30 min. As a control, 1%BSA-PBS-Azide was added instead of antibody. Stained cells were then washed twice with 1%BSA-PBS-Azide and resuspended with 50 µl of the same solution. Ten microlitres of sheep anti-mouse IgG coated beads (Dynal, Oslo, Norway) were added. The beads were washed once and resuspended in 1%BSA-PBS-Azide with 10%AB human serum prior to use. The blood/bead mixture was gently rotated on a platform shaker at room temperature for 1 h. After incubation, the mixture was gently resuspended and 10 µl of it was added to 10 µl of Turk's solution (2% acetic acid and 0.01% gentain violet). This was then loaded into the chamber of a hemacytometer for counting. Cells that had formed rosettes with three or more beads were counted as CD4 positive cells (Fig. 1). The percentage of CD4 positive cells was obtained by counting at least 200 white blood cells. The absolute CD4 positive cell counts were then calculated from the total number of white blood cells and the obtained percentage of CD4 rosetting cells.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. The PBMC were washed twice with 1% BSA-PBS-Azide and adjusted to a final concentration of 1 $x10^7$ cells/ml with the same solution.

Preparation of granulocytes

Granulocytes were isolated from the pellet of the Ficoll-Hypaque density gradient isolation of PBMC by the dextran sedimentation method. Cells were then washed twice and the contaminated red blood cells were removed by using hypotonic lysis with ammonium chloride buffered. To block non-specific Fc receptor-mediated binding of antibody, human AB serum was added to a final concentration of 10%.

Preparation of E^+ and E^- populations

To isolate E^+ and E^- populations, 2-aminoethylisothiouronium-hydrobromide treated sheep red blood cells (AET-SRBC)[14] were added to the PBMC, incubated at 37°C for 10 min, and then centrifuged at 600g for 2 min. Cells were kept at 4°C overnight. The rosette forming cells in the pellet were resuspended and isolated

further by Ficoll-Hypaque gradient centrifugation. E^+ and E^- fractions were collected and the sheep red blood cells were then lysed with ammonium chloride buffered. E^+ and E^- cells were then resuspended in 1%BSA-PBS-Azide with 10% human AB serum.

Statistical analysis

The CD4 positive cell counts obtained from flow cytometry and the manual rosetting method were compared by using the student paired t-test. The correlation coefficient (r) of both methods was established for all samples by linear regression analysis. Sensitivity was defined as the number of individuals with CD4 positive cell counts < 200 cells/μl by both methods divided by the number of individuals with CD4 cell counts < 200 cells/μl by flow cytometry. Specificity was defined as the number of individuals with CD4 positive cell counts > 200 cells/μl by both methods divided by the number of individuals with CD4 cell counts > 200 cells/μl by flow cytometry.

RESULTS

Comparison of the manual rosetting method and flow cytometric method

The manual rosetting method was developed for the enumeration of CD4 positive cells in countries where funds for flow cytometry are limited. Blood specimens from 24 HIV-seropositive and 16 HIV-seronegative donors were enumerated for CD4 positive cells by standard flow cytometry (Simulset) and the manual rosetting method. In the case of flow cytometry, the percentage of CD4 positive cells in white blood cells was calculated from the percentage of CD4 positive cells in the lymphocyte population (obtained from flow cytometric analysis), and the white blood cell count and leukocyte differential (obtained using standard laboratory procedures). A correlation plot comparing the percentage of CD4 positive cells in white blood cells obtained from both methods is shown in figure 2. Linear regression analysis resulted in a slope of 0.76 and an intercept of 1.67 % when data from the two methods were compared. The correlation coefficient (r) of this regression analysis was 0.93. The mean value of the percentage of CD4 positive cells for samples tested by standard flow cytometry and the manual rosetting method were 6.31 % and 6.48%, repectively. No significant difference in these values was seen ($p > 0.05$).

The absolute number of CD4 positive cells were then calculated from the percentage of CD4 positive cells and the number of white blood cells per µl. A correlation plot which compares between the absolute CD4 positive cell count obtained from the reference flow cytometric method and the manual rosetting method is shown in figure 3. The mean value for samples tested by standard flow cytometry and the manual rosetting method were 515.75 and 529.04 cells/µl, respectively. Linear regression analysis of both methods resulted in a slope of 0.80 and an intercept of 114.53 cell/µl. The correlation coefficient for this regression analysis was 0.95. Again, no significant differences or trends were seen in these values ($p>0.05$).

Since the CD4 positive cell count of less than 200 cells/µl was used as the critiria for AIDS in the case definition [3], the data obtained from both methods were also evaluated, based on the CD4 positive counts of more or less than 200 cells/µl. This analysis demonstrated that the manual rosetting method was 89% sensitivity and 96% specificity (Table 1).

Effect of granulocytes and monocytes on the manual rosetting method

Potential sources of interference with the rosetting method could be due to cell types other than CD4 positive T lymphocytes. To address this question, granulocytes, which do not express CD4 protein [15], were isolated from 4 normal donors and used as subjects for the manual rosetting method. By immunofluorescent analysis, the isolated granulocyte populations contained 0.6-5 % CD3 positive T cells, 0.3-1.4 % CD4 positive cells and 0-0.4% CD14 positive monocytes (Table II). Very few rosette forming cells, range 0.3-0.7 %, were observed in these granulocyte populations (Table II). These results indicated that granulocytes did not interfere with the manual rosetting method.

The interference of monocytes was also investigated. PBMC were separated into E^+ and E^- populations by sheep red blood cell rosetting [14]. The E^- population isolated from 4 normal donors consisted of more than 57% CD14 positive monocytes (range, 57-78%) and less than 3% of CD3 positive lymphocytes (range, 0.6-3%) (Table III). The rosette forming cells obtained from the manual rosetting method were barely detectable in these E^- populations (range, 1-4%). The results indicated that monocytes were not detected by the manual rosetting method. In contrast, in the E^+ population which contained a majority of CD3 positive cells and CD4 positive lymphocytes (Table IV), a high number of rosette forming cells were observed (Table IV).

DISCUSSION

The absolute number of CD4 positive cells is an important marker for the prognosis and classification of the state of the disease, and monitoring for the therapy of HIV infection[1-7]. The accepted standard method for the enumeration of CD4 positive cells is flow cytometry. However, problems facing the clinical laboratory are that flow cytometry generally does not lend itself to routine high-volume testing, and requires expensive equipment and reagents. In addition, specially trained personnel are required to operate flow cytometry instrumentation. This technology, is therefore, costly for adaptation as a routine method in laboratories in developing countries. Alternative methodologies for CD4 positive cell determination that should be simple, inexpensive and reliable are urgently needed for use in laboratories, especially in developing countries. To support this requirement, we introduce a non-flow cytometric method called the manual rosetting method for determination of CD4 positive cells.

The principle of the manual rosetting method is that lysed whole blood samples are pre-stained with CD4 mAb and the stained cells are then incubated with anti-mouse immunoglobulin coated beads. The CD4 rosette forming cells are then enumerated by a light microscope. The obtained percentage of CD4 positive cells are then calculated to the absolute number by multiplying with the white blood cell count. The manual rosetting method described here is simple, needs no any special instruments and is a cost-effective method for quantifying CD4 positive cells. A high degree of correlation between standard flow cytometry and the manual rosetting method has been found for both percentage and absolute CD4 positive cells (correlation coefficient, 0.93 and 0.95, recpectively). Furthermore, the manual rosetting method has high sensitivity and specificity for identifying individuals with less than 200 cells/μl of CD4 positive cells.

It is known that peripheral blood monocytes express CD4 molecules on their surface[16] and that these cells can interfere with and affect the accuracy of the flow cytometric measurement of CD4 positive lymphocytes[8,17]. Therefore, the standard flow cytometric method generally employs a T-cell specific antibody, CD3 antibody, to discriminate between target cells and contaminated cells[18,19]. In this study,

experiments were performed to confirm that the manual rosetting method was able to discriminate between the CD4 positive lymphocytes and monocytes. Four normal blood samples were separated into E^+ and E^- populations using a conventional AET-sheep red blood cell rosetting technique[14]. The E^- population, which contained 60-80% CD14 positive monocytes and less than 3% CD3 positive T cells was used to examine the issue of potential interference from monocytes. It was found that in the E^- population, by using the manual rosetting method, a very low number of rosette formimg cells (range 0.6-3%) were determined. In contrast, in the E^+ population, which contained more than 50% CD3 positive T cells, a high number of rosette formimg cells (range 44-60%) were determined. The resultant near-zero rosette formation in the E^- population indicated that there was no interfering effect from monocytes in the enumeration of CD4 positive cells by the manual rosetting method. This may be due to the low density CD4 expression on the monocyte surface[16]. Monocytes, therefore, do not form rosettes with anti-mouse immunoglobulin coated beads. Granulocytes were also tested for interference and their affect on the accuracy of the measurement of CD4 positive cells. As predicted, by the manual rosetting method, approximately 1% (range 0.6-1%) of CD4 rosetting cells were detected in these populations. These results indicated that granulocytes do not affect the accuracy of this manual rosetting method.

