CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS, REAGENTS AND INSTRUMENTS.

All materials and reagents used in this study are presented in appendix A and B. The instruments used are listed in appendix C.

2.2 Preparation of MT4 (CD4) monoclonal antibody

2.2.1 Culture of hybridoma cells

Hybridoma clone of which produces CD4 monoclonal antibody, named MT4 was used in this study. The hybridoma cells were grown in tissue culture dishes with IMDM (Iscove's Modified Dulbecco's Medium) supplemented with 10% fetal calf serum. The cells culture dishes were incubated in 37° C incubator with 5% CO_2 and the cells were carefully monitored daily under inverted microscope. The culture media was changed every 2-3 days. At the time that supernatant color changed from pink to yellow, supernatant whether containing antibody was collected to test for activity of antibody.

2.2.2 Preparation of hybridoma cells for ascitic fluid production

Hybridoma cells were washed three times by steriled PBS in 15 ml centrifuge tube. Cells were centrifuged at 1700 rpm, 20 °C for 5 minutes. Cells were checked for

its viability by trypan blue dye at dilution 1:2 and counted by using hemacytometer. Cells were then adjusted to $1x10^7$ cells in 600 µl of sterile PBS.

2.2.3 Immunization for ascitic fluid production

At one week before hybridoma immunization, 0.5 ml of pristane (2, 6, 10, 14-tetramethyl decanoic acid) was injected into intra-peritoneal (IP) cavity of Balb/c mice. Then, primed Balb/C mice were inoculated with $1x10^7$ hybridoma cells in 600 µl of steriled PBS. After 2-3 weeks, abdomen of mice was developed bigger as pregnancy size. Ascitic fluid was harvested from the peritoneal cavity. The fluid was centrifuged at 12,000 rpm for 10 minutes at 4°C. Clear ascitic fluid was collected and stored at –20 °C.

The indirect immunofluorescence staining was used for testing the activity of Ab in all obtained ascitic fluid before further purification.

2.3 Purification of MT4 monoclonal antibody by AKTA prime fraction collector

2.3.1 Sample preparation

To purify MT4 mAb, the sample was diluted with binding buffer to obtain final concentration of 0.8 M ammonium sulphate and 20mM phosphate. Diluted sample was clarified by centrifuging at 14,000 rpm, 4°C for 15 minutes.

2.3.2 Machine preparation

AKTA prime machine was turning on and then F1 was chosen for checking icons view. Washing and priming set up were done as below:

Firstly, for washing step, all lines (B, A, 2, 3) retaining in 20% ethanol were removed with ddH_2O. The word and application templates were chosen before washing system and buffer positioning was selected.

Secondly, before priming, the lines were removed into specific buffers and at least 40 ml of each specific buffer was replaced instead ddH_2O in all lines. Example line A1 and A3 were put into the binding buffer (0.8M ammonium sulphate, 20mM phosphate, pH 7.5), line B was put into eluting buffer 1 (20mM sodium phosphate, pH 7.0) at position 1 and line A2 into eluting buffer 2 (Isopropanol, pH 7.5) at position 2.

- Line B priming, template menu, manual run, method base and milliliter unit were subsequently selected. Setting commands of %B concentration to 100% was programmed. Then, the gradient, and select off commands were chosen. Flow rate of 20-40 ml/min, fraction base on milliliter and size of zero ml and pressure limit of 0.3 Mpa were set. Position valves were selected for example set buffer valve for position 1, and west command was selected for injecting valve position. Button O.K was pressed to start, to accept, or to select command. Line B priming was paused when volume reached at 40 ml and continued priming line A1.

- Line A1 priming, method base was set and milliliter unit was selected. Concentration of %B was changed from 100% to zero, and then gradient and select off mode were set. Flow rate of 20 ml/min was programmed, and then fraction base was set at zero milliliter and pressure limit at 0.3 Mpa. The position valves were set of which buffer valve at position 2 and waste mode was chosen for injecting valve position. Line A1 priming was paused when volume reached at 80 ml and continued priming line A3.

- Line A3 priming was performed same as line A1. Only buffer valve position was change from position 2 to 3. Line A3 priming was paused when volume reached at 120 ml and removed the line A3 from binding buffer into sample.

