

## CD4 Lymphocyte Enumeration in Patients with Human Immunodeficiency Virus Infection Using Three-Colour and Four-Colour Dual-Platform Flow Cytometry: An Inter-Laboratory Comparative Evaluation

L G Lau,\*<sup>MBBS, MRCP (UK)</sup>, G B Tan,\*\*<sup>BSc, Post-grad Dip (Med Tech), MSc</sup>, P Kuperan,\*\*\*<sup>FRCP (UK), FRCPA, FRCPath (UK)</sup>

### Abstract

**Introduction:** This study aims to evaluate the correlation and agreement between 2 methodologies of CD4 lymphocyte enumeration. **Materials and Methods:** Fifty-two blood samples from patients with human immunodeficiency virus (HIV) infection were sent for CD4 lymphocyte enumeration at 2 major hospitals using dual-platform flow cytometry where the absolute lymphocyte counts were determined on separate haematology analysers. CD4 cell enumeration was accomplished using 3-colour flow cytometry on the FACSCalibur cytometer (Becton Dickinson) in Hospital A and 4-colour flow cytometry on the Coulter Epic XL cytometer (Beckman Coulter) in Hospital B. The percentages and absolute counts of CD4 lymphocytes obtained were analysed using both linear regression and Bland-Altman plots. **Results:** On linear regression plots, the total white cell counts, absolute lymphocyte counts, CD4 lymphocyte percentages and absolute CD4 counts from the 2 hospitals correlated well with correlation coefficients,  $r > 0.95$ . On the Bland-Altman plots, there was a mean difference of  $0.13 \times 10^9/L$  and  $0.06 \times 10^9/L$  in the total white cell and absolute lymphocyte counts from the 2 hospitals respectively. The CD4 lymphocyte percentages revealed a mean difference of only 0.05% (95% limits of agreement, -3.6 to 3.7%) but there was a mean difference of 14/uL in the absolute CD4 lymphocyte counts (95% limits of agreement, -113 to 142/uL). **Conclusions:** The CD4 lymphocyte percentages obtained using the 3-colour and 4-colour flow cytometry correlated and agreed well. However, the absolute CD4 lymphocyte counts obtained using the dual-platform technique in both the hospitals did not agree well. Hence, absolute CD4 lymphocyte counts from the 2 hospitals cannot be used interchangeably in clinical practice due to poor inter-laboratory comparability.

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**Key words:** Agreement, Correlation, Dual-platform flow cytometry, Haematology analysers

### Introduction

Clinical flow cytometry provides a precise technology for the multiparameter analysis of the morphological (scatter) and immunofluorescence properties of stained leukocyte populations, as well as their percentages and absolute counts, at the single-cell level.<sup>1,2</sup> Flow cytometry is commonly used for the identification of human lymphocyte subsets, a process referred to as immunophenotyping. It involves the detection of antigenic determinants (which are unique to a particular cell type) on the surface of leukocytes using antigen-specific monoclonal antibodies that have been labelled with a fluorescent dye or fluorochrome. The fluorochrome-labelled cells are analysed by the flow cytometer, which categorises individual cells according to size, granularity, types of fluorochrome and intensity of fluorescence.<sup>3</sup> Immunophenotypic studies have

been increasingly applied in the clinical settings over the past 2 decades as a result of several developments in the field of flow cytometry. An increasing number of monoclonal antibodies specific for lymphocyte surface antigens have become available over the years. These reagents, together with the advancement in instrumentation, data analysis, computer technology and fluorochrome chemistry, have facilitated multiparameter analysis. More importantly, the appearance of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) has acted as the driving force behind the technological evolution of leukocyte immunophenotyping. The HIV/AIDS pandemic helps to move flow cytometry out of the research laboratory into routine clinical practice, and this has in turn provided further impetus for technical advances in the discipline of immunology.<sup>2,4</sup>

\* Registrar

Department of Haematology-Oncology  
National University Hospital

\*\* Principal Medical Technologist

\*\*\* Senior Consultant

Department of Pathology and Laboratory Medicine  
Tan Tock Seng Hospital

Address for Reprints: Dr L G Lau, Department of Haematology-Oncology, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074.  
E-mail: LauLeeGong@nuh.com.sg

