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Low-Cost CD4 Enumeration in HIV-Infected Patients in Thailand

Kovit Pattanapanyasat[1], Surada Lerdwana[1], Hla Shain[2], Egarit Noulsri[1], Charin Thepthai[3], Varipin Prasertsilpa[2], Achara Eksaengsri[2] and Krisana Kraisintu[2]

SUMMARY **In Thailand, over one million people have been infected with HIV since the beginning of the epidemic. This has created a great burden on the country's limited health care budget. Monitoring CD4+ T-lymphocytes is important to determine the success of any antiretroviral therapy as well as HIV vaccine trials. However, the high cost of CD4 counts makes monitoring of every HIV-infected patient impossible in Thailand. Therefore, the development of affordable strategies is necessary in order to allow more HIV infected persons to access CD4 testing to control the disease. The current standard methods for enumeration of CD4+ T-lymphocytes are performed on whole blood by flow cytometric immunophenotyping using the 6-tube 2-color and 3-tube 3-color panels recommended by the Centers for Diseases Control (CDC). In this study, percentage CD4+ T-lymphocyte values (from 142 HIV-seropositive patients and 26 anti-HIV negative adult blood donors) generated by the use of just 2 reagents (CD45/CD4) in a 1-tube 2-color panel employing side scatter/CD45 morphospectral gating were compared to those obtained by state of the art methods. We also compared the use of generic monoclonal antibody reagents with commercial reagents and found the results to be comparable with an overall correlation coefficient (*r*) of more than 0.95 for both CD4+ and CD8+ T-lymphocytes. Bland-Altman analysis of the mean CD4 values plotted against the difference in values between the generic reagents and the commercial reagents showed no bias. The 1-tube 2-color method using generic monoclonal antibody reagents potentially permits more affordable but reliable CD4 testing and therefore could increase access for more HIV-infected patients in resource-poor countries.**

Since the first case of HIV-infection which was recognized in 1985,[1] the disease has soared to epidemic proportions in Thailand. In 2001, the Thai Working Group on HIV/AIDS Projections reported that nearly one million people have been infected by HIV;[2] of these, 289,000 have died and nearly 700,000 are currently living with HIV or AIDS. It is estimated that another 55,000 will soon develop serious AIDS related illnesses that will require medical care. This AIDS epidemic has placed an enormous burden on the limited health care budget of a resource-poor country like Thailand.

CD4+ T-lymphocytes are primarily targeted by the human immunodeficiency virus and are depleted during the course of the disease.[3-5] CD4+ T-lymphocyte counts are thus a measurement of disease progress and relevant in dealing with HIV epidemics like in Thailand. The appropriate utilization of antiretroviral therapies and the success of HIV vaccine trials are critically dependent on the availability of CD4+ T-lymphocyte counts.[5-7] However, the cost for CD4 testing is relatively high, i.e. 500-1,000 Baht (US$12-24) per test. If one considers costs related to the whole antiretroviral therapy program that includes antiretroviral drugs, laboratory monitoring using expensive PCR based viral load and several CD4+ T-lymphocyte counts for all HIV+/AIDS individuals, the expense would be devastating and could easily exceed the current annual health care budget of Thailand.

In Thailand, a dual platform

From the [1]Center of Excellence for Flow Cytometry, Office for Research and Development, [3]Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, [2]Institute of Research and Development, Government Pharmaceutical Organization, Bangkok, Thailand.
Correspondence: Kovit Pattanapanyasat

