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Original Article

Time and temperature stability of T-cell subsets evaluated by a dual-platform method

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Abstract: Introduction: T-cell subset enumeration in HIV patients is routinely performed for monitoring infection stage and response to antiretroviral therapy. Studies have examined the effect of specimen refrigeration and age for single-platform (SP) methods, but there is limited data for time and temperature requirements of dual-platform (DP) methods. Methods: Using a DP method, we analyzed peripheral blood (PB) from 52 HIV patients at room temperature (RT) at 24, 72, and 96 hours. PBs from 34 HIV patients had baseline RT analysis within 24 hours, and then were refrigerated and analyzed at 24, 48, and 72 hours. The coefficient of variation (CV) and residuals (changes in lymphocyte subsets) were recorded at each time point and compared to assess the precision and bias under the various conditions. Testing performance under different conditions was compared by linear regression. Results: Mean CV was ≤7.3% and median residuals were <30/μl for absolute CD4 and CD8 determinations. There was good correlation between baseline analysis data at RT and at various time points, both at RT and 4°C. Conclusions: Our results are similar to those published for SP methods for aging or refrigerated specimens. The high level of agreement between measurements supports the robustness of this DP methodology.

Keywords: HIV, Absolute CD4 counts, flow cytometry, dual platform, specimen stability

Introduction

The hallmark of human immunodeficiency virus (HIV) infection is the progressive loss of CD4(+) T lymphocytes [1]. Enumeration of T-cell subsets in HIV patients is essential for clinical management and directs the initiation of highly active antiretroviral therapy (HAART) or the initiation of prophylactic therapy against opportunistic infections [2]. It is therefore important that CD4 (and CD8) counts be as reliable and precise as possible. The accurate enumeration of T-cell subsets, both as absolute counts and percentage values, is crucial not only in providing care to HIV-infected individuals, but also in assessing new antiretroviral drugs and therapeutic vaccines.

The evolution of multiparameter flow cytometry has made it possible to employ various gating strategies, with the purpose of increasing analytical robustness and precision, and decreasing costs associated with these analyses [2, 3]. Furthermore, dual platform methodologies that were historically the only option available to most laboratories performing subset analysis, can now be compared with single-platform methods that bypass the requirement of a hematology analyzer for white blood cell count determinations [4, 5]. It is well known that the gating strategy and the methodology (single vs. dual platform) can have significant impact on results [6, 7]. From a practical and logistical standpoint, results can be impacted even further by the age of the specimen. Not all laboratories have the capability of receiving and analyzing fresh infectious blood specimens (within 24 hours). The "1997 Revised Guidelines for Performing CD4+ T-Cell Determinations in Person Infected with Human Immunodeficiency Virus (HIV)" issued by the Centers for Disease Control and Prevention (CDC) acknowledge this issue and recommend performing the analysis within 48 hours of the time of draw [8]. The 1997 guidelines refer to dual-platform methods and have been updated in 2003 with recommendations specifically aimed at single-

platform methods that use CD45 gating [9]. While the time interval for analysis has been extended to 72 hours in the latter CDC report, it is still recommended to perform all studies at room temperature, since specimen refrigeration may have an adverse outcome on the analysis.

Studies have examined the effect of specimen refrigeration and age for single-platform methods [10], but there is only limited data for time and temperature requirements of dual-platform methods. Since the CDC recommendations allow individual laboratories to perform stability studies for time and temperature conditions that may exceed the general guidelines, we have studied the effect of specimen age and refrigeration on T-cell subsets in a cohort of HIV patients using a dual-platform method.