Human lymphocytes can be divided into 3 major populations based on their biologic function and cell-surface antigen (CD) expression — i.e., the T lymphocytes (CD3+), B lymphocytes (CD19+) and natural killer (NK) lymphocytes (CD16+/56+). Two subsets of T lymphocytes that are of particular clinical importance are the helper T cells and the suppresser T cells, which are CD4+ and CD8+, respectively. The primary clinical indications for immunophenotyping currently include enumeration of CD4 cell counts in HIV infection,<sup>3</sup> characterisation of primary immunodeficiency disorders,<sup>5,6</sup> evaluation of immune-mediated diseases,<sup>7</sup> and assessment of immune reconstitution in patients following stem cell transplantation.<sup>8,9</sup> The realisation that CD4 cell count in HIV-infected individuals is a reliable surrogate marker of both progression to AIDS and response to anti-viral therapy has created a need for the precise quantitation of CD4 cells in order to provide optimal clinical care.<sup>10,11</sup> As improvements in HIV therapy have altered the course of the disease, immunophenotypic studies have also shifted from simply monitoring the decline in CD4 cell counts to evaluation of immune reconstitution following highly active antiviral therapy (HAART) and identification of potential markers as prognostic indicators of HIV infection.<sup>12,13</sup>

There are significant variations among the different methodologies employed to determine the absolute CD4 cell percentages as well as numbers on the flow cytometry. The Center for Disease Control and Prevention (CDC) recommends using reagent combinations containing fluorochrome-labelled CD3 and CD4 monoclonal antibodies for determining the percentage of CD4 cells in HIV-infected subjects.<sup>3</sup> It was reported in 1995 that there were more than 1.6 million CD4 cell measurements performed yearly by approximately 600 testing laboratories in the United States.<sup>14</sup> Most of these measurements are made using dual-platform flow cytometric methods, although new single-platform methods are available and being increasingly used. The dual-platform technique utilises immunophenotypic data derived from the flow cytometer together with the total white cell, or total lymphocyte count, obtained from a haematology analyser. In contrast, the single-platform technique derives the absolute CD4 cell count directly from the flow cytometer, using either precision fluidics or microbead technology.<sup>15</sup> For both approaches, different types of panel of antibody combination can be used. This can range from 2-colour to 3-colour to 4-colour monoclonal antibody combination panels.<sup>3</sup> As there is as yet no one method considered as a “gold standard”, different laboratories employ different methodologies for CD4 lymphocyte measurement which can be single-platform or dual-platform in approach, using either 3-colour or 4-colour monoclonal antibody combination.<sup>3</sup> In this study, CD4 lymphocyte enumeration

using dual-platform flow cytometry with a 3-colour monoclonal antibody panel is compared with a 4-colour monoclonal antibody panel in 2 different hospitals to evaluate correlation and agreement between the 2 methodologies.

## Materials and Methods

### Samples

Fifty-two blood samples from HIV-infected patients were sent for CD4 cell measurement at 2 major hospitals in Singapore. All samples were collected in EDTA tubes and processed within 24 hours, using whole blood staining methods with a “lyse-no-wash” procedure. Briefly, in each tube, a fixed small quantity of anticoagulated whole blood was mixed with the appropriate amount of a suitable reagent containing the required fluorochrome-labelled monoclonal antibodies according to the manufacturers’ specifications. The mixture was then incubated at room temperature in the dark for 15 minutes. Next, a lysing solution was added; the mixture was vortexed gently and left to incubate for another 15 minutes at room temperature in the dark, after which the sample was ready for analysis on the flow cytometer. Both hospitals used the dual-platform technique where the total leukocyte and absolute lymphocyte counts were determined on the same blood samples using a separate automated haematology analyser. In Hospital A, the Coulter Gen S (Beckman Coulter) was used; whereas either the Advia 120 (Bayer) or the Sysmex SE 9000 (Sysmex) was used at Hospital B.

