CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals, antibodies, cell lines and instruments used in this study are shown in Appendix A-C.

2.2 Mycoplasma Detection

The aim of this study is to produce conditioned media for supporting hybridoma growth. Two cell lines, mouse myeloma and BW5147 mouse thymoma cells, were used in this study. To determine whether the cell lines used are contaminated with mycoplasma, nested PCR was performed as follows.

2.2.1 DNA preparation

The genomic DNA was isolated from mouse myeloma and BW5147 mouse thymoma using standard phenol-chloroform technique as described follows. $1x10^6$ cells were centrifuged at 550 g for 5 minutes with sterile phosphate buffered saline (PBS) for 3 times. Five hundred microlitres of TE 20-5 buffer was added and vigorously mixed. After that 25 µl of 10%SDS and 25 µl of proteinase K (2 mg/ml) were added and vigorously mixed. The mixture was incubated in 37°C water bath for overnight. After cell lysis, 300 µl of phenol and 300 µl of choloform-isoamyl alcohol were added. The solution was gently mixed by inverting the tube and then centrifuged at 12,000 rpm for 10 min. The lower layer containing organic solution was discarded and phenol/chloroform extraction was repeated. Then, 600 µl of choloform-isoamyl alcohol were added. The solution was gently mixed by inverting the tube and then

centrifuged at 12,000 rpm for 10 min. The lower layer containing organic solution was discarded and chloroform extraction was repeated. The DNA was precipitated by adding 45 µl of 4M NaCl and 900 µl of cold absolute ethanol. Then, DNA was centrifuged at 12,000 rpm for 10 min and washed with cold 70% ethanol. The DNA pellet was air dried. After that, 100-200 µl ddH_2O was added to dissolve the DNA pellet and the amount of DNA was determined by measuring the absorbance at wavelength of 260 nm. The DNA concentration was calculated by the following equation:

1 OD260/ml = 50 ng/µl genomic DNA

Concentration of DNA = O.D.260 x 50 ng/µl genomic DNA x dilution factor

2.2.2 Detection of mycoplasma contamination by PCR

In this experiment, the mycoplasma DNA was detected by using two primers, Ito-Myco F1 (5' ACA CCA TGG GAG CTG GTA AT 3') and Ito-Myco R1 (5' CTT CWT CGA CTT YCA GAC CCA AGG CAT 3'). The amplification reaction mixture was composed as shown in Table 2.1. The PCR cycling condition starts as one cycle at 94°C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. After 35 amplification cycles, the mixtures were incubated at 72°C for 5 minutes. The amplified product was then analyzed by 1.5 % agarose gel electrophoresis. Briefly, the amplified products were loaded in 1.5% agarose gel and separated at 100 volt. In order to visualize the DNA in the agarose gel, the gel was stained with 1% ethidium bromide (EtBr) for 20 minutes. Then the gel was destained with distilled water for 5 minutes. Consequently, the DNA bands were observed by UV transilluminator.

Table 2.1 The reaction mixture of mycoplasma DNA detection

Reaction mixture	**For 1 tube (µl)**
25 mM $MgCl_2$	2.5 µl
10X Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 µM Ito-Myco F1	0.5 µl
10 µM Ito-Myco R1	0.5 µl
Distilled water	13.4 µl
5 U/µl Taq DNA polymerase	0.1 µl
50 ng/µl DNA template	5 µl
Total volume	25 µl

2.3 Preparation of conditioned media

To produce conditioned media, two cell lines, mouse myeloma and BW5147 mouse thymoma cells, were used. $1x10^7$ cells of the cell lines were cultured in 10 ml of Iscove's Modified Dulbeco's Medium (IMDM) containing 10% fetal calf serum (FCS), gentamycin 40 mg/ml and fungizone 5 mg/ml (10% FCS-IMDM) at 37°C in a 5% CO_2 incubator in the presence or absence of 10 ng/ml phorbol myristate acetate (PMA). After 18 or 40 hours of cultivation, the culture supernatants were harvested by centrifugation at 550 g for 5 minutes and filtrated using 0.1 μm membrane filter. The culture supernatants were aliquot and stored at -20°C until used.

