulation : Paired comparison using self and commercial Q-prep for sample processing.

Monoclonal Antibody (n=12)	Type of Q-prep reagents used		
	Commercial (Coulter)	Self-prepared	Paired mean difference
T cells (CD3 ⁺)	56.3 ± 8.2	54.7 ± 7.2	-1.6 ± 5.0
T helper cells (CD3 ⁺ , CD4 ⁺)	30.5 ± 6.8	27.5 ± 5.3	-3.1 ± 4.7
T suppressor cells (CD3 ⁺ , CD8 ⁺)	23.6 ± 4.4	23.1 ± 4.7	0.5 ± 2.6

All comparisons were statistically Not Significant

Correlation CD3 80.0 p=0.003; CD4 72.3 p=0.012; CD8 83.6 p= 0.001.

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