### Reagents

At Hospital A, CD4 lymphocyte measurement was performed on the FACSCalibur cytometer (Becton Dickinson) using 2 tubes with a combination of 3 monoclonal antibodies in each tube (3-colour reagent). The reagents were TriTEST CD3-FITC/CD4-PE/CD45-PerCP and TriTEST CD3-FITC/CD8-PE/CD45-PerCP (Becton Dickinson), both 3-colour direct immunofluorescence reagents for the identification and determination of the percentages and absolute counts of mature human CD3+ lymphocytes as well as CD3+CD4+ and CD3+CD8+ lymphocyte subsets in erythrocyte-lysed whole blood. At Hospital B, CD4 lymphocyte measurement was performed on the Coulter Epic XL cytometer (Beckman Coulter) using one single tube with a combination of 4 monoclonal antibodies in the tube (4-colour reagent). The reagent was CYTO-STAT tetraCHROME (Beckman Coulter), a 4-colour counterpart containing CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5.

### Gating Strategy

On the FACSCalibur cytometer (Becton Dickinson) in Hospital A, the processed blood samples (in 2 tubes,

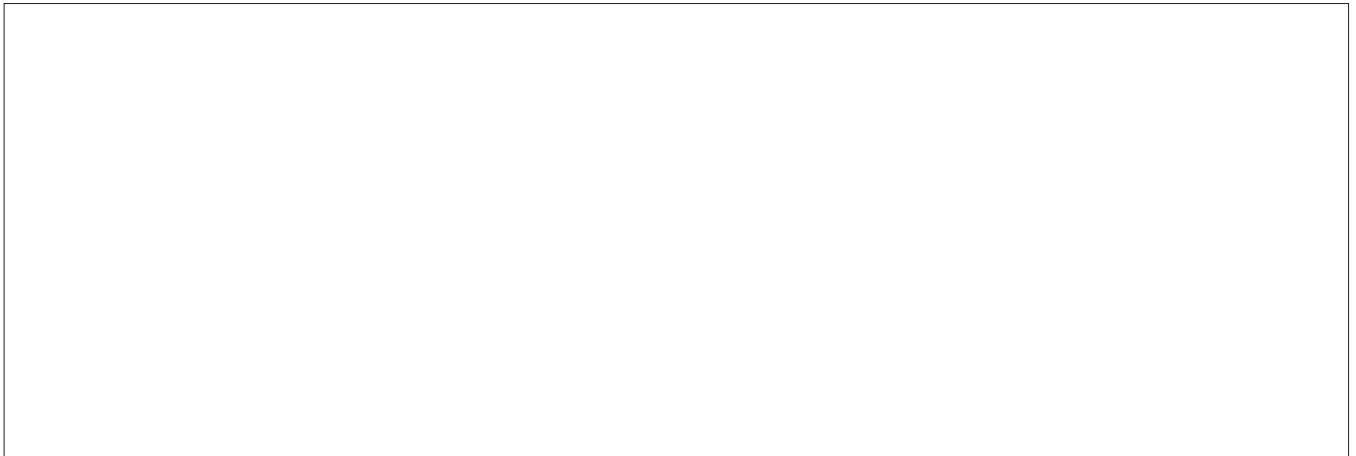


Fig. 1a. CD4 enumeration on 3-colour flow cytometry using TriTEST CD3-FITC/CD4-PE/CD45-PerCP and TriTEST CD3-FITC/CD8-PE/CD45-PerCP as the reagents in Hospital A.



Fig. 1b. CD4 enumeration on 4-colour cytometry using CYTO-STAT tetraCHROME CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 as the reagent in Hospital B.

stained with the CD45/CD3/CD4 antibody panel and the CD45/CD3/CD8 antibody panel) were acquired and analysed automatically using the MultiSET software (Becton Dickinson). In Hospital B, the processed blood samples (in one tube, stained with the CD45/CD3/CD4/CD8 antibody panel) were acquired and analysed by the tetraONE System software (Beckman Coulter) on the Coulter Epic XL cytometer (Beckman Coulter). Briefly, both softwares' lymph gate identified the lymphocyte population from a CD45 versus side scatter plot (as a CD45-bright and side scatter-low cluster). The CD3 and CD4 double-positive cell cluster was next identified on the lymphocyte-gated CD3 versus CD4 dot plot. Likewise, the CD3 and CD8 double-positive cell cluster could be identified on the lymphocyte-gated CD3 versus CD8 dot plot (Figs. 1a & 1b).

