ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (1998) 16: 185-192

Evaluation of Monoclonal Antibody Reagents from Three Different Manufacturers Using Flow Cytometric Two-Color Immunophenotyping

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SUMMARY **We evaluated a flow cytometric (FCM) two-color immunophenotyping of CD3+/CD4+ T-helper and CD3+/CD8+ T-suppressor lymphocytes in whole blood samples from HIV-infected individuals using monoclonal antibody reagents from three different manufacturers. Lymphocytes were firstly determined using CD45/CD14 in association with a forward scatter/side scatter gating strategy. CD3+/CD4+ and CD3+/CD8+ were then determined and compared. Reagents from all manufacturers showed good separation of lymphocytes, monocytes and granulocytes with high purity and recovery. There was a good correlation of the percentage of CD3+/CD4+ and CD3+/CD8+ lymphocytes amongst each of the manufacturer's reagents, but the fluorescent intensities of positive cells were not the same. This difference can result in poor discrimination of positive and negative non CD3 cells leading to erroneous results.**

The use of CD4+ T lymphocyte measurements has had a significant impact on the diagnosis, monitoring, and therapeutic control of HIV infection.[1-3] Immunophenotyping using lysed whole blood stained with monoclonal antibodies and analysed by flow cytometry (FCM) is the current standard method for determination of CD4+ T-cells.[4,5] Several guidelines addressing quality control of FCM CD4+ T-cell enumeration in persons with HIV infection have been developed.[3-7] These include the use of monoclonal antibody panels which contain appropriate monoclonal antibody combinations to enumerate CD4+ and CD8+ T-cells. In order to ensure the quality of the results, CD4+ and CD8+ T-cells must be identified as being positive for both CD3/CD4 and CD3/CD8, respectively.[6] The monoclonal antibody panel recommended by the National Institute of Allergy and Infectious Diseases, Division of AIDS (NIAID DAIDS) for the adult AIDS clinical trial[7] includes the following: CD45/CD14 to establish and verify light scatter gating of lymphocytes; IgG1/IgG2 as an isotype control for autofluorescence and nonspecific binding; CD3/CD4 to quantitate T-cells and T helper/inducer cells; and CD3/CD8 for T-cells and T suppressor/cytotoxic cells. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) are the two fluorochromes that are commonly used to conjugate monoclonal antibodies. Since the stability of these conjugates is a concern, the reagents from manufacturers must be optimized for fluorochrome/protein ratio with good quality control.

There are several manufacturers worldwide who provide two-color combinations of monoclonal antibodies for FCM immunophenotyping. In Thailand, reagents from many companies have been widely used by many FCM laboratories. However, most of these reagents have never undergone validation studies for their quality and stability. Despite this

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fact, Becton Dickinson Immunocytometry System (BDIS) products are extensively used for lymphocyte immunophenotyping in both normal[8,9] and disease states, including thalassemia[10] as well as HIV-infected individuals.[11] Webster *et al.*[8] was the first group who led efforts in Thailand to establish standards for clinical FCM, quality assurance, and normal reference values for lymphocyte immunophenotyping using BDIS monoclonal antibody panel. To be able to use these published reference ranges, particularly CD3+/CD4+ T cell values on Thais in the management of HIV/AIDS or other clinical applications, a FCM immunophenotyping equivalence study comparing BDIS reagents with those of other manufacturers was performed.

MATERIALS AND METHODS

Study population

Thirty-one HIV-1 positive patients from the Siriraj Hospital AIDS Cohort Study were investigated. The serological status of the patients was confirmed by ELISA and Western blot testing. All subjects gave full informed consent prior to their participation in this study. Peripheral blood specimens (5 ml) were collected by venipuncture into K_3EDTA-containing tubes and processed for FCM within 6 hours.