In summary, a non-flow cytometric method, named the manual rosetting method, was introduced for the enumeration of CD4 positive cells. The manual rosetting method is simple, inexpensive and meets the growing demand for CD4 counts, especially in developing countries where HIV prevalence is high.

ACKNOWLEDGMENT

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Table I. Sensitivity and specificity of the manual rosetting method compared with flow cytometry.

	Manual rosetting *		
	< 200 cells/μl	> 200 cells/μl	Total
Flow cytometry			
< 200 cells/μl	16	2	18
> 200 cells/μl	1	21	22
Total	17	23	40

*89% sensitivity and 96% specificity.

Table II. Interference of granulocytes with the manual rosetting method.

Sample	Immunofluorescence			Manual rosetting
No.	%CD3 pos.	%CD4 pos.	%CD14 pos.	%CD4 rosette
1	0.6*	0.3	0	0.8
2	0.6	0.3	0	0.4
3	4.6	1.4	0.4	1.1
4	1.7	0.8	0.2	0.6

Granulocytes were isolated from 4 healthy donors and were determined for percentages of CD3, CD4 and CD14 positive cells by indirect immunofluorescent technique. An aliquot of cells was enumerated for CD4 positive cells by the manual rosetting method.

* % positive cells.

Table III. Interference of monocytes with the manual rosetting method.

Sample	Immunofluorescence		Manual rosetting
No.	%CD3 pos.	%CD14 pos.	%CD4 rosette
1	1.0*	78	4.0
2	0.6	57	1.0
3	1.6	77	2.8
4	3.0	73	2

The E^- population was isolated from 4 healthy donors by sheep red blood cell rosetting and determined for the percentages of CD3 and CD14 positive cells by indirect immunofluorescent technique. An aliquot of cells was enumerated for CD4 positive cells by the manual rosetting method.

* % positive cells.

Table IV. Enumeration of CD4 positive cells in the E^+ population by the manual rosetting method.

Sample	Immunofluorescence			Manual rosetting
No.	%CD3 pos.	%CD4 pos.	%CD14 pos.	%CD4 rosette
1	95*	58	2	60
2	77	48	0.8	53
3	66	42	1.8	44
4	75	48	3.0	58

The E^+ population was isolated from 4 healthy donors by sheep red blood cell rosetting and determined for percentages of CD3, CD4 and CD14 positive cells by indirect immunofluorescent technique. An aliquot of cells was enumerated for CD4 positive cells by the manual rosetting method.

* % positive cells.

FIGURE LEGENDS:

FIG. 1. Representative picture in a hemacytometer chamber of CD4 positive cell binding beads.

FIG. 2. Correlation plot comparing the % CD4 positive cells measured with the manual rosetting method and the standard flow cytometric method. The linear regression is expressed by the equation $Y=1.67 + 0.76x$ ($r=0.93$).

FIG. 3. Correlation plot comparing absolute CD4 positive cell count measured with the manual rosetting method and the standard flow cytometric method. The linear regression is expressed by the equation $Y=114.53 + 0.80x$ ($r=0.95$).

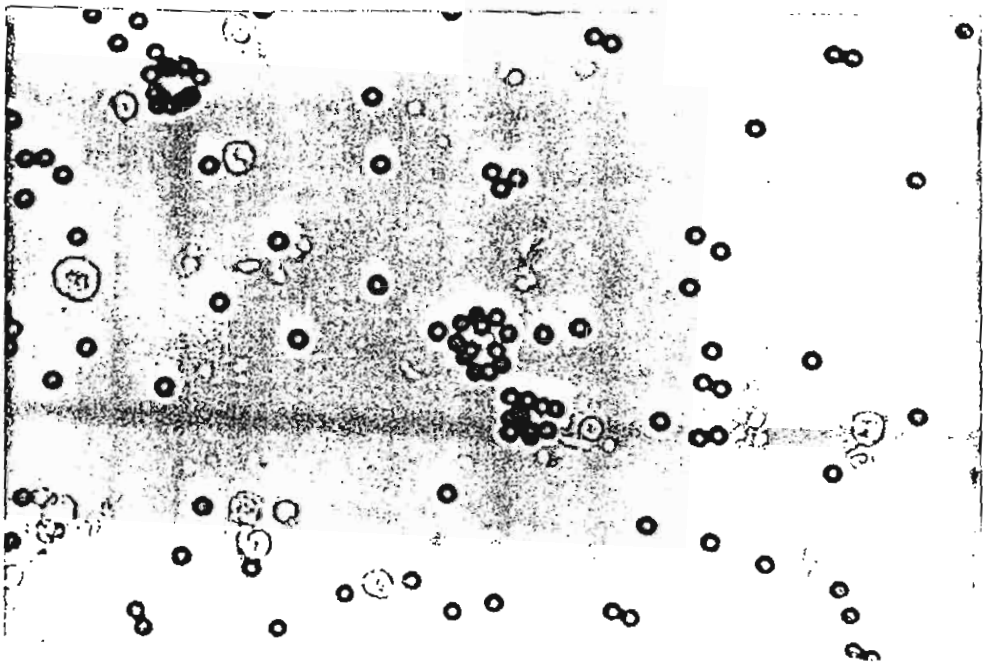

Fig.1

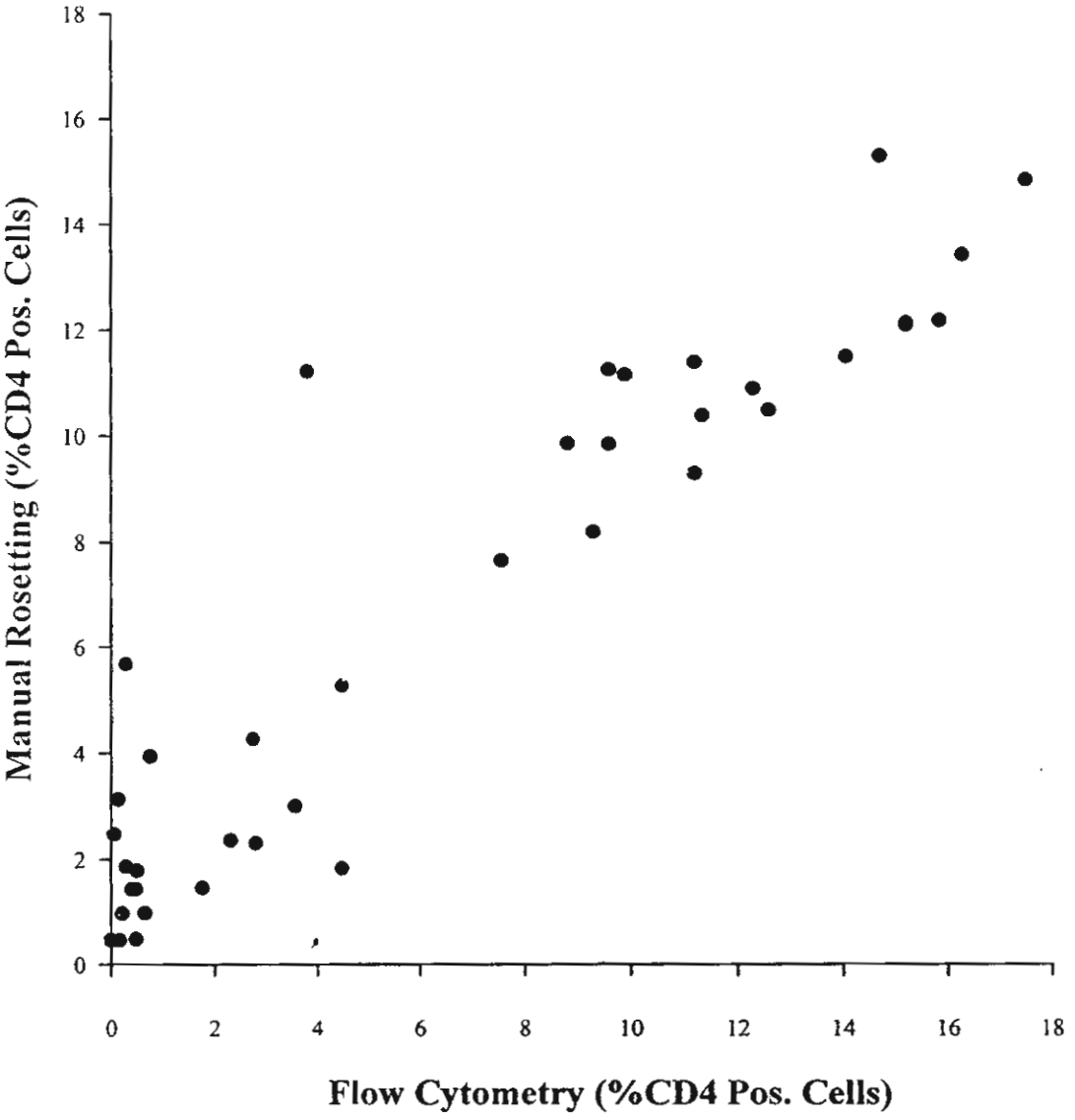
Manual Rosetting (%CD4 Pos. Cells)
Flow Cytometry (%CD4 Pos. Cells)
0
2
4
6
8
10
12
14
16
18

