CHAPTER IV

DISCUSSION AND CONCLUSION

In 2002, the total number of persons living with human immunodeficiency virus (HIV) is approximately 42 million and about 5 million new people worldwide became infection with the virus. Projections for the next 10 years suggest that the situation will become even more serious with possibly 100 million infected individuals. About 70% of HIV infected persons worldwide reside in poor countries. At the end of 2003, 400,000 affected by the HIV epidemic people living in resource-limited countries have benefit from antiretroviral treatment. To have 3 million people on effective ART promoted by World Health Organization at the end of 2005, the "3 by 5", to scale up to flight against HIV/AIDS in those countries (1, 3)

CD4+ T lymphocytes, also known as the helper T-cells, are the coordinators of the immune responses the body defense reaction against microorganism and some forms of cancer. These cells constitute as the primary target of HIV. The consequent destruction of CD4+ T cells is the main cause of the progressive weakening of the immune system in HIV infection, and leads ultimately to the acquired immune deficiency syndrome, AIDS. Therefore, Absolute CD4+ T-lymphocyte enumeration is an important immunodiagnostic measurement. It was used as surrogate marker for assessing the degree of immune deterioration and speed of progression towards AIDS (12, 13). Defining together with clinical information of patients infected with HIV for making a decision to initiate ART (17). The CD4+ lymphocyte number is also used for deciding the timing for prophylaxis of opportunistic infections (16) and monitors the efficacy of the treatment (14).

Flow cytometry is the gold standard technology for CD4 T lymphocyte enumeration. This technique is belonging to the automated accuracy counting. Its precision and reproducibility have made results reliable. However, this technique is expensive and requires sophisticated analysis performances as well as highly trained personnel. The instruments require regular of highly expensive maintenances. Additional, lacking of technical support has limited the application use of flow cytometer in resource-constrained countries (21, 22). Therefore, the alternative technologies to enumerate CD4+ T lymphocytes are urgently needed. The techniques for replacing flow cytometric method should be simple, reliable, inexpensive, and affordable and applicable for all laboratories in developing countries.

Currently several alternative assays which based on microscopes (optical or fluorescene) are available such as the Cyto-Spheres and the Dynabeads. Cyto-Spheres is relatively inexpensive by five times cheaper than conventional flow cytometry and requires no specialized equipment other than microscope with estimation cost only $6,000 compared to $50,000 of flow cytometer. This is still not including additional cost of equipment maintenance and training. However, Cyto-Sphere assay requires manual cell counting and only smaller number of cells were counted for estimating CD4+ cell numbers (25). In addition, the blocking reagent for demonstrating monocytes can be excluded only 85%. This would yield absolute CD4 counts higher than those of flow cytometry (26). By several studies in six sites in five countries of West Africa, the correlations between Cyto-Sphere assays with flow cytometry were between 0.8-0.93 (54). Dynabead, another microscopy based assay, showed highly correlated comparing to flow cyotmetry (28). However, this technique is a labor-intensive technology and requires time-consuming manual counting. Dynabead assay takes about 3 days to train a technician how to perform this method (23).

A number of alternative methods based on enzyme immunoassays such as TRAxELISA, Zymmune and Capcellia were developed. TRAx CD4+ ELISA counts were reported a considerable correlation when compared to that reference flow cytometry. However, this method is based on ELISA. The batch running system requires promptly lysate, therefore, the prepared lysate should be stored as frozen until all clinical specimens were prompt to run in a same batch (28, 31). This is taken an extended time in obtaining the result. In addition, the principle of this assay is based on the detection of total CD4 proteins in a whole blood lysate to predict CD4+ T cells per microliter. The results are not dependent on intact or viable cells as with other cytometric techniques. In addition, some diseases such as leukemia or lymphoma or rheumatoid arthritis may have biological factors perturb the ratio of CD4 molecules per cell. It may not possible to use the TRAx CD4 assay to predict the absolute CD4 count for all HIV-sero-positive patients. Today this method is taken off the market (31).

In this study, we have developed a new method, named MT4 method, for enumerating CD4+ T lymphocytes. By this technique, CD4 mAb coated megnatic beads are used to deplete CD4+ T cells in whole blood prior to count absolute lymphocyte by hematology analyzer. The CD4+ T lymphocyte numbers were then estimated by subtracting absolute lymphocyte number of CD4+ depleted and non-depleted blood.