Depending on the number of events acquired for each specific plot (CD3+CD4+ or CD3+CD8+), the percentages of CD4 lymphocytes and CD8 lymphocytes would then be reported. These percentages, together with the absolute lymphocyte counts determined on the Coulter Gen S (Beckman Coulter) haematology analyser in Hospital A, and either the Advia 120 (Bayer) or Sysmex SE 9000 (Sysmex) haematology analyser in Hospital B, were used to obtain the absolute CD4 and CD8 cell counts.

### Statistical Analysis

The absolute counts and percentages of CD4 lymphocytes obtained by the 2 different methods were analysed using both linear regression and Bland-Altman plots (which were performed by the software, Analyse-It version 1.31). The former gives information on the extent to which the results defined by the 2 methods are correlated. These values are necessarily high as the same parameters are measured, though with different methodologies. The Bland-Altman method examines, in a discriminate fashion, whether the 2 methods agree sufficiently well to be used interchangeably.<sup>16</sup> Here, the average of the values by the 2 methods is displayed on the x-axis and plotted against the difference between the 2 methods shown on the y-axis. The average difference between the 2 methods, referred to as the bias, and the 95% limits of agreement are shown on the graph as 3 horizontal lines.

### Results

#### Haematology Analysis

Table I shows the total white cell and absolute lymphocyte counts from Hospitals A and B. Briefly, the range of total white cell counts from Hospital A was 2.10 to 15.8 x 10<sup>9</sup>/L as compared to a range of 2.06 to 17.13 x 10<sup>9</sup>/L from

TABLE I: THE TOTAL WHITE CELL AND ABSOLUTE LYMPHOCYTE COUNTS AT HOSPITALS A AND B

|                                           | Total white cell counts |              | Absolute lymphocyte counts |             |
|-------------------------------------------|-------------------------|--------------|----------------------------|-------------|
|                                           | Hospital A              | Hospital B   | Hospital A                 | Hospital B  |
| Range (x 10 <sup>9</sup> /L)              | 2.10 – 15.8             | 2.06 – 17.13 | 0.67 – 4.65                | 0.45 – 4.67 |
| Mean (x 10 <sup>9</sup> /L)               | 6.08                    | 6.20         | 1.93                       | 1.87        |
| Standard deviation (x 10 <sup>9</sup> /L) | 2.93                    | 3.33         | 0.77                       | 0.83        |

TABLE II: THE PERCENTAGES AND ABSOLUTE CD4 COUNTS AT HOSPITALS A AND B

|                    | % CD4 lymphocytes |            | Absolute CD4 lymphocytes (per uL) |            |
|--------------------|-------------------|------------|-----------------------------------|------------|
|                    | Hospital A        | Hospital B | Hospital A                        | Hospital B |
| Range              | 1 – 38            | 1.3 – 42.9 | 5 – 877                           | 11 – 884   |
| Mean               | 14.62             | 14.56      | 289                               | 275        |
| Standard deviation | 9.47              | 9.59       | 221                               | 215        |

Hospital B. The mean total white cell counts were 6.08 and 6.20 x 10<sup>9</sup>/L from Hospital A and B, respectively. The range of absolute lymphocyte counts from Hospital A was 0.67 to 4.65 x 10<sup>9</sup>/L as compared to a range of 0.45 to 4.67 x 10<sup>9</sup>/L from Hospital B. The mean absolute lymphocyte counts were 1.93 and 1.87 x 10<sup>9</sup>/L from Hospital A and B, respectively. These 2 parameters were next evaluated with linear regression plots (data not shown). It was found that, despite the use of up to 3 different types of haematology analysers in the 2 hospitals, there were high degrees of correlation between the total white cell and absolute lymphocyte counts with R<sup>2</sup> of 0.96 and 0.92 [correlation coefficients (r) of 0.98 and 0.96], respectively.

On the Bland-Altman analysis (data not shown), there was a mean difference of 0.13 x 10<sup>9</sup>/L between the total white cell counts from Hospitals A and B, with the 95% limits of agreement ranging from -1.63 to 1.40 x 10<sup>9</sup>/L. It means that there may be a difference of up to 1.63 x 10<sup>9</sup>/L between the total white cell counts from 2 hospitals.

