CHAPTER II

MATERIALS AND METHODS

2.1. Purification of MT4 and MT14/3 monoclonal antibodies

2.1.1 Purification of MT4 mAb

Ascitic fluid containing CD4 mAb (MT4; IgM isotype) was diluted 1:4 with filtrated sodium phosphate buffer (PBS). The diluted ascitic fluid was centrifuged at 14,000 rpm 4°C for 15 minutes and the clear supernatant was collected. To purify MT4 mAb from ascitic fluid, rat anti-mouse IgM sepharose 4B column was used. The anti-mouse IgM sepharose was equilibrated with 50 ml of filtrated PBS and adjusted the solution to the bed volume. Then, 4 ml of the diluted ascitic fluid were added to the column, incubated for 5 minutes in order to support the binding of IgM with anti-mouse IgM. The unbound materials were re-passed into column for 4 times. The unbound proteins were then washed out by washing the column with 50 ml filtrated PBS. To elute antibodies from the column, 2.5 ml of elution buffer (0.2 M glycine-HCl, pH 2.8) were added into the column. One ml of eluate was collected as pre-elute. Then, the solution was drained out from the column by re-adding 2 times of elution buffer into the column. The eluate was collected as elute 1, 2 and 3 and adjusted to pH 7.0 with neutralization buffer (2 M Tris-HCl, pH 8.0). The column was then washed with 50 ml of filtrated PBS and retained in storage buffer (PBS containing 0.05% NaN_3) and stored at 4°C. The obtained eluates were concentrated

by speedvac concentrator and dialyzed against PBS overnight. The concentration of antibody was determined by measuring the absorbance at wavelength of 280 nm.

2.1.2 Purification of MT14/3 mAb

Ascitic fluid containing CD14 mAb (MT14/3; IgG1 isotype) was diluted 1:5 (or 1:10) with filtrated PBS 7.2 (or 8.0). The diluted ascitic fluid was centrifuged at 14,000 rpm 4°C for 15 minutes and the clear supernatant was collected. To purify MT14/3 mAb from induced ascitic fluid, protein A-sepharose column was used. The protein A-sepharose was equilibrated with 50 ml of filtrated PBS and adjusted the solution to the bed volume. Then, 2 ml of the diluted ascitic fluid were added to the column, incubated for 5 minutes. The unbound materials were re-passed into column for 4 times. The unbound proteins were then washed out by washing the column with 50 ml of filtrated PBS. To elute antibodies from the column, 1.5 ml of elution buffer (0.1 M citric acid, pH 3.0 and 6.5) were added into the column. One ml of eluate was collected as pre-elute. Then, the solution was drained out by re-adding 2 times of elution buffer into the column. The eluate was collected as elute 1, 2, 3, and 4 and adjusted to pH 7.0 with neutralization buffer (2 M Tris-HCl, pH 8.0). The column was then washed with 50 ml of filtrated PBS and retained in storage buffer pH 7.4 and stored at 4°C. The obtained eluates were concentrated by speedvac concentrator and dialyzed against PBS overnight. The concentration of antibody was determined by measuring the absorbance at wavelength of 280 nm.

2.2 Fluorescein isothiocyanate (FITC) labeling of MT4 mAb

One milligram of purified MT4 (from 2.1.1) was mixed with a volume of fresh prepared 10 mg/ml FITC in Dimethylsulfoxide (DMSO). The volume of added FITC was calculated from the following equation:

$$\text{Volume (ml) of 10 mg/ml FITC} = \frac{\text{mg antibody x 0.1}}{\text{MW of antibody}} (\text{R x MW of FITC})$$

Where R = molar incubation ratio of dye:antibody (20 and 200); MW of antibody = 180,000 Da; MW of FITC = 389.14 Da.

The FITC solution was added dropwise into antibody solution with slowly mixing the antibody. The antibody-FITC mixture was incubated at room temperature for 90 minutes with gentle stirring and protected from direct light. After that, the solution was dialyzed against PBS overnight to separate the coupled from uncoupled FITC. Finally, FITC to protein (F/P) ratio was determined by measuring the absorbance at wavelength of 495 and 280 nm.

