CHAPTER II

MATERIALS AND METHODS

2.1 Purification of immunoglobulins from pooled normal mouse serum

One milliliter of pooled normal mouse serum was centrifuged at 14,000 rpm 4°C for 10 minutes. Then, centrifuged serum was diluted with filtrated sodium phosphate buffer at ratio 1:4. For purification mouse immunoglobulins, Protein G Sepharose column was equilibrated with 50 ml filtrated sodium phosphate buffer and adjusted the solution to the bed volume. Then, 5 ml of the diluted mouse serum was added to the column. The un-bound materials were re-passed into column for 5 times. The unbound proteins were then washed out by washing the column with 50 ml filtrated sodium phosphate buffer. To elute antibodies from the column, 2 ml of elution buffer (0.1 M citric acid, pH 3.0) 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 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 filtrated sodium phosphate buffer and retained in storage buffer (PBS containing 0.05% NaN_3) and stored at 4°C.

The obtained eluates were concentrated by ultrafiltration in Vivaspin tube (MW cut off 30,000) and dialyzed against PBS overnight. The concentration of antibody was determined by measuring the absorbance at wavelength of 280 nm.

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2.2 Determination the purity of isolated mouse immunoglobulins with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The isolated mouse immunoglobulins were treated with non-reducing buffer and reducing buffer. The samples were boiled at 100°C for 5 minutes and kept on ice. The samples were analyzed by SDS-PAGE using 12.5% separating polyacrylamide gel. Then, gel was stained with Coomasie brilliant blue for 3 hours and destained with destain solution until the background was cleared. The molecular weights of proteins were determined by comparing with standard protein markers which were run in the same gel.

2.3 Determination of immunoglobulin isotypes

The isotype of isolated mouse immunoglobulins were determined by ELISA. Each well of the ELISA plate was coated with 50 µl of goat anti-mouse immunoglobulin isotypes (IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM) at diluted of 1:1000 in carbonate bicarbonate buffer (pH 9.6). After incubation at 4°C overnight, the plate was washed with 0.05% Tween 20 in PBS (PBS-Tween) 4 times. Then, 60 µl of PBS containing 2% bovine serum albumin (2% BSA-PBS) was added in each well and incubated at 37°C for 1 hour. After blocking step, 50 µl of 50 µg/ml isolated mouse immunoglobulins was applied and incubated at 37°C for 1 hour. Then, the plate was washed with PBS-Tween 4 times. The antigen-antibody complexes were monitored by adding 50 µl of horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulins at dilution 1:2000 and incubated at 37°C for 1 hour. After washing 4 times with PBS-Tween, 50 µl of

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orthophenylene diamine containing hydrogen peroxide (OPD-H_2O_2) substrate was added to each well and incubated at room temperature in the dark for 15-30 minutes. The reaction was stopped by adding 50 µl of 4N H_2SO_4 and the absorbance was determined by ELISA reader at 490 nm.

2.4 Immunization

Five-month-old egg laying Rosehorn hens, weighing approximately 1.5 kg, were kept in individual cages with free food and water throughout the time course of the study. Before immunization, blood and eggs were collected. For blood collection, 2 ml of blood were collected form wing vein using needle no 25. Obtained blood were let stand at room temperature for 1-2 hours. Sera were obtained from the blood by centrifugation at 13000 g for 3-5 minutes and stored at –20°C.

For immunization, at day 0, the first hen was injected *via* the pectoral muscle with 500 µg of mouse immunoglobulins in complete Freund's adjuvant. The second hen was injected *via* calf muscle with the same dose of immunoglobulins. Booster doses with 500 µg of mouse immunoglobulins in incomplete Freund's adjuvant were given twice, at days 14 and 28, after the first injection. For both hens, eggs and blood were collected every one and two weeks after first immunization, respectively.

