Novel Three-Color Reagent for Measurement of CD4 and CD8 Positive Lymphocytes by Flow Cytometry

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ABSTRACT

In HIV infection, the absolute number of CD4 positive lymphocytes is an important indicator for classification of the state of disease, prognosis, treatment decision and monitoring of therapy. Flow cytometric analysis is the current standard method for CD4+ lymphocyte measurement. This flow cytometric method generates accuracy and reliable results but the reagent used is very expensive. In this study, a novel three-color flow cytometric reagent for enumeration of CD4 and CD8 positive lymphocytes was developed. The developed reagent consists of FITC labeled CD3, PE labeled CD4 or CD8 mAb and 7-aminoactinomycin D (7-AAD) solution. 7-AAD is used to intercalate into DNA of white blood cells and allows white blood cells, but not red blood cells, to be detected with FL3 detector of flow cytometer. Fluorochrome labeled CD3, CD4 or CD8 mAb allows determination of CD4 and CD8 positive cells with FL1 and FL2 detector. Comparing to standard flow cytometric reagent, a very good correlation between the developed reagent and the reference reagent for both CD4 and CD8 positive lymphocyte counts was obtained. As the cost of the developed reagent is much lower than the commercially-available reagent, the developed reagent should be appropriate for using in low-income countries.

Key Words: Flow cytometry, Immunophenotyping, Three-color reagent, CD4 positive lymphocytes, CD8 positive lymphocytes, HIV

INTRODUCTION

Human immunodeficiency virus (HIV) is a retrovirus that infects cells which possess the CD4 receptor (Giorgi et al., 1987; De Wolf et al., 1988). This infection causes the depletion of CD4 positive lymphocytes which is a major clinical finding in progressive infection (Giorgi et al., 1987; Fahey et al., 1990). In HIV infection, the absolute number of CD4 positive lymphocytes is an important indicators for prognosis, classification of the state of disease, treatment decision and monitoring of therapy (Fahey et al., 1990; Volberding et al., 1990; Centers for Disease Control, 1992).

Immunophenotyping, using lysed whole blood stained with monoclonal antibody (mAb) panels and analyzed by flow cytometry is the current standard method for determination of CD4 positive lymphocytes (Nicholson, 1994). Recently, several types of immunophenotyping reagents for measurement of lymphocyte sub-populations have been developed and routinely used in clinical laboratory. Three-color immunophenotyping reagent is one of the common flow cytometric reagents for CD4 and CD8 positive lymphocyte determination. The commercially-available three-color reagent contains peridinin chlorophyll protein (PerCP) or phycoerythrin›cyanin 5.1 (PE-Cy5) labeled CD45 mAb for discrimination of white blood cells and red blood cells and contains fluorescein isothiocyanate (FITC) labeled CD3 and phycoerythrin (PE) labeled CD4 or CD8 mAb for enumeration of CD4 and CD8 positive lymphocytes, respectively (Nicholson et al., 1997). The three-color reagent generates sufficient accuracy and reliable lymphocyte counts but this reagent is expensive and not suitable in resource-poor countries. In Thailand, while less expensive (i.e. $1.00 per day) combination therapy is becoming more widely available, the cost of CD4 and CD8 positive lymphocyte monitoring remains prohibitive and may not be done unless cheaper reagents are developed.

Recently, several mAbs against various leukocyte surface molecules have been generated in our laboratory. By the generated mAbs, we have developed a new type of three-color reagent for enumeration of lymphocyte sub-populations. This reagent contains 7-aminoactinomycin D (7-AAD) which can intercalate into double-stranded DNA of white blood cells and allows white blood cells, but not red blood cells, to be detected with FL3 detector of flow cytometer (Kasinrerk, 2003). The reagent also contains FITC labeled CD3 and PE labeled CD4 or CD8 mAb, which allows determination of CD4 and CD8 positive cells with FL1 and FL2 detector. Comparing to standard flow cytometric reagent, the developed three-color reagent proves to be highly correlative to the commercially-available standard reagent in determining both CD4 and CD8 positive lymphocytes. As it is a country-made product, the developed reagent is cheap and suitable for implementation in Thailand.

