ORIGINAL ARTICLE

Comparison of Three Different Staining Methods for Lymphocyte Subset Analysis by Flow Cytometry

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ABSTRACT

Introduction: Only certain clinical laboratories offer lymphocyte subset immunophenotyping. Blood samples are typically collected at one site and sent to another for analysis. A late sample may delay processing until the next day, requiring overnight storage. Limited data exist regarding the impact of lymphocyte subsets when various staining preparation methods are utilised interchangeably, depending on sample availability. We aim to evaluate the variations in lymphocyte subset enumeration using three different staining procedures. **Methods:** Fifteen millilitres of peripheral blood were collected from 35 healthy adults. The blood was preserved in an ethylenediamine tetraacetic acid (EDTA) tube for isolating peripheral blood mononuclear cells (PBMC), lyse-wash (LW), and lyse-no-wash (LNW) methods. Lymphocyte subset enumeration was performed using flow cytometry, utilising PBMC, LW, and LNW methods. A comparative analysis of the lymphocyte subset enumeration was performed. **Results:** The three staining methods revealed significant variations in the mean percentages of T cells (CD3), B cells (CD19), and NK cells (CD56). In contrast, the mean percentages of helper T cells (CD4), naïve CD4 (CD45 RA), memory CD4 (CD45RO), cytotoxic T cells (CD8), naïve CD8 (CD45 RA), and memory CD8 (CD45 RO) displayed insignificant changes. **Conclusion:** The various staining preparation methods can be used interchangeably in an experiment to enumerate helper T cells, naïve CD4, memory CD4, cytotoxic T cells, naïve CD8, and memory CD8.
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INTRODUCTION

Many clinical laboratories do not offer lymphocyte subset enumeration by flow cytometry, so specimens must be sent to specialised reference laboratories. In clinical practice, it is common for blood samples to be collected at one site and transported to another for analysis. Specimens collected from the field site may arrive at the laboratory late in the day, making same-day processing impractical and necessitating overnight storage. Limited equipment availability among multiple users and shared access may also cause delays in specimen analysis, even when the test is performed in-house.

The peripheral blood mononuclear cells (PBMC) are more suitable for storing and analysing samples later. However, if a sample arrives early, the lyse-wash (LW) or lyse-no-wash (LNW) procedure is typically employed. Currently, a shift towards using the LW or LNW staining preparation method for analysis has occurred, given its simpler procedure compared to PBMC in the past. Nevertheless, when samples arrive late in the evening, there arises a need to store and process them the following day or later. Changes resulting from the staining preparation process and delays in preparation may impact lymphocyte enumeration.

Therefore, it is crucial to determine the impact of three distinct staining procedures on lymphocyte subset enumeration. The three staining preparation techniques include PBMC, which utilises density gradient centrifugation, LW and LNW of whole blood, which employ a lysing reagent to lyse the red blood cells (RBC). Isolating high-purity PBMC is straightforward but time-consuming, involving multiple steps. LNW effectively

maintains the cells during analysis; however, the lack of washing can result in high background fluorescence, potentially leading to a prolonged acquisition process. LW effectively removes RBC, facilitating the isolation and evaluation of white blood cells; however, it may cause cell loss during the washing steps.

Comparing the lymphocyte subsets using these three distinct staining preparation methods highlights the impact of each technique on the results. This study aims to determine whether there are any variabilities in lymphocyte subset enumeration using all three staining methods. If the results demonstrate no variability, these staining methods can be used interchangeably within an experiment.

MATERIALS AND METHODS

This experimental, laboratory-based study collected peripheral blood samples from 35 healthy individuals, consisting of healthy adults who met the inclusion criteria and volunteered to participate. The sample size was calculated using Hulley et al.'s formulation $N = [(Z\alpha + Z\beta)/c]2 + 3$, which suggested a minimum of 30 samples for clinical laboratory-based research (1). All participants were healthy adults with no chronic diseases, free from infections, not undergoing any inflammation-inducing procedures, and not taking any medications. The blood samples were stored at room temperature and processed within four hours of collection. A full blood count analysis was performed using a Sysmex automated haematology analyser (Sysmex Corporation, Japan). All the participants had a normal full blood count, including a normal lymphocyte count.

