CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Human blood samples

Blood samples of normal adult, normal cord blood, Hb Bart's hydrops fetalis, heterozygote Hb E were kindly provided by Asst. Prof. Dr. Thanusak Tatu, the Hematology and Health Technology Research Center, Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University.

2.1.2 Animal blood samples

Blood samples of dog and cat were kindly provided by D.V.M. Adul Saengthong, the Small animal hospital, Faculty of Veterinarian medicine, Chiang Mai University. Pig, cow and buffalo blood samples were purchased from Tonpayom market. Chicken blood was kindly provided by Mr. Apichai Khamrin, Faculty of Agriculture, Chiang Mai University.

2.1.3 Mouse

Female BALB/c mice used in this study were purchased from the National Laboratory Animal Center, Mahidol University. The mice were maintained under air condition at 25^{o}C in individual cage.

2.1.4 Cell fusions

X63-Ag8.653 myeloma cells were used for hybridoma cell production. The myeloma cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal calf serum (FCS) contained 40 mg/L Gentamicin and 2.5 mg/L Fungizone. The cell cultures were maintained in fully humidified atmosphere at 37^{o}C with 5% CO_2.

2.1.5 Chemicals

All chemicals used in this study were indicated in Appendix A

2.2 Methods

2.2.1 Preparation of hemolysates for monoclonal antibody production

Blood sample were collected using EDTA as an anticoagulant. Blood were centrifuged at 400g for 5 min 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 min by hand. The mixture was centrifuged at 2,000g for 10 min at room temperature. Thereafter, the red solution of hemolysate was collected and aliquot at 1 ml/tube. The obtained hemolysates were stored at -20^{o}C until used.

To measure the concentration of hemoglobins in the hemolysate, 20 μl of hemolysate was mixed with 5 ml Drabkin's solution and left stood at room temperature for 10 min. The concentration of hemoglobin was determined by

measuring the percent transmittance at 540 nm and compared to the standard calibration curve.

2.2.2 Purification of hemoglobins

2.2.2.1 Purification of Hb Bart's and Hb Portland

In order to prepare the purified Hb Bart's and Hb Portland, Hb Bart's and Hb Portland were separated from the hemolysate of Hb Bart's hydrops fetalis by cellulose acetate electrophoresis with an alkaline buffer solution. At the alkaline pH, all Hbs were negatively charged. The Hb Bart's and Hb Portland were separated from each other on the cellulose acetate membrane according to their net charge and size. By this technique, Hb Bart's hydrops fetalis hemolysate were added into sample well. The cellulose acetate membrane 60 x 76 mm was soaked in Tris-Borate-EDTA (TBE) buffer pH 8.6 for 3-5 min. Then the sample applicator was used to apply the hemolysate on the membrane and placing on the electrophoresis chamber by presenting 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. Each Hb was eluted from the membrane by soaked in 40 ml of phosphate buffer saline (PBS) pH 7.2 at 4^{o}C overnight. The obtained Hbs was concentrated by Vivaspin and measured the concentration as was described in 2.2.3

2.2.2.2 Purification of hemoglobins A, A_2, E and F

The hemolysates were prepared from normal adult (A_2A), normal cord blood (AF) and adult heterozygous beta thalassemia with hemoglobin E (AE) as was described in 2.2.1. The hemolysates were centrifuged at 14,000 rpm at 4^{o}C for 10 min 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 3-4 ml of hemolysates was added into the column. Changing of pH gradient by using the eluting buffer (THK buffer pH 6.5) caused the separation of different Hbs according to their net charge. The pH gradients were performed by increasing the percent of eluting buffer as shown in table 2.1. Normal adult hemolysate was eluted by increasing one percent in every six min of THK buffer pH 6.5 from 15-100%. Cord blood and Hb E heterozygote hemolysates were eluted by increasing one percent in every five min of the eluting buffer. The effluent Hb fractions were measured at 280 nm and collected by AKTA prime fraction collector. The concentration of Hbs was measured as described in 2.2.3.

Table 2.1. Eluting buffer gradients for each hemolysate

Hemolysate	THK buffer pH 6.5 gradients
• Normal adult (A_2A)	6 min/ 1% (15-100% within 510 min)
• Cord blood (FA)	6 min/ 1% (15-100% within 510 min)
• Hb E heterozygote (AE)	5 min/1% (15-100% within 425 min)