Materials and methods

Blood specimens

Peripheral blood (PB) specimens from 86 HIV (+) patients were evaluated. These were patients with a previously confirmed diagnosis of HIV infection (by ELISA, Western blot and viral load PCR), and their PB specimens were routinely analyzed in our laboratory. Specimens were collected in ethylenediamine tetraacetic acid (EDTA) vacutainer tubes (BD Biosciences, San Jose, CA). Using a sterile technique, all samples were aliquoted within 4 hours of phlebotomy. PB aliquots from 52 HIV patients were stored and analyzed at room temperature (RT) at 24, 72, and 96 hours. Also, PBs from 34 HIV patients had baseline RT analysis within 24 hours, and then were stored at 4°C and immunophenotyped again at 24, 48, and 72 hours. For all time points, flow cytometric testing was performed at RT. CBC data were determined at baseline (within 24 hours from draw) on a Bayer ADVIA120 automated hematology analyzer (Siemens Healthcare Diagnostics, Deerfield, IL).

Reagents and monoclonal antibodies

We used BD MultiTest, a commercial cocktail of monoclonal antibodies, for immunophenotyping. The monoclonal antibodies were conjugated with the following fluorochromes: CD3 (clone SK -7) with fluorescein isothiocyanate (FITC); CD8 (clone SK1) with phycoerythrin (PE); CD45 (clone 2D1) with peridinin chlorophyll protein (PerCP); and CD4 (clone SK3) with allophycocyanin (APC). FACS brand lysing solution (FACSLyse) was from BD, phosphate-buffered saline (PBS) was from Bio-Rad (Hercules, CA), and formaldehyde was from Electron Microscopy Sciences (Ft. Washington, PA).

Flow cytometry immunophenotyping

An aliquot of 100 µL of whole blood was mixed with 20 mL of monoclonal antibodies and incubated for 15 minutes in the dark at RT. The red blood cells were then lysed at RT for 10 minutes with 2 mL of FACSLyse solution, and centrifuged at 1250 RPM, for 5 minutes. The specimen was then washed with 2-3 mL of PBS (at 1000 RPM, for 5 minutes), fixed with 200-400 µL of 0.5% formaldehyde, and analyzed on a FACSCalibur flow cytometer with CellQuest acquisition software (BD). For analysis, we used a bright CD45, low side scatter lymphocyte gate. Subsequent analysis of CD3 vs. CD4 and CD3 vs. CD8 plots generated the percentages of CD3(+)/CD4(+) and CD3(+)/CD8(+) lymphocytes, respectively. For calculation of absolute lymphocyte subset counts, the absolute lymphocyte count was determined as the product of the white blood cell count and lymphocyte percentage derived from the hematology analyzer, and then multiplied by the percentage of CD4(+) and CD8(+) T-cells, respectively, established by flow cytometry.

Flow cytometric quality control and quality assurance

In addition to daily equipment maintenance, instrument quality control is tracked through the daily use of FACSComp and Calibrite beads (BD). Additionally, evaluations of CD-Chex cells (Streck Laboratories, Omaha, NE) are performed daily. Our laboratory successfully participates in the College of American Pathologists (CAP) CD4% and CD4 count proficiency surveys, and is fully certified in this CAP program. The immunophenotyping strategies employed ensure several quality assurance parameters, such as verification of lymphocyte recoveries of >95% by backgating; inclusion of <2% CD3(-)/CD4(dim +) monocytes in the lymphocytes gate, resulting in a lymphocyte purity of >98%; difference between the CD3 percentage and the sum of CD3 (+)/CD4(+) and CD3(+)/CD8(+) percentages (the check-sum difference) of <5%. Specimens with unusual staining patterns or that yield unusual results (<1% of total specimens tested in our laboratory) are re-stained to confirm the original results, or stained with additional anti-

Table 1. White blood cell (WBC) count and absolute lymphocyte count (ALC) at baseline in specimens analyzed at room temperature or following refrigeration.

For specimens stored at room temperature			
T cells	n	WBC[a]	ALC
All	52	4950 (3800-6075)[b]	1895 (1543-2430)
CD4≤300/μL	20	4000 (3025-5400)	1560 (1013-1895)
CD4>300/μL	32	5200 (4500-6925)	2150 (1823-2610)
For specimens stored at 4°C			
T cells	n	WBC	ALC
All	34	5450 (4875-6625)	1945 (1195-2590)
CD4≤300/μL	12	5150 (3075-10500)	1170 (478-1418)
CD4>300/μL	22	5700 (4975-6325)	2180 (1860-2755)

[a] cells/μL; [b] median (interquartile range)

bodies, to determine the cause of these unusual findings. This includes enumeration of B and NK cell percentages, or analysis of the proportion of CD4/CD8 double positive or double negative CD3(+) T-cells. Additional steps may include prewashing the whole blood specimen to remove interfering factors that cause artifactual staining.

