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Flow Cytometric Three-Color Determination of CD4 T-Lymphocytes on Blood Specimens from AIDS Patients Who Have a Large Number of Contaminating Non-Lymphocytes

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SUMMARY A three-color flow cytometric determination of CD4 T-lymphocytes on whole blood specimens from AIDS patients which contain a high proportion of non-lymphocyte elements is described. Peripheral blood cells were stained by a three-color method using monoclonal antibodies conjugated respectively with fluorescein isothiocyanate (FITC)-CD3, phycoerythrin (PE)-CD4 and peridinin chlorophyll protein (PerCP)-CD45. CD45 stains all leukocytes with the highest fluorescence expression of CD45 antigen in lymphocytes. By combining light scatter with CD45 in the fluorescence 3 (FL3) channel, a light scattering window can be drawn to include almost all bright CD45 lymphocytes. This live gate of lymphocytes was then acquired and analysed simultaneously using other irrelevant two-color (FITC/PE) antibodies of CD3 and CD4 in the FITC and PE channels, respectively. This method is easy and straightforward, and gives successful analysis of CD4 T-lymphocytes in AIDS blood specimens contaminated with an unusually large number of non-lymphocytic cells.

The CD4 lymphocyte is the primary target cell in HIV infection, and a substantial reduction in the number of these cells is required for development of AIDS.[1-3] In HIV infection, CD4 T-cell determinations are used for clinical prognosis, therapeutic monitoring, and for qualifying patients for clinical trials.[4-8]

The enumeration of absolute CD4 T-cell levels in whole blood involves three distinct laboratory measurements: the white blood cell (WBC) count, the lymphocyte differential (percent lymphocytes in WBC count), and the percentage of T-lymphocytes that are CD4 positive. The CD4 percentage is measured on whole blood by flow cytometric (FCM) immunophenotyping. Several sets of guidelines addressing quality control of CD4 T-cell enumeration have been developed.[6,9,10] These FCM immunophenotyping guidelines rely on detecting specific cell-surface antigens (eg CD4) by antigen-specific monoclonal antibodies that have been labeled with a fluorescence, such as FITC or PE. The fluorescent-labeled cells are analysed by flow cytometer which accurately distinquishes lymphocytes from other leukocyte population using the combination of fluorescence associated with CD45-FITC/CD14-PE and FSC/SSC.[11]

One of the major concerns in FCM immunophenotyping guidelines is the purity of lymphocytes in the FSC/SSC gate. The presence of non-lymphocytic cells in the acquisition gate may lead to erroneous results. The above mentioned guidelines suggest the proportion of all lymphocytes present in the samples that are contained within the boundaries of the lymphocyte light scatter gate must be at least 90%. However, in samples which contain a high proportion of non-lymphocytic cells (ie nucleated red cells or debris), such as occur in thalas-

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semia,[12] and AIDS, problems may be encountered in identifying pure lymphocyte clusters by the technique suggested by these guidelines.

We report here a simple FCM method for optimal light scattering gate delineation in acquisition of immunofluorescence from lymphocytes obtained from AIDS patients which contain a very high proportion of non-lymphocytic cells. We used three-color FCM of CD3-FITC, CD4-PE and CD45-PerCP (peridinin chlorophyll protein) in combination to achieve CD4+ T-lymphocyte determinations in lysed whole blood specimens.

MATERIALS AND METHODS

Patient population

Five AIDS male patients and 15 HIV-seropositive male patients were studied. All AIDS patients were from Ratchaburi Hospital, Ratchaburi, Central Thailand. They had been infected with HIV for a period of more than 3 years, and had recently been diagnosed with AIDS according to WHO and the CDC criteria.[13] Those who were HIV-seropositive patients were from Siriraj Hospital, Bangkok. They were classified according to the CDC recommendations into group II (asymptomatic). Their HIV-serology was determined by ELISA with confirmation by Western blot. All patients gave fully informed consent. Each patient donated a sample of 5 ml peripheral venous blood.

Phenotypic staining of peripheral blood

For each specimen, 7 tubes were set up (Table 1). The first 4 tubes were two-color (FITC/PE) Simultest reagent (Becton Dickinson Immunocytometry Systems, San Jose, CA), and the last 3 tubes were a combination of three-color reagent with PerCP as the third color. CD45-PerCP was also purchased from Becton Dickinson.

