



# THE CENTRAL AFRICAN JOURNAL OF MEDICINE

Vol. 47, No. 3

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March 2001

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# Enumeration of CD4 and CD8 T-cells in HIV infection in Zimbabwe using a manual immunocytochemical method

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## Abstract

**Objectives:** To enumerate CD4 and CD8 T-cells using the simple and cheap immuno-alkaline phosphatase (IA) method and to compare it with flow cytometry (FC); and to study the effects of duration of sample storage on the IA method results.

**Design:** Method comparison study.

**Setting:** Blair Research Laboratory, Harare, Zimbabwe.

**Subjects:** 41 HIV positive and 11 HIV negative men and women from Harare participating in HIV studies at Blair Research Laboratory, Zimbabwe

**Main Outcome Measures:** CD4 and CD8 T-cell counts by FC and the IA method.

**Results:** The IA method and FC were highly correlated for CD4 counts (Spearman  $r_s=0.91$ ), CD4 percentage ( $r_s=0.84$ ), CD8 count ( $r_s=0.83$ ), CD8 percentage ( $r_s=0.96$ ) and CD4/CD8 ratio ( $r_s=0.89$ ). However, CD4 cell counts and percentage measured by the IA method were (mean difference  $\pm$ SE)  $133 \pm 24$  cells/mL and  $6.7 \pm$

*Cent Afr J Med* 2001;47(3):64-70

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1.1% higher than those measured by the FC method ( $p < 0.0001$ ) respectively. CD8 counts and percentages by the IA method were lower than those by the FC method ( $p < 0.01$ ). Accordingly, the IA method gave a higher CD4/CD8 ratio ( $p < 0.01$ ). IA method CD4 counts  $< 300/\text{mL}$  best predicted FC CD4 counts  $< 200/\text{mL}$  while IA CD4%  $< 25$  best predicted FC CD4%  $< 14\%$ . IA method CD4/CD8 ratio  $< 0.8$  best predicted FC CD4/CD8 ratio  $< 0.5$ . Smears stored for up to 18 months gave results similar to fresh smears.

**Conclusion:** The IA method correlates well with but gives CD4 counts and percentages that are higher than those determined by FC. On the contrary, the IA method gives CD8 counts and percentage that are lower than FC values. The method is a cheap and reliable alternative to FC and allows storage of samples for extended periods before analysis, making it an appropriate technology for resource-poor countries.

## Introduction

The clinical manifestations of HIV infection are mostly dependent on the level of CD4 cells.<sup>1,2</sup> Where viral load assays are not available, a rise in the CD4 count is an acceptable indication of treatment efficacy. In addition, CD4 cell levels are very useful when deciding on the time to start or stop prophylaxis against certain opportunistic infections.<sup>1,2</sup>

The standard method for quantifying T-cell subsets is flow cytometry (FC). However, the method requires expensive instruments, maintenance and highly trained technologists. Thus this method of T-cell subset determination cannot be readily available for routine use in developing countries. Simple, reliable and inexpensive alternative methods are needed in these countries where HIV infection and AIDS are highly prevalent, and where financial and technical resources are limited. Furthermore, it would be useful if such methods allowed analysis of samples after relatively long periods of storage.

Several alternative technologies for CD4 and CD8 T-cell phenotyping have been developed and evaluated.<sup>3-11</sup> Among these methods is the immuno-alkaline phosphatase (IA) method.<sup>4,12</sup> This method is based on the principle that anti-CD4 or CD8 monoclonal antibodies react with CD4 or CD8 molecules on cells in a haematological smear.<sup>12</sup> This antigen-antibody reaction is then visualised by addition of an enzyme-labelled antibody raised against the anti-CD4 or CD8. The IA method has been shown to be reproducible and reliable in four African countries.<sup>4</sup> Furthermore, the method has been shown to be cheaper than FC and to allow examination of smears after long periods of storage.<sup>3</sup>

In Zimbabwe, although management of HIV infection is mostly palliative, the use of anti-retroviral drugs in treatment of HIV infection is increasing. This has resulted in an increased requirement for CD4 counts in clinical practice. Thus the need for cheaper methods of T-cell subset determination cannot be over emphasized. We, therefore, validated the IA method by comparing it with standard FC using the FACSCount system for potential use in research and clinical practice. The effect of storage of samples on the performance of the IA method was also evaluated.