Monoclonal antibodies

The monoclonal antibodies used from each manufacturer were directly conjugated with FITC or PE. Table 1 shows the combination of monoclonal antibodies used for enumerations of CD3+/CD4+ and CD3+/CD8+ T-cells, and the different clones. There was difference in the clone used by the manufacturers. In addition, the isotype control used by the companies varied so, in some experiments, a tube containing unstained patient cell was used in addition to the isotype control for evaluating autofluorescent and nonspecific properties of monoclonal antibody reagents in individual patient specimens.

Immunofluorescence staining

Twenty microliters of each monoclonal antibody reagent pair was added to 100 µl of whole blood in 12 x 75 mm test 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 (BDIS) was added to each tube and incubated for another 10 minutes. After centrifugation at 500 x *g* for 5 minutes and subsequent wash with 2 ml PBS, the cell pellets were resuspended in 0.5 ml of 1.0% paraformaldehyde in PBS with 0.1% sodium azide. The stained samples were kept at 4°C for 1, 3 and 4 days prior to FCM analysis. The stability of the monoclonal antibody reagents was also determined by comparing the percentage of CD3+/CD4+, CD3+/CD8+ and CD3+ T-cells obtained from the new lot of reagents with those that had been expired for 3 to 6 months.

Variable factors

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 washing and lysing buffer throughout. In addition, all of the immunostaining and procedure were performed by the

Table 1 Monoclonal antibody combinations used for CD3+/CD4+ and CD3+/CD8+ lymphocyte subset analysis

Tube no.	Monoclonal antibodies conjugated with		Manufacturer
	FITC (clone)	PE (clone)	
1	CD45 (2D1)	CD14 (MØP9)	BDIS[a]
2	IgG1 (X40)	IgG2a (X39)	BDIS
3	CD3 (SK7)	CD4 (SK3)	BDIS
4	CD3 (SK7)	CD8 (SK1)	BDIS
5*	CD45 (Immu19.2)	CD14 (RM052)	Immunotech[b]
6*	IgG1 (679.Mc7)	IgG1 (679.Mc7)	Immunotech
7*	CD3 (UCHT1)	CD4 (13B8.2)	Immunotech
8*	CD3 (UCHT1)	CD8 (B9.11)	Immunotech
9	CD45 (BRA55/2)	CD14 (BA-8)	Bio D[c]
10	IgG1 (HybIgG1)	IgG1 (HybIgG1)	Bio D
11	CD3 (BB12)	CD4 (GA20)	Bio D
12	CD3 (BB12)	CD8 (733)	Bio D

[a]San Jose, CA, U.S.A.
[b]Marseille, France
[c]Biotechnologie Diagnostici, Valenzano, Italy

*These monoclonal antibodies are an Opticlone product designed for multi-color immunostaining in manual no-wash sample preparation

same person and the FCM analysis was also performed by the same person. Moreover, instrumentation quality control was performed prior to sample analysis using a non-fluorescent and fluorescent particle (Calibrite bead; Becton Dickinson) to calibrate the instrument photomultiplier tube, sensitivity and fluorescent compensation.

Flow cytometry analysis

Data from the 12 tubes for each patient were acquired and analysed using the SIMULSET software on a FACScan (BDIS) flow cytometer equipped with a 15 mW argon ion laser tuned at 488 nm. Instrument setup and calibration, including photomultiplier tube, sensitivity and compensation adjustments, were performed using FACSComp software (BDIS) and Calibrite beads (BDIS) in accordance with manufacturer's recommendations. A minimum of 15,000 cells were acquired using the CD45/CD14 tube. Lymphocytes were identified automatically based on the intensity of the CD45/CD14 immunofluorescence and a corresponding forward and side light scatter (FSC/SSC) gate (lymphocyte gate) was drawn by the SIMULSET solfware. The recovery and purity of lymphocytes in the lymphocyte gate is defined as the proportion of lymphocytes that are found within the gate and the proportion of events within the gate that are lymphocytes, respectively. The percent gated cells that were lymphocytes was corrected for percent purity of CD45+ bright/CD14- lymphocytes and multiplied by 100.