2.3 Determination the activity of antibodies

2.3.1 Direct immunofluorescence assay for determination of the activity of FITC labeled MT4 mAb

Peripheral blood mononuclear cells (PBMC) were prepared from fresh heparinized blood by Ficoll-Hypaque gradient centrifugation technique. Ten millilitres of heparinized blood was diluted with PBS at ratio of 1:4, mixed thoroughly and underlayer with 10 ml of Ficoll-Hypaque solution, then centrifuged at 400xg for 30 minutes. The PBMC layer was collected into a 15-ml centrifuged tube, washed twice with PBS and then resuspended in 4 ml of PBS containing 1%BSA and

0.02%NaN_3 (1%BSA-PBS- NaN_3). The cells were counted with haemacytometer and adjusted to 1x 10^7 cells/ml with 1%BSA-PBS- NaN_3. Nonspecific Fc binding sites were blocked by adding human AB serum into the cell suspension at 10% volume of the PBMC. The cell suspension was incubated on ice for 30 minutes. After that, 50 µl of blocked cells were added to 50 µl of 20 µg/ml of FITC labeled MT4 mAb (from 2.2). After incubation on ice for 30 minutes, cells were washed 3 times with 1%BSA-PBS- NaN_3 and fixed with 350 µl of 1%paraformaldehyde in PBS. The stained cells were analyzed by flow cytometer.

2.3.2 Indirect immunofluorescence assay for determination of the activity of purified MT14/3 mAb

The PBMC were prepared by Ficoll-Hypaque gradient centrifugation and adjusted to 1x 10^7 cells/ml with 1%BSA-PBS-NaN_3. PBMC were blocked with human AB serum. After that, 50 µl of blocked cells were added to 50 µl of 20 µg/ml of purified MT14/3 mAb (from 2.1.2). After incubation on ice for 30 minutes, cells were washed twice with 1%BSA-PBS-NaN_3 and resuspended with 20 µl of 1%BSA-PBS-NaN_3. Then, 25 µl of 1:20 FITC conjugated rat anti-mouse immunoglobulins were added and incubated on ice for further 30 minutes. Cells were washed 3 times with 1%BSA-PBS-NaN_3 and fixed with 350 µl of 1%paraformaldehyde in PBS. The stained cells were analyzed by flow cytometer.

2.4 Determination the specificity of the purified antibodies

2.4.1 Transfection of CD4-DNA and CD14-DNA into COS cells by DEAE-dextran method

COS cells were collected from culture flasks by using PBS containing 0.5 mM EDTA (0.5 mM EDTA-PBS) and washed twice with MEM medium. Then, the COS cells were resuspended in 2 ml of MEM medium containing 10%FCS (10%FCS-MEM), counted with haemacytometer and adjusted to 1x 10^6 cells/ml with 10%FCS-MEM. One millilitre of the cell suspension was plated into a 6 cm dish, then 3 ml of 10%FCS-MEM was added and cultured in a CO_2 incubator at 37°C, fully humidified atmosphere with 5% CO_2. After overnight culturing, the culture medium was aspirated and discarded. Then, 4 ml of MEM medium were added into the COS cell dish and left in a CO_2 incubator. The transfection solution which contained 2 ml of MEM medium, 50 µl of DEAE-dextran stock solution (10 mg/ml), 80 µl of chloroquine diphosphate stock solution (10 mM) and 10 µl of plasmid DNA (200 µg/ml) was prepared. The medium from the COS cell dish was discarded and 2 ml of the transfection solution was added, then incubated in CO_2 incubator for 3 hours. The transfection solution was aspirated and 2 ml of PBS containing 10%DMSO (10%DMSO-PBS) was added into the COS cell dish, left for exactly 2 minutes at room temperature and then the DMSO-PBS solution was remove rapidly. The cells were washed once with 3 ml of MEM medium, added with 4 ml of 10%FCS-MEM and cultured in CO_2 incubator overnight. The medium was removed and 4 ml of fresh 10%FCS-MEM was added into the dish. The COS cells were then re-cultured for another 2 days in CO_2 incubator.

2.4.1.1 Direct immunofluorescence assay for determination of the specificity of FITC labeled MT4 mAb

CD4 transfected COS cells (from 2.4.1) were removed from the transfection dish by using 1.0 ml of 0.5 mM EDTA-PBS. Cells were washed twice with 1%BSA-PBS-NaN_3. The cell concentration was adjusted to 1x 10^7 cells/ml with 1%BSA-PBS- NaN_3. Aliquot of 50 µl of cells suspension was incubated with 50 µl of FITC labeled MT4 mAb (20 µg/ml) on ice for 30 minutes in dark. The cells were washed 3 times with 1%BSA-PBS-NaN_3 and resuspended with 14 µl of 1%BSA-PBS-NaN_3. The stained cells were analyzed by a fluorescence microscope.