## Materials and Methods

### Study Population.

The study population were men and women who knew their HIV serostatus through participating in HIV studies

at Blair Research Laboratory in Harare, Zimbabwe. Information about the study was provided and volunteers enrolled. Counselling was provided as part of the HIV studies in which the subjects were participating.

### Blood Collection and Processing.

Venous blood was collected in two EDTA tubes and in one plain tube. The anti-coagulated blood was used to prepare 6 haematological smears. The smears were air dried, wrapped in aluminium foil and stored at  $-20^{\circ}\text{C}$  until immunocytochemical analysis for CD4 and CD8 cells. The remaining anti-coagulated blood was used for total and differential white blood cell counts (Coulter MaxM, Denmark), and for CD4 and CD8 T-cell enumeration by FC. Serum obtained from the other tubes was tested for HIV antibodies using commercial enzyme-linked immunosorbent assay kits following manufacturer's instructions (Genelavia Mixt@, Sanofi, France and Recombigen, Trinity Biotech, Ireland).

### Flow Cytometry.

The FACSCount system (Becton Dickenson, NJ, USA) was used for automated CD4 and CD8 counts within 24 hours of blood collection. Anti-coagulated blood was added to reagent tubes containing fluorochrome conjugated monoclonal antibodies to CD3 and CD4 or CD8 (Becton Dickenson, NJ, USA) and incubated for two hours at room temperature. Fixative was added and samples incubated at room temperature for a minimum of two hours before enumeration. In the enumeration process, a known number of reference beads contained in each reagent tube functions as a fluorescence and quantitation standard for calculating the absolute CD4+, CD8+ and CD3+ T cells. The FACSCount system software collects up to 30 000 events per sample so that the precision of the measurements is not limited by statistical sampling variation. The FACSCount's usable range for CD4 T cells is 50 to 2 000 cells/ $\mu\text{L}$ , for CD8 100 to 2 000 cells/ $\mu\text{L}$  and for CD3 100 to 3 500 cells/ $\mu\text{L}$ . Manufacturer's instructions on quality control procedures were followed. CD4 and CD8 percentages were the proportion of total lymphocytes that were CD4+ or CD8+ expressed as a percentage. The total lymphocyte count was derived from the Coulter counts.

### Immuno-alkaline Phosphatase Method.

Stored smears were processed within one month of sample collection. The immuno-alkaline phosphatase method was used for CD4 and CD8 phenotyping as described in the World Health Organisation (WHO) manual for developing countries<sup>13</sup> and by Lisse *et al* (1997).<sup>4</sup>

Briefly, frozen blood smears were left to reach room temperature before they were fixed in acetone:methanol for 30 seconds. The smears were washed in 0.05M Tris buffered saline (TBS) pH 7.6 (Saarchem Pvt Ltd, South Africa) and incubated with 2% bovine serum albumin (BSA) in TBS for 10 minutes. Mouse anti-human CD4 or CD8 monoclonal antibody (Dako, Denmark) was added and the smears incubated in a moist chamber overnight at 4°C. After washing in TBS, biotinylated rabbit anti-mouse (Dako, Denmark) was added followed by avidin-biotin-alkaline phosphatase complex (Dako, Denmark) and Fast Red substrate (Dako, Denmark). The substrate stains the CD4<sup>+</sup> or CD8<sup>+</sup> cells red. Once the CD4<sup>+</sup> or CD8<sup>+</sup> cells were clearly visible under the microscope, smears were counter-stained with alcohol free Mayer's hematoxylin stain (Sigma, Germany) and mounted with glycerol (Dako, Denmark). Using a light microscope (x40 objective) the number of CD4<sup>+</sup> or CD8<sup>+</sup> cells per 200 lymphocytes were counted and the results reported as number of CD4<sup>+</sup> or CD8<sup>+</sup> cells per 200 lymphocytes. The absolute CD4 count was calculated as follows: CD4<sup>+</sup>/200 x WBC x %lymphocyte. The lymphocyte percentage and WBC values were derived from Coulter counts. Smears with known CD4 counts and percentage were included in all batches of smears stained to ensure quality of the immunostaining and enumeration procedures.