At least 2,000 lymphocytes were acquired for each tube after the CD45/CD14 tube. The isotype control tube determined nonspecific binding and set markers for distinguishing fluorescene-negative and positive cell populations. FCM results were reported as the lymphocyte subset percentage of total lymphocytes. For the purpose of analysis, total CD3+ T cells was calculated as the average percentage taken from the CD3+/CD4+ and CD3+/CD8+ tubes. The T helper/suppressor ratio was obtained from the CD3+CD4+ and CD3+CD8+ tubes.

Statistical analysis

Lymphocyte subset results obtained from the three manufacturers of monoclonal antibody reagents were correlated using linear regression lines. Other statistical significance of differences between the results was determined by the Mann-Whitney U-test.

RESULTS

Staining of HIV-seropositive peripheral blood with the CD45-FITC/CD14-PE reagent showed three major blood populations of lymphocytes, monocytes, and granulocytes (Fig. 1). Reagents from all manufacturers showed similar patterns. The recovery and purity of lymphocytes in the lymphocyte gate from the three manufacturers were about the same (Table 2) where all results were above 95%. The isotype controls, as indicated in tubes 2, 6 and 10 were used to determine where nonspecific and autofluorescence end and specific positive fluorescence from CD3/CD4 or CD3/CD8 begins on the fluorescence scale. After setting the quadrant cursor, all isotype controls showed a homogeneous population within 1 decade of logarithmic expression in the lower left quadrant with less than 0.5% in other quadrants (Fig. 2A, B and C). After defining the cursor with the isotype controls, lymphocyte subset data of T-helper (CD3+/CD4+) and T-suppressor cells (CD3+/CD8+) were determined using CD45-FITC/CD14-PE in combination with the FSC/SSC logical gate. A representative two-parameter dot plot of lymphocytes reactive for CD3-FITC/CD4-PE and CD3-FITC/CD8-PE are shown in the upper right quadrants (Fig. 2D, E and F for CD3+/CD4+ cells, and Fig. 2G, H and I for CD3+/CD8+ cells, respectively). When the percentage of CD3+/CD4+ and CD3+/CD8+ data was compared for each manufacturer's reagents, a good correlation was obtained (Table 3) with $P < 0.0001$. There was also no difference in the percentage of CD3+/CD4+ and CD3+/CD8+ T-cells after 1-4 days of fixation (Table 4). The stability of the monoclonal antibody reagents was still good after 3 or 6 months of the expiry date in which no significant difference in the percentage of CD3+/CD4+, CD3+/CD8+ and CD3+ T-cells was found (data not shown).

DISCUSSION

An important area of FCM immunophenotyping quality control is the purity of lymphocytes in the FSC/SSC gate. This depends a great deal on how the FSC/SSC lymphocyte gate is drawn. Ideally, all lymphocytes can be included in the light scatter gate, if a large FSC/SSC lymphocyte gate is drawn. However, because drawing a large lymphocyte gate allows for the inclusion of nonlymphocytes which may possess some of the same antigens as lymphocytes and thus, could lead to erroneous results.[10,11] To eliminate this biasing lymphocyte gate, we chose to use a lymphocyte gate drawn automatically by the SIMULSET soft-