Likewise, there was a mean difference of 0.06 x 10<sup>9</sup>/L between the absolute lymphocyte counts from Hospitals A and B on the Bland-Altman analysis, with the 95% limits of agreement ranging from -0.408 to 0.529 x 10<sup>9</sup>/L. This translates to mean that there may be a difference of up to 0.529 x 10<sup>9</sup>/L between the absolute lymphocyte counts from the 2 hospitals. Hence, we conclude that the total white cell and absolute lymphocyte counts from the 2 hospitals, using different haematology analysers, although seem to correlate well on the linear regression analysis, have wide 95% limits of agreement on the Bland-Altman analysis and thus, should not be used interchangeably in clinical practice.

Flow Cytometry Analysis

Table II shows the percentages and absolute counts of CD4 lymphocytes from Hospitals A and B. The CD4 lymphocyte percentages ranged from 1% to 38% at Hospital A using 3-colour reagents, with a mean of 14.62%, as

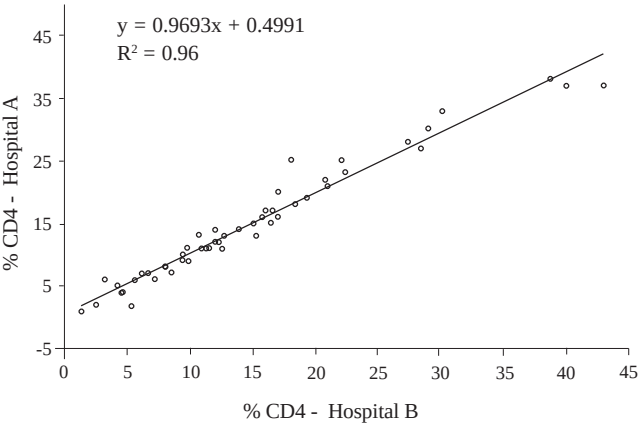


Fig. 2a. Linear regression plot of the CD4 percentages from Hospital A against those from Hospital B.

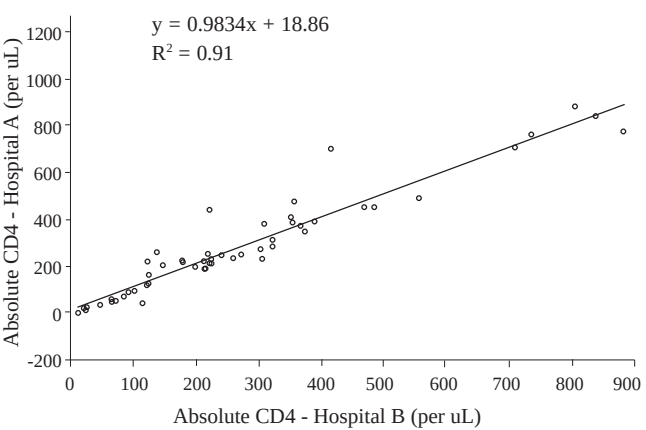


Fig. 2b. Linear regression plot of the absolute CD4 counts from Hospital A against those from Hospital B.

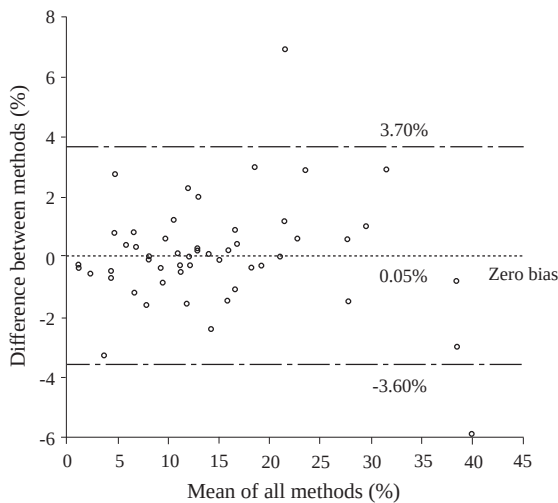


Fig. 3. Graph plotting differences between percentages of CD4 against the means of CD4 using the two different enumeration methods in Hospital A and B.

compared to a range of 1.3% to 42.9% at Hospital B using 4-colour reagents, with a mean of 14.56%. The absolute CD4 cell counts were obtained by multiplying the percentages of CD4 cells from the flow cytometry by the absolute lymphocyte counts from the haematology analysers. This gave a range of absolute CD4 cell counts of 5 to 877/uL at Hospital A (mean, 289/uL) as compared to a range of 11 to 884/uL at Hospital B (mean, 275/uL). The absolute counts and percentages of CD4 cells from the 2 hospitals were next evaluated using the linear regression plot (Figs. 2a & 2b). Again, high degrees of correlation between the percentages and absolute counts of CD4 cells were observed with  $R^2$  of 0.96 and 0.91 [correlation coefficients ( $r$ ) of 0.98 and 0.95], respectively.