approach utilizing either a two-color or three-color flow cytometric (FCM) immunophenotyping technique to generate a percentage CD4 (%CD4) among lymphocytes and a hematological analyzer to enumerate the absolute lymphocyte counts is used in most regional hospitals to calculate the absolute CD4 counts. Instead of the standard 6-tube 2-color method[8] which uses a panel of 12 reagents for the enumeration of CD4+ and CD8+ T cells, CD19+ B cells and CD16+/56+ NK cells, an abbreviated version of CD4 testing (utilizing a panel of CD45, CD14, CD3, CD4 and CD8 when CD8 values are requested) is in widespread use in an attempt to control costs. The 3-tube 3-color immunophenotyping technique[9-12] (CD3/CD4/CD45 for CD4 testing and CD3, CD8, CD45 for CD8 testing) is another strategy used in some hospitals. The cost of CD4 testing remains expensive and thus inaccessible to most HIV/AIDS patients. There are 2 main reasons for the high cost of CD4 and CD8 testing-1) the use of reagents for lymphocyte markers i.e. CD45, CD14 and CD3 in the 2-color protocol and CD45, CD3 in the 3-color protocol which are considered necessary for the separation of lymphocytes from other white cell elements, and 2) the use of commercial reagents which are expensive. Recently the use of simpler protocols in combination with generic monoclonal antibodies in order to increase cost-efficiency has been reported.[13]

The objective of this study was to find an affordable way of enumerating CD4 cells without compromising precision and accuracy, considering the local situation. Percentage CD4 among lymphocytes is commonly requested by physicians and is also an integral part of the FCM analysis of CD4 cells when performing the absolute CD4 count using the dual platform. In this study the accuracy and precision of %CD4 values (among lymphocytes) were assessed using the minimalist approach (1-tube 2-color CD45/CD4) and comparing the results with those obtained by the abbreviated Simultest 4-tube 2-color and the 3-tube 3-color protocols. The use of generic monoclonal antibody reagents, i.e. CD4 (RFT4), CD8(RFT8) and CD45(2D1) henceforth referred to as CD4, CD8, CD45 generic reagents was also evaluated in comparison with commercially available reagents.

MATERIALS AND METHODS

Patients and blood samples

Blood samples were collected from 26 normal healthy volunteers (21-45 years of age) and 142 HIV-seropositive patients. Two ml of venous blood were collected by venipuncture into K_3EDTA-containing tubes and processed for immunophenotyping within 6 hours. All HIV-infected blood samples were part of the routine clinical specimens obtained from the Department of Immunology, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand. HIV-1 infections were diagnosed by serologic testing with confirmation by western blot.

Monoclonal antibodies and reagents

The monoclonal antibodies used in this study were directly conjugated with FITC, PE or PerCP. Table 1 shows the combination of monoclonal antibodies, their manufacturers and the methods used for enumeration of CD4+ and CD8+ T-lymphocytes. The generic monoclonal antibodies CD45(2D1)-FITC, CD4(RFT4)-PE and CD8-(RFT8)-PE were a gift from Professor Janossy of the Royal Free and University College Medical School, London.

Immunophenotypic staining of peripheral blood cells

Twenty microliters of each combination of monoclonal antibody reagent were added to 100 µl (tube nos. 1-7) or 50 µl (tube nos. 8-11) of whole blood in 12 x 75 mm Falcon's polystyrene tubes. All tubes were gently mixed and incubated at room temperature for 15 minutes in the dark. Following the incubation period, 2 ml of (1x) FACS™ lysing solution (Becton Dickinson Biosciences; San Jose, CA, USA) were added to each tube and incubated for at least 15 minutes prior to FCM analysis. Then the lysed stained samples were centrifuged to remove the supernatant followed by washing with cold phosphate buffered saline containing 0.1% sodium azide. Finally the cell pellets were resuspended and fixed with 0.5% paraformaldehyde and kept at 4°C prior to the FCM analysis.

Variable factors and quality control

To ensure that the FCM immunophenotyping quality control was optimal and consistent, both at the instrument and personal performance level, this study was designed to use the same flow cytometer and the same lysing buffer throughout. In addition, all of the immunostaining procedures and the FCM analysis were performed by the same person. Moreover, FCM photomultiplier tube voltage, sensitivity and fluorescent compensation

settings were optimized prior to sample acquisition and analysis using non-fluorescent and fluorescent Calibrite™ beads (BDB) and FACSComp™ software (BDB).