One hundred microlitres of whole blood were mixed and incubated at room temperature with 20 µl of Simultest reagents or 30 µl of a solution containing equal volume of three antibodies for the three-color staining. After 15 minute incubation, red cells were removed by lysis through the addition of lysing buffer (Becton Dickinson) to the tubes for 10 minutes. After a centrifugation and subsequent wash with cold phosphate buffered saline containing 1% azide, the cell pellets were resuspended and fixed with 0.5% paraformaldehyde. The samples were stored at 4°C for at least 2 hours and analysed on a FACScan (Becton Dickinson) instrument equipped with an air-cooled Argon laser emitting 15 mW at 488 nm.

Flow cytometric analysis

Specimens stained in two-color immunofluorescence were analysed using SimulSET Software (Becton Dickinson). Lymphocytes were identified automatically by a combination of fluorescence associated with CD45/CD14 and FSC/SSC light scatter. The percent of gated events that were lymphocytes was corrected for percent purity of lymphocytes (CD45+bright/CD14$^-$) and multiplying by 100.

Data from the three-color staining were collected and analysed using the FACScan Research Software (Becton Dickinson) with linear amplification of the FSC and SSC signals, and logarithmic amplification of the FL1, FL2 and FL3 emission in the FITC (green), PE (orange) and PerCP (long red) regions of the spectrum, respectively. Cells stained with FITC, PE and PerCP conjugated antibodies were detected with FL1, FL2 and FL3, respectively. Throughout the course of this study, isotype control (Tubes No. 2 and 5) and CD3-FITC/CD19-PE/CD45-PerCP stained from a single healthy individuals were used to calibrate the instrument fluorescence compensation. Lymphocyte gating was performed using FSC/bright CD45+ cells and FSC/SSC. Data on 5,000-10,000 events per sample were acquired and stored in List mode.

RESULTS

Staining of HIV-seropositive peripheral blood with two-color LeucoGATE (CD45/CD14) reagent (Tube No. 1) showed three major populations of lymphocytes, monocytes and granulocytes (Fig. 1A), and that more than 95% of the cells in the gate would meet the criteria of being lymphocyte corresponded to the CD45-bright positivity of lymphocytes (Fig. 1B). However, in 3 out of 5 AIDS specimens, the

Table 1. Monoclonal antibody (MAb) combinations used in this study.

Tube No.	MAb FITC/PE/PerCP			Reactive lymphocyte subsets
1	CD45	/CD14	/-	All leukocytes
2	IgG1	/IgG2	/-	Isotype control for two-color assay
3	CD3	/CD4	/-	T cells/helper-inducer T cells
4	CD3	/CD8	/-	T cells/cytotoxic-suppressor T cells
5	IgG1	/IgG2	/IgG1	Isotype control for three-color assay
6	CD3	/CD4	/CD45	T cells/helper-inducer T cells
7	CD3	/CD8	/CD45	T cells/cytotoxic-suppressor T cells

combination of fluorescence associated with CD45/CD14 and FSC/SSC failed to identify lymphocyte populations in the lysed whole blood as there were many contaminating non-lymphocytes in the debris population (Fig. 2A). An attempt was made to reduce debris by increasing either the FSC boundary or the number of cells to be acquired, but we were still unable to draw an acceptable light scatter gate on lymphocytes.

Our three-color FCM staining of specimens that failed from the two-color assay is shown in Fig. 2B in which a live gate was set on FSC/CD45-PerCP bright (FL3) positive cells. 5,000–10,000 cells in the live gate were acquired and stored (Fig. 2C). Based on this live gate, the proportion of lymphocyte subsets in blood specimens could be identified using additional antibodies (Tubes No. 6, 7). A representative two-parameter plot of lymphocytes stained for CD3-FITC and CD4-PE is shown in Fig. 2D). $CD3^+/CD4^+$ lymphocytes are shown in quadrant 2. As can be seen, there was a considerable proportion of non-$CD3^+/CD4^+$ cells in this two-parameter plot (quandrant 1). These cells were dim $CD3^-/CD4^+$ which corresponded to monocytes and granulocytes (data not shown). However, after regating the live gate using FSC/SSC (Fig. 2E), these contaminating non-lymphocytes disappeared, leaving the pure $CD3^+/CD4^+$ in quadrant 2 and no dim $CD4^+$ cells in quadrant 1 (Fig. 2F). Moreover, the percentage of $CD3^+/CD4^+$ obtained with this regated system was lower than the value obtained with the live gate alone.