Statistical analysis

Statistical analyses of data were carried out in GraphPad Prism, version 5.0b (GraphPad Software, San Diego, CA); a p-value <0.05 was considered statistically significant. Differences in absolute CD4 or CD8 counts obtained at different time points were compared with baseline values using the Wilcoxon signed rank test. To assess the performance of subset measurements under different conditions, the mean, standard deviation, and coefficient of variation (CV) were calculated at RT from the 24, 72, and 96 hour aliquots, for the RT specimen aging stability. The same values were generated at RT and then at 4°C from the 24, 48, and 72 hour aliquots, for the refrigerated specimen aging stability. We also recorded the changes in lymphocyte subsets (residuals) at each time point and compared them to assess the precision and bias under the various conditions. We then performed linear regression analysis for CD4 and CD8 absolute counts and percentages, and compared the testing performance under different conditions with the baseline values obtained at RT within 24 hours.

Results

Patient characteristics

For the room temperature (RT) stability study, the patient cohort consisted of 42 men and 10 women with a median age of 42.5 years (range, 19-62 years). For the refrigeration (4°C) stability study, the patient cohort consisted of 25 men and 9 women with a median age of 43 years (range, 20-61 years). The white blood cell and absolute lymphocyte counts for the two patients groups, and separated according to CD4 strata, are summarized in **Table 1**.

Analysis of differences between absolute and percent T-cell counts in aging specimens

Median absolute CD4 counts performed on aging specimens at RT were higher at 72 hrs and 96 hrs, when compared to the baseline values (**Table 2**). Median absolute CD8 counts were essentially identical at RT and 72 hrs, and higher at 96 hrs, when compared with the baseline values (**Table 2**). Results obtained at different time points and RT are summarized in **Table 3** and **Figure 1A**. Mean CV was ≤7.3% for both absolute and percent CD4 and CD8 determinations at RT. Median residuals were <30/μL for absolute CD4 and CD8 determinations, and <2% for CD4 and CD8 percentage measurements.

Median absolute CD4 counts determined in aging specimens under refrigeration (4°C) were lower at all time points, when compared to the

Table 2. Comparison of absolute T-cell counts (cells/μL) determined in aging specimens stored at room temperature or at 4°C.

For specimens stored at room temperature					
CD4 subset			CD8 subset		
24 hrs	390 (219-534)[a]		24 hrs	1142 (808-1446)	
72 hrs	438 (246-597)	p < 0.005[b]	72 hrs	1140 (800-1514)	p = 0.49
96 hrs	436 (246-580)	p < 0.005	96 hrs	1159 (822-1509)	p = 0.03
For specimens stored at 4°C					
CD4 subset			CD8 subset		
24 hrs (RT)	422 (202-561)		24 hrs (RT)	867 (556-1305)	
24 hrs (4°C)	400 (205-530)	p < 0.005	24 hrs (4°C)	867 (541-1365)	p = 0.24
48 hrs (4°C)	403 (204-528)	p = 0.02	48 hrs (4°C)	890 (566-1421)	p < 0.005
72 hrs (4°C)	396 (204-547)	p < 0.005	72 hrs (4°C)	849 (568-1434	p < 0.005

[a] median (interquartile range); [b] Wilcoxon signed rank test (vs. baseline values)

Table 3. Comparison of measurements of CD4 and CD8 absolute counts and percentages in aging specimens at room temperature or refrigerated.