Flow cytometric analysis

Data from the 11 tubes for each sample were acquired and analyzed using the SimulSet™ software (tube nos. 1-4) or CellQuest™ software (tube nos. 5-11) on a FACSort™ flow cytometer (BDB) equipped with a 15 mW argon ion laser tuned at 488 nm. Cells stained with FITC-, PE- and PerCP-conjugated monoclonal antibodies were detected in the logarithmic amplification of fluorescence, FL1 (green), FL2 (orange) and FL3 (red), respectively. The forward scatter (FSC) and side scatter (SSC) were measured on a linear scale.

Simultest 4-tube 2-color protocol

For Simultest™ 2-color method (tube nos. 1-4), a minimum of 15,000 cells were acquired using a CD45/CD14 tube (tube no. 1). Lymphocytes were identified automatically based on the intensity of the CD45/CD14 immunofluorescence and a corresponding FSC/SSC gate (lymphocyte gate) drawn by the SimulSet™ software. The recovery and purity of lymphocytes in the lymphocyte gate exceeded 95%. If autogating failed (recovery and purity were less than 90%), usually due to lymphopenia of the samples or contamination by non-lymphocyte cells, a manual lymphocyte gate was drawn during data acquisition. At least 2,000 lymphocytes were acquired for each tube after the CD45/CD14 tube. Simultest™ isotype control (tube no. 2) was used for determination of nonspecific binding and to set markers for distinguishing fluorescent-negative and positive cell populations. Percentages of both CD3+/CD4+ T-lymphocytes and CD3+/CD8+ T-lymphocytes were determined by two-parameter plots of lymphocytes stained for CD3/CD4 and CD3/CD8.

3-tube 3-color protocol

For the 3-color CD45 gating method (tube nos. 5-7), a live gate was set on SSC/CD45-PerCP bright (FL3) positive cells. Cells in this gate were regarded as lymphocytes, while cells outside this gate represented monocytes and granulocytes. Once this was established, a minimum of 2,000 CD45-PerCP bright lymphocytes were acquired and stored using CellQuest™ software. For analysis, the new bivariant FSC/SSC light scatter gate was set to exclude non-lymphoid cells

Table 1 Monoclonal antibody reagent combinations and methods used for analyzing %CD4+ and % CD8+ T-lymphocyte subsets

Tube no.	Monoclonal antibody combinations			Method	Manufacturer
	FITC	PE	PerCP		
1	CD45	CD14	-	Simultest 2-color	BDB*
2	IgG_1	IgG_1	-	Simultest 2-color	BDB
3	CD3	CD4	-	Simutest 2-color	BDB
4	CD3	CD8	-	Simultest 2-color	BDB
5	IgG_1	IgG_1	CD45	3-color CD45gate	BDB
6	CD3	CD4	CD45	3-color CD45gate	BDB
7	CD3	CD8	CD45	3-color CD45gate	BDB
8	CD45	CD4	-	2-color CD45/CD4	RFUCMS**
9	CD45	CD8	-	2-color CD45/CD8	RFUCMS
10	CD45	CD4	-	2-color CD45/CD4	BDB
11	CD45	CD8	-	2-color CD45/CD8	BDB

*Becton Dickinson Biosciences, San Jose, CA, USA
**Royal Free and University College Medical School, London, UK

that may have been included in the SSC/CD45 lymphocyte gate. The cells in the FSC/SSC gate were then analyzed for percentages using a two-color isotype control (IgG_1/IgG_2), CD3-FITC/CD4-PE and CD3-FITC/CD8-PE. Percentages of CD3+/CD4+ and CD3+/CD8+ T-lymphocytes can also be analyzed using the BDB MultiSet™ software.

1-tube 2-color CD45/CD4 protocol using SSC/CD45 gating

The 2-color CD45/CD4 method using CD45-FITC/CD4-PE (tube nos. 8 and 10) is shown in Fig. 1. The method is a modification of the CD45-assisted Pan*Leuco*gating technique described by Glencross *et al.*[14] A total of 15,000 cells were first acquired on a SSC/CD45-FITC display. Lymphocytes were identified and gated by drawing a region around all bright CD45+ cells with low SSC (R1). Lymphocytes gated in R1 were further analyzed for CD4-PE by using SSC against CD4-PE. CD4+ T-lymphocytes were easily distinguished from non-CD4 T-lymphocytes, and thus no isotype control was needed in this gating protocol. An area R2 defining CD4+ T-lymphocytes was drawn on this two-parameter dot plot and % CD4+ T-lymphocytes was then obtained as a percentage of total R1 lymphocytes (Fig. 1B). The same analysis protocol was applied to CD8+ T-lymphocytes using CD45-FITC/CD8-PE (tube nos. 9 and 11, Fig. 1C).

