CORRELATION BETWEEN THE METHODS OF FLOWCYTOMETRY, MANUAL LIGHT AND FLUORESCENCE MICROSCOPES FOR CD4 CELL COUNT IN NORMAL ADULTS

Endang Retnowati*, Wira Santoso*, Nasronudin**

ABSTRACT

A laboratory study was conducted in order to assess the correlation and to compare between flowcytometry, manual light and fluorescence microscope methods used for enumeration of CD4 cell count. K3EDTA whole blood samples from 15 healthy volunteers were taken for determining CD4 cell count by flowcytometry, manual light and fluorescence microscope methods. The results of the manual light and fluorescence microscope methods showed a significant correlation to the flowcytometry method, r = 0.949 (p = 0.000) and r = 0.959 (p = 0.000) respectively. In addition, the manual light microscope method demonstrated a significant correlation to the manual fluorescence microscope method, r = 0.927 (p = 0.000). The comparison between the flowcytometry method, the manual light and fluorescence microscope methods was not significantly different, respectively p = 0.175 and 0.055. Furthermore, there was no significant difference between the manual light microscope and the fluorescence microscope, p = 0.768. In using the manual light and fluorescence microscope methods, the CD4 cell count were enumerated by three different laboratory technicians. The results showed a significant correlation between the manual light microscope and flowcytometry methods, r = 0.943 (p = 0.000), r = 0.943 (p = 0.000) and r = 0.935 (p = 0.000), respectively. Moreover, compared to the flowcytometry method the results were not significantly different, p = 0.167, 0.121 and 0.412, respectively. The manual fluorescence microscope method showed a significant correlation to the flowcytometry method, r = 0.961 (p = 0.000), r = 0.947 (p = 0.000) and 0.932 (p = 0.000), respectively. The comparison to the flowcytometry method showed that the results of the first and second technician were not significantly different p = 0.642 and 0.052, respectively, however, the result of the third technician was significantly different (p = 0.018). Based on the results of the data analysis obtained in this study, it can be concluded that the manual methods can be used to replace the flowcytometry method for the determination of CD4 cell count.

***Keywords**: flowcytometry, manual light, fluorescence microscopes for CD4 cell count*

INTRODUCTION

The problem of HIV/AIDS (Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) in Indonesia is becoming more serious. Recently, the dispersion of HIV epidemics, particularly among intravenous drug abusers, is on the increase. The increase of HIV/AIDS infection cases necessitates a serious and integrated management, since the infection may result in functional damage as well as progressive reduction of CD4. The reduction of CD4 is related to the increasing risk of opportunistic infection and malignancy (Mawle 1994, Horsburgh 1994, Klatt 2001, Center for Disease Control 1997).

CD4 cell count is an important parameter in monitoring and determining the prognosis and progress of antiretovirus treatment. The reduction of CD4 coincides with the occurrence of opportunistic infection. As an example, preventive treatment for *Pneumocystic carinii* is commenced when the CD4 cell count is below 200/ul (Fahey 1990, Horsburgh 1994, Bass 1997, Center of Disease Control 1997, Klatt 2001).

Current CD4 enumeration is as suggested by WHO (World Health Organization). This assessment uses flowcytometry method, which is highly expensive, particularly for people in developing countries, like Indonesia. The method requires sophisticated equipment, necessitating investment, treatment and reagents comprising various expensive monoclonal antibodies. The manual method using fluorescent staining requires fluorescent microscope that needs very costly maintenance (Nicholson 1994, Lydia 1997).

It is unfortunate that there is no manual method in Indonesia using a light microscope. Therefore, the authors tried to modify fluorescent staining method by using acridine orange with Turk solution, which is commonly and easily found in rural areas. This solution is used to enumerate leukocytes using common light microscope.