Protocol precision at room temperature (mean %CV)					
T cells	n	CD4 abs	CD8 abs	CD4%	CD8%
All	52	6.3	2.5	6.4	2.0
CD4≤300/μL	20	7.3	2.4	7.3	1.8
CD4>300/μL	32	5.6	2.6	5.8	2.2
Protocol precision at 4°C (mean %CV)					
T cells	n	CD4 abs	CD8 abs	CD4%	CD8%
All	34	4.6	2.6	3.5	1.9
CD4≤300/μL	12	5.8	3.0	4.8	2.1
CD4>300/μL	22	3.9	2.4	2.9	1.8

baseline values (**Table 2**). Median absolute CD8 counts were essentially unchanged after 24 hrs of refrigeration, were higher at 48 hrs, and lower at 72 hrs, when compared with the baseline values (**Table 2**). **Table 3** and **Figure 1B** compare results obtained at different time points and 4°C. Mean CV was ≤5.8% with absolute CD4 and CD8 determinations, and was ≤4.8% with percent CD4 and CD8 determinations at 4°C.

For specimens tested at RT, the median absolute CD4 count at 96 hours was 12% higher than at 24 hours. Therefore, 4 out of 52 (7.7%) patients that had an absolute CD4 count between 313 and 350/ μL at baseline, would have been resulted as >350/μL at 96 hours. Similarly, 1 out of 52 (1.9%) patients that had an absolute CD4 count between 446 and 500/μL at baseline, would have been resulted as >500/ μL at 96 hours. For specimens tested after refrigeration at 4°C, the median absolute CD4 count at 72 hours was 6.2% lower than at baseline (performed within 24 hours, at RT). Therefore, 2 out of 34 (5.6%) patients that had an absolute CD4 count between 350 and 372/μL at baseline, would have been resulted as <350/ μL at 72 hours.

Correlation of T-cell subset measurements in aging specimens

Comparison was established between baseline values and absolute counts obtained at later time points, either at RT or at 4°C. The values for r^2, slope, and y-intercept are presented in **Table 4** and **Figure 2**. As expected, the highest level of agreement between measurements was at earlier time points. Also, correlation coefficients were higher in measurements performed on refrigerated samples, as compared to similar time point values obtained at RT.

Discussion

In recent years, HIV lymphocyte immunophenotyping has evolved significantly. New fluorochromes and multicolor reagents, enhanced

