Change from dual- to single-platform reporting of CD4/CD8 values: experience from a small district general hospital laboratory

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ABSTRACT

Accurate and reliable CD4 and CD8 counts are essential for monitoring HIV disease progression or successful therapy. CD4 and CD8 counts can be determined on a flow cytometer by either single- or dual-platform technology. Dual-platform technology uses a haematology analyser to obtain a total white cell count and lymphocyte absolute count. CD4 and CD8 absolute values are then calculated from the CD4 and CD8 percentage positive results obtained from the flow cytometer. Single-platform technology uses latex beads of a predefined concentration, which are added to the blood sample immediately before flow cytometric analysis, thereby removing the need to use an additional analyser. Recent recommendations propose that single-platform technology should be the gold standard for CD4 measurement because it offers better inter-laboratory coefficients of variation (CVs). Before changing to single-platform technology in our department, CD4 and CD8 absolute counts, determined on 20 healthy volunteers, were used to establish new normal ranges for single-platform technology (Coulter epics XL), permitting absolute value data for dual-platform and single-platform technologies to be compared. Data obtained with single-platform technology was significantly higher for both CD4 and CD8 (P=0.001 and P=0.003, respectively). For CD4, mean single-platform value was 0.993 x 10^9/L (+SD = 0.510 – 1.376) and dual-platform value was 0.920 x 10^9/L (+SD = 0.500 – 1.340). For CD8, single-platform value was 0.483 x 10^9/L, (+SD = 0.207 – 0.756) and dual-platform value was 0.457 x 109/L (+SD = 0.222 – 0.692). Thus, the differences between dual- and single-platform absolute CD4 and CD8 results were small (8% and 6%, respectively) but significant. It is important, therefore, that clinicians closely monitoring CD4 and CD8 values and are informed of any laboratory changes.

KEY WORDS: CD4-positive T- lymphocytes. CD8-positive T-lymphocytes. HIV. Flow cytometry.

Introduction

Accurate monitoring of CD4 and CD8 absolute counts in human immunodeficiency virus (HIV)-positive patients is critical to the management of this disease, and there are a variety of reasons why the CD4 count may alter in these patients. Diurnal and non-pathological variations have been observed in CD4 measurements;[1] thus, technical differences in methodologies should be minimised in order to reduce the number of possible variables.

Possible changes in normal ranges, and how these might affect a clinician's judgement about patient treatment, is a concern. Often, those with progressive disease have very low CD4 counts, and a very small change may prove significant in the close monitoring of disease progression.

Currently, there are three main technologies available for absolute CD4 analysis by flow cytometry: the dual-platform, single-platform and volumetric approach. Before changing to a different CD4 absolute count methodology, the initial identification of lymphocytes should be considered.

Gating strategy, which identifies lymphocytes by their immunological characteristics using a CD45 side scatter (SS) gate has been recommended in recent UK guidelines.[2] This method enhances lymphocyte purity in the gate by reducing possible contamination from other white cells or debris, and laboratories that do not use it are more likely to produce unacceptable inter-laboratory results.[3]

Recommendations now propose single-platform technology as the preferred method for absolute counting to reduce inter-laboratory coefficients of variation (CVs).[4] Indeed, studies demonstrate a significant improvement in CD4 absolute counts between laboratories, with lower inter-laboratory variations, when comparing single-platform and dual-platform technologies.[5]

Dual-platform technology uses the mainstream haematology analyser to obtain either total white blood cell (WBC) count or absolute lymphocyte count to calculate absolute CD4 and CD8 values from percentages obtained by flow cytometry. However, haematology analysers used in this way frequently have been cited as the greatest source of variability in CD4-positive cell counts.[6]

Furthermore, lymphocyte counts determined on haematology instruments from different sites show significant differences.[1] These discrepancies may occur in the final CD4 and CD8 absolute count results because different haematology analysers apply different technologies when measuring total WBC and absolute lymphocyte counts,[7] and

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this creates an additional variable in any calculation of CD4 absolute count.

The Ortho Cytoron Absolute, a single-platform method that uses a volumetric approach, has produced the lowest overall inter-laboratory CV,[4] but many flow cytometers do not measure the volume of sample being analysed. The development of latex beads, however, enables a direct absolute count to be obtained from those flow cytometers that do not use this approach. This is known as the single-platform method and uses a defined number of latex beads, which are added directly to the prepared known sample volume immediately before flow cytometric analysis. The ratio between the beads and the number of CD4+ cells is then calculated by the flow cytometer to obtain an absolute CD4 count.