Data of $CD4^+$ and $CD8^+$ T lymphocytes from those 15 HIV-seropositive and 2 AIDS specimens, which passed the two-color test, were compared with the three-color assay outlined above. The results were comparable between the two methods (Fig. 3).

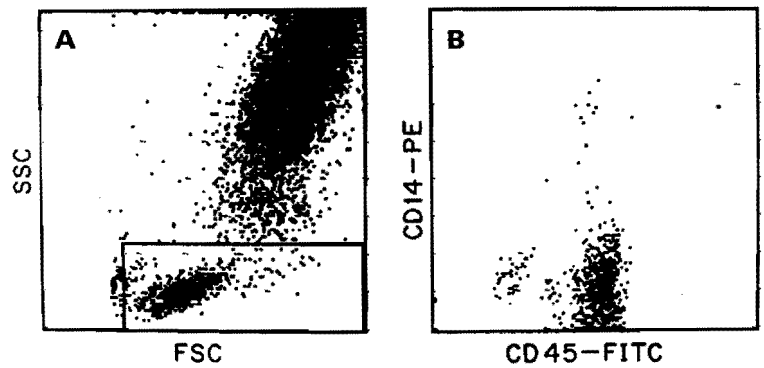

Fig. 1. Representative two-parameter dot plots of major leukocyte populations in HIV-seropositive lysed whole blood stained with CD45-FITC and CD14-PE antibodies. Lymphocyte gate was established from FSC/SSC data acquisition (A) which corresponded to bright $CD45^+/CD14^-$ (B).

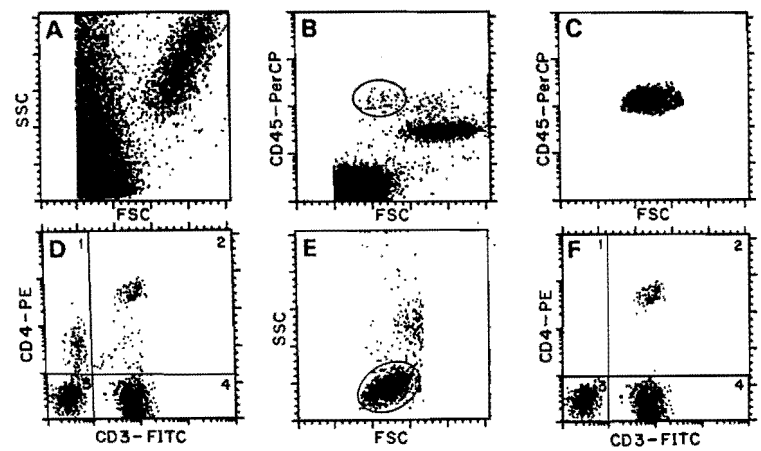

Fig. 2. Two-parameter dot plots showing identification of lysed whole blood of an AIDS patient, stained with CD3-FITC/CD4-PE/CD45-PerCP: A: Light scattering characteristics of FSC/SSC. B: Light scattering gate using FSC/bright $CD45\text{-}PerCP^+$ cells. C: FSC/bright $CD45\text{-}PerCP^+$ cells were live gated and acquired. D: Phenotypic identification of $CD3^+/CD4^+$ (quadrant 2) lymphocytes from FSC/$CD45\text{-}PerCP^+$ gated population showing some dim $CD4\text{-}PE^+$ cells. E: Light scatter gating analysis using FSC and SSC after FSC/$CD45\text{-}PerCP^+$ live gate. F: Phenotypic identification of pure $CD3^+/CD4^+$ (quadrant 2) lymphocytes from FSC/SSC regated population.