2.4 Study the utilization of conditioned media for hybridoma single cell cloning

2.4.1 Single cell cloning using stable hybridoma line

To study the use of the produced conditioned media in the single cloning of hybridomas, the limiting dilution was carried out. A stable hybridoma line, Thal-N/B, were counted and cell concentration was adjusted to 4 cells, 2 cells and 1 cell per 150 μl in 10% FCS-IMDM, 10% FCS-IMDM supplemented with vary concentrations of the produced conditioned media or 10% FCS-IMDM supplemented with 10% BM condimed H1 commercially condition medium. Then, 150 μl of each cell suspension were added in to 96-well tissue culture plate. The cells were cultured at 37°C in a 5% CO_2 incubator. After 5, 7, 10 and 15 days of cultivation, hybridoma clone numbers and size were determined under an inverted microscope**.** The clone size was scored into four levels, i.e. very small (VS), small (S), medium (M) and large (L) as described below.

Very small	clone size less than 1 in 8 of well
Small	clone size approximately 1 in 8 of well
Medium	clone size approximately 1 in 4 of well
Large	clone size more than 1 in 4 of well

To determine the appropriate conditioned medium source for supporting hybridoma growth, the number and size of single hybridoma clones were compared between the produced conditioned media.

To determine the optimal concentration of produced conditioned medium for supporting hybridoma growth, the number and size of single clones were compared between media supplemented with 10%, 20%, and 50% of conditioned medium.

2.4.2 Single cell cloning using unstable hybridoma line

To confirm that the produced conditioned medium could be used in single cell cloning of unstable hybridoma lines, cell fusion was performed and obtained hybridomas were used for this purposed. The procedure of hybridomas production will be described in 2.4.3. The number and sizes of single hyrbidoma clones were compared between using commercial BM condimed H1 and the produced conditioned medium. In addition, the antibody activity of the generated hybridoma clone using BM condimed H1 and conditioned medium was also compared.

2.4.3 Generation of hybridomas by immunoprecipitated bead immunization.

2.4.3.1 Preparation of immunoprecipitated beads

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. Briefly, whole

blood was diluted with an equal volume of phosphate buffered saline (PBS) in 50 ml centrifuge tube. Then, the diluted whole blood were overlayered on 10 ml of Ficoll-Hypaque solution and centrifuged at 400 g, 25°C for 30 minutes. The PBMCs were collected and washed three times using PBS. The PBMCs ($1x10^8$ cells) were lysed on ice for 30 minutes in 1 ml of lysis buffer (50 mM Tris-HCl pH 8.2, 100 mM NaCl, 2 mM EDTA, 0.02% NaN_3) containing 1% NP-40 as detergent and protease inhibitors (1mM phenylmethyl-sulphonylfluoride (PMSF), 5 mM iodoacetamide, 10 µg/ ml aprotinin). The clarified cell lysates were collected by centrifugation at 12,000 g, 4°C for 30 minutes. Then, cell lysates were precleared by adding sepharose 4B beads coated with normal mouse serum and rotated at 4°C for overnight. The precleared cell lysates were collected by centrifugation at 12,000 g, 4°C for 5 minutes. One milliliter of precleared cell lysates were added to OKT3 anti-CD3 mAb-coated sepharose beads and rotated at 4°C overnight. The OKT3 immunoprecipitated beads were washed with sterile PBS 10 times and resuspened in 500 µl of sterile PBS. The precipitated beads were then used as the immunogen for mouse immunization.

