Comparison of Two Alternative Methods for CD4⁺ T-Cell Determination (Coulter Manual CD4 Count and CyFlow) Against Standard Dual Platform Flow Cytometry in Uganda

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Background: In this study we evaluated alternative CD4⁺ T-cell counting methods in clients of a PMTCT Programme in rural Uganda.

Methods: The Coulter Manual CD4 Count method for CD4⁺ T-cell enumeration (Cyto-Spheres) and an automated method (volumetric, single-platform flow cytometry; CyFlow) were compared with a standard, dual-platform flow cytometry protocol (DPFC, FACScan).

Results: Correlation and precision of agreement were higher for the CyFlow method ($r = 0.929$ and $\eta = 0.08$) when compared to DPFC than for the Cyto-Spheres method ($r = 0.725$ and $\eta = 0.3$). Multiple linear regression analysis showed that CD4⁺ cell counts by the CyFlow method were a stronger predictor for results of DPFC than those of the Cyto-Spheres method ($r^2 = 0.864$ and $r^2 = 0.552$, respectively). When compared to DPFC the CyFlow method generated higher CD4⁺ cell counts than the Cyto-Spheres method, as expressed by a higher median and mean difference (+70 and +90 cells for CyFlow, +28 and -14 cells for Cyto-Spheres).

Conclusion: Both, the manual Cyto-Spheres method and the CyFlow method can be used for the enumeration of CD4⁺ cells in resource-limited settings. Under supervised conditions, the CyFlow method produced results more consistent with the reference method than the Cyto-Spheres method. © 2006 International Society for Analytical Cytology

Key terms: Cyto-Spheres; CyFlow; CD4⁺ T-cell determination; resource-limited setting; volumetric single platform flow cytometry; PMTCT Plus

The number of HIV infected people receiving antiretroviral treatment (ART) in developing countries will rise dramatically along with WHO’s initiative to treat 3 million people by the end of the year 2005 (“3 by 5 initiative”) (1). For the assessment of eligibility for ART as well as for monitoring of the response to therapy, the determination of CD4⁺ cell count is still the method of choice. Even though the availability of CD4⁺ cell counting is not considered as mandatory to start HIV treatment programmes in resource limited settings, WHO favors its uniform accessibility (2).

The standard method for CD4⁺ cell enumeration is flow cytometry based on either dual or single platform technologies (3,4). Because of the high cost of equipment and reagents, sophisticated and delicate technology, and the need for complex maintenance and qualified personnel, the applicability of these methodologies is very limited in areas of the world with insufficient health infrastructure (5–7).

A number of cheaper, more robust and simpler to use protocols for CD4⁺ cell analysis have been developed...
within the last years. Even though some of them show promising results, data evaluating their use and reliability in remote rural areas with low standards of health care are still scarce (3,5,7–13). The ideal method for CD4\(^+\) cell counting in such areas has not yet been identified.

We report data from a PMTCT Plus programme in Fort Portal, Western Uganda. CD4\(^+\) cell counts of HIV-infected clients were determined to assess ART indication and to monitor treatment outcome. CD4\(^+\) cell counts were analyzed using either a manual technique (Cyto-Spheres, Beckman Coulter, Miami, FL) or volumetric single-platform flow cytometry (CyFlow, Partec, Münster, Germany). Results were compared with a standard dual platform flow cytometry protocol (FACScan, Becton Dickinson, San José, CA).

**MATERIALS AND METHODS**

**Study Site**

Since 2001 the German Agency for Technical Cooperation has supported a Prevention of HIV Mother-To-Child Transmission (PMTCT) programme in three districts in Western Uganda. ART for women participating in the programme, their partners, and children was started in 2003 (PMTCT Plus approach) in the Regional Referral Hospital of Fort Portal. A repeatable CD4\(^+\) cell count below 350 cells/\(\mu\)l was chosen as cut-off for treatment indication. The local laboratory was equipped with the manual Cyto-Spheres technique and later with the CyFlow system. Laboratory personnel were trained on each method for 2–3 days. Dual platform flow cytometry (DPFC) was performed on a FACScan instrument in a reference laboratory in Entebbe, Uganda (Centers for Disease Control and Prevention, Uganda Virus Research Institute).

**Patients and Samples**

131 samples from 102 (76 women, 26 men) HIV-infected patients were analyzed by the manual Cyto-Spheres method and with DPFC. 128 samples from 121 (83 women, 32 men, 6 children > 6 years of age) HIV-infected patients were analyzed by volumetric, single-platform flow cytometry (VSPFC, CyFlow) and DPFC. CD4\(^+\) cell counts from children younger than 6 years of age were excluded from the analysis.