Fig. 2

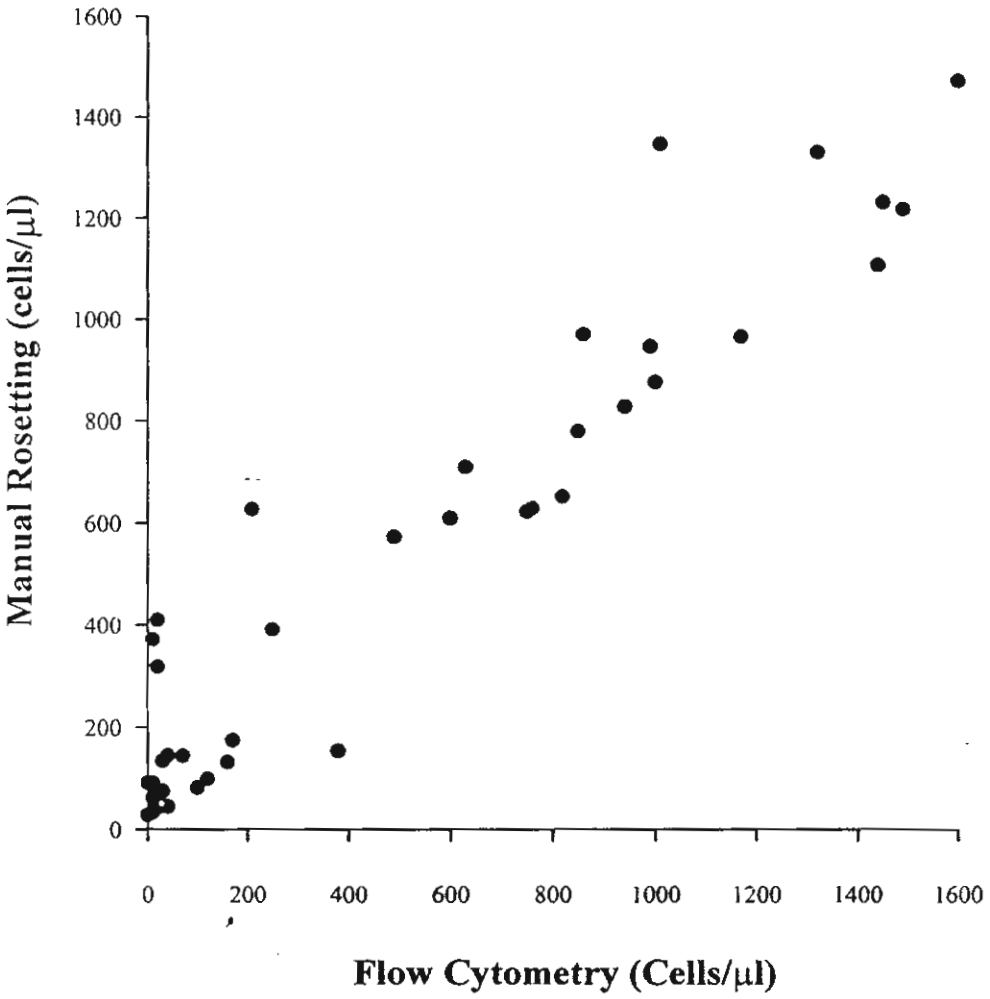
Manual Rosetting (cells/μl)
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
800
1000
1200
1400
1600
Flow Cytometry (Cells/μl)

Fig. 3

Production of CD4 monoclonal antibody and development of home made reagent for CD4+ lymphocyte determination

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Abstract

A hybridoma secreting monoclonal antibody (mAb) specific to CD4 protein was generated. This monoclonal antibody, named MT4, was proved to be specific to CD4 protein as it reacted with CD4-DNA transfected COS cells, CD4+ cell lines and CD4+ lymphocytes. Furthermore, MT4 mAb inhibited the binding of standard CD4 monoclonal antibodies to CD4 proteins on CD4+ cells. To develop a home made reagent for CD4+ lymphocyte determination by flow cytometry, fluorescein isothiocyanate (FITC) was conjugated to MT4 mAb. To evaluate the developed reagent, 30 HIV infected and 30 healthy individuals were determined for CD4+ lymphocytes by using both commercial Simultest™ reagent kit and home made FITC labeled MT4 mAb simultaneously. The study has shown that both percentages and absolute CD4+ lymphocyte counts obtained from both reagents were equivalent. The correlation coefficient for regression analysis was 0.995 and 0.996 for percentages and absolute CD4+ lymphocyte counts, respectively. The results suggest that home made FITC labeled MT4 reagent is an acceptable alternative reagent for monitoring CD4+ lymphocytes in blood samples by flow cytometry.

การผลิตโมโนโคลนอล แอนติบอดี ต่อ CD4 โปรตีน และพัฒนาเป็นน้ำยาเพื่อตรวจวัดระดับ CD4+ Lymphocytes

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ในการศึกษานี้สามารถเตรียมไฮบริโดมาที่ผลิตโมโนโคลนอล แอนติบอดีที่จำเพาะต่อ CD4 โปรตีนได้จำนวน 1 โคลน โมโนโคลนอล แอนติบอดีนี้ได้ให้ชื่อว่า MT4 และได้พิสูจน์ยืนยันความจำเพาะต่อ CD4 โปรตีนแล้วพบว่าแอนติบอดีนี้สามารถทำปฏิกิริยาได้กับ CD4-DNA transfected COS cells, CD4+ cell lines และ CD4+ lymphocytes นอกจากนี้แอนติบอดีนี้ยังสามารถยับยั้งการจับกันของ CD4 โมโนโคลนอล แอนติบอดีมาตรฐานกับ CD4 โปรตีนบนผิวของ CD4+ cells ได้ เพื่อผลิตน้ำยาตรวจนับ CD4+ lymphocytes ขึ้นมาใช้เอง จึงนำสาร fluorescein isothiocyanate (FITC) มาติดฉลากกับ MT4 แล้วนำไปใช้ตรวจนับจำนวน CD4+ lymphocytes โดยวิธี flow cytometry เพื่อประเมินประสิทธิภาพของน้ำยาที่เตรียมขึ้นมา ได้ทำการตรวจนับจำนวน CD4+ lymphocytes ในเลือดผู้ติดเชื้อเอดส์และคนปกติอย่างละ 30 ราย โดยทำการเปรียบเทียบระหว่างน้ำยา-FITC ติดฉลาก MT4 กับชุดน้ำยามาตรฐาน Simultest™ ผลการศึกษาพบว่าเปอร์เซนต์และค่า absolute CD4+ lymphocyte counts ที่ได้จากน้ำยาทั้งสองชนิดไม่แตกต่างกัน โดยมีค่าสัมประสิทธิ์ สหสัมพันธ์ เท่ากับ 0.995 และ 0.996 สำหรับเปอร์เซนต์และค่า absolute CD4+ lymphocyte counts ตามลำดับ ผลการศึกษาในครั้งนี้ชี้ให้เห็นว่า น้ำยา FITC ติดฉลาก MT4 ที่เตรียมขึ้นมาใช้เองนี้น่าจะเป็นน้ำยาอีกชนิดหนึ่งที่สามารถนำมาใช้ตรวจวัดระดับ CD4+ lymphocytes ในเลือดโดยวิธี flow cytometry ได้

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Accurate and reliable measures of CD4+ lymphocytes are essential for the assessment of the immune system of human immunodeficiency virus (HIV)-infected persons[1-3]. The pathogenesis of acquired immunodeficiency syndrome (AIDS) is largely attributable to the decrease in CD4+ lymphocytes[4-8]. Progressive depletion of CD4+ lymphocytes is associated with an increased likelihood of clinical complications [9,10]. Consequently, the Public Health Service (PHS) has recommended that CD4+ lymphocyte levels be monitored every 3-6 months in all HIV-infected persons[11]. The measurement of CD4+ T lymphocyte levels has been used to establish decision point for initiating prophylaxis[12], anti-viral therapy[13] and monitoring the efficacy of treatment[14-16]. It is also used for prognostic indicators in patients who have HIV disease[17,18]. Moreover, CD4+ lymphocyte levels are a criterion for categorizing HIV-related clinical conditions by CDC's classification system for HIV infection and surveillance case definition for AIDS among adults and adolescents[19].