The success of monoclonal antibody production at the end of 1970s has facilitated the identification of lymphocytes with Immunophenotyping using flowcytometry to determine appropriately the cellular number and types (Nicholson and Landay 1994). Recently, a number of CD4 cell count methods have been developed, for example, by immunofluoresence, Enzyme immunoassay (EIA), cytospheres assay, and magneted beads. However, the one used for enumeration as a standard method and reference by WHO (World Health Organization) as well as CDC is flowcytometry (Isabelle et al 1992, Nicholson et al 1994, Carriere et al 1994, Carella et al 1995, Lyamuya et al 1996, CDC 1997, O,Gorman 1998).

Recently, the use of dynabeads, coated with monoclonal antibody to separate various blood cells resulting in a higher specificity and cell quality has been promoted (Lyamuya et al 1996). Dynabeads CD4, which are

**Department of Clinical Pathology*
***Department of Internal Medicine*
Airlangga University School of Medicine,
Dr Soetomo Teaching Hospital, Surabaya

uniform, present as polystyrene beads supersupramagnetic coated with primary monoclonal antibodies specific against membrane CD4 antigens, which is predominantly expressed by human subset T helper/inducer cell. Mouse IgG1 monoclonal antibody is attached to dynabeads using a secondary antibody. Mouse IgG1 monoclonal antibody may recognize 59 kDa glycoprotein of cell membrane. Cells that have been successfully isolated from blood with these beads reached more than 99% with a viability > 95% (Dynalbiotech).

The objective of this study was to obtain a simple, fast, accurate, and cost-saving CD4 cell count method that can be used by clinicians in determining the prognosis and therapy for the patients. The advantages of this study were to obtain an acceptable, appropriate, simple, and cost-saving method that can be employed in remote areas with simple laboratory facilities useful for clinicians to commence secondary prevention and provide definitive treatment as well as to monitor the outcome of therapy.

MATERIALS AND METHODS

This study used selective cross-sectional observational comparative method, carried out in normal adults at the Department of Clinical Pathology, Airlangga University School of Medicine, Dr Soetomo Hospital, Surabaya. Subjects were included if they were male or female healthy individuals with a white cell count of 4.3 - 11.3 x 109/L, more than 14 years old, not receiving therapy with Zidovudine and immunosuppressant, not smoking, and not having strenuous exercise. Subjects were excluded if they were unhealthy, less than 14 years old, pregnant, and in a immunocompromised condition.

Sample materials

Samples used in this study consisted of venous blood anticoagulated with K3 EDTA. Six ml of blood was taken and divided into 2 vacutainers containing liquid K3 EDTA. The first tube was used for manual CD4 cell count using fluorescent microscope examined at the Tropical Disease Center, Airlangga University, and light microscope at the Department of Clinical Pathology, Airlangga University School of Medicine, Dr Soetomo Hospital, Surabaya. The second tube was sent to Dharmais Hospital, Jakarta, for CD4 cell count examination using flowcytometry method as the reference standard.

Each blood sample was labeled for name, date, and the time when the sample was taken. Blood samples should be examined not more than 30 hours after being obtained. Samples should be prevented from hemolysis or should not be frozen in refrigerator, and if blood clotting was found, sample should not be used. Samples should be immediately delivered at 18 - 22°C to prevent cell damage, and should be kept inside a heat-resistant container, put inside another container containing ice and other absorbant materials.

CD4 cell count with light microscope

Blood EDTA was mixed by turning the tube up and down for 2 minutes at room temperature. Fill the new tube with 225 µL washing/dilution buffer, 250 µL blood and 25 µL Dynabeads M450 CD14 diluted 1/1 with washing/dilution buffer. The tube was stoppered and mixed by using a Dynal Sample Mixer for 10 minutes at room temperature, and put on a magnetic MPC-S for 2 minutes. The supernatant of 200 µL was removed into the new tube and 200 µL washing/dilution buffer and 25 µL Dynabeads M450 CD4, and mixed by incubating for 10 minutes at room temperature in Dynal sample mixer, and put on magnetic MPC-S for 2 minutes, and then the supernatant was removed. Isolated cells were washed with with 500 µL washing/dilution buffer and put on MPC-S, turned up and down, kept untreated for 2 minutes, and after removal of supernatant, washing was repeated once more. Resuspension was carried out with 50 µL lysing solution, vortexed, and kept for 5 minutes at room temperature. Finally, 50 µL Turk solution was added. The number of blue-stained nuclei was enumerated using Improved Neubauer counting chamber using light microscope with a magnification of 400 X. Each sample was enumerated up to 3 times by a different person and the mean of these three enumerations was used. As a result, the cell count/µL was N x 2.5.