Statistical analysis

Evaluation of lymphocyte subsets obtained by the different methods was performed by linear regression analysis. The Bland-Altman statistical bias method[15] for

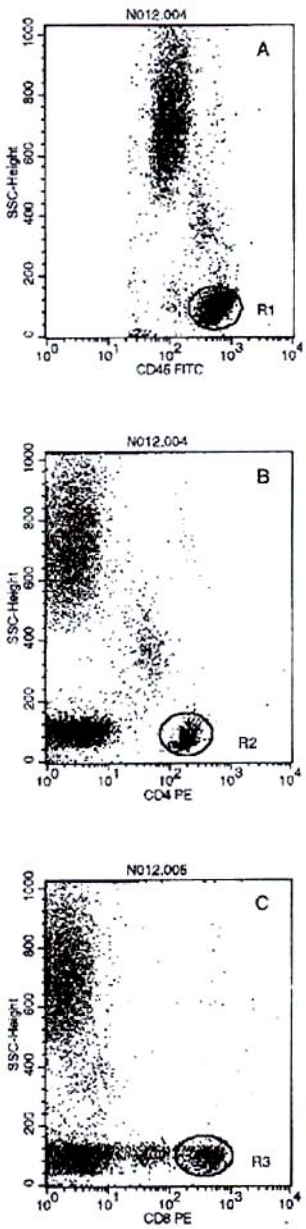

Fig. 1 Representative two-parameter dot plots of major leukocyte populations in peripheral blood stained with monoclonal antibodies reactive to CD45/CD4 and CD45/CD8. The lymphocyte gate for data acquisition from SSC-Height/CD45-FITC (**A**) was established by CellQuest software. Region 1 (R_1) represents a light scatter gate of lymphocytes. Phenotypic identification of CD4-PE+ T-lymphocytes (**B**) and CD8-PE+ T-lymphocytes (**C**) shown in R_2 and R_3 gated from R_1 was enumerated using regional statistics.

assessing agreement between methods was performed to verify whether the methods agreed sufficiently well to be used interchangeably.

RESULTS

Reproducibility of the 3 methods was assessed by analysing 10 HIV+ blood samples in triplicates by each method. The coefficient of variation (CV) for the replicates of both CD4 and CD8 percent values did not exceed 2.0%.

The mean percentages of CD4+ T-lymphocytes for 26 normal subjects determined by different FCM methods (i.e. Simultest 2-color method, 3-color CD45 gating method, 2-color CD45/CD4 generic reagents method, 2-color CD45/CD4 commercial reagent method) were 34.1 ± 7.6, 35.6 ± 8.6, 34.2 ± 7.9 and 34.4 ± 8.3, respectively (Table 2). The results for 142 HIV-1 infected individuals using the different methods were 12.5 ± 7.7, 13.1 ± 7.9, 12.6 ± 7.8 and 12.5 ± 8.1, respectively (Table 2). Correlation plots of CD4 percentages from all 168-blood samples derived from 2-color generic reagents method relative to the other three methods are presented in Fig. 2. The 2-color CD45/CD4 generic reagents method correlated well with the other three methods, with correlation coefficients (r) of 0.97, 0.98 and 0.99 (r^2 = 0.94, 0.96 and 0.98) for Simultest 2-color method, 3-color CD45 gating method and 2-color CD45/CD4 commercial reagents method respectively. Similar good correlations were obtained for CD8 percentages with r of 0.96, 0.95 and 0.96 (r^2 = 0.92, 0.90, and 0.92) when compared with Simultest 2-color method, 3-color CD45gating method and 2-color CD45/CD8 commercial reagents method. Bland-Altman plots comparing CD4 percentages generated by the 2-color CD45/CD4 generic reagents and Simultest 2-color methods, the 2-color CD45/CD4 generic reagents and 3-color CD45 gating methods, and the 2-color CD45/CD4 generic reagents and 2-color CD45/CD4 commercial reagents methods (Fig. 3) indicated no bias with 2 SD of -5.6 to +5.7, -4.0 to +3.9 and -5.6 to +4.2 for the 3 sets of comparisons. Of the 168 blood samples only 4 samples showed values exceeding 2 SD. For CD8 percentages, the Bland-Altman statistics showed good agreement, although the 2-color generic reagents method gave consistently lower values than the other three methods (Fig. 3) with 2 SD of -13.9 to 2.9, -14.7 to 1.8 and -10.4 to 4.4 when the 2-color generic reagents method was compared with the Simultest 2-color method, the 3-color CD45 gate method and the 2-color CD45/CD8 commercial reagents method. Only 6% of 168 blood samples showed values that were not within 2 SD.