The standard method for CD4+ lymphocyte enumeration involves the use of flow cytometric method[20,21]. This method, CD4+ lymphocytes in whole blood specimens are identified by immunophenotyping and analyzing the results using a flow cytometer. The results obtained in this manner are a percentage of CD4+ lymphocytes. These results must be combined with a hematology determination which provides the total white blood cell count and the percentage of lymphocytes (differen-tial) and calculated to the absolute CD4+ lymphocyte count. The standard fiow cytometric method, however, requires very expensive reagents, i.e., fluorescent dye labeled monoclonal antibodies specific to leukocyte surface molecules. This technique, thus, limits the availability of CD4 + lymphocyte enumeration in developing countries.

In this report, a monoclonal antibody (mAb) specific to CD4 protein was generated. The generated CD4 mAb was, then, developed to be a home made reagent for CD4+ lymphocyte determination by flow cytometer. This home made reagent could be used cheaply to enumerate CD4+ lymphocytes in blood samples as well as commercial products. The clinical utility of the reagent produced is very attractive in developing countries where HIV prevalence is high and funds for flow cytometry and services are limited.

MATERIALS AND METHODS

Production of monoclonal antibody to CD4 protein

Human T cell line, Sup T1, 1 x 10^7 cells were injected intraperitoneally into a BALA/c mouse. A booster immunization was followed a week later with intravenous injection of 1 x 10^6 Sup T1 cells . The animal was sacrificed 3 days after the booster and the spleen was removed. Spleen cells were then fused with myeloma cells X63-Ag8.653 using 50% PEG as previously described[22]. After that, cells were distributed into 672 wells of 96 well-plates. Two weeks later, hybridomas were identified by an inverted microscope . Cell culture supernatants from hydridoma containing wells were screened for antibody against CD4 protein. The positive clone was re-cloned three rounds by limiting dilution. To produce high concentration of CD4 mAb, the cloned hybridomas were injected intraperitoneally into BALC/c mice that were pre-treated with 2, 6, 10, 14-tetramethyl-pentadecan (Pristane). Ascitic fluid containing CD4 mAb was harvested, usually, 10-20 days after hybridoma inoculation.

Screening for CD4 specific monoclonal antibody

Hybridoma cell culture supernatants were firstly analyzed by indirect immunofluorescence using Sup T1 cells as antigens. The positive supernatants were screened further for antibody specific to CD4 protein by the same technique but using cDNA encoding CD4 protein transfected COS cells as antigens. In all experiments, COS cells transfected with cDNA encoding unrelated protein were used as negative control.

DEAE-Dextran transfection of COS cells

To prepare cDNA encoding protein of interests, the cDNAs, which had been constructed into an eukaryotic expression vector πH3M, were transformed into competent *E. coli* MC1061/p3. After that, plasmid DNAs were purified by cesium chloride-ethidium bromide density gradient ultracentrifugation[23]. The resulting DNAs were phenol/chloroform-extracted and ethanol precipitated, then resuspended in TE (10mM Tris, 1mM EDTA) pH 8.0. The Plasmid DNAs were transfected into COS cells using the DEAE-Dextran transfection method[24]. Briefly, 1x10^6 COS cells were

transferred to 6 cm tissue culture dishes (NUNC, Roskilde, Denmark) on the day before transfection. Cells were transfected in 2 ml of MEM containing 250 μg/ml DEAE-Dextran, 400 μM chloroquine diphosphate and 2 μg DNA. After 3 hours at 37°C, the transfection mixture was removed and the cells were treated with 10% DMSO in PBS for 2 min at room temperature. COS cells were then cultured overnight in MEM containing 5% FCS, washed once, and re-cultured with the same medium for another 2 days to allow expression of the encoded proteins.

Indirect immunofluorescence analysis

The specificity of antibody against CD4 protein was assessed by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin antibodies (Immunotech, Coultrer Corporation, Miami, FL). To block the non-specific Fc receptor mediated binding of the antibodies, cells were incubated for 30 minutes at 4°C with 10% human AB serum before staining. Blocked cells were then incubated for 30 minutes at 4°C with culture supernatants or mAb. After washing, cells were incubated with the FITC-conjugate for another 30 minutes. Membrane fluorescence was analyzed by flow cytometer (FACSCalibur, Becton Dickinson, San Jose, Ca).

Determination of isotype of monoclonal antibody

The isotype of mAb was determined by capture ELISA (Sigma, St. Louis, MO) in accordance with the recommended protocol. Goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM were used as capture antibodies, and peroxidase conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) were used as conjugate. The reactivity was visualized by using 3',3',5',5'-tetramethylbenzidine (TMB) as substrate.

Inhibition of standard CD4 monoclonal antibody binding by MT4 monoclonal antibody

CD4+ cells (peripheral blood lymphocytes or human T cell lines) were pre-incubated with MT4 mAb or irrelevant mAb for 30 minutes on ice. Phycoerythrin (PE)-labeled CD4 mAb Leu3a (Becton Dickinson) or FITC-labeled CD3 mAb Leu4 (Becton Dickinson) was then added to the pre-stained cells, and incubated for another

30 minutes. Membrane fluorescence was analyzed by a flow cytometer. The percent inhibition of fluorescence intensity was calculated from the mean fluorescence intensity of the sample in the presence and absence of first un-labeled mAb.

Fluorescent labeling of MT4 monoclonal antibody

MT4 mAb was purified from MT4 hybridoma induced ascitic fluid by using UltraLink™ Immunobilized Mannan Binding Protein column (Priece, Oud-Bejerland, The Netherlands) according to the recommended protocol (Priece). The concentration of purified MT4 was measured by reading the absorbance at 280 nm and adjusted to 2 mg/ml in PBS containing 0.1 M $NaHCO_3$. Fluorescein isothiocyanate (FITC; Sigma) ,dissolved in DMSO at a concentration of 10 mg/ml, 25 µl was slowly added to 1 ml of antibody (2mg/ml). The mixture was rotated at room temperature for 90 min. The free fluorescein dye was, then, removed by ultrafiltration using Centricon concentrator (MW cut-off 10,000; Amicon, Beverly, MA) and equilibrated with PBS. The ratio of fluorescein to protein was estimated by measuring the absorbance at 495 nm and 280 nm. The concentration of the FITC labeled antibody was measured by reading the absorbance at 280 nm.

Enumeration of CD4+ lymphocytes by FITC labeled MT4

One hundred microliters of K_3EDTA-whole blood was incubated at room temperature with 50 µl of FITC labeled MT4 (40 µg/ml). After 30 min room temperature incubation, 2 ml of lysing buffer (Becton Dickinson) was added and let stand at room temperature in the dark for 10 min for lysis of red blood cells. Cells were then washed once with 2 ml of PBS containing 0.1% sodium azide. Samples were subsequently fixed with 1% paraformadehyde and analyzed by using a FACSCalibur flow cytometer with CELLQuest software (Becton Dickinson). The lymphocyte population was gated according to their size and granularity using light scattering, i.e, forward scatter (FSC) and side scatter (SSC) with linear scale. The percentage of CD4+ lymphocytes in the gated population was determined by using FITC fluorescene-1 (FL1) and FSC. The absolute CD4+ lymphocyte count (cells/µl) was then calculated as the product of the total white blood cell count, percentage of lymphocytes, and the percentage of CD4+ lymphocytes.

In some cases, contamination of red blood cells occurred within the gated cells. The contaminated red blood cells were gated out from the lymphocyte population by making another gate using FL1 and FSC. The percentage of CD4+ cells in the lymphocyte population was then re-calculated from the number of lymphocytes in second gated population.

Enumeration of CD4+ lymphocytes by commercial Simutest™ reagent

One hundred microliters of K_3EDTA-whole blood was incubated at room temperature with 20 µl of each Simultest™ reagent panel (Becton Dickinson) in separate tubes. The Simultest™ reagent panel was composed of two-color reagent pairs of leukoGATE (CD45-FITC/CD14-PE), control IgG1-FITC/IgG2-PE, CD3-FITC/CD4-PE, and CD3-FITC/CD8-PE. After 15-30 min room temperature incubation, 2 ml of lysing buffer (Becton Dickinson) was added and let stand at room temperature in the dark for 10 min for lysis of red blood cells. Cells were then washed once with 2 ml of PBS containing 0.1% sodium azide. Samples were subsequently fixed with 1 % paraformadehyde and analyzed using a flow cytometer with Simultest IMK-lymphocyte software (Becton Dickinson). The absolute CD4+ lymphocyte count was then computed from the total white blood cell count and the percentage of lymphocyte.