0'Gorman *et al*. demonstrated that single-platform technologies generate reproducible CD4 results, both within and between laboratories,[5] supporting the view that progress to single-platform methodology should be made as soon as practicable.

Here, we establish in-house normal ranges using 20 healthy volunteers. The CD4/CD8 percentage positivity levels are measured on our flow cytometer, and absolute CD4 and CD8 values for single- and dual-platform methodologies compared. Furthermore, this study attempts to highlight possible discrepancies between the two methods that would indicate whether or not it is necessary to change CD4 absolute count normal ranges when progression from dual-platform to single-platform methodology takes place.

Materials and methods

Samples (4 mL) of EDTA-anticoagulated blood were obtained from 20 volunteers. All tests were conducted on a single blood sample taken from each individual. Normal control samples were not washed because we use the 'lyse no wash' method when preparing HIV samples. Three colour monoclonal antibodies were used in a single tube containing CD45/isotype control/isotype control, CD45/CD3/CD8 and CD45/CD3/CD4 (Beckman Coulter).

Using a positive displacement pipette, 100 µL of well-mixed whole blood was added to tubes containing 10 µL appropriate antibody, the tubes were mixed and incubated for 15 min. Samples were incubated in the dark at room temperature throughout the method described. Optilyse C (500 µL) was then added to the tubes and incubated for 10 min. Cold phosphate-buffered saline (PBS; 500 µL) was then added to each tube, mixed and incubated for 5 min.

Immediately before the samples were passed through the flow cytometer (Coulter epics XL), 100 µL flow count beads (Beckman Coulter) was added to the tubes containing CD4 and CD8 antibody, using the same positive displacement pipette. A CD45 side scatter (SS) gate was used to identify the lymphocyte population. This reduced the possibility of gate contamination by other blood cells from a different lineage or from debris.

Positive analysis was automatically set at 2% in the negative region for every sample tube. Calibration of the flow cytometer was performed and a process control (Beckman Coulter Immunotrol) was assayed with each batch of samples tested. CD3 percentage positivity values were within 3% of each other in every tube – a measure of tube-to-tube variability.

Each sample was assayed for total WBC and absolute lymphocyte count on the haematology analyser (Sysmex SE9500) within 1 h of the same sample being prepared and analysed by flow cytometry, which took place within 30 min of sample preparation, as recommended in the data sheet provided by the company. The same individual performed both methods on one sample.

Absolute CD4 and CD8 counts were calculated by obtaining the absolute lymphocyte value from the haematology analyser and multiplying this result by the flow cytometry CD4 and CD8 percentage positive results (dual-platform). In addition, absolute CD4 and CD8 values were obtained directly from the flow cytometer (single-platform).

Statistical analysis

The parametric Student's *t*-test was used to compare paired data from the two methods. Linear regression analysis was also performed. The slope and the y intercept values were also stated. $P<0.05$ was considered statistically significant.

Results

Means, standard deviations, ranges, *t* values and *P* values for CD4 and CD8 single- and dual-platform methodologies are listed in Table 1.

A strong correlation was seen between CD4 absolute counts obtained with the dual-platform and single-platform methods ($P=0.001$; Figure 1a) and between CD8 absolute counts with the two methods ($P=0.003$; Figure 1b).

When individual absolute lymphocyte counts were compared between the flow cytometer and the haematology analyser, a 3% difference overall between the two methods was found; however, this did not reach statistical significance ($P>0.3$).

The values obtained for single- and dual-platform methodologies on each individual test are shown for CD4 (Figure 2) and CD8 (Figure 3). The two highest individual CD4 values in Figure 2 show a larger discrepancy than the

Table 1. CD4 single versus dual statistical data

	Mean	SD	Median	Range	*t* value	*P* value
CD4 single	0.993	0.483	1.10	0.42 – 1.7	3.435	0.003
CD4 dual	0.920	0.420	1.01	0.46 – 1.5		
CD8 single	0.483	0.273	0.51	0.22 – 1.0	2.913	0.01
CD8 dual	0.457	0.235	0.47	0.24 – 0.90		