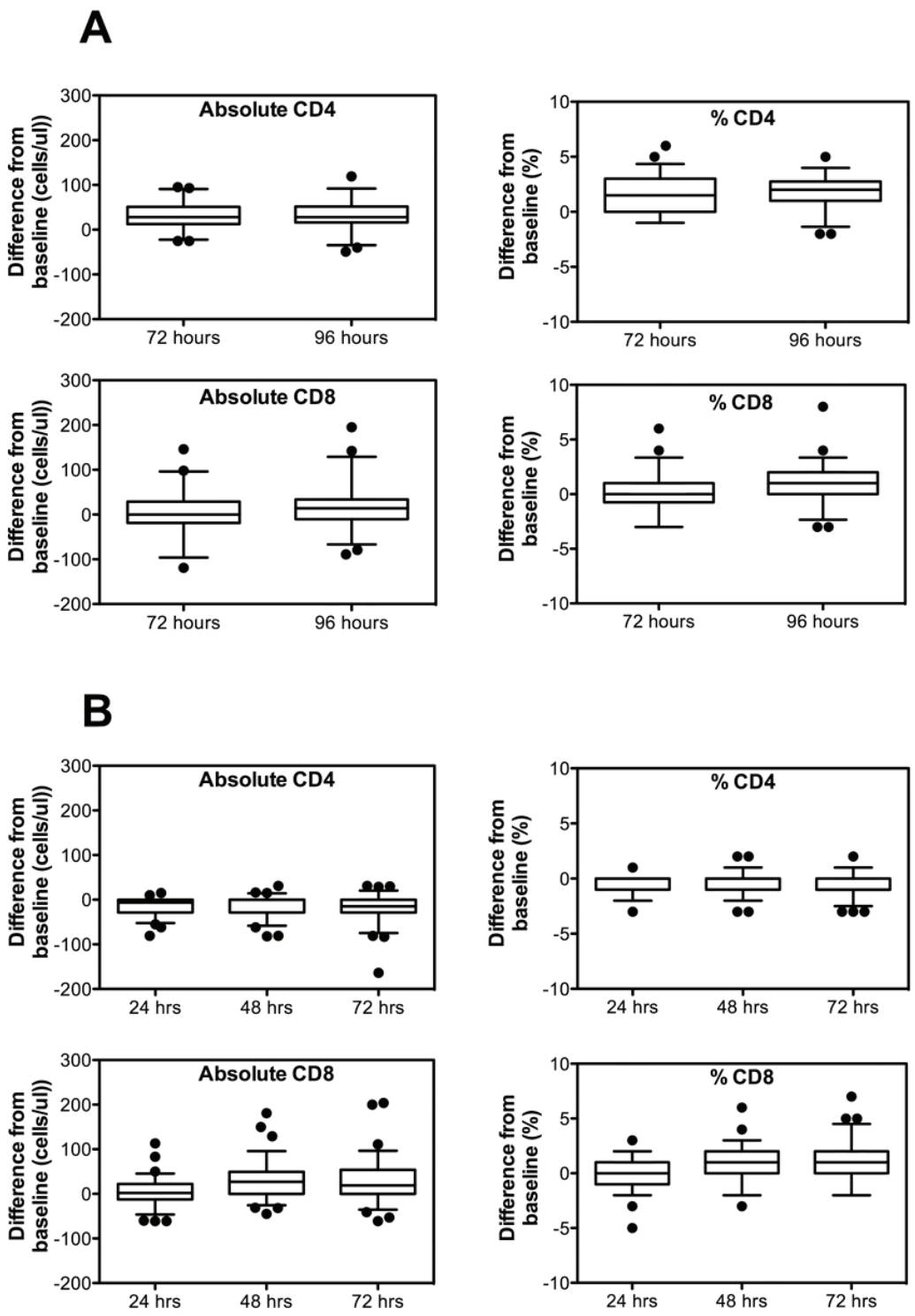

Figure 1. Changes in CD4 absolute count and percentages (top row) and in CD8 absolute count and percentages (bottom row) in aging specimens at room temperature (panel A) and at 4°C (panel B). Y-axis values represent the difference between results obtained at various time intervals and results obtained at baseline. The lower and upper limits in the box plot represent the 25th and 75th percentiles, respectively. The error bars above and below the box indicate the 90th and 10th percentiles. The horizontal line within the box marks the median value. The black circles represent residual values outside the error bar limits.

Table 4. Linear regression analysis results.

x-axis	y-axis	r^2	slope	y-intercept
CD4 abs				
24 hrs RT	72 hrs RT	0.9896	1.0360	16.79
24 hrs RT	96 hrs RT	0.9746	1.0902	12.82
CD8 abs				
24 hrs RT	72 hrs RT	0.9898	0.9856	21.54
24 hrs RT	96 hrs RT	0.9870	1.0007	10.98
CD4 abs				
24 hrs RT	24 hrs 4°C	0.9971	0.9499	5.74
24 hrs RT	48 hrs 4°C	0.9954	0.9395	12.99
24 hrs RT	72 hrs 4°C	0.9923	0.9169	15.86
CD8 abs				
24 hrs RT	24 hrs 4°C	0.9962	1.011	-3.66
24 hrs RT	48 hrs 4°C	0.9940	1.032	-2.67
24 hrs RT	72 hrs 4°C	0.9907	1.042	-12.37