2.4.3.2 Mouse immunization with OKT3 immunoprecipitated beads

A BALB/c mouse was used in this study. Blood was collected by tail-bleeding for using as pre-immunized serum. The mouse was intraperitoneally immunized with OKT3 immunoprecipitated beads in 500 µl of sterile PBS at one-week intervals. Two weeks after the third immunization, mouse serum was collected and stored at -20°C for antibody detection.

2.4.3.3 Determination of antibody response in the immunized mouse by indirect immunofluorescence staining

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. PBMCs were adjusted to 1 x 10^7 cells/ml in PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide (1%BSA-PBS-0.02%NaN_3). PBMCs were incubated at 4°C for 30 minutes with 10% AB serum for blocking of Fc receptor. Fifty microliters of the blocked cells were incubated with 50 µl of various dilutions of immunized mouse sera at 4°C for 30 minutes. After twice washing with 1%BSA-PBS-0.02%NaN_3, the cells were resuspended with 20 µl of 1%BSA-PBS-0.02%NaN_3. Then, 25 µl of sheep anti-mouse immunoglobulins-FITC were added into the cell suspension and incubated on ice for 30 minutes. Finally, cells were washed three times with 1% BSA-PBS-0.02%NaN_3 and fixed with PBS containing 1% paraformaldehyde. The cell suspensions were then analyzed by a flow cytometer.

2.4.3.4 Hybridoma technique

2.4.3.4.1 Preparation of spleen cells

The BALB/c mouse that produce the specific antibody response was boosted intraperitoneally (IP) with OKT3 immunoprecipitated beads in 500 µl of sterile PBS. Five days after boosting, the mouse was sacrificed. The spleen was aseptically removed and place in a 6-cm tissue culture dish containing 5 ml of IMDM medium. Spleen cells were isolated by homogenizing carefully. The cell suspensions were transferred to 50 ml centrifuge tube and let the cell debris settle down for approximately 10 minutes. Then, cell suspensions were collected and centrifuged at 550 g for 5 minutes. The supernatant were discarded. Red blood cells were lysed by 0.83% NH_4Cl hypotonic solution. The obtained splenocytes were washed twice with

IMDM medium. The number and viability of the spleen cells were counted in a hemacytometer using Turk's solution and 0.2% trypan blue.

2.4.3.4.2 Preparation of myeloma cells

Myeloma cells were collected from culture flasks and washed twice with IMDM medium. The number and viability of the myeloma cells were counted in a hemacytometer using 0.2% trypan blue.

2.4.3.4.3 Cell fusion

The splenocytes were fused with mouse myeloma cells using 50% polyethelene glycol (PEG) by standard hybridoma techniques. Briefly, mixture of splenocytes and myeloma cells at the ratio 2:1 was room temperature centrifuged at 300 g for 10 minutes. The supernatant were removed and the cells pellet was mixed and warmed at 37°C for 5 minutes. The fusion procedure was started by dropping 1.5 ml of 50% PEG into the cell mixture within 1 minute and gentle stirring for another 1 minute. Then, the cell mixture was suddenly diluted with IMDM medium by adding the medium and mixed throughly following from 1 ml within 1 minute, 3 ml within 1 minute and 16 ml within 2 minutes. The fused cells were then centrifuged at room temperature at 300 g for 5 minutes and warmed at 37°C for 5 minutes. After the supernatant was removed, the fused cells were resuspended in 100 ml HAT selection medium supplemented with 10% BM condimed H1. One hundred microliters of the cell suspension were seeded into each well of 96-well plate and cultivated at 37°C in a 5% CO_2 incubator. After five days of cultivation, 150 µl of HT medium supplemented with 10% BM condimed H1 were added into each well. The plates were incubated at 37°C in a 5% CO_2 incubator to expand the cells. The hybridomas were monitored by an inverted light microscope.

2.4.3.4.4 Screening of hybridomas by indirect immunofluorescence staining

The culture supernatant from the wells containing hybridoma cells were collected and screened for antibody reactivity against PBMCs using indirect immunofluorescence staining as was described in 2.4.3.3.