2.2.3 Measurement of isolated hemoglobin concentration

The purified Hbs obtained from both cellulose acetate membrane elution and column chromatography had very low concentration that unsuitable for storage and using in further experiments. Vivaspin concentrators were used in this study for concentration of hemoglobin. Vivaspin column was pre-wetted by adding of sterile distilled water and centrifuged at 2000g at 4°C for 10 min. Then the obtained Hbs were added into the upper chamber of the column and centrifuged at 4,000g at 4°C for 15 min. After centrifugation, buffered solution and other smaller molecules were passed through the column while the Hbs were retained in the upper chamber. The solution in the lower chamber was removed. The concentration of Hbs could be increased by adding more purified Hb and centrifugation cycles. The concentration of Hbs was measured by cyanmethemoglobinometry. The concentrations of Hbs were calculated from the following equation (Braunitzer *et al*., 1964).

$$\textbf{Hb concentration (mg/ml)} = \frac{\textbf{OD}_{540} \textbf{ x 64458 x Dilution factor}}{\textbf{44 x d x 1000}}$$

Where:

64458 = the relative molecular mass of the Hb tetramer
44 = ε^{540}, the millimolar absorbtivity of cyanmethemoglobin
d = Cuvette lightpath (cm), usually 1.000 cm.

2.2.4 Identification of hemoglobin by cellulose acetate electrophoresis

The cellulose acetate electrophoresis was used to confirm the types of blood samples and purity of the purified Hbs. The migration distances of the Hbs are according to their individual net charge and size. Ten microlitters of hemolysates or purified Hbs were added into the sample well of applicator. Then the cellulose acetate membrane was soaked in TBE buffer pH 8.6 for 3-5 min. The samples were applied onto the cellulose acetate membrane by applicator. After that the membrane was placed on the electrophoresis chamber by presenting the samples on the cathode side. The electrophoresis was performed at 160 V for approximately 2.30 hr. To visualize hemoglobin bands, the membrane was stained with Ponceau S for 5 min and destained with 5% acetic acid. For long term storage, the membrane was fixed in methanol and clearing solution. After that the membrane was dried by hair dryer.

2.2.5 Production of monoclonal antibodies

2.2.5.1 Mouse immunization with Hb Bart's hydrops fetalis hemolysate

Two female BALB/c mice at 6 weeks of ages, A and B, were used in this study. Blood were collected by tail-bleeding as pre-immunized serum. Mice were immunized by intraperitoneal route (IP) with 100 μg Hb Bart's hydrops fetalis hemolysate in 500 μl sterile PBS. The immunizations were repeated every 2 weeks. Blood were collected very 2 weeks after each immunization. Sera were isolated from the collected blood and keep at -20^{o}C until use.

2.2.5.2 Mouse immunization with purified hemoglobin Portland

A 6 week-age BALB/c mouse was used in this experiment. Blood was collected by tail-bleeding for using as pre-immunized serum. 100 μg purified Hb Portland in 300 μl sterile PBS mixing with 200 μl complete Freund's adjuvant were IP immunized in the 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 Hb Portland was mixed with sterile PBS and immunized without adjuvant. Two weeks after the third immunization, mouse serum was collected. Sera were isolated from the collected blood and keep at -20^{o}C until used.

2.2.5.3 Indirect ELISA for detection of polyclonal antibodies against hemoglobins

Twenty μg/ml of Hb Bart's hydrops fetalis hemolysate or purified Hb Portland were coated onto 96 well ELISA plate using carbonate/bicarbonate coating buffer pH 9.6 at 50 μl/well and incubated at 4^{o}C for overnight. After that the plate were washed with 0.05% Tween-PBS for 4 times and blocked with 60 μl/well of 2%BSA-PBS at 37^{o}C for 1 hr. The plate was washed once with 0.05% Tween-PBS. Mouse sera were diluted to various dilutions and 50 μl of the diluted sera were added to each well. After incubation at 37^{o}C for 1 hr, plates were washed with 0.05% Tween-PBS for 4 times and traced for antibody binding by adding 50 μl/well horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody at dilution 1:2,000 and incubated at 37^{o}C for 1 hr. Thereafter, the plates were washed

with 0.05% Tween-PBS for 4 times and 50 µl of 3,3' ,5,5'-tetramethyl benzidine (TMB) substrate was added into each well. After incubated in dark at room temperature for 20 minutes, the reaction was stopped with 1M HCl at 50 µl/well and measured the absorbance at 450 nm.