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2.5 Extraction of IgY from egg yolk by water dilution and salt precipitation method

Egg yolk was manually separated from white egg and washed thoroughly in distilled water. After that, the yolk membrane was punctured to collect its contents. Then, 5 ml of yolk was diluted with 9 volume of distilled water and mixed thoroughly. After kept at 4°C overnight, the yolk mixture was separated into 2 parts. Thirty milliliters of upper solution was collected and centrifuged at 4°C, 9000 g for 30 minutes. The supernatant was collected. Na_2SO_4 3.8 g was slowly added to 20 ml of supernatant. After 90 minutes stirring at room temperature, the precipitate containing IgY was pelleted by centrifugation at 4°C, 9000 g for 30 minutes. The precipitate was resuspended with 2 ml deionized distilled water and dialyzed against PBS at 4°C for overnight. The concentration of IgY was determined by measuring the absorbance at wavelength of 280 nm. Concentration of the isolated IgY could be obtained by the formula; 1.33 OD at 280 nm is equivalent to 1 mg/ml of IgY. The isolated IgY was adjusted to 1 mg/ml and stored at –20°C.

2.6 Determination the purity of chicken IgY by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

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Chicken IgY was diluted with non-reducing buffer and reducing buffer. The samples were boiled at 100°C for 5 minutes and kept on ice. The samples were subjected for SDS-PAGE using 12.5% separating gel. Then, gel was stained with Coomasie brilliant blue for 3 hours and destained with destain solution until the background was cleared.

The molecular weights of proteins were determined by comparing with standard protein markers.

2.7 Indirect ELISA for measurement of chicken anti-mouse immunoglobulins in serum and egg yolk

2.7.1 Titration of concentration of mouse immunoglobulins antigen for ELISA assay

The optimal concentration of mouse immunoglobulins for using as antigen for determination of antibody by ELISA was determined as follows. Each well of the ELISA plate was coated with 100 μl of mouse immunoglobulins at various concentrations ranging between 2.5 to 0.15625 μg/ml. After incubation at 4°C overnight, the plate was washed with PBS-Tween for 4 times. Plate were blocked with 2%BSA-PBS. Then, 100 μl of various dilutions of chicken sera, which obtained from pre-immunized and 6 weeks after immunization with mouse immunoglobulins, were applied and incubated at 37°C for 1 hour. Then, the plate was washed with PBS-Tween for 4 times. The antigen-antibody complexes were monitored by adding 100 μl of HRP conjugated rabbit anti-chicken immunoglobulins at dilution 1:4000. Then, the plate was incubated at 37°C for 1 hour. After washing 4 times with PBS-Tween, 100 μl of OPD-H_2O_2 substrate was added to each well and incubated at room temperature in the dark. The reaction was stopped by adding 100 μl of 4N H_2SO_4 and the absorbance was determined by ELISA reader at 490 nm.

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2.7.2 Titration of HRP conjugated **rabbit anti-chicken IgG**

The optimal concentration of HRP conjugated rabbit anti-chicken IgG was determined as follows. Each well of the ELISA plate was coated with 100 µl of mouse immunoglobulins at optimal concentration (from 2.7.1). After incubation at 4 °C overnight, the plate was washed with PBS-Tween for 4 times. Then, plate was blocked with 2%BSA-PBS. Then, 100 µl of various dilutions of chicken anti-mouse immunoglobulins sera were applied and incubated at 37°C for 1 hour. Plate was washed with PBS-Tween for 4 times. One hundred microlitters of various dilutions of HRP conjugated rabbit anti-chicken IgG were added. Plate was then incubated at 37°C for 1 hour. After washing 4 times with PBS-Tween, 100 µl of OPD-H_2O_2 substrate was added to each well and incubated at room temperature in the dark. The reaction was stopped by adding 100 µl of 4N H_2SO_4 and the absorbance was determined by ELISA reader at 490 nm.

2.8 Fluorescein isothiocyanate (FITC) labeling of chicken anti-mouse immunoglobulins antibodies

One milligram of chicken anti-mouse immunoglobulins was resuspended in 0.1 M $NaHCO_3$, pH 9.0 and mixed with different volumes of fresh prepared 10 mg/ml FITC in DMSO. The volume of FITC was calculated from the following equation:

Volume (ml) of 10 mg/ml FITC =

[(mg antibody x 0.1) / MW of antibody] x (R x MW of FITC)

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Where R = molar incubation ratio of dye:antibody; MW of antibody = 180,000 Da; MW of FITC = 389.4 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.