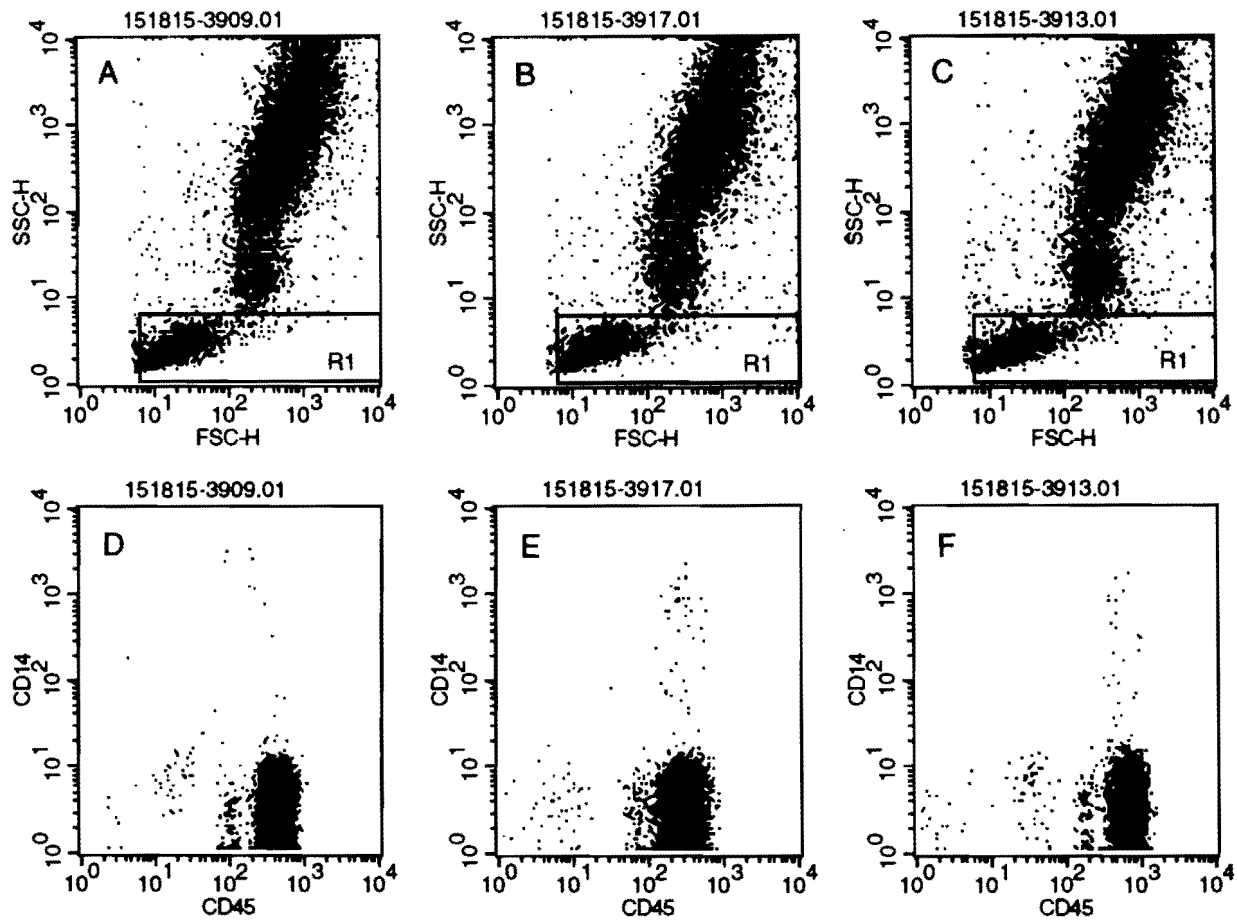

Fig. 1 Representative dot plots of major leucocyte populations in HIV-seropositive lysed whole blood stained with CD45 and CD14 monoclonal antibodies from BDIS (A and D), Immunotech (B and E) and Bio D (C and F). Lymphocyte gate (R1) for data acquisition from FSC/SSC was established by SIMULSET software using fluorescence parameter from CD45-FITC/CD14-PE tube.

