

CHAPTER IV

DISCUSSION AND CONCLUSION

CD4 lymphocyte count is an important marker for classification the status of the disease (12), the prognosis (13-14) and monitoring for the therapy of HIV infection (4-5). The standard method for enumeration of CD4 lymphocytes is flow cytometry (3). This technique measures the absolute CD4 lymphocyte count in whole blood by a dualplatform, three-stage process: the white blood cell count, the percentage of lymphocyte in white blood cells and the percentage of CD4 lymphocyte in lymphocyte population. Measuring the percentage of CD4 lymphocytes is performed by immunophenotyping and analyzed by a flow cytometer. Two-fluorescent color reagent using monoclonal antibody panel was developed and is acceptable for enumeration of CD4 lymphocytes. However, this method requires very expensive reagents. Whole blood specimen must be stained in four separated tubes with the two-color immunofluorescence using directly conjugated monoclonal antibody pairs followed by lysis of erythrocytes and analysis by flow cytometer. By this method, specimens that red blood cells are incompletely lysed and purities of lymphocyte analysis gate from each stained tube are different or less than 95%, the obtained CD4 lymphocyte number will be erroneously decreased (36).

In this study, the three-color immunofluorescence technique was developed for enumerating CD4 lymphocytes in blood samples by using three fluorochromes conjugated mAbs including anti-CD45, MT4 and MT14/3 mAb. MT4 mAb, an

antibody against CD4 protein and MT14/3 mAb, an antibody against CD14 protein were generated by Dr. Watchara Kasinrerak at the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University (37-38). MT4 mAb and MT14/3 was IgM and IgG1 isotype respectively (37-38). To develop the three-color reagent, purified MT4 and MT14/3 were needed. To obtain purified MT4 mAb, MT4 hybridoma induced ascitic fluid was purified by affinity chromatography using rat anti-mouse IgM sepharose 4B column. In the purification processes, 0.15 M PBS pH 7.2 was used as binding buffer and 0.2M glycine-HCl pH 2.8 was used as elution buffer. By affinity chromatography, approximately 1 mg of antibody was obtained from 1 ml of ascites. To purify IgG1 mAb from ascitic fluid, affinity chromatography using Protein A-sepharose was suggested (39-41). In this study, MT14/3 hybridoma induced ascitic fluid was therefore purified by Protein A sepharose CL-4B column. Protein A sepharose CL-4B is protein A, extracted from *Staphylococcus aureus* which consists of five IgG binding domain and immobilized by the CNBr method to sepharose CL-4B. IgG from most species binds Protein A sepharose CL-4B at neutral pH and physiological strength. By this method, 20 mM sodium phosphate, pH 7.0 or 50 mM Tris buffer, pH 7.0 are recommended as binding buffers. To elute IgG from protein A sepharose CL-4B, it is normally necessary to lower the pH to about 3.0 depending on the sample. Mouse IgG1 may use pH 6.5, for IgG2a use pH 4.5 and for IgG2b and IgG3 use pH 3.0 as a general method and 0.1M glycine buffer pH 3.0 or 0.1M citric acid pH 3.0 are recommended as elution buffers. This study, we compared 0.15M PBS pH 7.2 and 8.0 as binding buffers, then 0.1M citric acid pH 3.0 and 6.5 were used as elution buffers. It was found that when 0.15M

PBS pH 7.2 and 0.1M citric acid pH 3.0 were used the yield was 2-3 folds higher than another method (0.15M PBS pH 8.0 and 0.1M citric acid pH 6.5).

After purification, purified MT4 mAb was then conjugated to FITC. Several methods have been described for FITC-antibody labeling (42-45). Amine-reactive label, labeling at the amine side is the most widely used method (46). Antibodies contain a large number of lysines which are the amine site for reacting with the acylating compounds or acylating fluorophores such as isothiocyanate and yielding thiourea bonds at pH 9-9.5. However, for IgM molecules, it is not recommended to perform at pH more than 7.2 (47). In this study, we therefore did not dissolve purified MT4 mAb in 0.1M sodium bicarbonate as suggested in standard method, but labeling in PBS pH 7.2. The FITC coupled MT4 was then separated from free FITC by dialysis. In this study, labeling of MT4 with FITC was performed 2 times. At the first time, MT4 mAb was labeled with FITC using R ratio of 20. After labeling and dialysis, F/P ratio was determined. It was found that F/P ratio was 0.3 that was rather low compare to the recommended ratio for the optimal conjugation of FITC to antibody (0.5-1.0). By this labeling, it was found that F/P ratio increased to 0.59 and this ratio was in the recommended ratio (48).