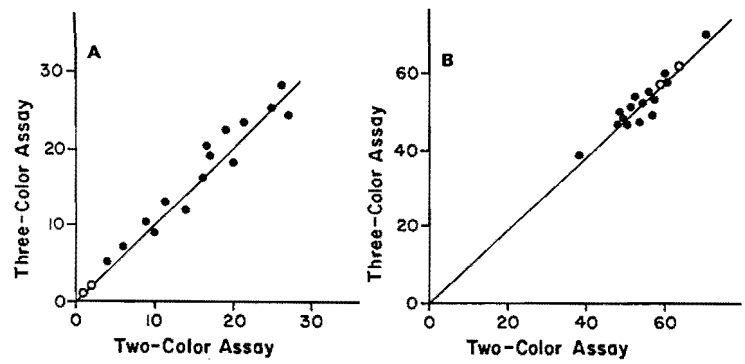

Fig. 3. Correlation of % CD4 (A) and % CD8 (B) T-lymphocyte results from HIV-seropositive (closed circle) and AIDS patients (open circle) using two-color and three-color analyses. Correlations (r^2) are 0.9578 and 0.9703 for A and B, respectively.

DISCUSSION

Two-color FCM analysis of lymphocyte surface antigens has become a standard method for detection of cell subsets. Providing the cell specimens contain a high proportion of lymphocytes, FCM immunophenotyping of lymphocyte subsets can be easily achieved by a FSC/SSC light scattering gate on lymphocytes.[14] In contrast, when there is a high proportion of non-lymphocytes, problems may be encountered in identifying pure lymphocyte cluster by the light scatter analysis alone. The use of CD45/CD14 in combination with FSC/SSC helps define with certainty the lymphocyte population.[11] Once a FSC/SSC gate is drawn on cells expressing bright CD45 but negative for CD14, virtually all the events in the gate will be lymphocytes (Fig. 1). This back-gate technique, however, does not apply to some blood specimens from AIDS patients with incomplete red cell lysis and contain an unusually large number of non-lymphocytes (Fig. 2A). An attempt to acquired more events (up to 100,000 cells) in FSC/SSC gate was also failed, because there were neither enough lymphocytes in the analysing gate nor acceptable light scatter gate to include ≥90% lymphocytes. Several observations suggest that certain patients variables (eg medications, biological factors, etc.), could affect FCM light scatter resolution.[9,10,12]

In order to optimize immunophenotyping in these abnormal AIDS specimens, we have used in combination to two-color (FITC/PE) antibodies an additional antibody of CD45-PerCP to gate lymphocytes. Our technique is simple, reliable, and is based on an assumption that CD45 is present only on leukocytes, and that the cells of specific leukocyte lineages express different densities of CD45 antigen, with the highest fluorescence expression of CD45 antigen seen for lymphocytes.[11,15] Using PerCP-positive CD45 cells with the highest fluorescence signal as live gating criteria helps ensure that all lymphocytes are included in the gate. This live gate effectively distinquished lymphocytes from non-lymphocytes (negative CD45) that scatter with the same FSC. Whilst events outside this live gate with higher FSC but dim PerCP-positive CD45 cells, represent granulocytes and monocytes.

Although, the gating combination of CD45-PerCP with FSC was far superior in identifying the lymphocytes visually (Fig. 2B), it sometimes included some events with the same bright CD45-PerCP cells but dim CD4-PE positivity (Fig. 2D). These dim $CD4^+/CD3^-$ cells corresponded to monocytes (Fig. 2E) and could be easily gated out using FSC/SSC scatter gate. In our method for $CD4^+$ T-lymphocyte determination, it is very important to use CD4 with CD3 so that any contamination of the lymphcyte gate of FSC/CD45-PerCP with monocytes (dim $CD4^+/CD3^-$) will be easily localized and excluded from the $CD4^+$ results.

When results from our FCM three-color assay were compared with data obtained from a standard SimulSET two-color assay, there was good correlation between the two methods (Fig. 3) suggesting that this three-color assay might be a promising assay for monitoring $CD4^+$ T-cells in HIV-seropositive whole blood. However, we do not recommend using this three-color assay to replace the standard two-color assay, as an additional cost of using a third antibody may make this three-color assay less than ideal FCM immunophenotyping for some laboratories. We suggest that, if the quality of a specimen is suspected of being contaminated with non-lymphocytes, a blood smear should be performed to assess contamination by non-leukocyte cells before a decision is made concerning using either the standard SimulSET two-color assay or the three-color assay.

In conclusion, the three-color analysis described will help in identifying with certainty the lymphocyte cluster in AIDS specimens which contain a high proportion of non-

lymphocytes and cannot be analysed with the existing standard two-color assay.

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