2.2.5.4 Hybridoma technique

The BALB/c mice that appeared to produce the specific antibody response were boosted IP with 100 µg of the corresponding antigen. Five days after boosting, mouse was sacrificed and spleen was taken. Spleen cells were isolated by homogenizing carefully. The obtained spleenocytes were counted with Turk's solution using hematocytometer. Myeloma cells were collected from culture flasks and counted by 0.2% trypan blue. Then the spleenocytes were fused with mouse myeloma cells using 50% polyethelene glycol (PEG) by standard hybridoma techniques with some modification. Briefly, mixture of spleenocytes and myeloma cells at the ratio 2:1 was centrifuged at room temperature 300g for 10 min. Then, supernatant were removed, the cells pallet were mixed by hand and warmed at 37^{o}C for 5 min. The fusion procedure was started by dropping 1.5 ml of 50% PEG into the cells mixture within 1 min and gentle stirring for another 1 min. Then the cells mixture was suddenly diluted with IMDM medium by continue adding the medium and mixed thoroughly following from 1 ml within 1 min, 3 ml within 1 min and 16 ml within 2 min. The fused cells were then centrifuged at room temperature at 300g for 5 min and warmed at 37^{o}C for 5 min. After the supernatant was removed, the fused cells were resuspended in HAT selection medium and transferred to 96 well culture plates at 10 plates/fusion. The hybridoma cells were

incubated at 37°C in 5% CO_2 incubator for 5 day. After that the culture plates were added with HT supplement medium and monitored the hybridoma clone by inverted light microscope.

The culture supernatant from the wells containing hybridoma cells were collected and screened for antibody reactivity with the corresponding antigens by indirect ELISA as was described in 2.2.5.3. Briefly, for screening of antibodies against Hb Bart's hydrops fetalis hemolysate, firstly, ELISA plates were coated with Hb Bart's hydrops fetalis hemolysate. The positive clones were selected for further screening using normal adult and normal cord blood hemolysate as antigen. For screening of anti-Hb Portland, the ELISA plated were coated with purified Hb Portland and purified Hb F. The clones that were positive with purified Hb Portland and negative with purified Hb F were selected for further characterization.

2.2.5.5 Single cell cloning by limiting dilution

In order to clone the hybridoma, hybridoma clones were stained with 0.2% trypan blue and counted for viable cells by hematocytometer. Then the cells were diluted in IMDM medium containing 10% FCS and 10% BM condimed at final concentration of 400 cells/15 ml. After that the hybridoma cells were diluted in serial two-fold dilution into 3 concentrations: 400 cells/7.5 ml, 200 cells/7.5 ml and 100 cell/15 ml respectively. Then the cell suspension were added into 96 well culture plates at 150 µl/well, the final concentration of each well was 4 cells/well, 2 cells/well and 1 cell/well respectively. The culture plates were incubated at 37°C in 5% CO_2 incubator for 2 weeks. Cell culture supernatants were collected from wells containing single clone or at least double clones and determined their activity by indirect ELISA.

2.2.6 Isotyping of monoclonal antibodies

An isotyping ELISA kit from Sigma was used for determined the isotype of the generated mAbs. Goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM at dilution 1:1,000 were coated using carbonate/bicarbonate coating buffer pH 9.6 into 96 well ELISA plate at 50 µl/well and incubated at 4^{o}C for overnight. After that the plates were washed with 0.05% Tween-PBS for 4 times and blocked with 60 µl/well of 2% BSA-PBS at 37^{o}C for 1 hr. The plates were washed once with 0.05% Tween-PBS. Cell culture supernatants were added to each well. After incubation at 37^{o}C for 1 hr, the plate were washed with 0.05% Tween-PBS for 4 times and determined for antibody binding by adding 50 µl/well horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody at dilution 1:2,000 and incubated at 37^{o}C for 1 hr. Thereafter, the plates were washed with 0.05% Tween-PBS for 4 times and added 50 µl TMB-substrate into each well. After incubated in dark at room temperature for 20 minutes, the reaction was stopped with 1M HCl at 50 µl/well and measured the absorbance at 450 nm.

2.2.7 Study of the specificity of monoclonal antibodies with different hemolysates

Twenty µg/ml of various hemolysates, including Hb Bart's hydrops fetalis, normal cord blood and normal adult hemolysate were coated using carbonate/bicarbonate coating buffer pH 9.6 onto 96 well ELISA plate at 50 µl/well and incubated at 4^{o}C for overnight. The specificity of mAbs were determined by indirect ELISA as was described in 2.2.6.

2.2.8 Study of the specificity of monoclonal antibodies with purified hemoglobins

Beside of hemolysate, the purified Hbs were also used to characterize the specificity of the mAbs. Purified hemoglobin, including Hb A, A_2, E, F, Bart's and Portland at the concentration 20 μg/ml were coated using carbonate/bicarbonate coating buffer pH 9.6 onto 96 well ELISA plate at 50 μl/well and incubated at 4°C for overnight. The specificity of mAbs were determined by indirect ELISA as was described in 2.2.6.

2.2.9 Study of the cross reactivity of monoclonal antibodies between human and various animal hemoglobins

Blood were collected from various animal including dog, cat, pig, cow, buffalo, chicken. Hemolysates were prepared as was described in 2.2.1. The hemolysates obtained from animals and human blood at the concentration of 20 μg/ml were coated using carbonate/bicarbonate coating buffer pH 9.6 into 96 well ELISA plate at 50 μl/well and incubated at 4°C for overnight. The cross reactivity of mAbs with various animal hemoglobins were determined by indirect ELISA as was described in 2.2.6.