CD4 cell count with fluorescent microscope

The procedure was similar to that of the examination using a fluorescent microscope. However, at the final stage, Turk solution is replaced with acridine orange. Orange fluorescent nuclei were counted using an Improved Neubauer counting chamber using a light microscope with a magnification 400 x. Each sample was enumerated up to 3 times by different persons and the mean of these three enumerations was used. As a result, the cell count/µL was N x 2.5.

CD4 cell count with Flowcytometry (Facscan® Becton Dickinson)

Samples were delivered to the Dharmais Cancer Hospital, Jakarta, and examined less than 24 hours using a Flowcytometry Facscan.

RESULTS

Between June and July 2002 15 samples that met the inclusion and exclusion criteria were successfully examined. Those samples have been subjected to the methods as mentioned above. Results revealed 15 subjects, 53% males and 47% females, with 22 - 44 years old (mean 30.80 years and SD = 7.33). Leucocyte count was ranging between 4900/µL and 10.000/µL, with mean 6933.33/µL and SD = 1428.12/µL.

Results of CD4 enumeration

Table 1. Results of CD4 cell count enumeration using manual method with light microscope, fluorescent microscope, and flowcytometry

No of samples	CD4 count (per µL)		
	Light microscopoe	Fluorescent microscope	Flowcytometry
1	835	850	734
2	1055	1001	971
3	720	885	837
4	490	505	449
5	835	855	794
6	642	640	633
7	785	740	762
8	1007	987	978
9	885	835	851
10	827	782	806
11	570	525	549
12	655	740	665
13	715	737	721
14	702	697	708
15	640	652	636
Mean	757.53	762.07	739.60
SD	154.75	146.39	143.92
CV (%)	20.42	19.20	19.45
SE of Mean	39.96	37.80	37.16

Notes:
SD : Standard Deviation
SE of Mean : Standard Error of Mean
CV : Coefficient of Variation

Results of CD4 cell count using manual method with light microscope revealed values ranging from 490/µL to 1055/µL, mean 757.53/µL, SD = 154.75/µL, using manual method with fluorescent microscope, the values ranged between 505 /µL and 1001/µL, mean 762.07/µL and SD = 146.39/µL, and using flowcytometry, the range was 449/µL- 978/µL, mean 739.60/µL and SD = 143.92/µL.

Table 2. Results of CD4 cell count using manual method with light microscope by three persons.

Sample no.	CD4 count (per µL)			
	Light I	Light II	Light III	Mean
1	814	834	858	835
2	1040	1087	1038	1055
3	702	743	715	720
4	505	489	477	490
5	855	885	764	835
6	650	647	630	642
7	787	762	805	785
8	1019	988	1015	1007
9	899	905	851	885
10	837	815	830	827
11	565	585	560	570
12	659	667	639	655
13	704	700	741	715
14	720	712	675	702
15	629	616	675	640
Mean	759.00	762.33	751.53	757.53
SD	154.46	159.55	154.58	154.75
CV (%)	20.35	20.92	20.56	20.42
SE of Mean	39.88	41.20	39.91	39.96

Results of CD4 cell count using manual method with light microscope showed that the reading from observer I (light I) ranged between 505/µL and 1040/µL, mean 759.00/µL and SD = 154.46/µL, observer II (light II) ranged between 489 /µL and 1087/µL, mean 762.33/µL and SD = 159.55/µL, and observer III (light III) ranged between 477/µL and 1038/µL, mean 751.53/µL and SD = 154.58/µL.