5 ml of whole blood each were taken in two EDTA vacutainer tubes. One tube was transferred to the local laboratory and CD4\(^+\) cell analysis was done within 6 h either by the Cyto-Spheres method or by the CyFlow method. The other EDTA tube was sent to the reference laboratory in Entebbe at ambient temperature and processed within 24 to 30 h by dual platform analysis. Clotted samples as well as samples older than 6 h in case of analysis by the Cyto-Spheres and CyFlow methods or older than 48 h in case of the dual platform analysis were discarded.

**Manual CD4\(^+\) Cell Counting (Cyto-Spheres)**

In the Cyto-Spheres assay, monoclonal antibody-coated latex spheres bind to cells expressing CD4 surface antigen. Spheres coming into contact with a CD4\(^+\) cell form a typical rosette. After lysis of erythrocytes, cell-sphere rosettes can be identified and counted under a light microscope in a counting chamber. Monocytes expressing also CD4 surface antigen are blocked by beads coated with MY4 (CD 14) antibodies.

In detail, 100 \(\mu\)l of EDTA blood was placed in a test tube and mixed for 2 min with 10 \(\mu\)l of a monocyte blocking reagent (MY4 antibodies). Thereafter, 10 \(\mu\)l of CD4 antibody-coated latex spheres were added and the mixture shaken for 2 min. 10 \(\mu\)l of this mixture was transferred to a second tube containing 100 \(\mu\)l of a lysing reagent. The tube was shaken for 15 s to lyse the red blood cells. Both chambers of a 0.1 mm deep hemacytometer were filled with the mixture and examined under a light microscope. Cells bearing three or more latex spheres were counted. The result had to be multiplied by a factor of 7.3 to account for dilution and to receive the absolute number of CD4\(^+\) T lymphocytes.

**Volumetric Single Platform Flow Cytometry (CyFlow)**

The CyFlow Counter used in this study was an automated two parameter flow cytometer. In the volumetric, single-platform protocol, CD4\(^+\) cells are marked with a single monoclonal fluorochrome (phycoerythrin)-conjugated antibody in a known volume of blood. The fluorochrome marked CD4\(^+\) cells are counted by excitation with a green solid-state laser operating at 532 nm and by side scatter analysis. An erythrocyte lysing procedure is not required (no-lyse-no-wash procedure).

In detail, 50 \(\mu\)l of EDTA blood were placed in a test tube and mixed with 10 \(\mu\)l monoclonal CD4 antibody (clone EDU-2, DIATEC, Oslo, Norway). After an incubation period of 15 min at room temperature in the dark, 800 \(\mu\)l of no-lyse dilution buffer (phosphate buffered saline) was added. A total sample volume of 860 \(\mu\)l was then analyzed with the CyFlow Counter. The results were indicated on the display.

**Dual Platform Flow Cytometry (FACScan)**

In this method, absolute CD4\(^+\) cell counts are obtained from a combination of results from flow cytometry and hematoanalysis. A complete blood cell count (CBC) was performed using the Act 5Diff instrument (Beckman Coulter). Flow cytometry was performed with a FACScan instrument and MultiSET software (Becton Dickinson) modified to accept manual entry of the total white cell count from the CBC and the percentage of lymphocytes derived from the Attractors software (Becton Dickinson), which reports a three-part differential based on cell-surface markers and side scatter. By use of this dual-platform approach, the MultiSET software reported the absolute CD3\(^+\) CD4\(^+\) cell counts for the specimen (14).
In detail, TriTEST reagents (CD3 FITC/CD4 PE/CD45 PerCP) were used to stain peripheral blood mononuclear cells according to the protocol of the manufacturer. After lysis of erythrocytes (for 10 min using FACS Lysing Solution) the cells were washed twice, fixed with a formalin-based buffer, and analyzed by flow cytometry.

**Statistical Analysis**

Statistical analysis was performed using the SPSS programme version 11.5 (SPSS, Chicago, IL).

The mean and median of the CD4⁺ cell counts were calculated. In addition, the median differences between CD4⁺ counts by the reference and the alternative methods were determined and compared to a hypothetical median difference of 0 using the Wilcoxon signed rank test.

Correlation between the methods was analyzed by linear regression. A model of multiple linear regression was applied to evaluate associations with other variables.

To analyze mean differences and 95% limits of agreement between the methods, the difference of each CD4 pair (CD4 alternative method – CD4 reference method) was plotted against its average (CD4 alternative method + CD4 reference method/2), as suggested by Bland and Altman (15). The mean differences and the 95% limits of agreement (bias ± 1.96 SD) were used to assess agreement of the different methods.