#### Effect of Duration of Storage.

In order to assess the effect of storage on detection of the CD4 T cell marker, a batch of smears from two HIV positive and two HIV negative individuals with known CD4 percent was stored. Once every six months, one smear from each of the individuals was retrieved and included in the staining procedures. The CD4 percentages were then compared to determine the effect of storage on the immunophenotyping process using the IA method.

#### Data Analysis.

The Mann-Whitney U-test was employed to compare between groups. The Wilcoxon Signed Rank test was used for paired analysis of the two methods. Spearman's rank correlation ( $r_s$ ) assessed the degree of association between the results of the two methods. Means and standard deviation of differences between the two methods were used to determine the agreement of the methods.<sup>14</sup> The 95% limits of agreement were defined as mean difference  $\pm$  1.96 SD. Various cut-offs of IA CD4 counts and percentage were used to evaluate the sensitivity and specificity of the IA method to identify low FC CD4 counts (< 200/mL) or percentage (<14%).

## Results

Paired FC and IA CD4 counts were available from 52 individuals (median age 26.5, range 18 to 42 years). Thirty five (67.3%) were females, 29 of whom were HIV positive, while 12 (70.6%) of the 17 males were HIV positive. Males were older than females (median age, 35.0 versus 25.2 years,  $p < 0.0001$ ). Overall, based on FC results, CD4

counts ranged from 17 to 1 981 cells/ $\mu$ L with a median of 408 cells/ $\mu$ L. Median CD8 count was 899, range 157 to 1 924 cells/ $\mu$ L while CD4/CD8 ratio ranged from 0.05 to 2.94 with a median 0.47. IA method and FC results obtained showed that CD4 counts, percentages and CD4/CD8 were lower and CD8 counts and percentage were higher in HIV positive than in negative individuals ( $p < 0.01$ ).

#### Correlation Between FC and IA CD4 and CD8 T-cell Measurements.

Figure I shows scatter plots of IA against FC values for CD4 and CD8 counts and percentages and for CD4/CD8 ratio, with the line of equality describing the relationship. The IA CD4 counts were highly correlated with FACS CD4 counts (Spearman  $r_s = 0.91$ ;  $p < 0.0001$ ). Similarly IA method CD4 percent ( $r_s = 0.86$ ), CD8 counts ( $r_s = 0.83$ ), CD8% ( $r_s = 0.96$ ) and CD4/CD8 ratio ( $r_s = 0.89$ ) were highly correlated with FACS results ( $p < 0.0001$  for all).

Table I: Mean and standard deviations (SD) of IA and FC CD4 and CD8 counts, percentage and ratio, with mean differences (SD) between the methods.

	Methods		Difference*
	IA	FC	
<b>CD4 (n=52)</b>			
Cells/ $\mu$ L	688 (565)	555 (473)	133 (170)
Percent	33.2 (17.0)	26.5 (15.9)	6.7 (8.0)
<b>CD8 (n=27)</b>			
Cells/ $\mu$ L	814 (341)	890 (384)	-76 (183)
Percent	40.1 (14.3)	45.2 (18.3)	-5.0 (5.8)
<b>CD4/CD8 ration (n=27)</b>	1.10 (0.95)	0.85 (0.75)	0.25 (0.37)

\*Difference = IA - FC.

#### Agreement of Methods.

Table I shows the mean (SD) of CD4 and CD8 counts, percentage and CD4/CD8 ratio, and the mean differences (SD) between the IA and FC methods. IA CD4 counts and percentages were significantly higher than FC values (688 versus 555 cells/mL and 33.2 vs 26.5%; Wilcoxon Signed Rank test,  $p < 0.0001$  respectively). Thus the IA method gave CD4 counts that were higher than FC counts by (mean difference  $\pm$  SE)  $133 \pm 24$  cells/mL;  $p < 0.001$ ) and the 95% limits of agreement of the methods were (mean difference  $\pm$  SD)  $133 \pm 170$  or -198 to 464 cells/mL. There was no difference in agreement between the two methods at FC CD4 counts below and above 500/mL (mean difference  $\pm$  SD,  $105 \pm 118$  vs  $178 \pm 226$  cells/mL;  $p = 0.13$ ) (Figure I). The mean difference  $\pm$  SE between the IA and FC method CD4% was  $6.7 \pm 1.1$ . The 95% limits of agreement between the two methods were  $67 \pm 8.0\%$  or -9.0% to 22.4%. The agreement was also similar at FC CD4% below and above 25% ( $6.9 \pm 6.6$  vs  $6.4 \pm 9.5$ ;  $P = 0.85$ ) (Figure II).