Table 3. Results of CD4 enumeration using manual method with fluorescent microscope by three persons

Sample no.	CD4 count (per µL)			
	Fluorescent I	Fluorescent II	Fluorescent III	Mean
1	805	895	850	850
2	1059	985	960	1001
3	915	850	894	885
4	480	523	512	505
5	810	850	905	855
6	600	640	680	640
7	724	754	742	740
8	1020	990	952	987
9	812	850	842	835
10	755	791	801	782
11	487	520	567	525
12	704	711	805	740
13	725	752	735	737
14	670	697	725	697
15	620	672	665	652
Mean	745.73	765.33	775.67	762.07
SD	168.42	143.67	132.84	146.39
CV (%)	22.68	18.77	17.12	19.20
SE of Mean	43.49	37.09	34.30	37.80

Results of CD4 cell count using manual method with fluorescent microscope showed that the reading from observer I (light I) ranged between 480/µL and 1059/µL, mean 745.73/µL and SD = 168.42/µL, observer II (light II) ranged between 520 /µL and 990/µL, mean 765.33/µL and SD = 146.67/µL and observer III (light III) ranged between 512/µL and 960/µL, mean 775.67/µL and SD = 132.84/µL.

Statistical analysis

Results were subjected to descriptive analysis, Pearson correlation test, to find the presence of difference among the results of the three methods paired T test was used. The significance level in this study was determined as 0.05 (5%), so that the correlation is regarded as significant if $p < 0.05$, and not significant if $p = 0.05$. Results of correlation of CD4 cell count between flowcytometry and light microscope can be seen in Figure 1, while Figure 2 describes the correlation of CD4 cell count between flowcytometry and fluorescent microscope.

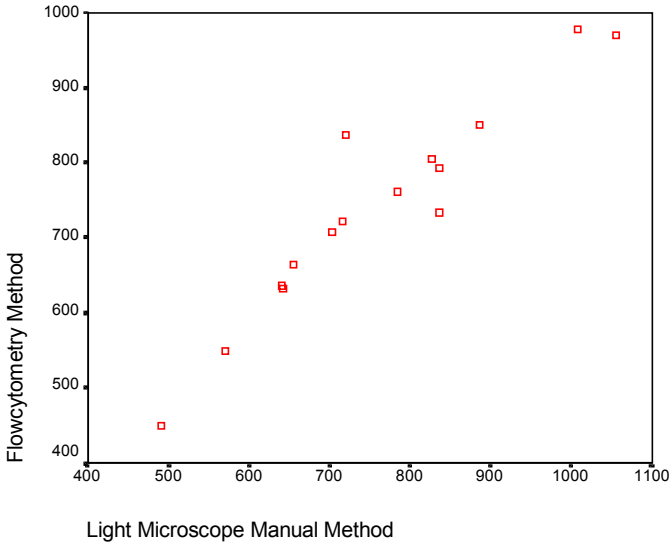

Figure 1. Scatterplot of the correlation of CD4 cell count between flowcytometry method and manual light microscope with r = 0.949 ($p < 0.05$).

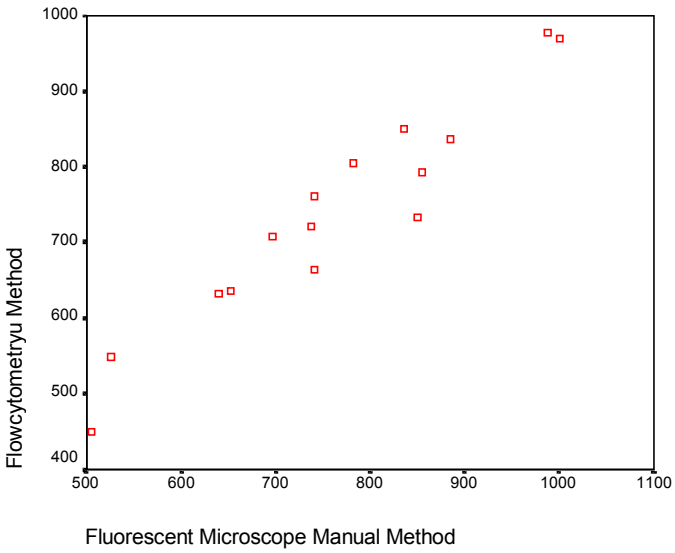

Figure 2. Scatterplot of the correlation of CD4 cell count between flowcytometry method and manual fluorescent microscope with r = 0.959 ($p < 0.05$).