For the isolation of PBMC, LW and LNW procedures, approximately 15 ml of peripheral blood was collected from each participant, stored in an ethylenediamine tetraacetic acid (EDTA) tube, and kept at room temperature before analysis. Each sample was run three times a day, near-consecutively, for each preparation method (LW, LNW, and PBMC) to obtain the mean percentage values for lymphocyte subset enumeration. The enumeration of lymphocyte subsets obtained from the three staining methods was compared.

Lyse Wash (LW)

20 µL of monoclonal antibodies (BD Bioscience, USA) were added to 100 µl of whole blood. The tube was vortexed and incubated in the dark at room temperature (20° to 25°C) for 15 minutes. Subsequently, 2 ml of 1x FACS lysing solution (BD Bioscience, USA) was added, vortexed, and incubated under the same conditions for 10 minutes. After incubation, the sample was centrifuged at 500 x g for 5 minutes, after which the supernatant was removed. Then, 2 ml of PBS buffer (Cellgro Mediatech, USA) was added and centrifuged at 500 x g, then the supernatant was discarded. Finally, 0.5 ml of stain buffer was added, mixed thoroughly, and subjected to flow cytometric analysis.

Lyse No Wash (LNW)

To 50 µL of whole blood, 20 µL of monoclonal antibodies (BD Bioscience, USA) were added. The tube was vortexed and incubated at room temperature for 15 minutes. Subsequently, 450 µL of lysing solution (BD Bioscience, USA) was added, thoroughly mixed, and subjected to flow cytometric analysis.

Peripheral blood mononuclear cells (PBMC) isolation.

10 ml of blood was carefully layered over Ficoll-Hypaque TM PLUS (GE Healthcare, UK). The sample was centrifuged at 2000 rpm for 20 minutes at room temperature. The buffy coat was aspirated and transferred into a new tube. Subsequently, 10 ml of PBS (GE Healthcare, UK) was added for washing, followed by centrifugation at 2000 rpm for 10 minutes at room temperature. After removal of the supernatant, the cell pellet was gently resuspended in 1 ml of PBS. The cell concentration was adjusted to 1×10^7 cells/ml.

Immunophenotyping markers/sample processing

The markers incorporated in the panel were selected to identify the lymphocyte lineage in the blood (B cells, T cells and NK cells). The antibodies used were CD45 as the leukogate with forward and side scatter, CD3 (T cell), CD4 (T helper cell), CD45RA (Naıve CD4), CD45RO (Memory CD4), CD8, CD45RA (Naıve CD8), CD45RO (Memory CD8), CD19 (B cell) and CD56 (NK cell). These methods, which are standard for flow cytometry, have previously been described (2). All the antibodies were purchased from BD Pharmingen, USA. The data were analysed using the FACSDiva analysis software in the FACS Canto II (BD Biosciences) flow cytometry.

Statistical Analysis

The data were analysed using SPSS Statistics v27 (IBM Corporation, USA). The means and standard deviations of each subset's percentages were determined. The mean differences of the three methods were determined using the coefficient of variation and one-way ANOVA.

RESULTS

Participants characteristics

The participants' ages ranged from 18 to 40. The majority were in the 26 to 35 age group, consisting of 19 participants (54.3%). Ten participants (28.6%) were aged 18 to 25, while six (17.1%) were over 35. There were more males, with 19 participants (54.3%), than females, who made up 16 (45.7%) (Table 1).