RESULTS

Production of CD4 monoclonal antibody

After fusion, cell culture supernatants from hybridoma containing wells were tested for reactivity with surface antigens of Sup T1 cells by indirect immuno-fluorescence technique. Forty-three culture supernatants showed clearly positive. To screen further for hybridomas that produced CD4 specific antibody, all positive supernatants were tested again by the same technique, but CD4 transfected COS cells were used as the antigen. One of these culture supernatants reacted with CD4 transfected COS cells, but not with mock transfections. The hybridomas in this culture well were then re-cloned three times by limiting dilution. A final clone (3E8) that gave the same reaction pattern with transfected COS cells was propagated and re-named MT4. By using capture ELISA for isotype characterization, MT4 mAb was proved to be of IgM isotype.

Characterization of MT4 monoclonal antibody specificity

To confirm the specificity of generated mAb, MT4 mAb was used to stained CD4+ T cell lines, Sup T1[25] and Molt4[26], and CD4- cell lines, K562[27] and U937[28] by indirect immunofluorescence and analyzed by flow cytometry. As predicted, MT4 mAb reacted to both CD4+ cell lines, but not to CD4- cell lines. Then, MT4 mAb was used to stain peripheral blood lymphocytes by the same technique. The results were compared to those obtained by using standard CD4 mAb Leu3a. As shown in Table 1, percentages of positive cells in 5 donors obtained by using both antibodies were very similar.

To characterize the specificity of MT4 mAb further, MT4 was used to inhibit the binding of standard CD4 mAb to CD4 proteins on both peripheral blood lympho-cytes and CD4+ cell lines. Peripheral blood mononuclear cells from 3 normal donors were, firstly, incubated with MT4 or control antibodies. Then, PE labeled CD4 mAb Leu3a or FITC labeled CD3 mAb Leu4 was added and the fluorescence intensity was determined by a flow cytometer. It was found that MT4 mAb inhibited the binding of standard CD4 mAb Leu3a in all 3 donors tested with the percent inhibition of 97, 98 and 97, respectively. The irrelevant mAb control, M6, had no inhibitory effect with the

percent inhibition of 4, 8 and 3, respectively. In contrast, MT4 mAb did not inhibit the binding of standard CD3 mAb Leu 4 (% inhibition of 1, 1 and 0, respectively). The FACS profiles were similar for each donor and one of which is shown in Fig. 1. CD4+ cell lines, Sup T1 and Molt4, were also used to confirm these results by the same technique and it was found that MT4 strongly inhibited the binding of standard CD4 mAb (PE labeled Leu3a) to both cell lines (Fig. 2). Whereas, irrelevant antibodies had no effect (Fig. 2).

Enumeration of CD4+ lymphocytes by FITC labeled MT4 monoclonal antibody

In order to develop a home made reagent for enumerating CD4+ lymphocytes in blood samples by using the generated CD4 mAb, fluorescent dye (FITC) was conjugated to the MT4 mAb. The FITC labeled MT4 was then used to determine CD4+ lymphocytes in blood samples. For performing CD4+ lymphocyte determination using the home made reagent, Blood sample was incubated with FITC labeled MT4, after that, red blood cells were lysed and the stained cells were analyzed by a flow cytometer with CELLQuest software. By flow cytometric analysis, the lymphocyte population was firstly gated using FSC and SSC (Fig. 3A and 3C). Fluorescent labeled cells in the gated lymphocytes were then determined according to their fluorescence intensity by using FL1 and FSC (Fig. 3B and 3D). The results obtained in this manner were the percentages of CD4+ cells in the lymphocyte population. In some cases, a number of red blood cells were contaminated in the lymphogate (Fig. 3E). Since red blood cells are smaller in size and less fluorescent than lymphocytes (Fig.3E), those contaminated could be gated out by making another gate using FL1 and FSC (Fig. 3F; R2). Then, the percentage of CD4+ cells in the lymphocyte population was re-calculated from the number of cells in the second gated population (Fig. 3F).

By the method mention above, the lymphocyte populations were gated out from other cells according to their size and granularity. It was possible that some non-lymphocytes could have been contaminated in the lymphogate and affect the accuracy of the flow cytometric measurement of CD4+ lymphocytes. To address this question, 40 blood samples (20 healthy and 20 HIV infected persons) were stained with PE labeled CD14 /FITC labeled CD45 mAb and analyzed for non-lymphocytes in the lymphocyte population that had been gated by using FSC and SSC. As shown in Table

2, very few monocytes and granulocytes were detected in the gated lymphocytes. The FACS profile from one donor is shown in Figure 4.

To evaluate the accuracy of the home made reagent, CD4+ lymphocytes from 30 healthy and 30 HIV infected persons were determined by using home made FITC labeled MT4 and standard Simultest™ reagent kit. As shown in Table 3, both percentages and absolute CD4+ lymphocyte counts obtained by both reagents were very similar with no statistics significantly difference. A correlation plot comparing the percentages and absolute number of CD4+ lymphocytes obtained from both methods are shown in Figure 5 and 6, respectively. Linear regression analysis resulted in a slope of 0.971 and an intercept of 0.933 when the percentage of CD4+ lymphocytes from the two methods were compared (Fig.5). The correlation coefficient of the percentage CD4+ lymphocytes obtained from both methods was 0.995 (Fig 5). When the absolute CD4+ lymphocyte counts were compared, linear regression analysis resulted in a slope of 0.996, an intercept of 8.914 and the correlation coefficient obtained from both methods was 0.996 (Fig. 6). These results indicated that home made FITC labeled MT4 reagent can be used to enumerate CD4+ lymphocytes in blood samples in equivalent to those given by the commercial reagent.

DISCUSSION

The absolute number of CD4+ lymphocytes is an important marker for the prognosis and classification of the state of the disease, and monitoring for the therapy of HIV infection[12-19]. CD4+ lymphocyte counts must be monitored every 3-6 months in all HIV-infected persons[11]. The accepted standard method for the enumeration of CD4+ lymphocytes is flow cytometry[20,21]. By this technique, the CD4+ lymphocyte number is the product of three laboratory techniques: the white blood cell count, the percentage of lymphocytes and the percentage of CD4+ lymphocytes. Measuring the percentage of CD4+ lymphocytes is carried out by immunophenotyping and analyzed by a flow cytometer. However, a problem facing the clinical laboratory is that flow cytometry requires very expensive reagents. This technology is, therefore, costly for adaptation as a routine method in laboratories in developing countries. In this part of the world, inexpensive and reliable reagent is urgently needed. To support this requirement, anti-CD4 monoclonal antibody was generated in our laboratory. The generated monoclonal antibody was then conjugated to fluorescein isothiocyanate (FITC) and used as a homa made reagent for enumerating CD4+ lymphocytes.

By using conventional hybridoma technique[22], in this study, a hybridoma producing CD4 specific monoclonal antibody, named MT4, was obtained. The specificity of MT4 mAb was confirmed as it reacted with CD4-DNA transfected COS cells, CD4+ cell lines and CD4+ lymphocytes. Furthermore, MT4 mAb inhibited the binding of standard CD4 monoclonal antibodies to CD4 proteins on CD4+ cells.

The MT4 mAb was then conjugated to FITC by alkaline reaction. The ratio of fluorescein to protein was estimated by measuring the absorbance at 495 nm and 280 nm. In this study, the ratio of 0.6 was obtained and this ratio was in the recommended ratio for the optimal conjugation of FITC to antibody[29].

The home made FITC labeled MT4 was then used to enumerate CD4+ lymphocytes by flow cytometer using CELLQuest software. By flow cytometric analysis, the lymphocyte population was firstly gated according to their size and granularity using FSC and SSC. The percentages of CD4+ lymphocytes in the gated lymphocytes were then determined by using FL1 and FSC parameters. It is known that monocytes also express CD4 molecules on their surface[30]. Therefore, if monocytes were contaminated in the lymphogate, these cells can affect the accuracy of the flow

cytometric measurement of CD4+ lymphocytes. The commercial Simultest™ reagent kit, thus, generally employs PE labeled CD14/FITC labeled CD45 (leukoGate) for setting gate around the lymphocyte cluster. It also employs a T lymphocyte specific antibody, CD3 mAb, to discriminate between CD4+ lymphocytes and contaminated monocytes. By using the home made FITC labeled MT4 mAb, however, lymphocytes were gated according only to their size and granularity. To clarify whether non lymphocytes had been contaminated in the lymphocyte population that had been gated by FCS and SSC, 40 blood samples were stained with PE labeled CD14/FITC labeled CD45 and analyzed for monocytes and granulocytes in the lymphogate. It was found that very few monocytes and granulocytes were in the gated lymphocytes. The results indicated that parameters FSC and SSC can be used to gate the lymphocyte population.