Finally, to set up collector machine and program for protein purification, micro-tubes were filled into the fraction collector rack. The sensor had been checked that the position of the white plate was fit on fractionation arm against the first tube. Commands were programmed as same as line B set. Only flow rate was changed from 20 ml/min to 1 ml/min and load command was replaced waste command for injecting valve position. The column was connected between port 1 on the injection valve and the UV flow cell. Sample loop was connected by appropriated injecting volume selection and interest sample volume was programmed.

2.3.3 Purification step

To start purification, the command of application template was selected, then followed by selection of command for HiTrap IgM Purification. The processes of protein purification were displayed on monitor.

2.3.4 Dialysis

The fractions of purified MT4 mAb were assessed for protein concentration by measuring optical density at wavelength of 280 nm. The fractions were dialyzed overnight against PBS (pH 7.4). Protein concentration was re-determined by absorbance at 280 nm. The purified antibody was stored at –20°C.

2.4 Verify the activity and specificity of purified MT4 mAb

2.4.1 Indirect immunofluorescent assay

Peripheral blood mononuclear cells (PBMC) were prepared from anticoagulated blood by Ficoll-Hypaque gradient centrifugation techniques. Briefly, 10 ml of heparinized blood was diluted with 30 ml of PBS in 50 ml centrifuge tube, mixed throughly and over-layered on 5 ml of Ficoll-Hypaque solution, then centrifuged at 400xg for 30 minutes at 25°C. The PBMC layer was collected into a 15 ml centrifuge tube, washed twice with PBS and then resuspended in 4 ml of PBS-1%BSA-0.02% NaN_3 . The cells were counted using haemacytometer and adjusted to $1x10^7$ cells/ml with PBS-1%BSA- 0.02% NaN_3.

The indirect immunofluorescent staining and flow cytometry were performed to verify the activity and specificity of purified MT4 mAbs. To block nonspecific Fc receptor, PBMC were pre-incubated at 4°C for 30 minutes with 10% AB serum. After that, 50 µl of cells at concentration of $1x10^7$ cells/ml were incubated with MT4 mAb at concentrations 20 and 50 µg/ml at 4°C for 30 minutes. Cells were then washed twice with 1%BSA-PBS- 0.02% NaN_3, and stained with FITC-conjugated sheep-anti-mouse immunoglobulin antibody, then incubated at 4°C for 30 minutes in dark. The stained cells were washed three times with 1%BSA-PBS-NaN_3 and fixed in 350 µl of 1% paraformaldehyde. The stained cells were analyzed by FACSCalibur flow cytometer.

2.4.2 SDS-PAGE

The purity of purified MT4 mAb was tested by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The gel-set was first assembly. 12.5% of separating gel was prepared and loaded gently into the assembled gel casting, and let stand at room temperature for 1 hr. Then, 4% of stacking gel was prepared and loaded on the top of separating gel and stood at room temperature for 2 hrs. Samples were prepared by pipetting 10 μg of protein in PBS into a microtube. Then reducing or non-reducing buffer was added to obtain final concentration of 1X in the total volume of 30 μl. The mixture was heated at 90° C for 4 min. The treated samples were kept on ice until use. The comb was removed gently from the stacking gel and bubbles were removed from the well. Running buffer was prepared and filled up into the electrophoresis tank carefully. Treated samples were loaded gently with the same volume. Power supply was connected and turned on, then the current was adjusted to 500 mA per 1.5 mm thick gel and the voltage was adjusted to 120 V. Proteins were run about 2 hours until the dye reached the bottom of the gel, turned the power supply off and disconnect the power cables. Gel was then stained using Coomassie Brilliant Blue for 4 hrs with slowly shaking. To remove the bulk, the excess stain was destained using destaining solution I (40% methanol, 7% acetic acid) for 30 minutes with slowly shaking. Then, the destaining solution II (7% acetic acid, 5% methanol) was replaced until the background was clear. The immunoglobulin bands of heavy chain and light chain at about 50 and 25 kDa were observed, respectively.

2.5 Development of reagent for CD4+ T-lymphocyte enumeration

2.5.1. MT4 bead reagent preparation

The vial of M450 Epoxy magnetic bead solution was horizontal re-suspended. A interest volume of magnetic beads was pipetted into a tube and 0.1 M phosphate buffer, pH 7.4 (buffer A) was added. The mixture was mixed well using vertex mixture. Then, the tube was placed on a magnet particle concentrator (Dynal MPC) for 1 minute. Washing solution was pipetted off leaving beads undisturbed and re-washing the beads with the same solution for three times. The washed beads were resuspended in 0.5 ml buffer A and ready for use.