DISCUSSION

Although Thailand has had substantial success in the prevention and control of HIV infections, nearly 30,000 new infections are estimated to occur each year. At present, about three-quarters of a million are living with HIV/AIDS[2]

Table 2 Mean percentage ± SD (ranges) for CD4+ and CD8+ T-lymphocyte values in normal and HIV-1 infected subjects determined by different reagents and flow cytometric methods

			BDB*	BDB	RFUCMS**	BDB
Subject	Subset	n	Simultest 2-color method	3-color CD45gate method	2-color CD45/CD4 or CD45/CD8 method	2-color CD45/CD4 or CD45/CD8 method
Normal	CD4+	26	34.1 ± 7.6 (16.0-51.0)	35.6 ± 8.6 (15.9-51.2)	34.2 ± 7.9 (15.9-51.2)	34.4 ± 8.3 (18.5-53.1)
Normal	CD8+	26	28.0 ± 7.0 (8.0-44.0)	28.0 ± 7.3 (7.9-46.4)	23.9 ± 6.6 (7.5-42.2)	24.0 ± 6.9 (6.9-42.5)
HIV-1	CD4+	142	12.5 ± 7.7 (0.1-34.0)	13.1± 7.9 (0.2-33.3)	12.6 ± 7.8 (0.3-30.7)	12.5 ± 8.1 (0.2-31.8)
HIV-1	CD8+	142	52.9 ± 11.1 (23.0-82.0)	54.0 ± 11.6 (23.1-86.5)	47.1 ± 11.1 (20.4-81.6)	50.6 ± 11.2 (22.0-87.4)

*Becton Dickinson Biosciences, San Jose, CA, USA
**Royal Free and University College Medical School, London, UK