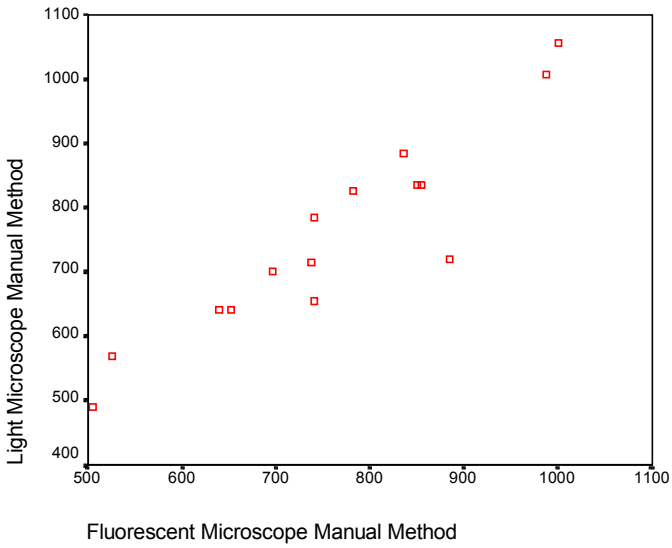

Figure 3. Scatterplot of the correlation of CD4 cell count between manual method using light and fluorescent microscopes with r = 0.927 ($p < 0.05$).

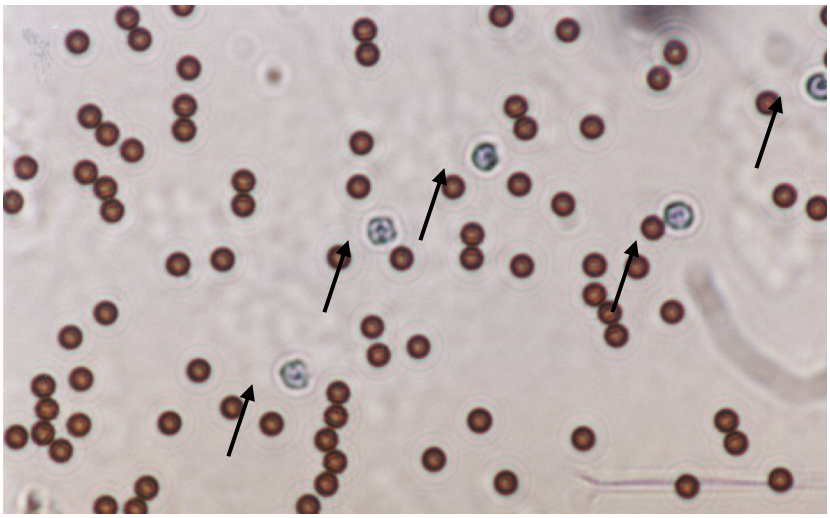

Figure 4. CD4 nuclei (arrows) observed with light microscope (400 x)