2.4.1.2 Indirect immunofluorescence assay for determination of the specificity of purified MT14/3 mAb

CD14 transfected COS cells (from 2.4.1) were removed from the transfection dish by using 1.0 ml of 0.5 mM EDTA-PBS. Cells were collected, washed and adjusted to 1x 10^7 cells/ml with 1%BSA-PBS-NaN_3. Aliquot of 50 µl of cell suspension was incubated with 50 µl of tested antibodies (20 µg/ml) on ice for 30 minutes in dark. The cells were washed twice with 1%BSA-PBS-NaN_3 and resuspended with 20 µl of 1%BSA-PBS- NaN_3. Then, 25 µl of 1:20 FITC conjugated rat anti-mouse immunoglobulins were added and incubated on ice for further 30 minutes. The cells were washed 3 times with 1%BSA-PBS-NaN_3 and resuspended with 14 µl of 1%BSA-PBS-NaN_3. The stained cells were analyzed by a fluorescence microscope.

2.5 Titration for optimal concentration of monoclonal antibodies used in the development of three-color reagents

2.5.1 Titration of FITC labeled MT4 mAb

One hundred microlitres of K_2EDTA-whole blood were added to 20 µl of FITC labeled MT4 mAb (from 2.2) at various concentrations (5, 10, 20, and 40 µg/ml) of in separate tubes. Then 40 µl of 1%BSA-PBS-NaN_3 were added and mixed gently. After 30 minutes incubation at room temperature in dark, 2 ml of FACS™ lysing solution was added and left for 10 minutes for lysis of red blood cells. Cells were then washed once with 2 ml of PBS containing 0.1% NaN_3 (PBS-NaN_3) and fixed with 500 µl of 1%paraformaldehyde in PBS. The stained cells were analyzed by flow cytometer.

2.5.2 Titration of purified MT14/3 mAb and phycoerythrin (PE) conjugated goat anti-mouse immunoglobulin G (IgG)

One hundred microlitres of K_2EDTA-whole blood were added to 10 µl of various concentrations (25, 50, 100, and 200 µg/ml) of purified MT14/3 mAb (from 2.1.1) in separate tubes. Then, 10 µl of various dilutions (1:4, 1:8, and 1:16) of PE conjugated goat anti-mouse IgG (commercial reagent) were added. Afterward, 40 µl of 1%BSA-PBS-NaN_3 were added in each tube. Cells were incubated at room temperature in dark for 30 minutes. Two ml of FACS™ lysing solution was added and left for 10 minutes. Cells were then washed once with 2 ml of PBS-NaN_3 and fixed with 500 µl of 1%paraformaldehyde in PBS. The stained cells were analyzed by flow cytometer.

2.5.3 Titration of peridinin chlorophyll protein (PerCP) conjugated anti-CD45 monoclonal antibody

One hundred microlitres of K_2EDTA-whole blood were mixed with various volumes (5, 10, and 20 µl) of PerCP conjugated anti-CD45 mAb (commercial reagent) in separate tubes. Then the volume of each tube was adjusted to 60 µl with 1%BSA-PBS-NaN_3. Cells were incubated at room temperature in dark for 30 minutes. Then 2 ml of FACS™ lysing solution was added and left for 10 minutes. Cells were then washed once with 2 ml of PBS-NaN_3 and fixed with 500 µl of 1%paraformaldehyde in PBS. The stained cells were analyzed by flow cytometer.

2.6 Enumeration of CD4 lymphocytes by the developed three-color reagent

One hundred microlitres of K_2EDTA-whole blood were incubated at room temperature with 20 µl of FITC labeled MT4 mAb (40 µg/ml), 10 µl of purified MT14/3 mAb (100 µg/ml) and 10 µl of PE conjugated anti-mouse IgG (1:8) for 30 minutes. After that, red blood cells were lysed by using 2 ml of FACS™ lysing solution and washed once with 2 ml of PBS-NaN_3. Twenty microlitres of PerCP conjugated anti-CD45 mAb was added and incubated for 5 minutes. Cells were fixed with 500 µl of 1%paraformaldehyde in PBS and analyzed by a flow cytometer using CellQuest program for obtaining of percentage of CD4 lymphocytes in lymphocyte population. The absolute number of CD4 lymphocytes was then calculated from the total white blood cells count, the percentage of lymphocytes and the percentage of CD4 lymphocytes in lymphocyte population.