In some samples, contamination of red blood cells within the lymphogated was occurred. This contamination could also affect the accuracy of the flow cytometric measurement of CD4+ lymphocytes. To correct this affect, the contaminated red blood cells had to be gated out. According to their size and auto fluorescent, contaminated red blood cells could be easily gated out by making an additional gate using FL1 and FSC. The percentage of CD4+ cells in the lymphocyte population was then re-analyzed from the second gated population.

In this study, CD4+ lymphocytes from a total of 60 blood samples were evaluated by both the home made FITC labeled MT4 reagent and the standard Simultest™ reagent kit. It was concluded that the home made reagent provides results which are equivalent to those given by the commercial Simultest™ reagent kit. A very high degree of correlation between both reagents has been found in both percentage and absolute CD4+ lymphocytes. The results suggest that home made FITC labeled MT4 reagent is an acceptable alternative reagent for monitoring CD4+ lymphocytes in blood samples.

In summary, a home made reagent for determining CD4+ lymphocytes in blood samples by flow cytometry has been developed. This reagent is more cost effective than available commercial reagents. Therefore, it is appropriate for use in measuring CD4+ lymphocytes in either asymptomatic HIV infected persons or AIDS patients. We

believe that this home made FITC-labeled MT4 meets the growing demand for CD4 counts, especially in developing countries where HIV prevalence is high.

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Table 1. Percentage of positive cells in peripheral blood lymphocytes determined by staining with MT4 mAb and standard CD4 mAb Leu3a.

Donor No.	Monoclonal antibody		
	MT4	Leu3a	Myeloma control[a]
1	32[b]	32	0
2	37	39	0
3	30	32	0
4	33	33	0
5	24	25	0

[a] Myeloma induced ascitic fluid which was used as negative control.

[b] Percentage of positive cells was determined by flow cytometric analysis.

Table 2. Determination of monocytes, granulocytes and lymphocytes in the lymphogate using FSC and SSC.

Donor[a] no.	% cells in lymphogate			Donor no.	% cells in lymphogate		
	Mono.	Gran.	Lymph.		Mono.	Gran.	Lymph.
1	0.3	4.7	95.0	21	0.3	6.5	93.2
2	0.0	6.7	93.3	22	0.9	3.8	95.3
3	0.2	5.8	94.0	23	1.1	3.0	95.9
4	0.1	4.4	95.5	24	0.8	4.1	95.1
5	0.2	3.6	96.2	25	1.1	2.5	96.4
6	0.2	4.7	95.1	26	0.8	4.4	94.8
7	0.2	5.9	93.8	27	0.8	3.1	96.1
8	0.1	7.4	92.5	28	1.0	5.0	94.0
9	0.1	6.7	93.1	29	0.5	4.7	94.8
10	2.0	4.2	93.8	30	0.4	7.1	92.5
11	0.3	5.9	93.7	31	1.0	2.4	96.5
12	0.5	4.0	95.5	32	0.2	3.3	96.5
13	0.6	7.3	92.1	33	1.0	6.0	93.0
14	0.9	4.9	94.2	34	0.4	2.2	97.4
15	1.2	3.4	95.3	35	1.0	5.2	93.8
16	0.8	4.9	94.2	36	0.5	5.5	94.0
17	0.2	5.3	94.5	37	0.6	3.4	96.0
18	1.1	5.2	93.6	38	0.2	6.4	93.4
19	0.1	2.9	97.0	39	1.2	3.8	95.0
20	0.5	5.1	94.4	40	0.9	4.8	94.3

[a] Donor no. 1-20 were healthy donors. ; Donor no. 21-40 were HIV infected donors.

Table 3. Percentages and absolute CD4+ lymphocyte counts determined by home made FITC labeled MT4 and Simultest™ reagent kit.

Donor no.	%CD4+ lymph[c]		Abs. CD4+ lymph[d]	
	Simul.[b]	MT4[b]	Simul.	MT4
H1[a]	23	23	441	441
H2	24	26	507	549
H3	36	35	661	643
H4	24	27	513	578
H5	16	17	256	272
H6	13	15	346	400
H7	17	17	371	371
H8	15	15	322	322
H9	19	22	512	593
H10	3	4	18	23
H11	22	22	779	779
H12	25	27	378	408
H13	7	7	101	101
H14	12	13	277	277
H15	1	1	5	5
H16	23	24	883	921
H17	11	12	721	786
H18	1	1	17	17
H19	22	23	408	427
H20	12	11	297	272
H21	4	5	73	91
H22	3	2	25	27
H23	1	1	6	6
H24	1	1	8	8
H25	22	23	409	428
H26	38	37	717	699
H27	15	15	151	151
H28	11	13	257	304
H29	10	11	192	212
H30	0	0	0	0
Mean	14.37	15.00	321.70	337.03
SD	10.30	10.48	258.66	268.27

Donor no.	%CD4+ lymph		Abs. CD4+ lymph	
	Simul.	MT4	Simul.	MT4
N1[a]	33	32	1433	1390
N2	35	34	947	920
N3	35	35	1062	1062
N4	32	31	594	575
N5	28	28	902	902
N6	34	32	993	934
N7	32	32	737	737
N8	32	34	609	647
N9	39	39	1024	1024
N10	24	24	504	504
N11	40	37	963	891
N12	42	40	816	777
N13	39	36	888	820
N14	29	29	1107	1107
N15	35	35	735	735
N16	28	26	908	843
N17	45	46	1035	1058
N18	47	47	954	934
N19	32	32	730	730
N20	36	37	647	665
N21	32	35	860	941
N22	38	39	1098	1127
N23	33	35	1200	1273
N24	35	35	635	635
N25	44	41	687	640
N26	36	36	1109	1109
N27	24	24	714	714
N28	30	32	744	793
N29	42	41	1005	982
N30	28	30	1540	1650
Mean	34.63	34.47	906.00	903.97
SD	5.85	5.54	239.41	251.21

[a] Donor H1-30 were HIV infected donors. ; Donor N1-30 were healthy donors.

[b] Simul.; Simultest™ reagent kit / MT4; home made FITC labeled MT4

[c] %CD4+ lymphocytes

[d] Absolute CD4+ lymphocyte count (cell/cu.mm.)

Figure legends:

Fig. 1. Inhibition of standard CD4 mAb binding to lymphocytes by MT4 mAb. Peripheral blood lymphocytes were pre-incubated with MT4 mAb (B and D) or without mAb (A and C). PE-labeled CD4 mAb Leu3a (A and B) or FITC labeled CD3 mAb Leu4 was added to the pre-stained cells. The membrane fluorescence intensity was analyzed by flow cytometry.

Fig. 2. Inhibition of standard CD4 mAb binding to CD4+ cell lines by MT4 mAb. Molt4 (A-C) or Sup T1 (D-F) were pre-incubated with MT4 mAb (B and E), M6 mAb (C and F) or without mAb (A and D). PE-labeled CD4 mAb Leu3a was added to the pre-stained cells and the membrane fluorescence intensity was analyzed by flow cytometry.

Fig. 3. Flow cytometric analysis of CD4+ lymphocytes using FITC labeled MT4 mAb. Lymphocytes in the blood samples were gated according to FSC and SSC (A and C; gate R1). The percentages of CD4+ lymphocytes were determined by FL1 and FSC (B and D). In the case of red blood cell contamination (E), the contaminated red blood cells were gated out according to FL1 and FSC (F; gate R2). Samples in A, B and C, D were taken from healthy and AIDS patient, respectively. The percentage of CD4+ lymphocytes detected in each sample was indicated.

Fig. 4. Determination of monocytes and granulocytes in the lymphocytes gated by using FSC and SSC. Lymphocytes in the blood samples were gated according to FSC and SSC (A). The gated cells were analyzed for bright CD45+ lymphocytes, weak CD45+ granulocytes and CD14+ monocytes according to their fluorescent reactivity (B). The percentage of cells in each quadrant was indicated.

Fig. 5. Scattergram of percentage of CD4+ lymphocytes from 60 blood samples as determined by home made FITC labeled MT4 and commercial Simultest™ reagent.

Fig. 6. Scattergram of absolute CD4+ lymphocyte counts from 60 blood samples as determined by home made FITC labeled MT4 and commercial Simultest™ reagent.