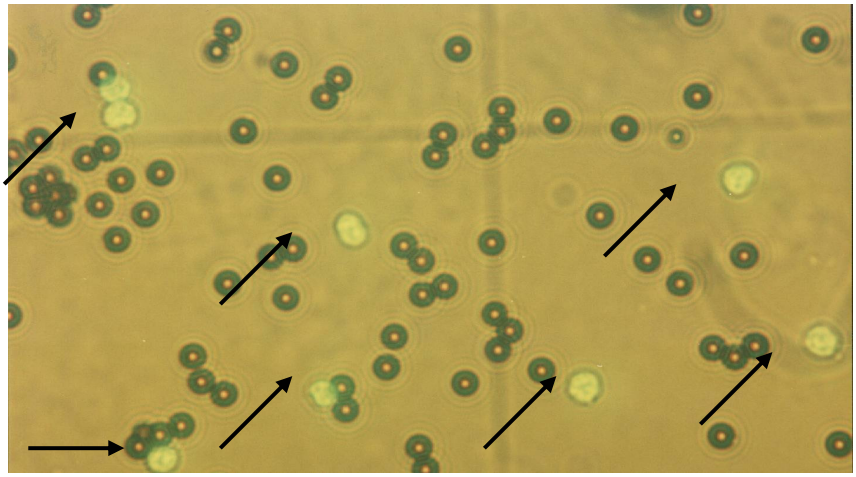

Figure 5. CD4 nuclei (arrow) observed with fluorescent microscope (400 x)

Results of correlation of CD4 cell count between manual methods with light and fluorescent microscope can be seen in Figure 3. CD4 nucleus can be observed with light (Figure 4) and fluorescent microscope (Figure 5).

DISCUSSION

CD4 cell count in this study was performed by three method, namely, flowcytometry method and two other manual methods using light and fluorescent microscope. The manual method using light microscope is a new method. It is simple and cost-saving, so that it can be carried out by simple small laboratory. The method using fluorescent microscope, despite its simpleness, requires expensive equipment and maintenance costs. Both methods need skilled technicians, either in the operation or enumeration. Observed samples are also limited in number, as it requires a longer time, approximately 40 - 50 minutes for one examination with maximally 6 samples. Similarly, although being the referred method, not all laboratories have a flowcytometer as it requires high investment and maintenance cost, as well as highly-skilled technicians. Nevertheless, due to its automated process, this equipment can examine a larger number of samples.

Another manual method is the use of dynabeads binding monoclonal antibodies against CD14 and CD4 molecules. To obtain CD4, during the examination monocytes should be separated by using CD14 beads to bind monocytes, thereby, this method produces absolute CD4 results. Lysed samples should be immediately enumerated. Otherwise, the nuclei will aggregate, rendering uneven distribution of the cells and difficulties in the enumeration due to the overlapping cells. It is therefore recommended that samples be quickly enumerated in less than one hour after being lysed and stained.

This study revealed that in general, both manual method using light microscope and fluorescent microscopes, showed a significant correlation with flowcytometry method, r = 0.949 and p = 0.000 for light microscope and r = 0.959 and p = 0.000 for fluorescent microscope. Significant correlation of r = 0.927 and p = 0.000 was also found between manual methods with light microscope and fluorescent microscope. The results of this study confirmed those of Lyamuya et al (1996), which showed a remarkable correlation between the manual method using light microscope and flowcytometry (r = 0.939), and those of Thorstensson et al (1993) who proved a remarkable correlation between flowcytometry method and methods using fluorescent (r = 0.84) and light (r = 0.90) microscopes.

Comparison between CD4 cell count using flowcytometry method and manual method using light and fluorescent microscope showed no significant difference, mean 757.53/µL and SD = 154.75/µL (p = 0.175) for manual method with light microscope, and mean 762.07/µL and SD = 146.39/µL (p = 0.055) for fluorescent microscope. Similarly, no significant difference was found between light and fluorescent microscope with p = 0.768. CD4 cell count using manual method with light microscope by three observers revealed the following results: the first observer found a mean of 759.00/µL and SD = 154.46/µL with r = 0.943 (p = 0.000), the second observer, mean 762.33/µL and SD = 159,55/µL with r = 0.943 (p = 0.000) and the third observer, mean 757.53/µL and SD = 154.75/µL with r = 0.935 (p = 0.000). Compared to the results of flowcytometry, these results showed no significant difference, p = 0.167, 0.121 and 0.412. It can be concluded, therefore, that the

results of the enumeration carried out by the three observers using light microscope were similar to those using flowcytometry.