MATERIALS AND METHODS

Blood samples

Normal and HIV-infected blood samples were obtained from the Medical Technology Service Center, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai and Siriraj Hospital, Mahidol University, Bangkok, Thailand. Venous blood was collected by venipuncture into K3EDTA-containing tubes (Becton Dickinson, San Jose, CA) and processed for immunophenotyping within 6 hours.

Antibodies and reagents

The two-color immunofluorescence reagent (Simultest) for enumeration of lymphocyte sub-populations was obtained from Becton Dickinson (San Jose, CA). This reagent contains four dual-fluorochrome conjugated mAb pairs including FITC-CD45/PE-CD14, FITC-IgG1/PE-IgG2b (isotype matched control), FITC-CD3/PE-CD4 and FITC-CD3/PE-CD8.

A three-color reagent was developed in the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University. This reagent contains fluorochrome conjugated mAbs including FITC-CD3, PE-CD4 or PE-CD8 and 7-AAD solution. 7-AAD was acquired from Sigma (St. Louis, MO) and dissolved in DMSO at a concentration of 10 mg/ml.

Immunofluorescence staining

For enumeration of lymphocyte sub-populations in blood samples by two-color reagent (Simultest), 20 µl of each mAb reagent pairs was added to 100 µl of EDTA-anticoagulated blood in separate 12 x 75 mm test tubes. All tubes were gently mixed and incubated at room temperature for 15-30 minutes in the dark. Following the incubation period, 2 ml of RBC lysing solution (Becton Dickinson) was added and incubated for another 10 minutes. After centrifugation at 500 x g for 5 minutes and subsequently washed with 2 ml PBS containing 0.1% sodium azide, the cell pellets were resuspended in 0.5 ml of 1% paraformaldehyde in PBS. The stained cells were then analyzed by a flow cytometer (FACScalibur, Becton Dickinson), using the Simulset software analysis program (Becton Dickinson).

For three-color reagent, 10 µl of 7-AAD solution, FITC-CD3 and PE-CD4 or PE-CD8 mAb were added into 100 µl of EDTA blood in a 12 x 75 mm tube. The tube was gently mixed and incubated at room temperature for 15-30 minutes in the dark. Following the incubation period, 2 ml of RBC lysing solution (Becton Dickinson) was added and incubated for another 10 minutes. After centrifugation at 500 x g for 5 minutes and subsequently washed with 2 ml PBS containing 0.1% sodium azide, the cell pellets were resuspended in 0.5 ml of 1% paraformaldehyde in PBS. The stained cells were then analyzed by a flow cytometer, using CellQuest software program (Becton Dickinson).

Flow cytometric analysis

Samples stained with two-color reagent were analyzed, using Simulset software. A minimum of 2,000 lymphocytes was initially acquired, using CD45/CD14 tube. By this software, lymphocyte population was identified automatically, based on the intensity of the CD45/CD14 immunofluorescence and a corresponding forward and side light scatter (FSC/SSC). The lymphocyte gate (lymphogate) was drawn. The purity of lymphocytes in the lymphogate is defined as the proportion of events within the gate that are lymphocytes. At least 2,000 lymphocytes were acquired for other tubes after the CD45/CD14 tube. The isotype control tube determined non-specific binding and set marker for distinguishing fluorescence-negative and-positive cell populations. The CD3/CD4 and CD3/CD8 tubes were analyzed for the number of CD3 positive and CD4 positive lymphocytes and CD3 positive and CD8 positive lymphocytes based on their FITC and PE fluorescence, using FL1 and FL2 respectively. By this flow cytometric analysis, results were reported as the percentages of the lymphocyte sub-populations in total lymphocytes.

For CellQuest software, a minimum of 10,000 cells were measured. By monitoring of FL3 and SSC, RBC were gated out from the 7-AAD-stained WBC, having bright red fluorescence by the FL3 threshold. The remaining WBC were analyzed, using FSC/SSC, and lymphocyte population was gated according to their size and granularity. The percentages

of CD3 positive, CD4 positive and CD8 positive lymphocytes were determined from the CD3/CD4 and CD3/CD8 tubes, using FL1 and FL2.

Statistical analysis

Data obtained by the two methods were compared by linear regression analysis. Bland-Altman plot was used to assess the agreement (Bland and Altman, 1986).