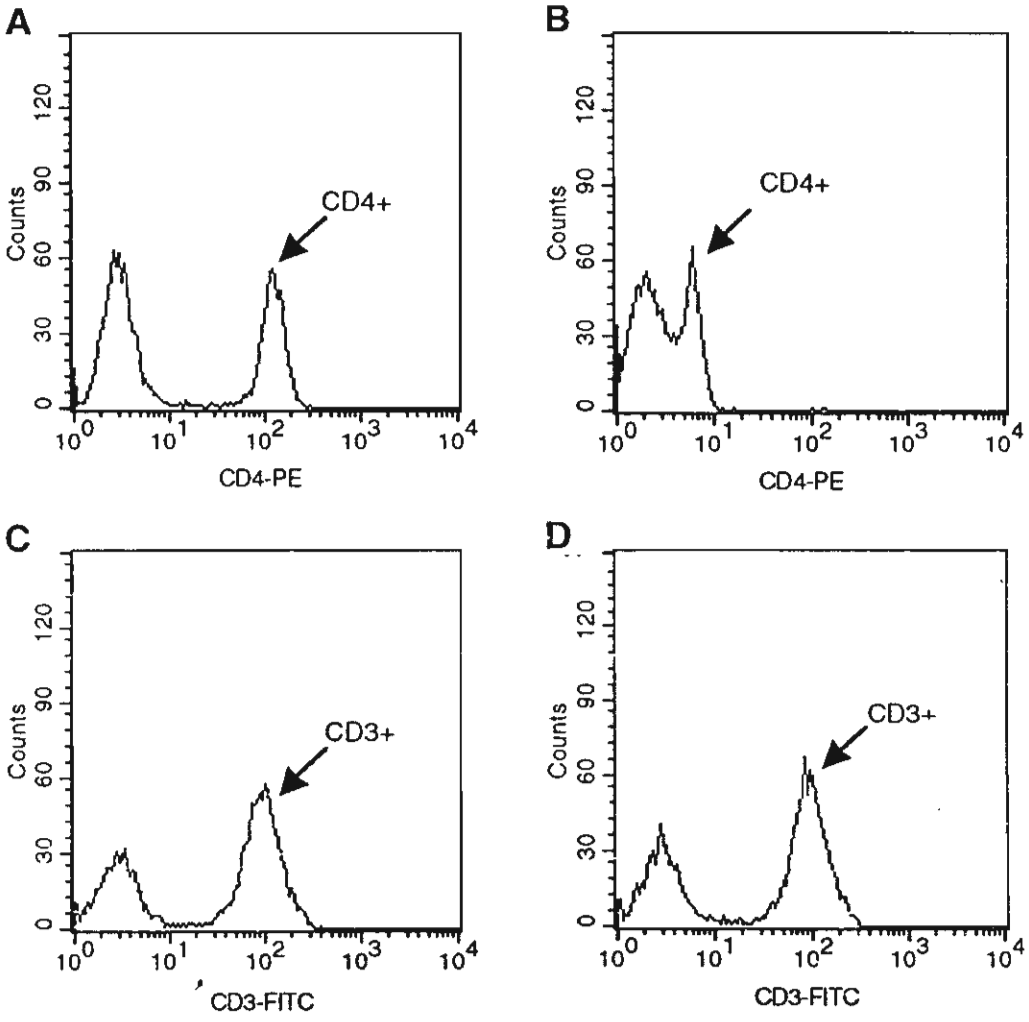
A
Counts
CD4+
CD4-PE
B
Counts
CD4+
CD4-PE
C
Counts
CD3+
CD3-FITC
D
Counts
CD3+
CD3-FITC