Table I: The characteristics of participants (n=35)

	Frequency(n=35)	Percentage %
Age group		
18-25	10	28.6
26-35	19	54.3
>35	6	17.1
Gender		
Male	19	54.3
Female	16	45.7
Race		
Malay	14	40
Chines	2	5.7
Indian	3	8.7
Others	16	45.7

A significant difference was observed in the enumeration of T cells (CD3), B cells (CD19), and NK cells (CD56) across the three staining methods. The percentage of CD3 was highest when using the PBMC method compared to the LW and LNW methods. In contrast, the percentages of CD19 and CD56 were lowest when the PBMC method was used.
Helper T cells (CD4), naıve CD4 (CD45RA), memory CD4 (CD45RO), cytotoxic T cells (CD8), naıve CD8 (CD45RA), and memory CD8 (CD45RO) did not show any significant changes. The absence of a significant difference indicates that all three staining methods can be used interchangeably to determine these parameters.

DISCUSSION

Reliable and accurate assessment of lymphocyte subsets is essential for evaluating initial immune status, managing HIV-infected patients, predicting outcomes for those with metastatic melanoma undergoing chemoimmunotherapy, addressing post-transplant lymphoproliferative disorders, and diagnosing acute leukaemia and chronic lymphoproliferative disorders.
The accuracy of lymphocyte subset measurements depends on proper procedure validation, standardisation, and the quality of the blood sample. Delays in reaching the testing laboratory and issues related to transport and storage conditions could impact the results.

Various biological and technical factors influence the analysis of lymphocyte subsets (3). Most previous studies evaluate the effects of storage, different anticoagulants, and varying temperatures (3,4,5). Results may also be influenced by sample handling, cell stabilisers, laboratory practices, and various instruments (4). Pre-analytical methods should be reassessed, considering the latest advancements in flow cytometry, reagents, and instruments for immunophenotyping.

Limited information exists regarding the effects of lymphocyte subsets using three different staining methods simultaneously. Therefore, our study aimed to evaluate the enumeration of lymphocyte subsets employing these three staining methods at once.

Significant difference in the lymphocyte subset enumeration

As indicated by our results, significant differences in the mean values were observed among the three staining methods for CD3 (T cells), CD19 (B cells), and CD56 (NK cells). The mean percentages showed a significant difference when comparing the PBMC method to the LW and LNW methods (Table 2). The PBMC method demonstrated the highest percentage of T cells compared to the LW and LNW methods. Conversely, the LW staining method showed the highest enumeration percentage for B and NK cells.

Table II: The differences in the mean of the extended lymphocyte subset using PBMC, LW and LNW

Extended lymphocyte	PBMC	LW	LNW	
subset	Mean % (SD)	Mean (SD)	Mean (SD)	p value
T cell (CD3)	70.2(7.04)	65.2(7.2)	64.3(7.3)	<0.001*
Helper T cell (CD4)	50.4(8.6)	49.3(8.6)	48.5(8.6)	0.17
Naıve CD4 (CD45 RA)	50.5(8.5)	51.5(8.6)	52.5(8.6)	0.24
Memory CD4(CD45 RO)	43.0(7.6)	40.0(7.6)	39.1(7.6)	0.17
Cytotoxic CD4 (CD8)	28.6(5.0)	27.6(6.5)	26.6(6.5)	0.09
Naıve CD8 (CD45 RO)	53.0(9.6)	52.0(9.6)	51.1(9.7)	0.33
Memory CD8 (CD45RO)	38.9(6.6)	38.1(6.7)	37.0(6.7)	0.12
B cell (CD19)	19.7(3.4)	23.7(5.1)	22.7(4.9)	0.02*
NK cell (CD56)	18.9(3.5)	25.8(5.3)	21.9(5.1)	0.02*

*Significance p value, p<0.05

The interchange between the staining methods in an experiment for these three lymphocyte subsets was shown to be highly variable. Therefore, to measure CD3, CD19, and CD56, it is essential to maintain a consistent single-staining method throughout the experiments. Altering staining methods during a single experiment is not advisable, as this indicates that the values of the lymphocyte subsets were significantly different.