Precision of agreement (η) of the different methods was calculated using the following formula (16):

\[
\eta = \frac{VAR(Y - (\alpha + \beta X))}{VAR(Y) + VAR(X)|\beta|^2}
\]

(VAR = variance; Y = CD4⁺ cell counts by the alternative method; X = CD4⁺ cell counts by the reference method; \(\alpha\) and \(\beta\) = regression coefficients). \(\eta\) was chosen as a measure for precision of agreement with a range from 0 to 1 (0 \(\leq\) \(\eta\) \(\leq\) 1). Since \(\eta = 1\) would indicate that the methods compared were completely independent, high values for \(\eta\) indicate low precision of agreement. Similarly, \(\eta = 0\) would indicate perfect agreement.

The sensitivity, specificity, positive and negative predictive values were analyzed to classify CD4⁺ cell counts correctly below a certain threshold.

In diagnostic tests which are not producing dichotomous results but results based on continuous parameters as in this study, a threshold value has to be determined. The choice of this threshold value is crucial, since sensitivity and specificity depend strongly on it. Receiver Operating Characteristics (ROC) graphs were created by plotting the sensitivity against the specificity of the different methods in order to select an optimal threshold value. A value on the ROC graph closest to the point of 100% sensitivity and 100% specificity was considered as optimal threshold and determined by the minimal Euclidean distance to this point. Geometrically, this optimal value was found when a circle constructed with the centre at this point touched the ROC curves.

**RESULTS**

Comparison of the Manual CD4⁺ Cell Counting Method (Cyto-Spheres) with Dual-Platform Flow Cytometry (FACScan)

Among the 131 samples analyzed by dual-platform flow cytometry (DPFC), 44 (34%) revealed CD4⁺ counts below 200 cells/µl, 23 (18%) between 201 and 350, 24 (18%) between 351 and 500, 27 (20%) between 501 and 750, and 13 (10%) above 750 cells/µl.

The Cyto-Spheres method generally generated higher CD4⁺ cell counts than DPFC (median CD4⁺ cell count 343/µl and 332/µl, respectively).

The overall median difference between the Cyto-Spheres method and DPFC was +28 cells/µl (+50.5 for CD4⁺ cell counts below 200, +59.5 for CD4⁺ cell counts between 201 and 350, +21.5 for CD4⁺ cell counts between 351 and 500, −69 for CD4⁺ cell counts between 501 and 750, and −250 for CD4⁺ cell counts above 750).

When comparing the overall median difference to a hypothetical median difference of 0 no statistically significant difference was detected (\(P = 0.19\)).

The correlation coefficient comparing both methods was \(r = 0.725\) (\(P < 0.01\)). The regression analysis, including the best regression line \(y = a + b x; (i = 1, 2, 3, \ldots, n)\) is illustrated in Figure 1a. Multiple linear regression was used to scan for additional covariates. Among the covariates included (Cyto-Spheres method, age, gender), the Cyto-Spheres method and age were significantly associated with DPFC (\(r^2 = 0.552, P < 0.01\) for the Cyto-Spheres method, \(P < 0.09\) for age). While both covariates could predict the results of the standard DPFC method, the Cyto-Spheres method was a stronger predictor (\(b = 0.7\)) than age (\(b = -1.6\)).

Bland-Altman analysis showed a mean difference of −1.4 CD4⁺ cells/µl (95% CI −3.4, −31.3) between the Cyto-Spheres method and DPFC. 95% limits of agreement were +369 cells/µl (95% CI +313.5, +425.6) and −372 cells/µl (95% CI −428.4, −316.5) (Fig. 2a).

If only the range clinically relevant for HIV+ patients was analyzed (CD4⁺ cell count between 0 and 500 cells/µl), the mean difference was +61 cells/µl (95% CI +34.5, +88.4) between the methods and the 95% limits of agreement decreased to +315 cells/µl (95% CI +269, +361.5) and −192 cells/µl (95% CI −238.7, −146.1) (Fig. 2b).

The analysis demonstrated an increasing variability of the differences (plotted on y-axis) with an increasing average (plotted on x-axis), indicating that at least one of the methods depended on the magnitude of the measurement (CD4⁺ cell count) (Figs. 2a and 2b).

The precision of agreement (\(\eta\)) of the methods was 0.3 (with 0 \(\leq\) \(\eta\) \(\leq\) 1, \(\eta = 0\) indicating high and \(\eta = 1\) indicating low precision of agreement).