Table 2 Lymphocyte recovery and purity using different manufacturers' reagents

Parameter	BDIS		Immunotech		Bio D	
	Recovery	Purity	Recovery	Purity	Recover	Purity
Mean ± SD (Range)	99.5 ± 0.7 (98-100)	95.3 ± 1.6 (91-98)	99.5 ± 0.6 (98-100)	95.1 ± 3.3 (90-98)	99.4 ± 0.7 (97-100)	95.2 ± 2.0 (90-98)

Table 3 Flow cytometric immunophenotyping comparison of results between the reference BDIS and Immunotech or Bio D

Subset	BDIS	Immunotech	Bio D			
	Mean ± SD (range)	Mean ± SD (range)	Mean ± SD (range)	r^a	r^b	r^c
CD3+/CD4+	24.8 ± 6.7 (11-35)	25.1 ± 6.4 (10-35)	24.9 ± 6.8 (9-37)	0.96	0.94	0.94
CD3+/CD8+	42.5 ± 10.1 (27-66)	42.6 ± 9.3 (28-66)	41.2 ± 9.7 (26-65)	0.97	0.95	0.95
CD3+	67.4 ± 8.8 (59-85)	67.7 ± 7.3 (58-84)	66.4 ± 8.2 (59-81)	0.92	0.90	0.90
CD4+/CD8+[d]	0.6 ± 0.3 (0.17-1.14)	0.7 ± 0.3 (0.15-1.04)	0.6 ± 0.2 (0.14-1.33)	0.97	0.97	0.96

[a]Correlation coefficient between BDIS reagents *vs.* Immunotech reagents
[b]Correlation coefficient between BDIS reagents *vs.* Bio D reagents
[c]Correlation coefficient between Immunotech reagents *vs.* Bio D reagents
[d]Ratio of CD3+/CD4+ to CD3+/CD8+ T cells

Table 4 Flow cytometric immunophenotyping comparision of CD3+/CD4+, CD3+/CD8+ and CD4+/CD8+ ratio analysed after 1, 3 and 4 days fixation

Manufacturer	Subset	N	Day		
			1	3	4
BDIS	CD3+/CD4+	9	21.6 ± 7.0	21.4 ± 7.4	21.8 ± 6.9
	CD3+/CD8+	9	50.8 ± 10.7	50.2 ± 10.3	50.6 ± 10.1
	CD4+/CD8+	9	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
Immunotech	CD3+/CD4+	9	21.6 ± 7.4	20.8 ± 7.8	21.1 ± 7.5
	CD3+/CD8+	9	51.2 ± 9.3	50.8 ± 10.1	51.0 ± 9.6
	CD4+/CD8+	9	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
BIO D	CD3+/CD4+	9	20.8 ± 6.8	21.0 ± 7.0	20.9 ± 7.1
	CD3+/CD8+	9	50.3 ± 9.9	50.5 ± 10.0	50.1 ± 9.7
	CD4+/CD8+	9	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2