RESULTS

In the present study, a novel three-color reagent for determining CD4 and CD8 positive lymphocytes in peripheral blood was developed. The developed reagent contains 7-AAD for discrimination of nucleus containing WBC from non-nucleus-containing RBC (Kasinrerk, 2003) and fluorochrome labeled mAbs for determination of lymphocyte sub-populations. To determine CD4 and CD8 positive lymphocytes by using the developed three-color reagent, whole blood was stained with the reagent as described earlier and analyzed by flow cytometry, using CellQuest software. By monitoring of FL3 and SSC, RBC were gated out from the 7-AAD-stained WBC, having bright red fluorescence by the FL3 threshold (Figure 1A). The remaining WBC were analyzed, using FSC/SSC and lymphocyte population was gated according to their size and granularity (Figure 1B). By monitoring FL1 and FL2, percentages of CD4 (CD3+/CD4+) and CD8 (CD3+/CD8+) positive lymphocytes (Figure 1C and D) were determined. Absolute CD4 and CD8 positive lymphocyte counts were then obtained by multiplying the percent positive cells by the lymphocyte counts.

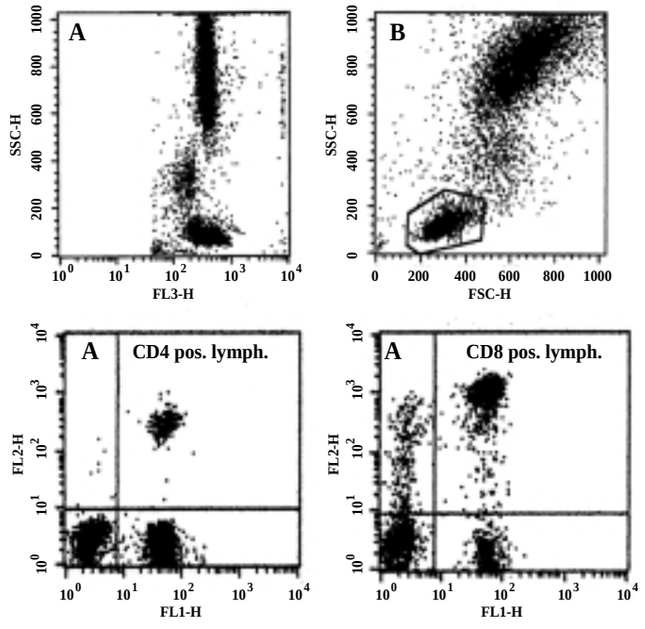


Figure 1. FACS analysis of CD4 and CD8 positive lymphocytes by the developed three-color reagent. By FL3 and SSC acquisition, the FL3 threshold was adjusted to gate out RBC from the 7-AAD-stained WBC which have bright red fluorescence (A). By FSC and SSC monitoring, WBC were displayed and lymphocyte population was gated according to their size and granularity. By FL1 and FL2 monitoring, cells in the acquisition lymphocyte gate were displayed. CD4 and CD8 positive lymphocytes were indicated in C and D, respectively.

To determine the accuracy of the developed three-color reagent, the reagent was distributed to two laboratories for evaluation. Enumerations of CD4 and CD8 positive lymphocytes were compared between the use of developed three-color reagent and reference two-color reagent (Simultest) of Becton Dickinson. For the first laboratory at Chiang Mai University, blood samples from 24 healthy and 29 HIV-infected individuals were studied. Regression analysis and Bland-Altman plots of the comparing data are shown in Figure 2. Results obtained by the two methods correlated well with regression R^2 of 0.991 for CD4 positive lymphocyte counts and 0.958 for CD8 positive lymphocyte counts. Mean difference and 2 two standard deviations of difference were 16 ± 45 cells/μl for CD4 positive lymphocytes and -49 ± 217 cells/μl for CD8 positive lymphocytes.