IA CD8 counts and percentages were lower than FC values (814 vs 890 cells/mL,  $p = 0.029$  and 40.1 vs 45.2%,  $p < 0.001$  respectively). The mean difference  $\pm$  SE were  $-76 \pm 36$  cells/mL and  $-5.0 \pm 1.1\%$  respectively. The limits of agreement for CD8 counts and percentage were  $-76 \pm 183$

Figure I: Scatter plots of CD4 and CD8 counts and %, and CD4/CD8 ratio by the IA against FC method, with line of equality.

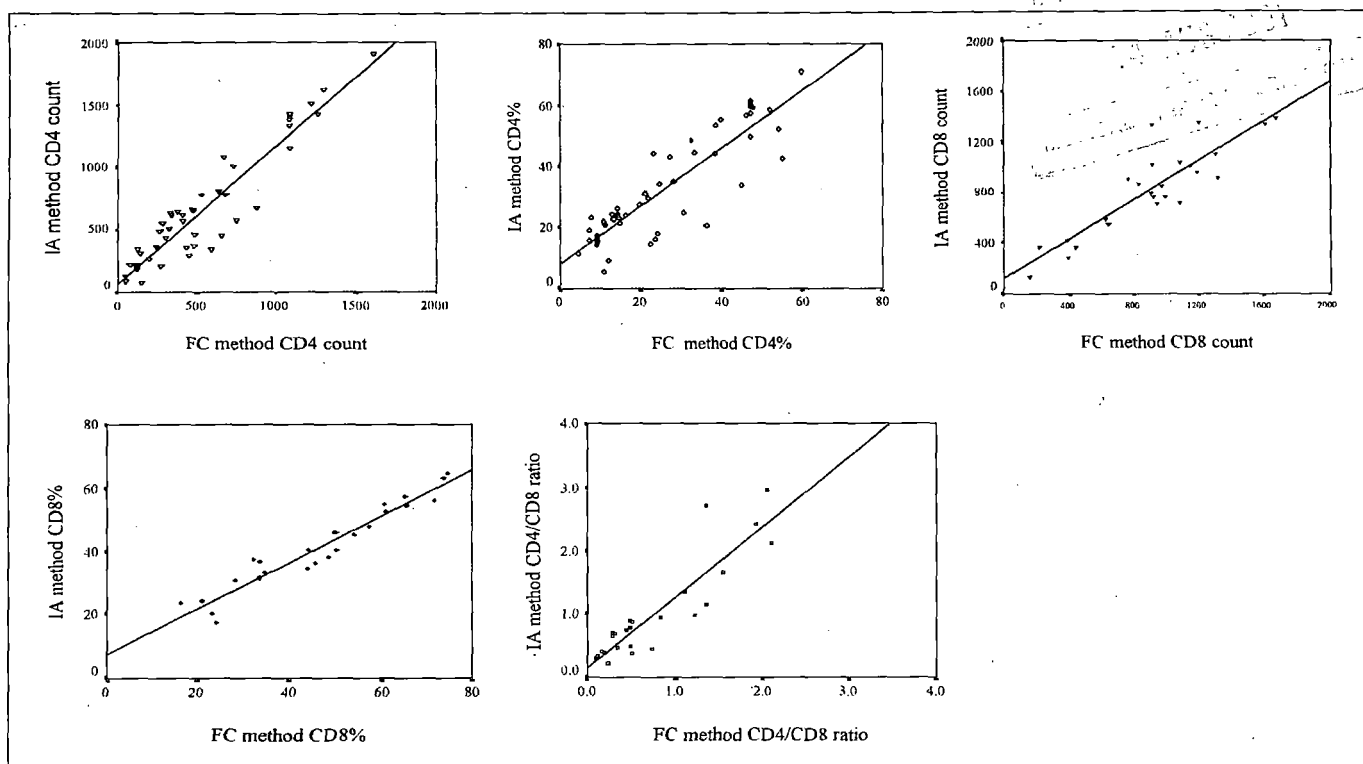
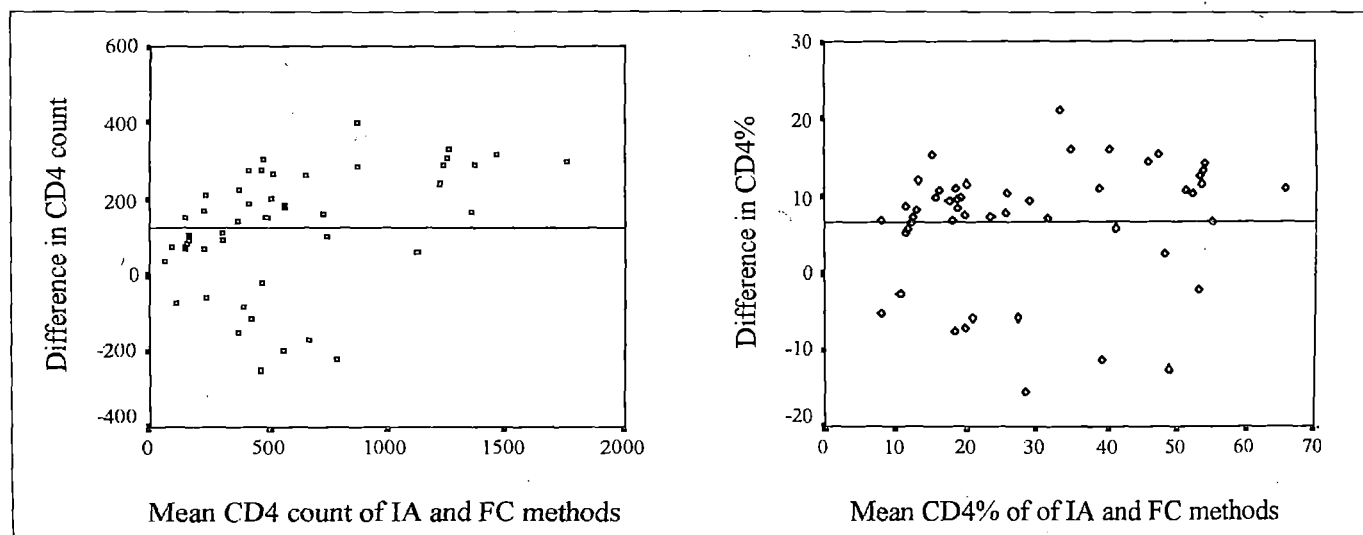