2.4.3.4.5 Single cell cloning

The hybridoma clones those were positive with lymphocyte subpopulation were selected for single cell cloning as described in 2.4.1. In this study, the number and sizes of single clones were compared between using commercial BM condimed H1 and the produced conditioned medium. The antibody activity produced by hybridoma cultured in both commercial BM condimed H1 and conditioned medium were determined by indirect immunofluorescence staining as was described in 2.4.3.3 and compared.

2.5 Study the utilization of conditioned media for generation of hybridomas by hybridoma technique

2.5.1 Preparation of Hb Portland and Hb A_2

2.5.1.1 Preparation of hemolysates

EDTA blood was centrifuged at 400 g for 5 minutes at room temperature. The plasma was discarded and red blood cells (RBCs) were washed with 0.9% NaCl isotonic solution for 3 times. Then, packed RBCs were lysed by adding an equal volume of distilled water and mixed vigorously to release Hbs. After that, the organic carbon tetrachloride (CCl_4) was added to a half volume of the mixture and mixed for 5 minutes. The mixture was centrifuged at 2,000 g for 10 minutes at room temperature.

Then, the upper layer containing hemolysate was collected and stored at -20°C until used.

In this study, Bart's hydrops fetalis' blood was used to prepare Hb Portland and normal blood was used to prepare Hb A_2.

2.5.1.2 Preparation of purified Hb Portland

In order to prepare the purified Hb Portland, Hb Portland was separated from the hemolysate of hemoglobin Bart's hydrops fetalis by cellulose acetate electrophoresis with an alkaline buffer solution. By this technique, the cellulose acetate membrane 60 x 76 mm was soaked in Tris-Borate-EDTA (TBE) buffer pH 8.6 for 3-5 minutes. Then, the sample applicator was used to apply the hemoglobin Bart's hydrops fetalis hemolysate on the membrane and placing on the electrophoresis chamber by presenting of the hemolysate on the cathode side. The electrophoresis was performed at 160 V until the band of Hb Bart's was clearly separated from Hb Portland. After electrophoresis, the bands of those two hemoglobins were cut apart from each other. Hb Portland was eluted from the membrane by soaked in 40 ml of phosphate buffer saline (PBS) pH 7.2 at 4°C overnight. Then, Hb Portland was concentrated by ultrafiltration using Vivaspin.

2.5.1.3 Preparation of purified Hb A_2

The hemolysates were prepared from normal adult blood. The hemolysates were centrifuged at 14,000 rpm at 4°C for 10 minutes to eliminate the unfavorable protein debris. Then, the supernatants were dialyzed with the binding buffer (Tris-HCl-KCN or THK pH 9.0) for overnight. The C10/10 column which contains DEAE Sepharose beads was equilibrated with THK buffer pH 9.0 for 2-3 column volumes at room temperature. Then, hemolysates were added into the column. The hemolysate

was eluted by increasing one percent in every 6 minutes of THK buffer pH 6.5 from 15-100%. The Hb A_2 fractions were collected by AKTA prime fraction collector.

2.5.2 Mouse immunization with Hb Portland and Hb A_2

BALB/c mice were used in this study. Blood were firstly collected from the mice by tail-bleeding for using as pre-immunized serum. One hundred micrograms of purified Hb Portland or Hb A_2 in 300 µl sterile PBS mixing with 200 µl complete Freund's adjuvant were IP immunized in each mouse. The immunizations were repeated every 2 weeks. For the second immunization, incomplete Freund's adjuvant was used in stead of complete Freund's adjuvant. At the third immunization, the Hbs were mixed with sterile PBS and immunized without adjuvant. Two weeks after the third immunization, mice sera were collected and stored at -20°C until used.