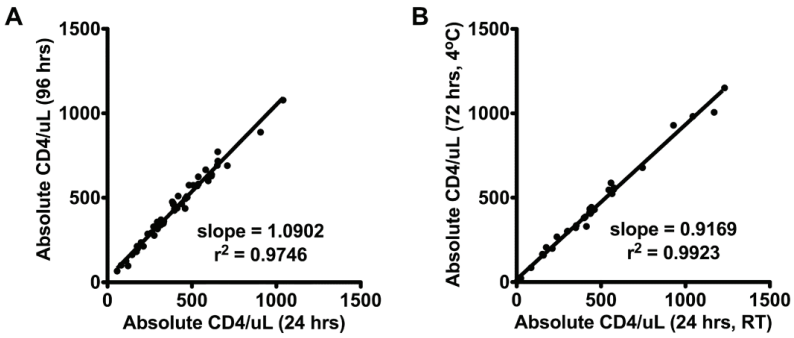

Figure 2. Linear regression analysis results for CD4 absolute counts generated from specimens stored for 96 hours at room temperature (panel A) and for 72 hours at 4°C (panel B), when compared to baseline measurements.

instruments, and the capacity to provide absolute cell counts using single-platform techniques have all contributed to the reliability of T-cell subset measurements. Previous studies in aging specimens have shown that the choice of gating strategy had a dramatic impact on immunophenotyping results in specimens that were analyzed beyond 48 hours [10]. Specifically, the largest deviations from baseline values occurred at 96 hours and gating protocols that included dual light scatter gates provided the greatest shift of T-cell subset values over time. In contrast, gating protocols that were based exclusively on cell lineage-specific gates gave the most robust T-cell values up to 96 hours, thus demonstrating that by selecting the most appropriate gating protocol, the temporal integrity of specimens can be extended up to 4 days [10]. Because there is only limited literature data for time and temperature requirements of dual-platform methods, we performed an internal study regarding the effect of specimen age and refrigeration on T-cell subsets. The impetus for performing this analysis stemmed from the fact that, while hematology analyzers were available around the clock, no immunophenotyping was available during off shifts. Thus, peripheral blood specimens would potentially have to be analyzed for T-cell subsets beyond the recommended 48-hour window, even though complete blood count data could have been performed within 24 hours from the draw.

When comparing absolute CD4 and CD8 counts obtained from aging specimens store at RT, the CD4 subset showed at positive bias at both 72 and 96 hours, while the CD8 subset demonstrated a similar trend only at 96 hours. Of note, reported acceptable intralaboratory absolute count ranges are within 75 cell/μL for CD4 and within 150 cells/μL for CD8 [11], which are similar to the differences seen in our study at

RT. Aging specimens preserved at 4°C showed a negative bias and less variation in the CD4 absolute counts, as compared to specimens held at RT. CD8 absolute counts demonstrated a negative trend at 72 hours of refrigeration, as well. Of note, the proportions of outliers were higher for the cold-stored specimens; however, the outliers did not occur preferentially at either high or low absolute T-cell counts. When comparing mean % CV for subset determinations at different time points, the values were similar to values reported for aging specimens on single-platform methods [10]. Once again, measurements performed on refrigerated specimens showed a lower % CV compared to those performed on samples stored at RT. One limitation of the study is that the number of cold-stored specimens with CD4 absolute counts <300 cells/μL was relatively small (n=12).

Our findings support the robustness of T-cell subset enumeration by a dual-platform methodology in aging specimens, up to 96 hours at RT, and up to 72 hours after refrigeration at 4°C. Similar to results reported in other, single-platform studies, our gating strategy takes advantage of the fact that lymphocytes maintain a uniform, high-density expression of CD45 at least up to 96 hours. This is unlike traditional, dual light scatter gates, in which there is significant drift of lymphocytes outside the initial gate when the specimen is older than 24 hours. Also, while initial studies have reported that single-platform methods resulted in lower interlaboratory variations of absolute T-cell counts (as compared to dual-platform methods) [12, 13], a recent report actually did not show statistical differences between the two methods [11]. At least in this context, dual-platform methods show similar performance characteristics to single-platform techniques, thus suggesting the successful employment of either methodology in achieving accurate results.

From a logistical standpoint, our results suggest that T-cell subset immunophenotyping can be performed on aging specimens stored at RT or at 4°C, with results generated within published acceptable ranges. This allows off shift processing of specimens, which in our laboratory account for <10% of the total subset immunophenotyping volume.

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