Figure II: Plots of differences in CD4 counts and % between the IA and FC methods against the average values for the two methods.



or -435 to 283 cells/ $\mu$ L and  $-5.0 \pm 5.8$  or -16.5 to 6.3% respectively. Because of the higher CD4 and lower CD8 percentages, the IA method gave CD4/CD8 ratio that was (mean difference  $\pm$ SE)  $0.25 \pm 0.07$  higher than that by the FC method ( $p \leq 0.001$ ) (Table I). The 95% limits of agreement were  $0.25 \pm 0.37$  or -0.48 to 0.98.

**Sensitivity and Specificity of the IA Method.**

Sensitivity and specificity of the IA method to identify low CD4 counts (<200/mL by FC method) was evaluated at IA CD4 counts <200, <250 and <300 cells/mL. Similarly,

sensitivity and specificity of IA CD4% <14, <16, <20 and 25 to detect FC CD4% <14 are shown in Table II. The ability of the IA method to correctly identify FC counts <200 cells/ $\mu$ L was highest with an IA method cut-off of 300 cells/ $\mu$ L, and lowest when a cut-off of <200 cells/ $\mu$ L. An IA CD4 percent cut-off of <14% could only correctly identify 18.8% of FC values <14% whereas IA CD4% <25 best predicted FC CD4 <14%. On the other hand, an IA CD4/CD8 ratio <0.8 had over 90% sensitivity and specificity in identifying FC CD4/CD8 ratio <0.5.