2.5.3 Determination of antibody response in the immunized mice by indirect ELISA

Antibodies against Hb Portland and Hb A_2 in mice sera were determined by indirect ELISA. Briefly, 20 µg/ml of purified Hb Portland or Hb A_2 were coated into 96-well ELISA plate using carbonate/bicarbonate coating buffer pH 9.6 at 50 µl/well and incubated at 4°C for overnight. After that the plate was washed with PBS containing 0.05% Tween20 (0.05% Tween-PBS) for 4 times and blocked with 60 µl of 2%BSA-PBS at 37°C for 1 hour. The plate was washed once with 0.05% Tween 20-PBS. Mice sera were diluted to various dilutions and 50 µl of the diluted sera was added to each well and incubated at 37°C for 1 hour. The plate was washed with 0.05% Tween 20-PBS for 4 times and 50 µl of horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody at dilution 1:2,000 were added to each well and incubated at 37°C for 1 hour. Thereafter, the plate was washed with 0.05%

Tween-PBS for 4 times and 50 µl of tetramethylbenzidine (TMB) substrate was added into each well. After incubation in dark at room temperature for 20 minutes, the reaction was stopped by adding 50 µl of 1N HCl and measured the absorbance at 450 nm.

2.5.4 Hybridoma technique

2.5.4.1 Preparation of spleen cells

The BALB/c mice that appeared to produce the specific antibody response were boosted IP with 100 µg of the Hb Portland or Hb A_2. Five days after boosting, immunized mouse was sacrificed. The spleen cells were prepared as described in 2.4.3.4.1.

2.5.4.2 Preparation of myeloma cells

Myeloma cells were prepared as described in 2.4.3.4.2..

2.5.4.3 Preparation of HAT and HT medium

In this study, two types of HAT and HT medium were used. The first media is the medium that supplemented with 10% BM condimed H1 and another is the medium that supplemented with 20% of the produced conditioned medium. The BM condimed H1 was the standard conditioned medium.

2.5.4.4 Cell fusion

The splenocytes were fused with mouse myeloma cells using 50% (PEG) by standard hybridoma techniques as described in 2.4.3.4.3, in contrast, the fused cells were resuspended in 50 ml HAT selection medium supplemented with 10% BM condimed H1 and 50 ml HAT selection medium supplemented with 20% produced conditioned medium. One hundred microliters of the cell suspension were seeded into each well of 96-well plate and cultivated at 37°C in a 5% CO_2 incubator. After five

days of cultivation, 150 µl of HT medium supplemented with BM condimed H1 and HT selection medium supplemented with produced conditioned media were added into each well. The plates were incubated at 37°C in a 5% CO_2 incubator to expand the cells. The hybridomas were monitored by an inverted light microscope.

2.5.5 Screening of hybridomas by ELISA

The culture supernatant from the wells containing hybridoma cells were collected and screened for antibody reactivity against Hb Portland or Hb A_2 using ELISA as was described in 2.5.3. In this study, the number of hybridomas and number of antibody positive wells were compared between media supplemented with BM condimed H1 and produced conditioned medium. The clones those were positive with purified Hb Portland or Hb A_2 were selected for single cell cloning by limiting dilution.

2.5.6 Single cell cloning

The hybridoma clones those were positive with purified Hb Portland or Hb A_2 were selected for single cell cloning as described in 2.4.1. The number and sizes of single clones were compared between medium supplemented with BM condimed H1 and produced conditioned medium. The antibody activity was also determined by indirect ELISA and compared between medium supplemented with BM condimed H1 and produced conditioned media.

2.6 Analysis of protein in the produced conditioned medium by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The produced conditioned medium, BM condimed H1, and 10% FCS-IMDM were serial two-fold diluted into 1:2, 1:4, and 1:8. The diluted samples were boiled in

50 µl of reducing SDS-PAGE sample buffer for 5 minutes. The proteins were separated by SDS-PAGE using 12.5%, 10% and 7.5% separating gel and 4% stacking gel at 120 V (constant volt). After electrophoresis, the gels were stained with Coomassie Blue.