CD4 cell count using manual method with fluorescent microscope by three observers revealed the following results: the first observer found a mean of 745.73/μL and SD = 168.42/μL with r = 0.961 (p = 0.000), the second observer, mean 765.33/μL and SD = 143.67/μL with r = 0.947 (p = 0.000) and the third observer, mean 775.53/μL and SD = 132.84/μL with r = 0.932 (p = 0.000). Compared to the results of flowcytometry, the results from the first and second observer showed no significant difference, with p = 0.642 and 0.052, while those of the third observer showed a significant difference, with p = 0.018. This might be due to less careful enumeration, uneven distribution of the cells, or too early enumeration before all cells precipitated on the base of counting chambers. The results of enumeration using manual method either with light or fluorescent microscope showed a higher mean, which was likely due to the manual characteristic of the enumeration itself, while flowcytometry used automatic instruments and fragments of cellular nuclei were also enumerated as one single cell. Manual method may become more difficult to perform when the number of cells to be counted is higher, for example, more than 1000/μL, leading to uneven cell distribution. To overcome this problem, sample should be diluted earlier during sample processing.

CONCLUSION

Manual methods for CD4 cell count can be used routinely. The results of these methods had a significant correlation with flowcytometry, correlation with light microscope showed r = 0.949 ($p < 0.05$), and r = 0.959 ($p < 0.05$) for the correlation with fluorescent microscope. Similarly, significant correlation with r = 0.927 ($p < 0.05$) was found between manual method using light microscope and fluorescent microscope. Results of statistical analysis using paired T test also showed no significant difference between flowcytometry method and manual light microscope, $p > 0.05$ (0.175) and manual fluorescent microscope $p > 0.05$ (0.055). In conclusion, manual methods using light and fluorescent microscope can be used to enumerate CD4.

RECOMMENDATION

This study was carried out in normal adults. Therefore, further studies in adult patients with positive HIV or AIDS, not done in this study due to limited fund and time, should be undertaken. Further studies regarding the reproducibility of these manual methods should also be carried out.

REFERENCES

Bass HZ, Fahey JL, Nishanian P et al (1997). Relation Impaired Lymphocyte Proliferative Function to Other Major Human Immunodeficiency Virus Type 1 - Induced Immunological Changes: Clinical and Diagnostic Laboratory Immunology: 4:1: 64 - 69

Bird AG (1995). Quantification of CD4-positive T Lymphocytes. in Karn (editor) HIV, Virology and Immunology A Practical Approach, Volume 1: Oxford University Press. New York. 211 - 219

Carella AV, Moss MW, Provost V and Quinn TC (1995). A Manual Bead Assay for The Determination of Absolute CD4+ and CD8+ Lymphocyte Counts in Human Immunodeficiency Virus - Infected Individuals. Clinical and Diagnostic Laboratory Immunology:2 :5: 623 - 625

Carriere D, Fontaine C, Berthier AM et al (1994) Two Site Enzyme Immunoassays of CD4 and CD8 Molecules on the Surface of T Lymphocytes from Healthy Subjects and HIV-1-infected Patients: Clinical Chemistry : 40: 1: 30 37

Center for Disease Control (1997) Revised Guidelines for Performing CD4+ T-Cells Determinations in Persons Infected with Human Immunodeficiency Virus (HIV): MMWR: 46: RR-2

Dynalbiotech. Dynabeads T4 - T8 System. A complete system for the Counting of T4 and T8 Lymfocytes from Whloe blood. Dynal Biotech ASA.Oslo

Fahey JL, Taylor JMG, Detels R et al (1990). The Prognostic Value Cellular and Serologic Markers in Infection with Human Immunodeficiency Virus Type 1: The New England Journal Medicine: 322: 3: 166 - 172