The sensitivity of the Cyto-Spheres method to predict CD4⁺ cell counts below 350 cells/µl (cut-off for treatment indication) correctly was 84% (95% CI 73–90%), the specificity 80% (95% CI 68–88%), the positive predictive value 81% and the negative predictive value was 82%.
Comparison of Volumetric Single Platform Flow Cytometry (CyFlow) with Dual Platform Flow Cytometry (FACScan)

Among the 128 samples analyzed by DPFC, 44 (34%) revealed CD4⁺ counts below 200 cells/μl, 39 (23%) between 201 and 350, 19 (15%) between 351 and 500, 28 (22%) between 501 and 750, and 8 (6%) above 750 cells/μl. VSPFC (Cyflow) generally generated higher CD4⁺ cell counts than DPFC (median CD4⁺ cell count 369/μl and 288/μl, respectively).

The overall median difference between the CyFlow method and DPFC was 68 cells/μl (+70 for CD4⁺ cell counts below 200, +92 for CD4⁺ cell counts between 201 and 350, +32.5 for CD4⁺ cell counts between 501 and 750, and +178 for CD4⁺ cell counts above 750).

When comparing the overall median difference to a hypothetical median difference of 0, a statistically significant difference was detected ($P < 0.0001$).

The correlation coefficient comparing both methods was $r = 0.929$ ($P < 0.01$). The regression analysis, including the best regression line $y = a + bx_i$ ($i = 1, 2, 3, \ldots, n$) is illustrated in Figure 1b. Multiple linear regression was used to scan for additional covariates. Among the covariates included (CyFlow method, gender, age), only the CyFlow method was significantly associated with DPFC ($r^2 = 0.864$, $P < 0.01$) and could be used to predict the results of the standard DPFC method ($\beta = 0.93$).

Bland–Altman analysis showed a mean difference of +369 cells/μl (95% CI +65.1, +120.4) between the CyFlow method and DPFC, 95% limits of agreement were +402 cells/μl (95% CI +355.1, +450.9) and -217 cells/μl (95% CI -264.4, -169.7) (Fig. 3a).

If only the range clinically relevant for HIV⁺ patients was analyzed (CD4⁺ cell count between 0 and 500 cells/μl), the mean difference was +103 cells/μl (95% CI +76.3, +129.6), the 95% limits of agreement decreased.
to +355 cells/µl (95% CI +309.7, +401.2) and −149 cells/µl (95% CI −195.2, −103.7) (Fig. 3b). No trend for a variability of the differences was detected.

The precision of agreement (η) of both methods was 0.08 (with 0 ≤ η ≤ 1, η = 0 indicating high and η = 1 indicating low precision of agreement).

The sensitivity of the CyFlow method to predict CD4⁺ cell counts below 350 cells/µl (cut-off for treatment indication) correctly was 71% (95% CI 60–80%), the specificity 93% (95% CI 83–97%), the positive predictive value 93% and the negative predictive value was 71%.

**ROC Graph and Euclidean Distance**

The ROC graphs for the manual Cyto-Spheres and for CyFlow methods are illustrated in Figure 4. A circle was constructed with the centre at the point of 100% sensitivity and 100% specificity (left upper corner of the rectangle) in order to visualize the minimal Euclidean distance of values on the ROC graphs to this point. The values closest to this point are considered as optimal threshold values and illustrated in Figures 5a and 5b. The curve illustrated in Figure 5a has a clear minimum at a CD4⁺ cell count of 400/µl, which expresses the optimal threshold value for the CyFlow method. The curve illustrated in Figure 5b does not show a unique, distinct minimum. The optimal threshold value for the Cyto-Spheres method is difficult to determine and lies between 400–500 cells/µl.

**Discussion**

In this study we evaluated two techniques for CD4⁺ cell enumeration under field conditions in Uganda; a manual method (Cyto-Spheres assay) and a volumetric, single-platform flow cytometry (automated 2 parameter CyFlow Counter) against standard dual-platform flow cytometry (FACScan). Because of simplified protocols and analyzing procedures, both methods are cheaper and easier to handle than conventional techniques (standard single or dual-platform flow cytometry) and thus are deemed to be very useful in resource limited areas.

Our study showed a higher correlation for the CyFlow method (r = 0.929) than for the manual Cyto-Spheres method (r = 0.725) when compared to the reference method. These findings are in line with other studies. Recently, a multicenter study demonstrated a high correlation of CD4⁺ counts obtained by the CyFlow method when compared to either standard single or dual-platform flow cytometry (overall mean correlation for all centers; r = 0.944) (13). Other studies compared the Cyto-Spheres assay with dual-platform flow cytometry (DPFC) and detected intermediate to high correlations (r = 0.74 to r = 0.93) (8,17–20), whereas only one study found a substantially lower correlation (r = 0.45) (6).