The most probable cause of the variation was differences in sample preparation techniques, leading to variable cell losses (6). The choice of staining method significantly influenced the measurements of these parameters. The method used to obtain the lymphocyte subsets, whether PBMC, LW, or LNW, requires careful consideration due to their impact on cell yield, viability, and functional integrity.

Nicholson et al. have shown that while the T and B cells remained stable in the LW method, a significant increase in T cells and a decrease in B cells were observed when PBMC was used (7). Again, their experiment utilised stored blood for 24 hours at room temperature, and they did not investigate whether the staining methods could be used interchangeably (7).

Other researchers found lower CD3, CD4, and CD8 counts using PBMC, with no significant difference between those obtained using LNW and LW methods (8). Our results indicated that CD19 and NK cell enumeration were significantly lower in PBMC compared to the LNW and LW methods, but not for CD4 and CD8. Conversely, we observed that CD3 was significantly higher in PBMC than in the LW and LNW methods.

Lymphocyte isolation using Ficoll-Hypaque can alter specific subsets and properties (9). During centrifugation, mononuclear cells separate from granulocytes and erythrocytes based on density, Ficoll-Hypaque characteristics, and the natural aggregation of red blood cells. Some researchers discourage the use of PBMCS due to the risk of selective cell loss (6). Two possible explanations for the lower percentage of CD19 and NK cells might be their higher density compared to other cells or their tendency to form aggregates with red blood cells, leading to precipitation during centrifugation and cell loss.

According to Lao et al., storage and transportation influence the percentages of CD3, CD4, CD8, B cells, NK cells, and HLA-DR, particularly if the blood sample is delayed for more than 24 hours before arriving at the laboratory (4).

No significant difference in the lymphocyte subset enumeration

However, we found no significant differences in the enumeration of CD4 T helper cells, naïve CD4 cells, memory CD4 cells, CD8 cells, naïve CD8 cells, and memory CD8 cells (Table 2). The absence of significant differences in these lymphocyte subsets suggests that all staining methods can be used interchangeably within an experiment. In certain instances, when sending specimens from remote locations, blood may need to be stored for short periods before staining, thus necessitating a change in the preparation methods. Even though the staining methods may be changed, adhering to one procedure throughout the experiment is still preferable to minimise variability.

Utilising validated procedures is crucial, as they can save time and resources while ensuring better compatibility across studies conducted in different laboratories. Before being undertaken, these results must be further confirmed, and the optimal method must be determined in individual laboratories (2). Addressing this source of variability can enhance the consistency and reliability of cell population measurements in future assay runs (10). It also highlighted the importance of interpreting results based on sample preparation and staining protocols, which may influence the outcome (10). One should consider these findings when selecting the most appropriate method for cellular analysis needs.

Lysis methods are widely preferred for erythrocyte removal due to their speed and minimal impact on white cell populations (11). Furthermore, leukocyte recovery through erythrocyte lysis is significantly higher than that achieved via density gradient separation (11). This approach typically does not affect the staining pattern of lymphoid markers and preserves white blood cell viability (11). This method enhances lymphocyte immunophenotyping by removing most granulocytes, significantly reducing sample acquisition time (11).

Our results indicated that sample preparation techniques are another factor that can influence the consistency of results. This is essential for standardising lymphocyte enumeration, as cell loss is unavoidable, particularly when centrifugation is involved. Thorough assay validation and sample preparation methods must be assessed for the variables that require optimisation (12).

CONCLUSION

This study suggests that the staining preparation method for certain lymphocyte subsets can be interchanged within an experiment, as it did not show any significant changes in the mean percentages of CD4 T helper cells, naïve CD4 cells, memory CD4 cells, CD8 cells, naïve CD8 cells, and memory CD8 cells.

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