To coat the purified MT4 mAb directly onto the surface of the beads, the interested volume of washed M450 epoxy beads was pipetted. Then, 10 μg of purified MT4 mAb was added to 10^7 washed beads in 0.5 ml of final volume coating solution, then mixed it well. The tube was put on a rotator and incubated the mixture for 60 minutes at room temperature. After that, the bead surfaces were blocked with 1%BSA-PBS-0.02%NaN_3 by incubating overnight for about 16-20 hours at 4°C. Then, the coated beads were washed three times with PBS, pH 7.4. The coated bead concentration was adjusted to 10^7 beads/ml by 1%BSA-PBS-0.02% NaN_3. The coated beads, named MT4 beads, were stored at 2-8°C.

2.5.2 Checking the activity of coated bead

To check the activity of the MT4 beads reagent, $5x10^5$ beads were stained with 25μl of FITC-conjugated rabbit anti-mouse immunoglobulins by incubation for 30 minutes at room temperature in the dark. The solution was mixed every 10 minutes.

After that, stained beads were washed three times with 1%BSA-PBS-0.02% NaN_3 by centrifugation at 12,000 rpm for 1 minute. Then the stained beads were fixed in 1% paraformaldehyde, and analyzed under fluorescent microscope and FACScalibur flow cytometer.

2.6 Enumeration of CD4+ T lymphocytes in whole blood

2.6.1 The developed MT4 beads reagent

To enumerate the CD4+ T lymphocytes in whole blood, 200 µl of K_2EDTA-whole blood was incubated with 200 µl of MT_4 beads reagent for the test tube and 200 µl of PBS-0.5% NaN_3 for the control tube. Both tubes were slowly shaken on a rotator at room temperature for 30 minutes. The tubes used in this study were plastic micro tube (size 1.8 ml) with cap. After that, the test tube was placed on a magnetic particle concentrator for 5 minutes. CD4+ T lymphocytes, which bound with MT4 beads were adhered on the tube wall at the magnetic particle concentrator. Then, the blood was transferred to a new tube leaving bound cells-beads undisturbed. The diluted blood from the control tube was transferred to a new tube. The depleted and non-depleted CD4+ T lymphocytes blood were then counted for complete blood count using COULTER^R-MAXM hematology analyzer to obtain the absolute lymphocyte numbers. The absolute CD4+ T lymphocyte number was calculated by subtraction the absolute lymphocyte count of the control tube from the test tube.

2.6.2 Standard flow cytometric method

For each specimen, flow cytometry was performed with FACScalibur flow cytometer and hematological counts were done on a COULTER^{R}-MAXM hematology analyzer. Fifty microlite of K_2EDTA-whole blood were incubated with ten microliters of Simultest™ reagent panel in four separation tubes. The monoclonal antibodies of the Simultest™ reagent panel incubated with CD14-PE/-CD45-FITC, CD4-PE/CD3-FITC and CD8-PE/-CD3-FITC. Then, the mixtures were incubated in the dark for 30 minutes at room temperature. After incubation, 1 ml of 1XFACS™ lysing solution was added, mixed well, and left for 10 minutes in dark, centrifuged at 1700 rpm for 5 minutes, discarded supernatant and then cell pellet was washed with 2 ml of PBS-0.02% NaN_3. After that, stained cells were fixed with 500 µl of 1% paraformaldehyde in PBS. The stained cells were analyzed for CD4+ T lymphocytes with a flow cytometer using SimulSet software.

2.6.3 Validation of the developed method

To validate the developed method, blood samples obtained from normal subjects and HIV infected persons were used. CD4+ T lymphocytes from the blood samples were determined by using developed method (2.6.1) and standard flow cytometry (2.6.2). The obtained CD4+ lymphocytes were compared as was indicated in 2.7.

2.7 Data analysis

Several statistics analyses were performed for comparing the results obtained by the developed MT4 beads method and standard flow cytometry. The correlation coefficient between the two techniques was calculated and a model of regression then applied to the data. Further analysis using Bland Altman plots was also used to assess if there was any systematic bias between absolute counts derived from each method. The mean absolute difference (bias) and the limits of agreement at 95% confidential interval are calculated. The sensitivity was calculated from the results which CD4+ T lymphocyte counts fell below 200 or 350 cells/μl, while specificity was calculated from the results which CD4+ T lymphocyte counts were greater than 200 or 350 cells/ μl.