Multiple linear regression analysis showed that CD4⁺ cell counts by the CyFlow method were a stronger predictor for results of DPFC than those of the Cyto-Spheres method (r² = 0.864, β = 0.93 and r² = 0.552, β = 0.7, respectively). Whereas for the CyFlow method about 86% of the observed variance was explained, this was the case for only 55% of the observed variance in the Cyto-Spheres method.

When compared to the reference method, the precision of agreement was higher for the CyFlow (η = 0.08) than for the Cyto-Spheres method (η = 0.3).

Generally, the CyFlow method generated higher CD4⁺ cell counts than DPFC (median CD4⁺ cell count +369 and +288 cells/µl, respectively). This trend was less pronounced for the Cyto-Spheres method when compared to DPFC (median +343 and +332 cells/µl, respectively). In addition, the overall median difference of CD4⁺ cell counts was higher for the CyFlow method than for the manual method when compared to DPFC (+68 and +28 cells/µl, respectively). A statistically significant difference of the overall median difference to a hypothetical median difference of 0 was detected only for the CyFlow method, not for the Cyto-Spheres method.

Similarly, the mean difference assessed by Bland–Altman analysis was higher for the CyFlow method when compared to DPFC than for the Cyto-Spheres method (−92 and 1.4 cells/µl, respectively). This difference, how-
ever, decreased when only the clinically relevant range for HIV+ patients was analyzed (CD4⁺ cell count between 0 and 500 cells/µl; mean difference +103 and +61 cells/µl, respectively).

Results of Bland-Altman analysis from a multicenter study were similar to our findings: the mean difference between the CyFlow method and DPFC was +123, +44, +39 and −68 cells/µl according to the different study centres (13).

Specificity and positive predictive value (ppv) to classify CD4⁺ cell counts correctly below a threshold set for treatment indication (CD4⁺ cell count <350/µl) were higher for the CyFlow method when compared to DPFC than for the Cyto-Spheres method (specificity 93% and 80%, ppv 93% and 80%, respectively). In contrast, sensitivity and negative predictive value (npv) were higher for the Cyto-Spheres method than for CyFlow method (sensitivity 84% and 71%, npv 82% and 71%, respectively).

With an optimal relationship between sensitivity and specificity, as analyzed on the basis of ROC graphs, the best threshold value would be at 400 CD4⁺ cells/µl for the CyFlow method. In contrast, no clear threshold value could be demonstrated for the Cyto-Spheres method.

Taken together, comparing VSPFC (CyFlow) and the manual method (Cyto-Spheres), our findings would favor the CyFlow method to be used as a substitute for reference DPFC. The generally higher counts obtained with the CyFlow method compared with those obtained by the reference method can at least partly be explained by the lack of an erythrocyte lysing procedure in the CyFlow protocol. This needs to be taken into account when using this technique and requires appropriate calibration (13). This systematic difference also contributes to the higher median and mean difference and to the lower sensitivity of the CyFlow method as compared with the Cyto-Spheres method.

Advantages and Disadvantages of the Manual Cyto-Spheres Method and the CyFlow Method

The equipment for the Cyto-Spheres assay is minimal and relatively cheap (about € 3,000 for a microscope).
whereas the price per test kit is relatively high (about € 7–14). The method is robust and easy to handle under field conditions, maintenance is minimal and uncomplicated. Training for laboratory technicians requires 1–2 days. The number of samples which can be analyzed per day is relatively low (about 2–4/h) due to the time consuming counting procedure. In addition, the quality of the results depends on individual skills and experience of the laboratory technician. Results may also be less accurate due to the low number of total cells counted (250) per analysis (6,8,17–20).

The price for a two-parameter modus-based CyFlow to perform VSPFC is moderate (about € 20,000) and the price per test kit and reagents is relatively low (€ 1.75–2).

The analyzing procedure of the CyFlow counter is automated and training for laboratory technicians requires 1–2 days. The number of samples which can be analyzed per day is higher (about 10/h). In addition, generic antibodies can be used which may further decrease running costs. The technical expertise required for VSPFC using a CyFlow counter is, however, higher and maintenance is more complicated. Repair procedures usually require the attendance of a specialist from outside and experience with the technique under field conditions is still scarce.

Our results show that both, the manual Cyto-Spheres method and the CyFlow method can be used for the enumeration of CD4⁺ cells in resource limited settings. The choice of the technique should depend on the local conditions. Under supervised conditions as in our study, the CyFlow method produced more reliable results than the Cyto-Spheres method. Furthermore, the CyFlow method processes a higher number of samples. Whatever technique is used, regular quality control with a standard method should be performed.

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LITERATURE CITED