Next, a Bland-Altman analysis was performed to evaluate agreement. The comparison of the CD4 cell percentages (Fig. 3) revealed a mean difference of only 0.05% between samples analysed in the 2 hospitals, with the 95% limits of agreement ranging narrowly from -3.6% to 3.7%. There was little indication of a tendency to increased bias at low or high lymphocyte percentages as displayed on the graph. It means that there may be a difference of, at most, less than 4% between the CD4 lymphocyte percentages obtained in the 2 hospitals by the 2 different flow cytometric methodologies.

However, there was a mean difference of 14/uL in the final absolute CD4 lymphocyte counts, with the 95% limits of agreement ranging from -113 to 142/uL (Fig. 4). This will translate into a difference of up to 142/uL of CD4 lymphocytes between the 2 hospitals. Hence, while the CD4 percentages from the 2 hospitals, determined using 3-colour and 4-colour flow cytometers, agree well and can be used interchangeably, the absolute CD4 lymphocyte counts

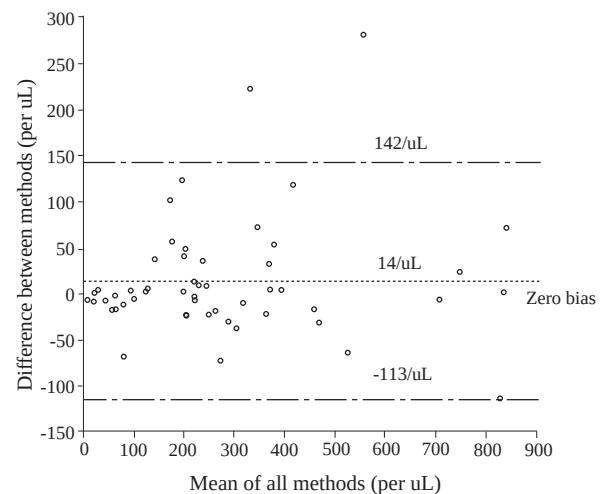


Fig. 4. Graph plotting differences between absolute CD4 counts against the means of CD4 counts using the two different enumeration methods in Hospital A and B.

from the 2 hospitals, determined using the dual-platform technique, do not agree sufficiently well to be used interchangeably in clinical practice. It can be clearly seen that the lack of agreement between the absolute CD4 lymphocyte counts arises from the total white cell and absolute lymphocyte counts obtained using different types of haematology analysers in the 2 hospitals.

## Discussion

Accurate and reliable measures of CD4 lymphocytes are essential to the assessment of the immunological status of HIV-infected individuals. The pathogenesis of AIDS is largely attributed to the decrease in T lymphocytes that bear the CD4 receptor; the progressive depletion of CD4 cells is associated with an increased likelihood of clinical complications including opportunistic infections.<sup>3</sup> CD4 cell counts are a criterion for categorising HIV-related clinical conditions by CDC's classification system for HIV infection and surveillance case definition for AIDS among adults and adolescents.<sup>17</sup> The measurement of CD4 cell counts has been used to establish decision points for initiating *Pneumocystis carinii* pneumonia prophylaxis<sup>18</sup> and prophylaxis for several other opportunistic infections,<sup>19</sup> antiviral therapy<sup>20</sup> and to monitor the efficacy of treatment.<sup>21,22</sup> CD4 lymphocyte counts are also used as prognostic indicators in HIV-infected patients.<sup>23</sup>

Currently, lymphocyte subset analysis is routinely undertaken by flow cytometry. There are significant variations among the different methodologies employed to determine the absolute CD4 cell numbers as well as cell percentages on the flow cytometry. The CDC recommends using reagent combinations containing fluorochrome-labelled CD3 and CD4 monoclonal antibodies for

determining the percentage of CD4 lymphocytes in HIV-infected subjects.<sup>3</sup> We, thus, compared CD4 lymphocyte enumeration using 3-colour and 4-colour dual-platform flow cytometry in 2 different hospitals to evaluate inter-laboratory correlation and agreement between the 2 methodologies.