2.9 Determination the activity of FITC labeled chicken anti-mouse immunoglobulins

U937 cell line was adjusted to $1x10^7$ cells/ml in PBS containing 1%BSA and 0.02% NaN_3 (1% BSA-PBS-NaN_3). To block Fc receptor, human AB serum was added to the cell suspension at final dilution of 1:10 and incubated on ice for 30 minutes. After that, 50 µl of blocked cells were added to 50 µl of 20 µg/ml of various isotypes of CD147 mAbs. After incubation on ice for 30 minutes, cells were washed twice with 1% BSA-PBS-NaN_3 and resuspended with 40 µl of 1% BSA-PBS- NaN_3. Then, 50 µl of 100 µg/ml FITC conjugated chicken anti-mouse immunoglobulins (from 2.8) were added. After incubation on ice for 30 minutes, cells were washed with 1% BSA-PBS-NaN_3 3 times and were fixed with 350 µl of 1% paraformaldehyde in PBS. The stained cells were analyzed by flow cytometry.

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2.10 Titration of FITC conjugated chicken anti-mouse immunoglobulins antibodies for using in indirect immunofluorescence assay.

Cell samples were adjusted to $1x10^7$ cells/ml in 1% BSA-PBS-NaN_3. To block Fc receptor, human AB serum was added to the cell suspension at final dilution of 1:10 and incubated on ice for 30 minutes. After that, 50 µl of blocked cells were added to 50 µl of 20 µg/ml mAbs specific to various proteins on cell surfaces. The mAbs used in this experiment were various isotypes, i.e., IgG1 (OKT3), IgG2a (M6-1E9), IgG2b (MT14/2), IgG3 (M6-1B9) and IgM (M6-1D4). After incubation on ice for 30 minutes, cells were washed twice with 1% BSA-PBS-NaN_3 and resuspended with 40 µl of 1% BSA-PBS-NaN_3. Then, 50 µl of various concentration of FITC conjugated chicken anti-mouse immunoglobulins were added. After incubation on ice for 30 minutes, cells were washed with 1% BSA-PBS-NaN_3 3 times and were fixed with 350 µl of 1% paraformaldehyde in PBS. Then, the stained cells were analyzed by flow cytometry.

2.11 Determination of leukocyte sub-populations by using FITC conjugated chicken anti-mouse immunoglobulins

Fifty microlitters of EDTA-whole blood were added to 50 µl of 20 µg/ml various mAbs specific to various proteins on leukocyte surface. The mAb used including OKT3 (CD3), MT14/2(CD14), VIMD5(CD15) and MEM-97(CD20). Cells were then incubated on ice for 30 minutes. After incubation, cells were washed twice with 1% BSA-PBS-NaN_3. Then, cells were resuspended with 40 µl of 1% BSA-PBS-NaN_3 followed by

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addition of 50 μl of 500 μg/ml FITC conjugated chicken anti-mouse immunoglobulins . After incubation on ice for 30 minutes, 1 ml of FACS lysing solution was added into cell suspension and incubated at room temperature for 10 minutes. Cells were washed with 1%BSA-PBS-NaN_3 2 times. Then, cells were fixed with 350 μl of 1% paraformaldehyde in PBS and analyzed by flow cytometry using CellQuest software.

2.12 Determination of recombinant protein expression on COS cells using FITC conjugated chicken anti-mouse immunoglobulins antibodies

COS cells were transfected with cDNA encoding CD147 (M6-DNA) and CD14 (CD14-DNA) by DEAE-Dextran transfection technique. Fifty micolitters of $1 x 10^7$ cells/ml of transfected COS cells were added into 50 μl of 20 μg/ml of CD147 mAbs (M6-1D4 and M6-1E9, M6-1B9) and CD14 mAbs (MT14/3 and MT14/2). Cells were incubated on ice for 30 minutes. After that, 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 500 μg/ml FITC conjugated chicken anti-mouse immunoglobulins were added and incubated on ice for 30 minutes. After incubation, cells were washed with 1%BSA-PBS-NaN_3 3 times. Then, cells were resuspended with 1% BSA-PBS-NaN_3 20 μl and analyzed by a fluorescence microscope.

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