2.7 Enumeration of lymphocyte subset by Simultest™ reagent

One hundred microlitres of K_2EDTA-whole blood were incubated with 20 µl of Simultest™ reagent panel in four separate tubes. The Simultest™ reagent panel composes of two-color monoclonal antibody pairs including: leukogate (FITC labeled CD45/PE labeled CD14), isotype control (FITC labeled IgG1/PE labeled IgG2), FITC labeled CD3/PE labeled CD4 and FITC labeled CD3/PE labeled CD8. After 30 minutes room temperature incubation, 2 ml of FACS™ lysing solution was added and left for 10 minutes. The cells were then washed once with 2 ml of PBS-NaN_3 and fixed with 500 µl of 1%paraformaldehyde in PBS. The stained cells were analyzed by using a flow cytometer with SimulSet software. Lymphocyte subset as the percentage and the absolute number were calculated from the total white blood cells count and the percentage of lymphocytes.

2.8 Preparation of red blood cell lysing solution

2.8.1 Enumeration of lymphocyte subset by Simultest™reagent using 0.83%ammonium chloride lysing solution

One hundred microlitres of K_2EDTA-whole blood were incubated with 20 µl of Simultest™ reagent panel in four separate tubes. After 30 minutes room temperature incubation, 2 ml of 0.83%ammonium chloride was added and left for 10 minutes. The cells were then washed once with 2 ml of PBS-NaN_3 and fixed with 500 µl of 1%paraformaldehyde in PBS. The stained cells were analyzed using a flow cytometer with SimulSet software. Lymphocyte subset as the percentage and the

absolute number were calculated from the total white blood cells count and the percentage of lymphocytes.

2.8.2 Enumeration of lymphocyte subset by Simultest™ reagent using ammonium chloride tris lysing buffer

One hundred microlitres of K_2EDTA-whole blood were stained with Simultest™ reagent as described in 2.8.1 but red blood cells were lysed with 2 ml of ammonium chloride tris lysing buffer.

2.8.3 Enumeration of lymphocyte subset by Simultest™ reagent using hypotonic ammonium chloride lysing buffer

One hundred microlitres of K_2EDTA-whole blood were stained with Simultest™ reagent as described in 2.8.1 but red blood cells were lysed with 2 ml of hypotonic ammonium chloride solution.

2.8.4 Enumeration of lymphocyte subset by Simultest™ reagent using 0.83%ammonium chloride containing 3% formaldehyde (3% formaldehyde-NH_4Cl) lysing solution

One hundred microlitres of K_2EDTA-whole blood were stained with Simultest™ reagent as described in 2.8.1 but red blood cells were lysed with 100 µl of 3%formaldehyde-NH_4Cl solution and 1 ml of deionized water was added immediately after adding of 3% formaldehyde-NH_4Cl and left for 5 minutes.

2.8.5 Enumeration of lymphocyte subset by Simultest™ reagent using PBS containing 0.83% ammonium chloride and 3% formaldehyde (3% formaldehyde-NH_4Cl-PBS) lysing solution

One hundred microlitres of K_2EDTA-whole blood were stained with Simultest™ reagent as described in 2.8.1 but red blood cells were lysed with 100 µl of 3% formaldehyde-NH_4Cl-PBS solution and 1 ml of deionized water was added immediately after adding of 3% formaldehyde-NH_4Cl-PBS and left for 5 minutes.

2.9 Comparison of the developed reagents with the commercial reagents

2.9.1 Comparison of the developed three-color reagent with Simultest™ reagent

In order to evaluate the accuracy of the developed three-color reagent, the developed reagent and Simultest™ reagent were used to determine CD4 lymphocytes in 60 blood samples (30 healthy and 30 HIV infected persons) as described in 2.6 and 2.7. The percentage and absolute number of CD4 lymphocytes obtained from both reagents were compared using linear regression and paired t-test.

2.9.2 Evaluation of the developed red blood cell lysing solution

In order to evaluate the accuracy of home made red blood cell lysing solutions, lymphocyte subset of 20 donors were determined using the developed red blood cell lysing solution. Lymphocyte subset as percentage and absolute number were compared with the results obtained from standard Simultest™ reagent kit using linear regression and paired t-test.