Before using FITC labeled MT4 and purified MT14/3 mAbs, the activity and specificity of the obtained mAbs were confirmed by immunofluorescent staining assay. FITC labeled MT4 mAb reacted directly to CD4 lymphocytes from PBMC, which expressed CD4 molecules on their surface (49). The second labeling antibody (F/P ratio 0.59) showed higher positive reactivity compared to the first labeling (F/P ratio as 0.3). The purified MT14/3 mAbs strongly reacted to monocyte from PBMC, which expressed CD14 molecules on their surface (49). These results indicated that

all of the purified mAbs still have its binding activity. The purified mAbs were further proved for their specificity by staining with the CD4 and CD14 expressing COS cells. The FITC labeled MT4 mAbs reacted to CD4 expressing COS cells and MT14/3 mAb strongly reacted to CD14 expressing COS cells but not to the mock transfectants indicating these purified mAbs were antibodies directed against CD4 and CD14 molecule, respectively.

The three-color immunofluorescence technique used commercial CD45-PerCP/CD14-PE/CD4-FITC was developed for enumerating CD4 lymphocytes in blood sample. FITC labeled MT4 mAb and PerCP conjugated anti-CD45 mAb were directly used to stain whole blood. Purified MT14/3 mAb was used to indirectly stain whole blood, as primary mAb, and PE conjugated goat anti-mouse IgG antibody was used as a secondary mAb. The optimization concentration of the antibodies used was determined before used. It was found that 40 $\mu\text{g/ml}$ of FITC labeled MT4 mAb, 20 μl of PerCP conjugated anti-CD45 mAb, 100 $\mu\text{g/ml}$ of purified MT14/3 mAb and counterstained with PE conjugated goat anti-mouse IgG antibody at dilution 1:8 were the optimal concentrations. For cell staining, however, it can not stain whole blood with anti-CD45 mAb and MT14/3 mAb at the same time. As both mAbs were IgG isotype, the secondary mAb (PE conjugated anti-mouse IgG antibody) will then bind to both mAbs. Therefore, in this study, whole blood must be stained with antibodies in two steps as described in materials and methods.

By flow cytometric analysis, the monocyte population was firstly gated as R1 using color-gating technique, the granulocyte population was gated as R2 in dot plot display of FSC and SSC. Then the lymphocyte population was gated as R3 in dot plot display of FL3 and SSC. It is known that monocytes also express CD4 molecules on

their surface (50). The momentous problem, if monocytes were contaminated in the lymphogate these cells can affect the accuracy of the measurement of CD4 lymphocytes. By limited gating of the lymphocyte population, lymphocytes were not contaminated with other cells determined according to fluorescence intensity by using FL3 and FL2. Then fluorescence labeled lymphocytes were determined for percentage of CD4 lymphocytes according to fluorescence intensity by using FL3 and FL1. In this study, the lymphocyte population rather separated clearly from the monocyte population in dot plot display of FL3 and SSC. Therefore CD45 versus SSC gating strategy was the preferable method for identifying lymphocytes accurately and reproducibly as the preceding authors found (51-57). CD4 lymphocytes from a total of 57 blood samples were evaluated by both the developed three-color and the standard two-color, Simultest™ reagent kit. A very high degree of correlation between both reagents was found in both percentage and absolute CD4 lymphocytes from 47 blood samples. There were 10 blood samples from Simultest™ reagent kit when considered lymphocyte analysis gate did not meet the QC-criteria. The results of both reagent were higher different than these obtained from 47 blood samples. The results of 10 blood samples stained with the standard two-color method may not be accurate.

In addition, this study attempted to produce a home made red blood cell lysing solution. Several solutions were prepared and used to lyse red blood cells after stained with antibodies and then analysed by flow cytometry. The 3% formaldehyde-NH₄Cl lysing solution provided appropriate cellular distribution and accuracy compared to the standard FACS™ lysing solution. Lymphocyte subsets as CD4, CD8 and CD3 of 20 healthy donors were determined using the prepared lysing

solution. Both percentage and absolute number of CD4, CD8 and CD3 lymphocytes obtained from the prepared and commercial lysing solution were similar with no statistically significant difference. Although, using this lysing solution the monocyte population was rather near the lymphocyte population. However, the lymphocyte purities obtained from this lysing solution was very high (92%-97%). The 3%formaldehyde-NH₄Cl-PBS lysing solution was another lysing solution provided appropriate cellular distribution. Using this lysing solution, the monocyte population was rather far from the lymphocyte population. This 3%formaldehyde-NH₄Cl-PBS lysing solution was used to evaluate the accuracy compared to FACS™ lysing solution. Lymphocyte subsets as CD4, CD8 and CD3 of 10 healthy donors and 10 HIV infected persons were determined. A high degree of correlation between both reagents was found in both percentage and absolute number of CD4, CD8 and CD3 lymphocytes. The lymphocyte purities obtained from this lysing solution was also very high (93%-97%). It was concluded that the home made red blood cell lysing solutions provides results which are equivalent to those given by the commercial FACS™ lysing solution.

In conclusion, three-color immunofluorescence reagent and red blood cell lysing solution for determining CD4 lymphocytes in blood sample were developed. These reagents were accurate, more cost effective than available commercial reagent and appropriate for use in measurement of CD4 lymphocytes in healthy and HIV infected blood samples.