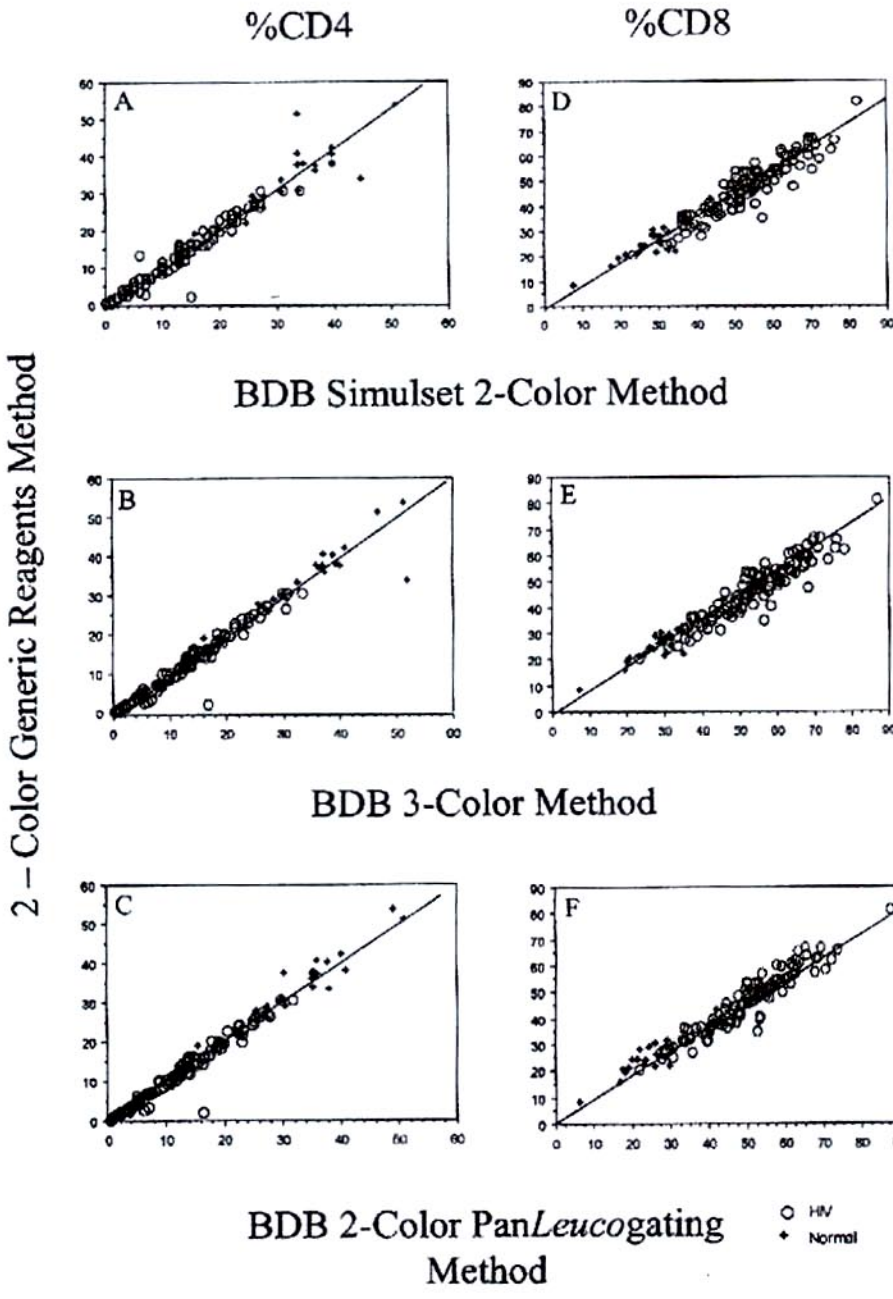

Fig. 2 Correlation between 2-color generic reagents for the Pan*Leuco*gating method (Y-axis) and BDB Simulset 2-color, 3-color and 2-color for Pan*Leuco*gating methods (X-axis). Left column, **A, B, C**: correlation plots for % CD4+ T-lymphocytes. Right column, **D, E, F**: correlation plots for % CD8+ T-lymphocytes.