This study demonstrated excellent correlation and agreement of CD4 percentages obtained using 3-colour and 4-colour flow cytometry from 2 different hospitals. Linear regression analysis revealed good correlation (with  $r = 0.98$ ) and Bland-Altman analysis revealed a less than 4% mean difference between the values of CD4 percentages using the 2 different methodologies. Thus, these values from the 2 hospitals could be used interchangeably in the clinical management of HIV-infected patients. However, as both hospitals used the dual-platform technique of CD4 cell enumeration, where the absolute lymphocyte counts were determined from separate haematology analysers, the absolute CD4 cell counts, though well-correlated ( $r = 0.95$ ), did not agree sufficiently well on the Bland-Altman analysis to be used interchangeably. This was principally due to the wide 95% limits of agreement, although the average difference of the absolute CD4 cell counts was only 14/uL. With the 95% limits of agreement ranging from -113 to 142/uL, it could mean a difference in CD4 cell count of up to 142/uL, a difference that is clearly unacceptable in the clinical management of HIV-infected patients. For example, a patient may be considered for enrollment into a particular treatment protocol based on his or her CD4 cell count at one institution, but be precluded from treatment at another institution due to a different CD4 cell counts obtained at that institution. Given the inter-laboratory variability in absolute CD4 cell counts between institutions, it is possible that a patient would have AIDS at one institution and not at another, according to the CDC classification system for case definition for AIDS. It can be clearly appreciated from the presented data that the lack of agreement between the absolute CD4 cell counts arises from the total white cell and absolute lymphocyte counts obtained by the different types of haematology analysers in the 2 hospitals.

Despite having many conceptual and technical limitations, dual-platform flow cytometry is traditionally recommended as the preferred method for absolute cell count enumeration, including CD4 lymphocytes.<sup>3</sup> It has long been recognised that a major factor contributing to inter-laboratory variation in the absolute CD4 cell counts using dual-platform flow cytometry is the poor reproducibility of the absolute lymphocyte counts generated by different haematology analysers.<sup>24-26</sup> In a recent study, the variability of results obtained from the same samples on the different flow cytometers (i.e., percentages of CD4 and CD8 lymphocytes)

was less than the variability of the results obtained for the different haematology measurements (i.e., absolute lymphocyte counts). These results suggest that a significant amount of inter-laboratory variability of CD4 and CD8 cell measurements made by the dual-platform flow cytometry is contributed by the haematology measurements.<sup>27</sup> Similar findings are obtained in the present study; there is excellent agreement between the CD4 percentages from the 2 different flow cytometers, but the haematology values from the different haematology analysers agree poorly with each other. As a result, the development of single-platform technologies has recently attracted attention with several different technical approaches now being available. These include precision fluidics and microbead technology.<sup>28-30</sup> These single-platform approaches, which eliminate the use of total white cell and absolute lymphocyte counts from the haematology analyser, have superior precision and less sources of variability. A number of reports clearly demonstrate that they provide better inter-laboratory coefficients of variation (CV) in multicentre studies and a lower chance to generate aberrant results.<sup>15,31</sup> This technique has also been shown recently to be useful in CD34 stem cell enumeration.<sup>15,32,33</sup>

In conclusion, we found excellent correlation and agreement between the percentages of CD4 lymphocytes obtained using the 3-colour and 4-colour flow cytometry. The total white cell and the absolute lymphocyte counts which are analysed using different haematology analysers, though correlate reasonably well, fail to agree satisfactorily. As a result, the absolute CD4 lymphocyte counts as performed using the dual-platform technique in both the hospitals, fail to agree well and thus, cannot be used interchangeably in the clinical management of HIV-infected patients. It may be that in future CD4 cell measurement should be performed using single-platform flow cytometry rather than the dual-platform technique as used in this study for less inter-laboratory variation and thus, better inter-laboratory comparability.

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