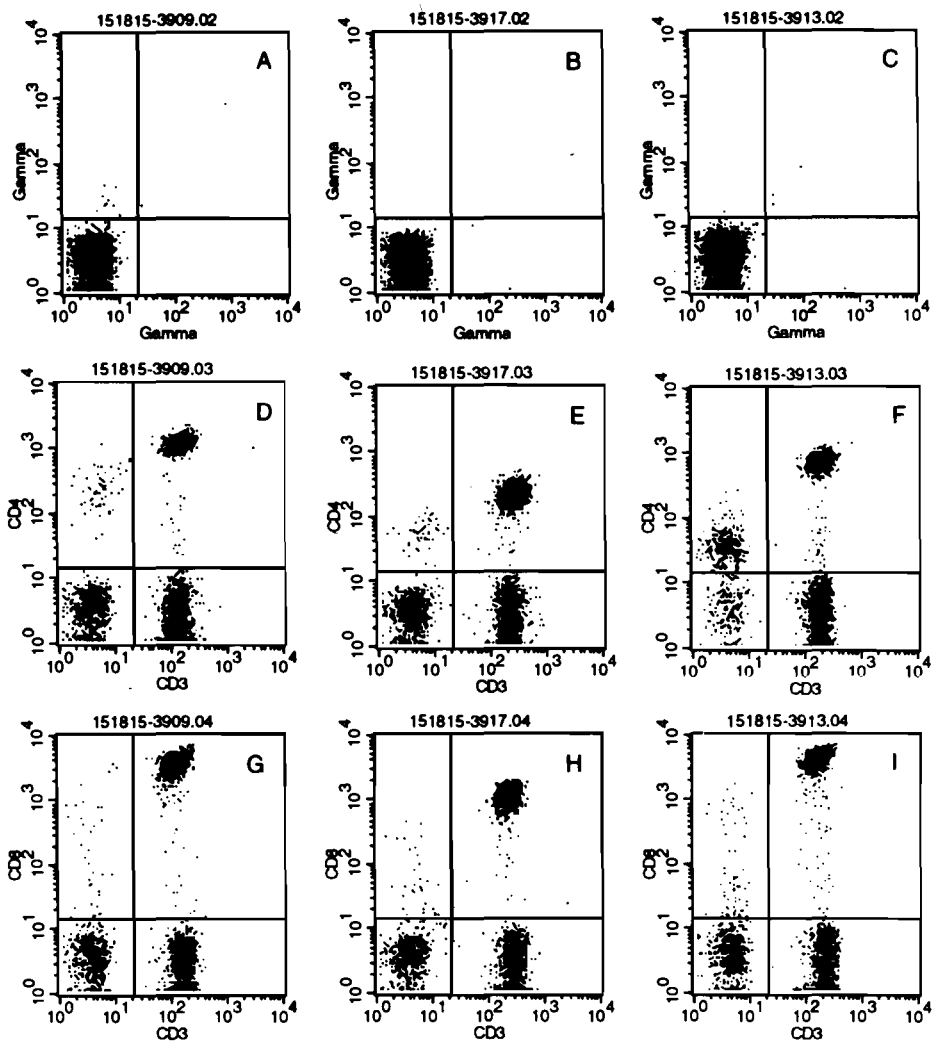

Fig. 2 Representative dot plots showing identification of lysed whole blood of a HIV-seropositive patient, stained with monoclonal antibodies from BDIS (A, D, G) or Immunotech (B, E, H) or BIO D (C, F, I) which are reactive for isotype control (A,B, C), CD3-FITC/CD4-PE (D, E, F) and CD3-FITC/CD8-PE (G,H,I). CD3+/CD4+ and CD3+/CD8+ are shown in upper right quadrant of D, E, F and G, H, I, respectively.

ware using the CD45/CD14 tube. The use of CD45-FITC/CD14-PE in combination with low FSC/SSC helps define, with certainty, the lymphocyte population.[10,11] Once a gate is automatically drawn using bright CD45+/CD14-, virtually all the cells in the gate will be lymphocytes (Fig. 1D, E, and F). From our study, CD45/CD14 reagents from all manufacturers showed similar lymphocyte recovery and purity, with low contamination of nonlymphocytes in the gate. All showed similar CD45/CD14 fluorescence patterns with the same brightness of CD45 positivity. This suggests that CD45 and CD14 monoclonal antibodies from the three different manufacturers efficiently differentiates leucocyte populations based on fluorescence intensity, and thus, are suitable for FSC/SSC lymphocyte gating.

The isotype controls from all manufacturers showed similar negative cell populations (Fig. 2). This implies that the isotype controls from each manufacturer are optimal for controlling nonspecific and autofluorescence, and are appropriate for use with test monoclonal antibody reagents from other manufacturers.