To develop the MT4 method, in this study CD4 (MT4) mAb coated magnetic beads was first developed. For the MT4 bead development, a CD4 monoclonal antibody, specific binding only to CD4+ T lymphocyte, clone MT4, (IgM isotype) was generated by Assoc. Prof. Dr. Watchara Kasinrerk at Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand was used. The ascitic fluid containing MT4 mAb was first produced in a

Balb/C mouse by inoculation of hybridoma clone MT4. By the technique used in this study, large amount (8 ml) of ascitic fluid was obtained. The MT4 mAb, which is IgM isotype, was then purified from the obtained ascitic fluid by affinity chromatography using Sepharose coated with rat monoclonal antibody specific to mouse IgM.

After purification, the activity and specificity of the obtained purified mAb was confirmed by immunofluorescent staining assay. The purified MT4 mAb reacted to lymphocyte sub population in PBMC and also reacted to Sup T1 human T cell line, which express CD4 molecules on their surface. This result indicating that after purification, MT4 mAb have its binding activity. The purified MT4 mAb was also confirm its purity by SDS-PAGE. Only immunoglobulins bands could be observed. Therefore, the purified MT4 mAb could be used in the further step.

The M450 epoxy beads purchased from Dynal, Norway were then coated with the purified MT4 mAb. After bead coating, the MT4 coated beads reagent was checked whether beads were coated by immunofluorescence staining assay. MT4 mAb coated beads were homogenous strongly positive with FITC conjugated anti mouse-Igs antibody which indicated the success of beads coating. In addition, the MT4 bead reagent was used for the negative selection of CD4+ cells from human peripheral blood. By using magnetic particle concentrator, about 90% of CD4+ lymphocytes were depleted. In contrast, neutrophils and monocytes were not depleted. The MT4 bead reagent, therefore, was specifically binding with CD4+ T lymphocyte subpopulation and can be used in development of method for enumeration of CD4+ lymphocytes.

To develop new non-flow cytometry for enumeration of CD4+ cells in whole blood, the suitable condition of the method was optimized. In this study, a new method for enumeration of CD4+ lymphocytes is set up as follows: In test tube, 200 µl of blood was mixed with 200 µl of MT4 bead (concentration of 10^7 beads/ml). In control tube: 200 µl of blood was mixed with 200 µl of PBS. Both tubes were then incubated

at room temperature for 30 minutes with constant rotation. After that, the test tube was placed on magnetic particle concentrator (MPC) for 5 minutes, then blood were removed to a new microtube leaving attached cells-beads-magnet undisturbed. In this tube, CD4+ T lymphocytes were depleted. In the control tube, the diluted blood was transferred to a new micro-tube. In this tube, there was no CD4+ T lymphocyte depletion. CBC analysis was performed for both tubes. An absolute CD4+ lymphocytes was calculated by the following formula:

Absolute CD4+ T cells (cells/μl) = Abs. Lym. in control tube - Abs. Lym. in test tube

To validate the developed MT4 method, enumeration of CD4+ T lymphocytes of normal and HIV infected persons were determined by the developed MT4 method and compared the results to the standard flow cytometric method. In this study, total of 195 samples including 17 healthy donors and 178 HIV infected patients were compared. It was shown that the developed method generated the comparable results to the standard flow cytometric method. Analyzed data were summarized (Table 4.1)

In summary, in this study, MT4 bead reagent and a new non-flow cytometry to enumerate CD4+ T lymphocyte, named MT4 method, were developed. This MT4 method is a new practical procedure that can be applied to hematology analyzer that is already available in most laboratories in developing counties. The introduced method could be used as an alternative to flow cytometric method with sufficient accuracy, reliability, and simplicity. The cost of CD4+ T lymphocytes count by MT4 method is approximately five times cheaper than conventional flow cytometry. Within one hour, 30 samples could be tested when the 6 holders type of magnetic particle concentrator was used. Therefore, the developed assay is suitable for several testing number a day for assisting HIV/AIDS monitoring in resource limited setting area.

Table 4.1: Summary of data analysis of MT4 method development compared to flow cytometry for enumeration of CD4+ lymphocyte in whole blood.

Indicator	Improved MT4 method
Population	17 blood donors 178 HIV infected persons
Regression line analysis and correlation to flow cytometry	slope of 1 intercept of -0.09 r=0.91
Classifying at the treshold of	
≤ 200 cells/μl	
Sensitivity	86%
Specificity	85%
Accuracy	86%
PPV	74%
NPV	93%
≤ 350 cells/μl	
Sensitivity	93%
Specificity	83%
Accuracy	89%
PPV	90%
NPV	87%
% Discrepancy when	
<200 cells/μl	11.10%
200-499 cells/μl	11.30%
>500 cells/μl	12.80%
Bland-Altman analysis (bias)	9 cells/μl
% population within 2SD	95%