Horsburgh Jr, CR (1994). Prognostic Indicators For Progression of HIV Disease. in Schochetman (eds) AIDS Testing, A Comprehensive Guide to Technical, Medical, Social, Legal and Management Issues. Second Edition: Springer - Verlag. New York. 196 - 205

Isabelle BF, Philipe P and Jose S (1992) Flow Cytometry as Reference Method to Validate A New CD4 / CD8 Lymphocyte Counting Assay Based on Immuno-magnetic Separation (IMS). Biology Cell:76:2: 244 - 250

Klatt, EC (2001). Human Immunodeficiency Virus. In Pathology of AIDS version 10: Department of Patology University of Utah. Utah. 5 - 29

Kresno SB (2001). Unsur - unsur yang Berperan Dalam Reaksi Imunologik. dalam Imunology: Diagnosis dan Prosedur Laboratorium edisi keempat: Fakultas Kedokteran Universitas Indonesia. Jakarta 14 - 101

Lyamuya EF, Kagoma C, Mbena EC et al (1996). Evaluation of the FACScount, TRAx CD4 and

Dynabeads methods for CD4 Lymphocyte Determination. Journal of Immunological Methods: 195: 103 - 112

Lydia A (1997). Hubungan Hitung Limfosit total dengan Hitung CD4+ pada penderita AIDS. Majalah Kedokteran Indonesia: 47: 2: 65 - 70

Mawle AC and McDougal JS(1994). Immunology of HIV Infection. in Schochetman (eds) AIDS Testing, A Comprehensive Guide to Technical, Medical, Social, Legal and Management Issues. Second Edition: Springer - Verlag. New York. 32 - 51

Nicholson JKA and Landay AL (1994). Use of Flow Cytometry to Enumerate Lymphocyte Populations in HIV Disease. in Schochetman (eds) AIDS Testing, A Comprehensive Guide to Technical, Medical, Social, Legal and Management Issues. Second Edition: Springer - Verlag. New York.171 - 195

Nicholson JKA, Velleca WM, Jubert S, Green TA and Bryan L (1994) Evaluation of Alternative CD4 Technologies for the Enumeration of CD8 Lymphocytes: Journal of Immunological Methods. 177: 43 - 54.

O'Gorman M (1998). Evaluation of Single Platform Technologies for Absolute CD4+ and CD8+ Cells. Presented in 1998 Conference on the Laboratory Science of HIV. Chicago

Romeu MA, Mestre M, Gonzales L, Valls A, Verdaguer J, Corominas M et al (1992).Lymphocyte Immunophenotyping by Flowcytometry in Normal Adults: Comparison of Whole Blood Lysis Technique, Ficoll-Paque separation and Cryopreservation. Journal of Immunological Methods 154: 7 - 10.

Stein DS, Korvick JA and Vermund SH (1992). CD4+ Lymphocyte Cell Enumeration for prediction of Clinical Course of Human Immunodeficiency Virus Disease: A Review. The Journal Of Infectious Diseases: 165: 352 - 363

Streicher HZ, Reitz Jr MS, and Gallo RC (2000). Human Immunodeficiency Virus. In Mandell (eds) Principles and Practice of Infectious Diseases, 5th Edition: Churchill Livingstone Inc. Edinburgh. 1874 - 1885

Tamul KR, O'Gorman MRG, Donovan M, Schmitz JL and Folds JD (1994). Comparison of a Lysed Whole Blood Method to Purified Cell Preparations for Lymphocyte Immunophenotyping: Differences Between Healthy Controls and HIV-positive Specimens. Journal of Immunological Methods: 167: 237 - 243.

Thorstensson R, Vetterly L, Rudolfson FR, Gaiens H and Biberfeld G (1993). Evaluation of Alternative methods for determinationof Absolute CD4+ and CD8+ Lymphocyte Counts. Presented in The African AIDS Congress. Marakesh 1993.

Vergis EN and Mellors JW (2000). Natural History of HIV-1 Infection: Infectious Disease Clinics of North America: 14: 4