Analysis of CD3/CD4 and CD3/CD8 lymphocyte subsets with the isotype-set cursors showed clear separation of the negative and positive staining cells. When the percentage of CD3+/CD4+ or CD3+/CD8+ T-lymphocytes was analysed with each manufacturer monoclonal reagents, there was good correlation with all of the reagents (Table 3). Further FCM quality control on the variability of CD3 in each of the analysis tubes for CD4 and CD8 showed no difference between CD3 from the CD3+/CD4+ tube and CD3+/CD8+ tube. The spread of replicate CD3 in the two tubes was less than 3%. This suggests that the monoclonal antibodies for CD3/CD4 and CD3/CD8 from these three manufacturers can be used for T-helper and T-suppresor lymphocyte determination. In addition, there was also no difference in CD3-FITC fluorescent intensities from all reagents tested. There was also no difference in fluorochrome labeling when a different IgG subclass of monoclonal antibodies was used. Both mouse anti-human IgG1 and IgG2 monoclonal antibodies showed the same homogeneous negative within the 1 decade logarithmic fluorescent population. This indicates that monoclonal antibody combinations from different manufacturers with different isotypes can be used in CD3/CD4 and CD3/CD8 determination. However, the staining fluorescent intensities of positive cells for CD4 or CD8 are not the same, particularly with the PE-conjugated CD4 antibody. Amongst the three manufacturers, the BDIS reagent showed the brightest PE fluorescent intensities for CD4 (Fig. 2D). For CD8-PE, both BDIS and Bio D showed brighter fluorescent intensites than Immunotech (Fig. 2G, I). Immunotech reagents that we used are Opticlone monoclonal antibodies which are originally designed for lysed-no wash sample preparation method. Our lysed and washed method may interfere with the PE intensity (in separate experiment, using Immunotech IO test with lysed and washed technique showed the same fluorochrome intensity when compared to BDIS reagents, data not shown). In this study, we also observed that the BIO D CD4-PE reagent showed poor separation between the CD3-/CD4- cell population (lower left quadrant) and the CD3-/CD4+ cell population (upper left quadrant) (Fig. 2F). Moreover, the percentage of CD3-/CD4+ cells obtained from Bio D is significantly higher than the other two manufacturers reagents with $P < 0.001$ ($9.45 \pm 4.0\%$ compared to $3.32 \pm 1.17\%$ and $2.97 \pm 0.84\%$ from BDIS and Immunotech, respectively). This means that CD4 and CD8 antibody reagents from different manufacturers separate positive and negative lymphocyte subsets differently, based on PE fluorescent intensity. This observation could be due to cross-reaction of CD4 antibody to monocytes, and clumping or aggregation of CD3-/CD4+ cells with CD3-/CD4- cells. The other explanation could be a difference in fluorochrome/protein ratio. Conjugation of CD4 antibody with fluorochrome from the manufacturer may not be optimal, but here, we used the manufacturer's recommended amount of antibody which should give optimal positive/negative resolution and optimal positive fluorescent intensity. This difference is particularly important when applied to antigen with a continuous distribution of fluorochrome, and no clear demarcation between positively and negatively labeled cells, as seen with CD16/CD56 (natural killer cell) and HLA DR reagents. Further evaluation of other monoclonal antibodies, ie. CD16/CD56, CD69 or CD38 needs to be done in both normal and HIV-infected individuals to assess the accuracy and interlaboratory variability of the CD4 T-cells determination.

In conclusion, this study demonstrates a good correlation with all of the three manufacturers' monoclonal antibody reagents in determining CD3+/CD4+ and CD3+/CD8+ T lymphocytes. However, the quality of the reagents in term of fluorescent intensity was not the same, particularly the PE-conjugated monoclonal antibody to CD4. In order to use the existing lymphocyte immunophenotyping reference ranges in healthy Thais[8] when using reagents other than BDIS products, each investigator has to validate the equivalency of the reagents with BDIS products. Different clones of monoclonal antibodies, and different methods for sample preparation and staining have to be considered with care.

ACKNOWLEDGEMENTS

This work was financially supported by the Division of AIDS, Ministry of Public Health, Thailand, and by Becton Dickinson Immunocytometry Systems, U.S.A.. The authors thank Dr. Kyle Webster for his helpful discussion and suggestions; and Becton Dickinson Immunocytometry System, Immunotech, and Biotechnologie Diagnostici for kindly providing the monoclonal antibody reagents.

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