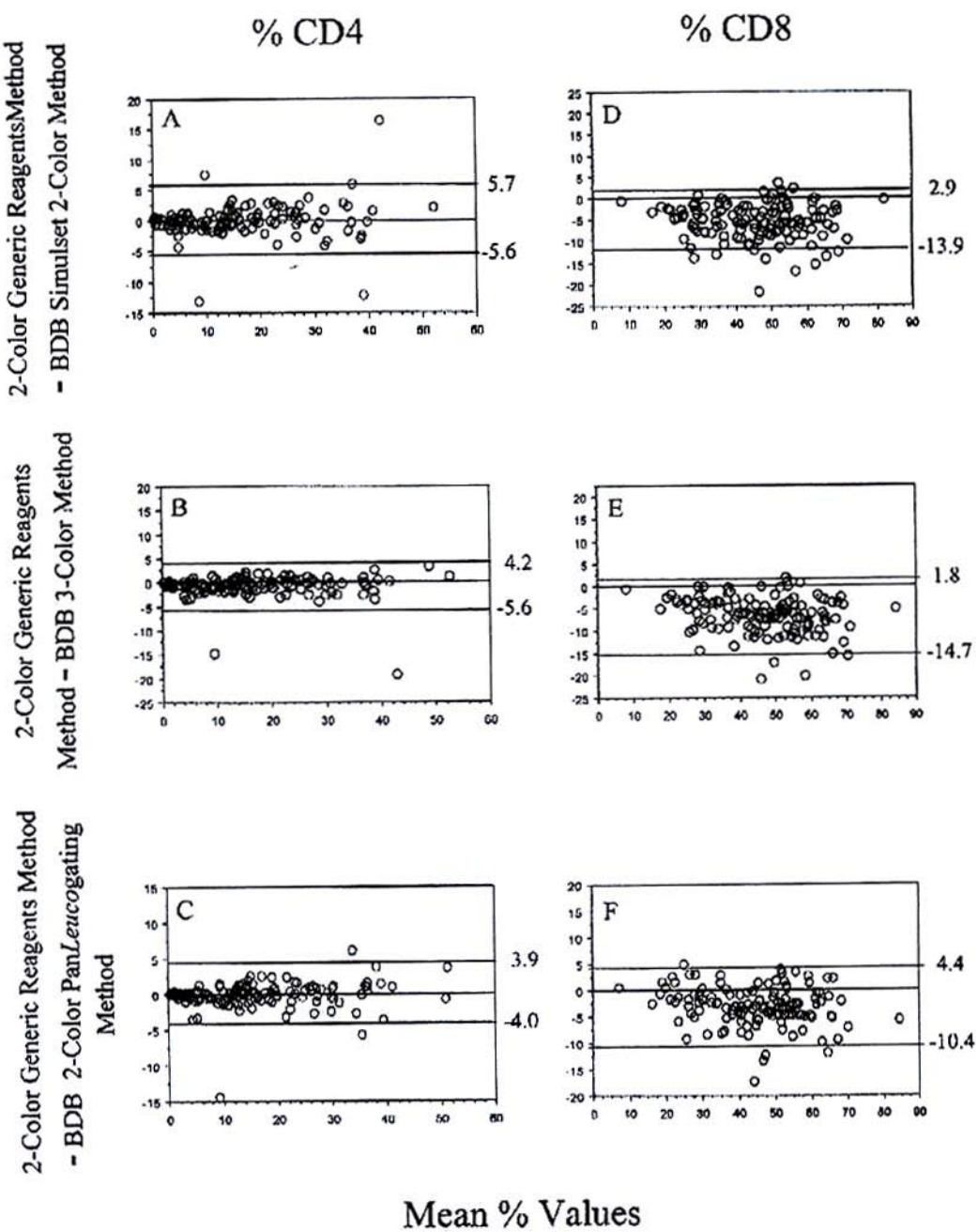

Fig. 3 Bland-Altman plots to establish the difference between 2-color generic reagents for the Pan*Leuco*gating method and BDB Simulset 2-color, three-color and 2-color Pan*Leuco*gating methods for % CD4+ T-lymphocytes (**A, B, C**) and % CD8+ T-lymphocytes (**D, E, F**).

causing a heavy burden on the health care system. The recent introduction of cheap anti-retroviral drugs by the Government Pharmaceutical Organization of Thailand has allowed many HIV-infected patients access to anti-retroviral therapy but the cost of CD4 enumeration for diagnosis and monitoring of therapy remains high. The use of the standard "state of the art" FCM 6-tube 2-color method which contains the quality control measures recommended by CDC[8] also allows the "Lymphosum" to be measured by identifying all T-lymphocytes (CD3+), all B lymphocytes (CD19+) and all NK cells (CD16+/56+). However, physicians who manage HIV/AIDS are only interested in the absolute CD4 cell counts and especially in children, in the percentage of CD4+ T-lymphocytes. Other parameters including CD3, CD19, CD16/56 and CD45 cell levels are therefore usually clinically irrelevant. The use of the Simultest 2-color method to generate just the absolute CD4 count and % CD4 value would still require CD45, CD14, CD3, CD4 and the isotype controls. The alternative 3-tube 3-color method[9-12] requires the use of CD45, CD3, CD4 and isotype controls.