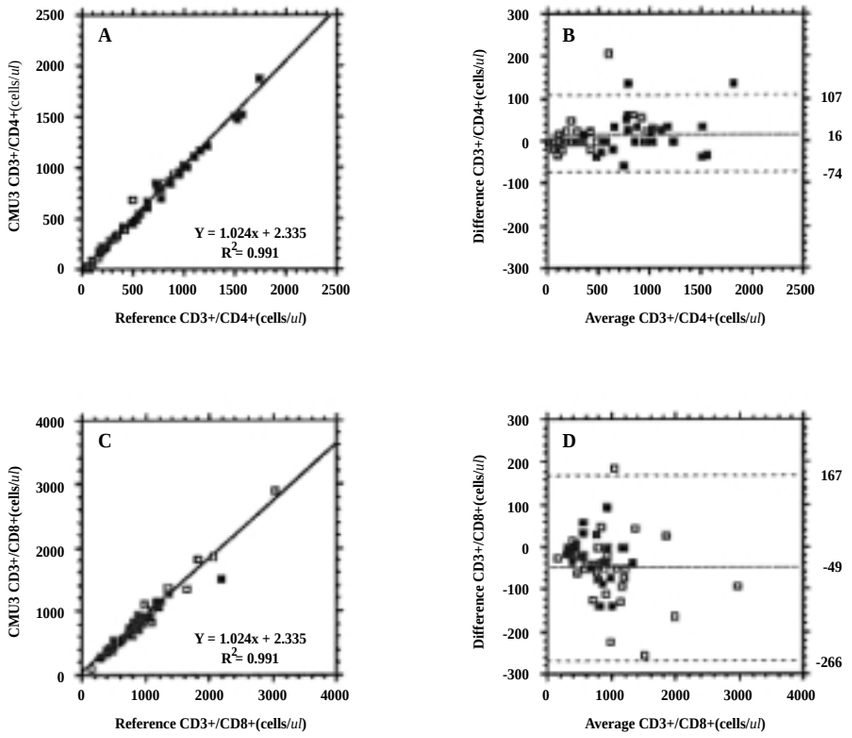


Figure 2. Correlation and Bland-Altman analysis of results obtained from Laboratory I. Absolute CD4 positive and CD8 positive lymphocyte counts obtained by the developed three-color reagent (CMU3) were compared with those obtained by the reference two-color regent (A and C, respectively). Agreement by the 2 methods was assessed by Bland-Altman plots (B, absolute CD4 positive lymphocytes and D, CD8 positive lymphocytes). The actual mean difference (solid line) is shown together with the 2SD of the difference (dashed line). Values are shown to the right. Data from 24 normal healthy volunteers (filled square) and from 29-HIV infected persons (empty square) are included.

External evaluation of the developed reagent was performed at the Center of Excellence for Flow Cytometry, Office of Research and Development, Siriraj Hospital, Mahidol University. Results are summarized in Figure 3. Data from 10 normal healthy volunteers and 41 HIV-infected persons were analyzed. Good correlation with the regression $R^2 = 0.989$ and 0.968 for absolute CD4 and CD8 positive lymphocytes, respectively, was obtained. Bland-Altman analysis revealed mean difference and 2 two standard deviations of difference were of -12 ± 68 cells/μl for CD4 positive lymphocytes and -82 ± 160 cells/μl for CD8 positive lymphocytes.