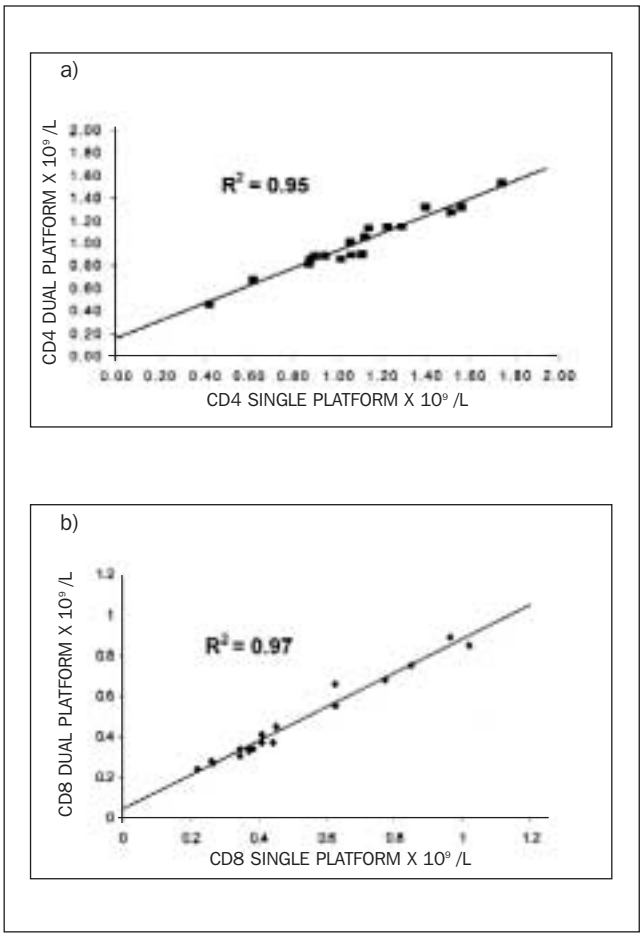

Fig. 1. Comparative analysis of a) absolute CD4 and b) absolute CD8 single versus dual data in peripheral blood of normal individuals. Results are expressed as x10⁹/L.

lower CD4 values, demonstrating a difference of 0.21 and 0.23, respectively.

There was a statistically significant difference between the single-platform and dual-platform methodologies for both CD4 and CD8 values, with the mean values for single-platform technology slightly higher than for dual-platform technology. The percentage difference in CD4 and CD8 values between the methods were 8% and 6%, respectively.

Values for the single-platform methodology, in our hands, appeared to be slightly higher than similar comparative studies, possibly because of the low numbers involved, which can lead to a type I statistical error.

Discussion

Accurate measurement of CD4 counts is critical to the choice of diagnostic and therapeutic interventions in the care of HIV patients. One of the main objectives of the present study was to determine whether or not changes in single-platform CD4 and CD8 normal ranges, however small, might alter a clinician's approach to treatment for existing patients. Furthermore, although the proposed single-platform method was likely to be more accurate, the clinician might be unsure about which method provided the 'true' CD4 level, as the trend in CD4 level over time is of paramount importance.

Although individual differences in CD4 and CD8 values were minimal when comparing single- and dual-platform results, overall there was a small but statistically significant difference between the two methods, with the single-platform method producing slightly higher overall absolute values for both cell types.

It is well documented that laboratory bias in CD4 results depends on the type of haematology analyser used to produce absolute lymphocyte counts.[8] The difference in absolute lymphocyte and total WBC counts between haematology analysers and flow cytometers is likely to be due to the different technologies employed by each for measuring absolute numbers. A fundamental difference between the two technologies is that analysers use a fixed sample volume, whereas flow cytometers analyse a predefined number of cell events;[6] thus, discrepancies between the two would be highlighted in severely leucopenic patients.

Most haematology analysers do not use monoclonal antibodies for lymphocyte selection or gating; however, Loken *et al*.[9] found that monoclonal antibodies could identify leucocyte populations more precisely than identification by forward and light scatter characteristics. Furthermore, the gating strategy for selecting lymphocytes on a flow cytometer can influence final CD4 results, as it has been shown that laboratories that do not use CD45 gating have two to three times more chance of providing inaccurate CD4+ results.[3] Indeed, the use of CD45/SS gating, to replace FS/SS gating for CD4+ lymphocyte analysis, has been strongly recommended in recent UK guidelines.[2]

In the present study, in some individual normal samples, it was found that the higher CD4 results were more prone to discrepancy between the two methods. Many HIV patients, however, have low CD4 counts, and it was this group that was of particular concern. It is now well accepted that

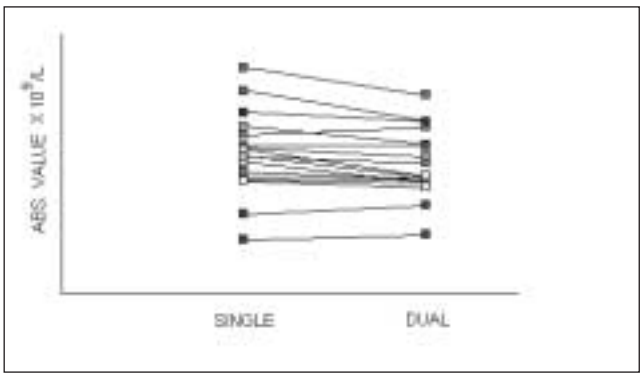

Fig. 2. Individual comparisons of CD4 single versus dual data.