On the dual platform, the reference lymphocyte populations need to be defined with the greatest precision. When lymphocytes are identified by morphological gating using FSC versus SSC, large lymphocytes tend to be inadvertently excluded and non lymphoid cells (monocytes, basophils and unlysed red cells) have been shown to contaminate the gates.[16] A method for assessing lymphocyte gate purity and lymphoid cell recovery on the basis of differential CD45 antigen density expression was described by Loken *et al.*[17] This method also known as fluorescence backgating used 2 reagents (CD45-FITC/CD14-PE): CD45 is a panleucocyte marker expressed at different intensities on white cells (granulocytes CD45+; monocytes CD45++; lymphocytes CD45+++) while the CD14 antigen is selectively expressed by monocytes.

The identification of lymphocytes on the basis of CD45 expression and SSC (morphospectral gating) has been described.[14] Discrimination from granulocytes (SSC+++, CD45+) and monocytes (SSC++, CD45++) can be achieved readily due to the high CD45 expression and low SSC (SSC+, CD45+++) of lymphocytes. A % CD4 among lymphocytes can be generated by this method using just 2 reagents CD45 and CD4. In this paper we have shown that a reliable % CD4 value can be obtained by the SSC/CD45 protocol and that the additional markers and internal checks for consistency do not provide any extra advantage when CD4 analysis is performed with modern FCM analysis. Since the use of SSC in combination with CD45 and CD4 allows the accurate discrimination between CD4+ T-lymphocytes and monocytes, a saving in the cost of reagents can be achieved by omitting CD14. Arguably CD3, the specific T cell marker is also not required to identify CD4+ T-lymphocytes.[13,14,18] A quality control measure recommended by the CDC but not included in the SSC/CD45 protocol is the use of isotype controls. However cursors can be set without the use of isotype controls since the cell populations in the lymphocyte subset analysis are discrete.[19]

When the SSC/CD45 method was used to obtain % CD8 values, the results of a few samples were outside the 2 SD values in the Bland-Altman plot. This discrepancy might be a gating problem. Unlike the Simultest 2-color method and the 3-color CD45 gating method where CD8+ T-lymphocytes were obtained from both dim and bright CD3+/CD8+ T-lymphocytes, the SSC/CD45 technique used only bright CD8 expression to identify CD8+ T-lymphocytes. This would explain the lower % CD8 values obtained by the SSC/CD45 method when compared to the Simultest 2-color and the 3-color methods. One major criticism of the recommended standard 6-tube 2-color method using replicate tubes is the difficulty in controlling tube to tube variability, since the analysis gate is set in tube 1 which may not be identical with the other tubes which contain the lymphocyte markers.[14]

At present all CD4+ T-lymphocyte enumerations in Thailand are performed using Standard Guidelines and expensive commercial reagents renowned for their quality. A simpler protocol using fewer lymphocyte markers and cheaper reagents would represent substantive savings and thus relieve some of the economic burden of the HIV/AIDS epidemic in Thailand. We have chosen the SSC/CD45 gating protocol using just 2 reagents-CD45/CD4 in our search for a cost effective CD4 analysis that would be suitable in our local situation. There have been recent reports of the use of generic monoclonal antibody reagents in CD4 analysis.[13,14,18,19] Although, the evaluation of the generic reagents seems promising when compared with the world leading manufacturer's rea-

gents in determining CD4 counts, the batch to batch quality of the generic reagents have to be considered if they are to replace the commercial reagents.

In summary, we have investigated three generic monoclonal antibody reagents using the SSC/CD45 protocol and have found them to be as good and effective as commercial reagents. The cost of CD4 testing can be reduced from US$ 12-24 to less than US$ 3 by the use of a simplified protocol which requires the use of only 2 reagents. This together with the use of generic or locally produced reagents will no doubt make CD4 testing in resource-poor countries like Thailand more affordable and therefore available to most HIV-infected Thai patients.

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