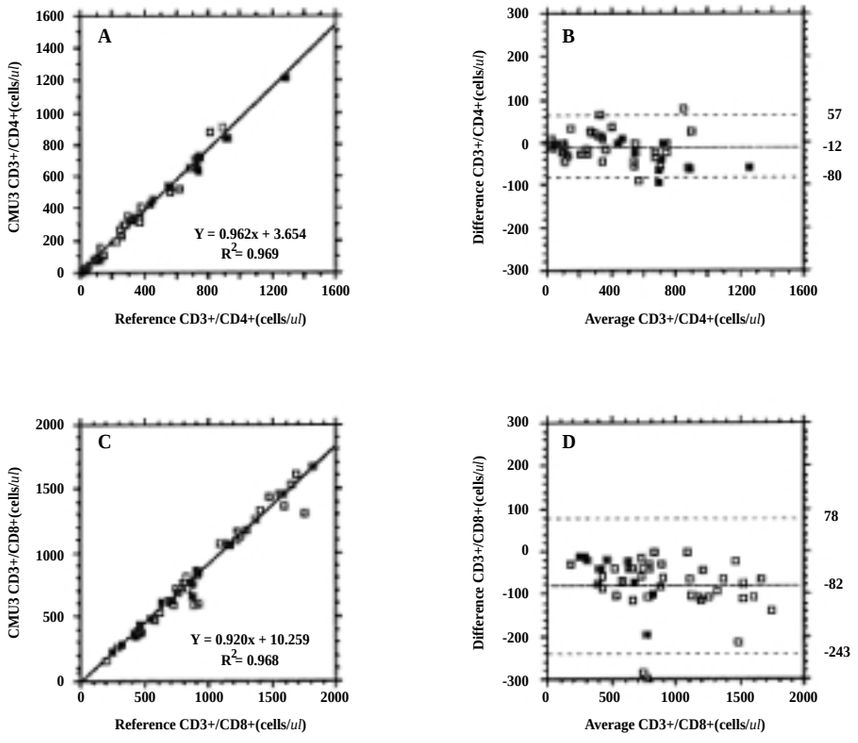


Figure 3. Correlation and Bland-Altman analysis of results obtained from Laboratory 2. Normal healthy volunteers (filled square, n = 10) and HIV-infected persons (empty square, n = 41) are included. See Figure 2 for details.

These comparison results indicated that the developed three-color reagent could be used to enumerate CD4 and CD8 positive lymphocytes in blood samples, equivalent to those given by the commercial standard reagents.

DISCUSSION AND CONCLUSION

The use of CD4 positive lymphocyte measurements has a significant impact on the diagnosis, monitoring, and therapeutic control of HIV infection (Fahey et al., 1990; Volberding et al., 1990; Centers for Disease Control, 1992). Immunophenotyping using lysed whole blood stained with fluorochrome labeled color monoclonal antibody panels and analyzed by flow cytometer is the current standard method for determination of CD4 positive lymphocytes (Nicholson , 1994). The results obtained by this flow cytometric method is accurate and reliable, however, the flow cytometric reagent is very expensive. In Thailand, the cost of antiretrovirals has recently been reduced for more than 10 folds. However, the cost of CD4 positive lymphocyte counts remains unaffordable by the majority of the patients. Research for the cost reduction is warranted.

In the present report, a novel three-color immunofluorescent reagent for CD4 and CD8 positive lymphocyte counts was developed. The developed reagent consisted of FITC labeled CD3 mAb, PE labeled CD4 or CD8 mAb and 7-AAD. 7-AAD, a DNA- binding fluorescein dye, is efficiently excited by the 488 nm laser line commonly used in flow cytometer. After excitation, it yields fluorescence emission into the far red spectrum (approximately 650 nm) which can be detected by FL3. 7-AAD is excluded by viable cells but can penetrate cell membranes of dying or dead cells. As 7-AAD intercalates into double-stranded nucleic acid, therefore, this reagent is widely used for dead cell discrimination (Schmid et al., 1992). In our developed reagent, 7-AAD is used to discriminate lysis-resistant RBC from WBC in the lymphocyte gate in the measurement of lymphocyte sub-populations.

In our developed reagent, whole blood was stained with fluorochrome labeled mAb panels and 7-AAD solution and RBC were lysed by lysing solution that contained fixative. By the RBC lysing solution, blood cell membrane was permeabilized and allows allowed 7-AAD to penetrate. As WBC contain nucleic acid but not for RBC, WBC were therefore stained with 7-AAD. On flow cytometric analysis, after excitation with 488 nm, WBC were emitted with 670 nm which could be detected by FL3. Thus, WBC could be separated from RBC according to their FL3 fluorescence patterns. The remaining white blood cells were then analyzed, using FSC/SSC and lymphocyte population was gated according to their size and granularity. The CD4 positive or CD8 positive lymphocytes were than then measured, using FL1 and FL2.

For validating the developed reagent, the reagent was used to enumerate CD4 and CD8 positive lymphocytes and compared with the reference method using two-color Simultest reagent. In this validation, two different laboratories at Chiang Mai University and Mahidol University independently studied. Both laboratories demonstrated a very good correlation between the developed reagent and the reference reagent for both CD4 positive and CD8 positive lymphocyte counts. These results suggested that the developed reagent is an acceptable alternative reagent for monitoring CD4 and CD8 positive lymphocytes in blood samples.

As the reagent is was developed in our laboratory, the cost is 5 times lower than that of the commercially-available reagent. The developed three-color reagent is therefore appropriate for using in low-income countries including Thailand.

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