Fig 1

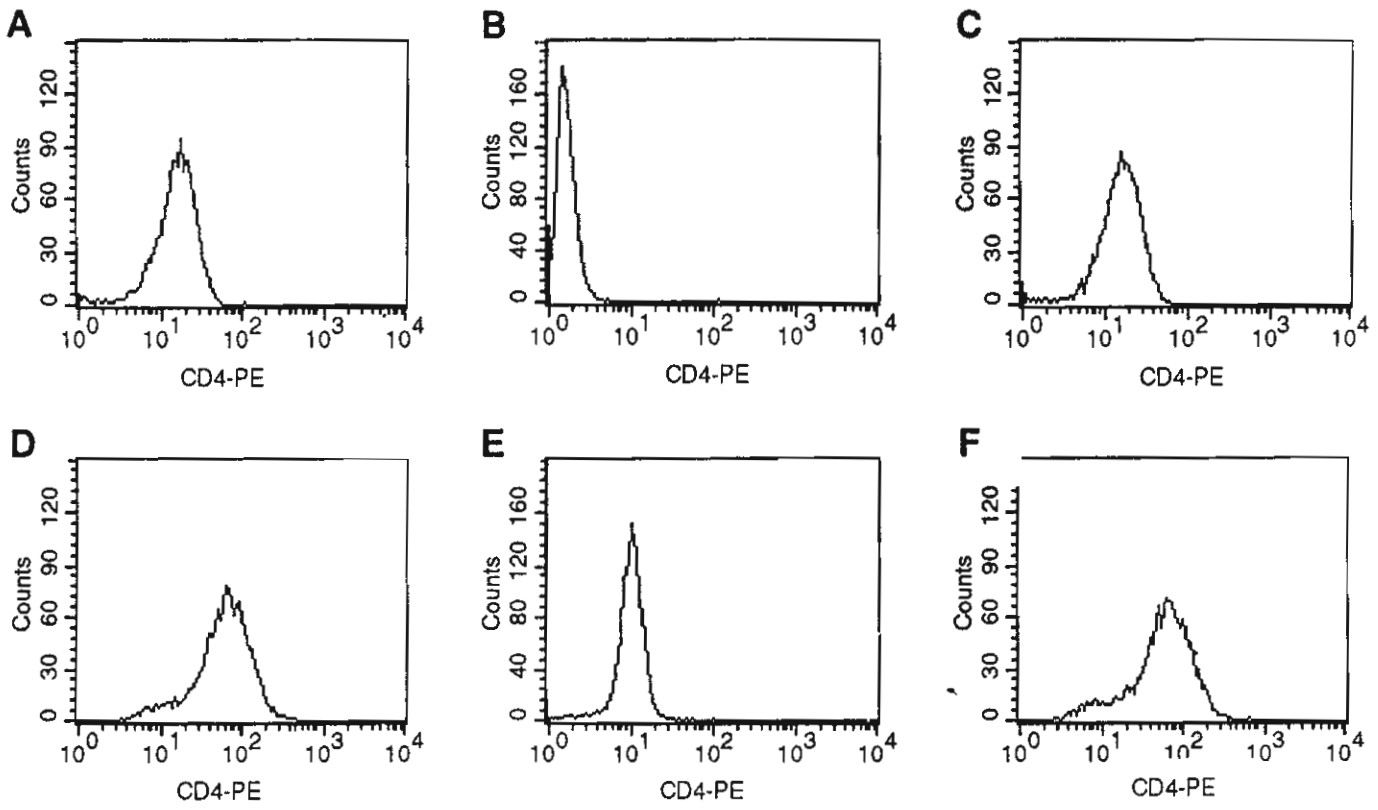

Fig. 2

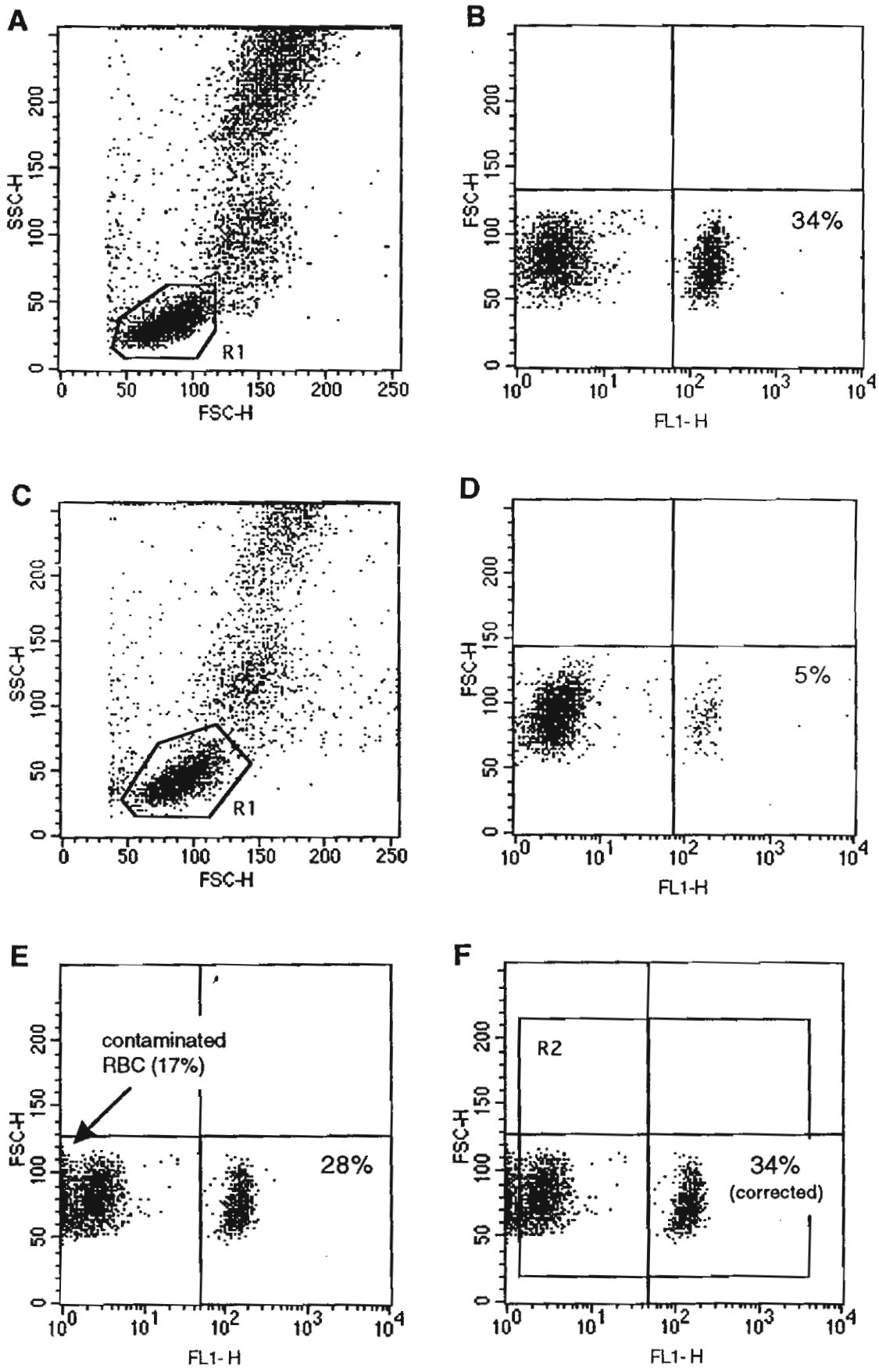
A
SSC-H
FSC-H
R1
B
FSC-H
FL1-H
34%
C
SSC-H
FSC-H
R1
D
FSC-H
FL1-H
5%
E
contaminated
RBC (17%)
FSC-H
FL1-H
28%
F
R2
FSC-H
FL1-H
34%
(corrected)

Fig. 3

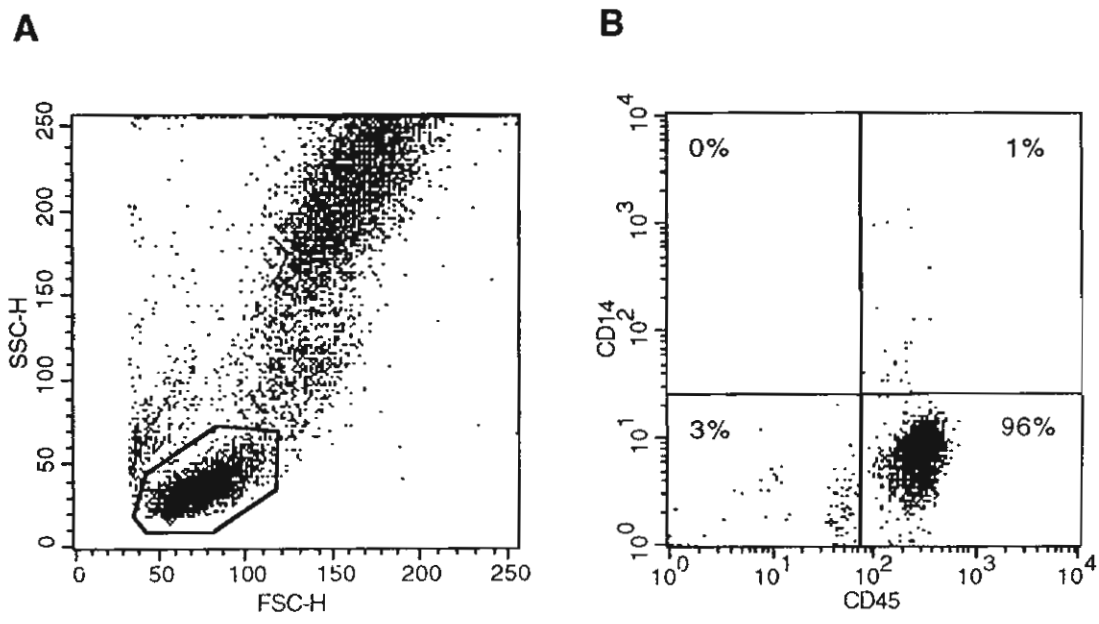
A
B
SSC-H
FSC-H
0
50
100
150
200
250
CD14
CD45
0%
1%
3%
96%
10^0
10^1
10^2
10^3
10^4

Fig. 4

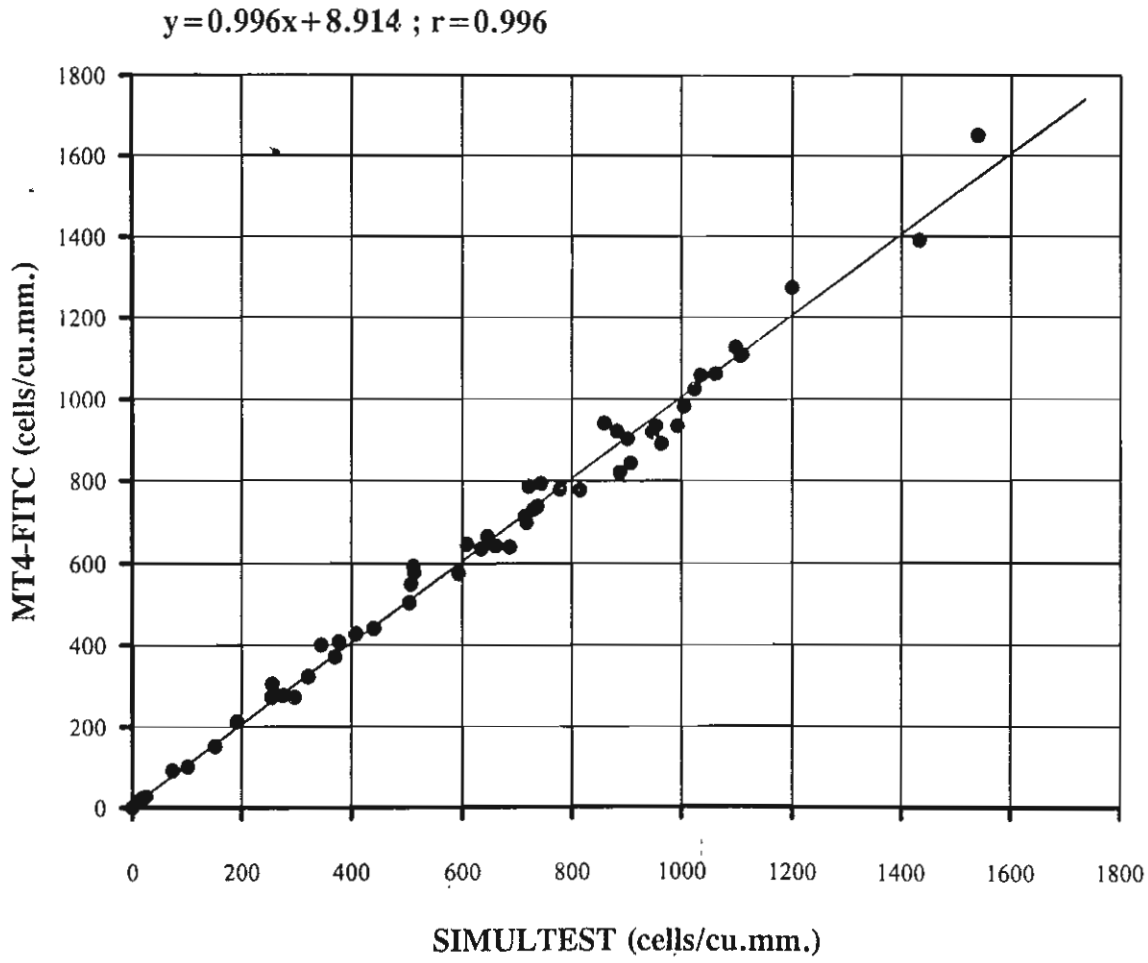
y=0.996x+8.914 ; r=0.996
MT4-FITC (cells/cu.mm.)
SIMULTEST (cells/cu.mm.)
0
200
400
600
800
1000
1200
1400
1600
1800

Fig. 6