**Table II: Sensitivity and specificity of the IA method in classifying low (<200 cells/ $\mu$ L) FACS count CD4 counts and percent, and CD4/CD8 ratio.**

IA CD4 level	Sensitivity*	Specificity**
<b>CD4 count (cells/<math>\mu</math>L)</b>		
<200	50.0% (7/14)	100% (38/38)
<250	78.6% (11/14)	97.3% (37/38)
<300	85.7% (12/14)	94.7% (36/38)
<b>CD4%</b>		
<14	18.8% (3/16)	100% (36/36)
<16	43.8% (7/16)	97.2% (35/36)
<20	62.5% (10/16)	91.7% (33/36)
<25	100% (16/16)	75.0% (27/36)
<b>CD4/CD8 ratio</b>		
<0.5	57.1% (8/14)	91.7% (11/12)
<0.8	92.9% (13/14)	91.7% (11/12)
<1.0	100% (14/14)	66.7% (8/12)

\* The figures in parentheses are the proportion of FACScount CD4 values <200/ $\mu$ L correctly identified by the appropriate IA cut-off.

\*\* The figures in parentheses are the proportions of FACScount CD4 values  $\geq$ 200/ $\mu$ L correctly identified by the appropriate IA cut off.

### Observer Variation.

The positive methodologic control used showed that precision of the IA method was 5.4%. Intra- and inter-observer variation of the IA enumeration of CD4 cells was evaluated by double reading by one and two observers, respectively. The coefficient (SD) of intra-observer variation was 6.3 (8.3)% and that of inter-observer variation was 8.9 (5.7)%. Intra-observer variation was lower when CD4 counts were <200/ $\mu$ L compared to >200/ $\mu$ L (2.1% versus 6.8%, Mann-Whitney U-test;  $p=0.001$ ) but inter-observer variation was similar for CD4 counts <200/ $\mu$ L compared to >200/ $\mu$ L (10.8% versus 8.5%;  $p=0.09$ ).

### Effect of Storage on CD4 Count.

To evaluate effect of storage on antigenicity of CD4, smears from four subjects were stained and enumerated within one month and after six, 12 and 18 months of storage. The mean (SD) CD4% at these time points was 28.1 (13.7), 26.1 (16.8), 30.9 (11.6) and 23.4 (11.6) respectively. There was no difference in CD4% between the specified time points (Kruskal Wallis,  $p=0.60$ ) indicating no significant change in CD4%. However, counts at 18 months tended to be lower than at earlier times.

## Discussion

The present study has shown that the IA method CD4 and CD8 counts, percentage and ratio correlate well with FC values. However, the two methods are not interchangeable because the IA method gives consistently higher CD4 counts and percentages as has been observed by others.<sup>3,4</sup> There is no obvious explanation for the higher counts but this has been attributed to a qualified identification of lymphocytes by the manual method, whereas FC could miss CD4 T-cells weakly stained by the fluorochrome.<sup>3,15</sup> Alternatively, in the IA method, CD4 positive monocytes could be mistaken for CD4 T-cells.

That CD8 counts and percentage by the IA method were lower than the FC CD8 T-cell values is in agreement with a recent study comparing the results of the IA method performed in four countries.<sup>4</sup> However, the same authors previously reported higher CD8 T-cell percentage by the IA compared to FC.<sup>3</sup> This variation in CD8 T-cell values may be partially dependent on the monoclonal antibodies used and the heterogeneity of the CD8 molecules.<sup>4</sup> Nonetheless, because of the higher CD4 and lower CD8 T-cell percentage, we found that the IA method consistently gave higher CD4/CD8 ratios than those derived from FC. The wide limits of agreement suggest wide variation in individual measurements by the IA method and would likely be much lower with larger sample sizes. Indeed a total of only 200 lymphocytes were counted in the IA method. In contrast, the FACScount systems records up to 30 000 events per sample in the counting process such that the precision of the measurement is not limited by statistical sampling variation. It is, therefore, important that the IA method be evaluated further with larger sample sizes that will also enable conversion factors to be determined to allow estimation of FC equivalents where this is deemed necessary.