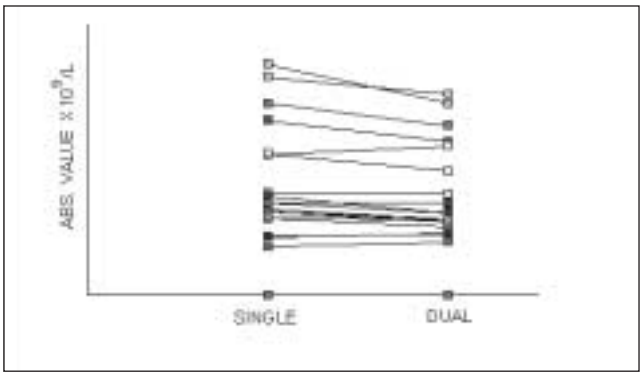

Fig. 3. Individual comparisons of CD8 single versus dual data.

therapeutic intervention with antiviral drugs may be necessary to reduce the likelihood of opportunistic infection when CD4 count falls to $<0.2 \times 10^9$/L.[10] Thus, during the changeover, it was necessary to ensure that existing patients with low CD4 counts continued to receive appropriate therapeutic advice. For example, a patient with a low CD4 count, which would be considered borderline for combination chemotherapy by dual-platform methodology, could be interpreted as a having a slight increase in CD4 count by the proposed single-platform method.

Although the advent of single-platform technology has reduced the reliance on haematology analysers for absolute lymphocyte counts, some technical considerations need to be addressed. Accurate pipetting technique is of paramount importance when using the single-platform method, and it is well documented that positive displacement pipettes should always be used. In fact, some companies now produce premeasured bead samples to minimise the risk.

Beads used for absolute counting must be thoroughly mixed by the operator each time they are used. Therefore, it is important that all operators apply the same technique when mixing the beads prior to their use in single-platform technology. Recently, the 'vanishing bead phenomenon' has been reported,[11] whereby vortex-mixing is thought to have charged the beads and caused them stick to the side of the tube. This allows the formation of doublets and reduces the number of beads counted by the flow cytometer. This phenomenon can be overcome by adding protein (e.g., bovine serum albumin) to the sample.[11]

UK guidelines for CD4 counting propose that reference limits be obtained from a minimum of 70 normal individuals.[2] However, as the present study was conducted in a small district general hospital, the cost in time and reagents prohibited the use of such a large number. Ideally, the samples should also have been tested in duplicate to assess intra-laboratory precision.

Recent study to compare inter- and intra-laboratory CD4 results from single- and dual-platform methodologies confirms that the single-platform approach reduces inter- and intra-laboratory CVs.[12] This supports the view that single-platform methodology is the preferred method for absolute CD4 analysis.

One of the main objectives of the present study was to ascertain whether or not changes in methodology would affect patients currently monitored by dual-platform results. Although NEQAS CD4 data demonstrated a noticeable improvement in performance scores since the change to a single-platform methodology, the impact of the new procedure was discussed with the clinician, who expressed concern about continued monitoring of existing patients.

During the transition from dual- to single-platform technology, some laboratories report two sets of results on existing patients for a period of time, and this approach was discussed with the clinician. An alternative is to provide a factor that enables conversion of the new single-platform CD4 value to what it would have been by the dual-platform method. Finally, it was agreed that a memorandum would be issued when the technology used was changed and that two sets of results would be issued on existing patients for a period of six months.

It is clear that single-platform technology is the way forward, particularly in patients with very low CD4 counts, where rare event analysis requires a high level of skill to obtain the precision and accuracy required. Indeed, it has been suggested that improvements in the quality of routine CD4 analysis will mean that biological factors may influence lymphocyte counts more than technical variations.[13]

We recommend that all laboratories should compare normal ranges between the two methodologies before issuing single-platform results to their clinicians. Possible discrepancies, and their impact, can then be identified and discussed prior to the changeover. Furthermore, recently it was proposed that laboratories intending to switch from dual- to single-platform methodology should also evaluate patients with CD4 absolute counts $<0.2 \times 10^9$/L.[14]

Currently, methodologies (and normal ranges) for CD4 counts vary between laboratories. Although variations are minimal, standardisation of the normal ranges used for CD4 monitoring of HIV patients should be a national goal. This would minimise the risk of patients with very low, clinically significant CD4 counts at the borderline therapeutic window being classified differently across the country. □

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