2.2.10 Study of the specificity of monoclonal antibodies with various globin chains by Western blot analysis

In order to specify the reactivity of mAbs whether to the whole molecule of hemoglobin tetramer or to the globin chains, Acid-Urea-Triton X-100 polyacrylamide gel electrophoresis (TAU-PAGE) was used to separate the globin chains from hemoglobin tetramer (Alter *et al*., 1980). At the acid pH, all globin chains were positively charged and migrated from the anode to cathode according to their individual charge and molecular size. The gel was prepared by using 12% polyacrylamide in acid urea and allowed to complete polymerization for 2 hr at room temperature. Pre-electrophoresis were performed with 5% acetic acid running buffer, without adding of samples, for 45 min at 200 V with the anode on top. After pre-electrophoresis, the buffer was removed and replenished with new buffer. The slab gel was overlaid with 10 μl mercaptoethanol and performed the second pre-electrophoresis for 60 min at 150 V. After that, 10 μg of Hb Bart's hydrops fetalis hemolysate which contain ζ, $^{A}\gamma$, $^{G}\gamma$-globin chain, purified Hb A_2 which contain δ, α-globin chain and normal adult hemolysate which contain β, α and small amount of δ-globin chain were mixed with sample buffer (8M urea; fresh prepared 1 ml, glacial acetic acid 100 μl, 2-mercaptoethanol 100 μl, Pyronin Y dye 2-3 pieces) at the ratio 1:20 and applied into each lane of the slab gel. The voltage was started from 100 V for 1 hr and then 160 V for 3 hr, respectively.

The separated globin chains were transferred to a nitrocellulose membrane by blotting buffer using semi-dry blotting apparatus at 40 mA for 2 hr. Then the membrane was blocked with 2% BSA-PBS at 4^{o}C for overnight. The nitrocellulose membrane were rotated at room temperature for 2 hr and washed twice with PBS pH

7.2 for 5 min each. After that the purified mAb were added at a final concentration of 10 μg/ml into the tray and rotated the membrane with mAb for 1 hr at room temperature. The membrane was washed again for 5 times with 0.1% Tween-PBS, 5 min each. Then the secondary antibody, horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody was added at dilution 1:5,000 and rotated at room temperature for 1 hr. Finally the membrane was washed twice with 0.1% Tween-PBS and PBS pH 7.2 for 3 times, 5 min each. The protein bands were visualized by using the chemiluminescence detection system.

2.2.11 Large scale production of monoclonal antibodies and purification of monoclonal antibodies

The BALB/c mice were injected by intraperitoneal route with 500 μl pristane oil solution. Seven days after injection, $5x10^6$-$1x10^7$ hybridoma cells in sterile PBS were injected with the same route into the mice. After 1-2 weeks of the hybridoma cell injection, the ascitic fluid were collected and centrifuged at 12,000 rpm at 4°C for10 min. The supernatant was collected and stored at -20°C until used.

To purify the mAbs from the mouse ascitic fluid, the ascites was diluted in binding buffer (20 mM Sodium phosphate buffer pH 7.0 (1:2) for IgG or 4X 0.8M Ammonium sulfate pH 7.5 (1:4) for IgM, see Appendix B). The column was equilibrated with the binding buffer corresponding to each type of immunoglobulin classes as mention above for 2-3 column volumes. Then the mAb was purified by Purification column (Protein G for IgG or Hitrap™ IgM for IgM) using AKTA Prime fraction collector.

The antibodies were eluted using eluting buffer. For IgG, the antibodies were eluted by 0.1 M Glycin-HCl pH 2.7 and immediately adjusted the pH by neutralizing buffer (1 M Tris-HCl pH 8.0). For IgM, the antibodies were eluted by 20 mM Sodium phosphate buffer pH 7.5. The purified mAb fractions were measured at 280 nm and collected by AKTA prime fraction collector. The purified mAb fractions were pooled and dialyzed in PBS at 4^{o}C for overnight and determined the concentration by measured the absorbance at 280 nm.

The purity of the purified mAbs was determined by SDS-PAGE. Briefly, 12.5 μg of purified mAbs were mixed with reducing and non-reducing sample buffer (see Appendix B) and then loaded into each lane of 12.5% SDS-polyacrylamide gel. The electrophoresis was performed at 120 V for 2 hr. The heavy chain and light chain of mAbs were migrated according to their molecular weight. Proteins on the slab gel were visualized by staining with Coomassive Brillant Blue R250 for overnight. After that the gel was destained with destaining gel solution I and II, respectively.