Flow cytometry CD4 T-cell counts below 200/ $\mu$ L are associated with the immunosuppression characteristic of AIDS in HIV infected individuals.<sup>1,2</sup> Thus monitoring of CD4 cell counts guides management of HIV infected patients. Since the IA method gives higher CD4 values, it is essential that the appropriate cut-offs be determined to enable clinical application of the IA method results. In the present study, the IA CD4 T-cell level of <300/ $\mu$ L gave the highest sensitivity in the identification of FC CD4 counts <200/ $\mu$ L and therefore in classifying AIDS patients. This is in accordance with the observed bias of 133 cells/ $\mu$ L between the two methods. The sensitivity of 86% achieved by the IA CD4 count cut-off of <300/ $\mu$ L is similar to that of Lisse *et al.* at 81%<sup>4</sup>. It has been suggested that CD4% is a more reliable parameter than absolute counts in assessing immune status,<sup>16</sup> and a FC CD4 percentage below 14% (low CD4%) roughly corresponds to an absolute count of <200 CD4 cells/ $\mu$ L. In the present study maximum sensitivity of the IA method was achieved by a cut-off of <25%. Furthermore, CD4% of <25% was associated with IA absolute counts <300/ $\mu$ L and FC values <200/ $\mu$ L (data not shown). Our data suggests that the IA method cut-offs have adequate diagnostic sensitivity and specificity for classification of HIV disease in our setting.

T-cell phenotyping is subject to significant technical and physiologic variability,<sup>17-20</sup> and correct interpretation of results depends on the precision and reproducibility of the method used. With the IA method, variation in measurements could arise from differences in affinity of monoclonal antibodies in different lots or by different manufacturers, or due to observer error. Alternatively, results could also depend on the method used to evaluate the total lymphocyte and white blood cell count. Manually determined total lymphocyte counts have been shown to be

higher than those from an automated haematology analyser.<sup>3</sup> We assessed intra- and inter-observer variation and found them to be within acceptable ranges compared to other alternative CD4 technologies.<sup>6,9</sup> This suggests that observer error is unlikely to hamper use of the IA method, provided the microscopists are well trained. The method has been shown to be reproducible in different settings in Africa and Europe.<sup>3,4</sup> However, even using FC there is considerable variation in results due to differences in instruments, reagents and processing of specimens.<sup>15</sup>

We have also shown that blood smears can be kept at -20°C for up to 18 months and still give results that are similar to fresh smears. However, this was assessed on only four subjects and may need to be verified with larger sample sizes.

The use of CD4 counts is increasing in developing countries as HIV infected individuals become more aware of the importance of the parameter in prognosis, and clinicians battle to improve the management of ever increasing numbers of AIDS patients in the absence of anti-retroviral drugs. The present study has shown that the IA method could be relied upon as an alternative to flow cytometry and could offer expanded availability of CD4 evaluation to individuals with HIV infection in Zimbabwe. We have determined CD4 count and percentage cut-offs that are applicable with the IA method thereby allowing application of the method in classification of HIV disease and other immuno-deficient conditions in the Zimbabwean setting. The cost of the IA method is six-fold cheaper than FC kits based on costs of reagents only (data not shown). Clearly, if the costs of equipment repair and maintenance were added, then the IA method would be many times cheaper than FC.

In conclusion, the IA method does not require expensive instruments or laboratories and enables samples to be analysed long periods after collection. The method is, therefore, appropriate for use in developing countries with poor resources. However, the results of the IA method should be interpreted in cognisance of the fact that the method gives CD4 counts that are about 100 cells/ $\mu$ L higher than those of flow cytometry and that the accuracy of the method is heavily dependent on the microscopists. Hence the need for well trained microscopists cannot be over-emphasized.

### Acknowledgements

We thank the participants in the study, and the technical staff of the Communicable and Infectious Diseases Unit of Blair Research Institute, in particular White Soko, Wislon Mashange, Kerina Duri and Tsitsi Chirewa for their support. Flow cytometry was performed with the assistance of Simon Madzime at the Department of Obstetrics and Gynaecology, University of Zimbabwe. We are grateful to the Director of Blair Research Institute, the Secretary for Health and the Medical Research Council of Zimbabwe for granting permission to conduct the study. Danish

International Development Assistance and the Danish Bilharziasis Laboratory, Denmark funded the study.

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