CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and instruments used in this study are shown in Appendix A

2.2 Characterization of monoclonal antibodies against hemoglobins

Various monoclonal antibodies (mAbs) against hemoglobins were kindly provided by Assoc. Prof. Dr. Watchara Kasinrerk. These mAbs were produced from BALB/C mouse immunized with Bart's hydrops fetalis hemolysate, then cell fusion were performed by standard hybridoma technique using 50% polyethylene glycol. The hybridoma clones producing anti-hemoglobin Bart's mAbs were selected by screening using an indirect ELISA.

2.2.1 Hemolysate preparation

Blood samples were collected from normal, normal cord blood and stillborn with Hb Bart's hydrops fetalis using EDTA as an anticoagulant. Hematological data and hemoglobin typing of the blood samples were determined by automatic cell counter and cation-exchange HPLC, respectively (Jintaridth P, 2005). Then, blood samples were centrifuged at 2000g, 4°C for 10 min. After plasma was discarded, red blood cells were washed three times in 0.9% NaCl solution. The packed red cells were lysed by adding 2 volume of distilled water and 1 volume of toluene, then, vigorously mixed. The solution was centrifuged at 2000g, 4°C for 30 min, then, toluene layer was removed. The clear hemolysate solution was collected and hemoglobin concentration was determined by Cyanmethemoglobin method.

Briefly, 20 μl of hemolysate was added into 5 ml of Drabkin's solution. The solution was mixed and incubated at room temperature for 10 min. Then, hemoglobin concentration was measured by a spectrophotometer at 540 nm.

2.2.2 Characterization of monoclonal antibodies against hemoglobins by indirect ELISA

Fifty microlitres of 50 μg/ml of Hb Bart's hydrops fetalis hemolysate, normal cord blood hemolysate or normal adult hemolysate in 0.1 M Carbonate bicarbonate buffer, pH 9.6 (coating buffer) was coated into 96-well plate at 4°C for overnight. The plate was washed four times with 0.05% Tween-PBS. Then, 60 μl of 2% bovine serum albumin in PBS (2% BSA-PBS) was added in each well and incubated at 37°C for 1 hr. After washing, 50 μl of monoclonal antibodies were added and incubated at 37°C for 1 hr. After that 96-well plate was washed four times with 0.05% Tween-PBS, then, 50 μl of rabbit-anti mouse immunoglobulins-HRP (1:2000) was added in each well and incubated for 1 hr. After washing, 50 μl of TMB substrate (3,3,5,5-TetramethylBenzidine) was added and incubated in dark room temperature for 20 min. The reaction was stopped with adding 50 μl of 1N HCl and plate was then read by an ELISA reader at 450 nm.

2.3 Single cell cloning

To obtain single clone of hybridoma producing interested mAbs, the limiting dilution technique was performed. Hybridoma clones were counted and added into 96-well plate at 4 cells/well, 2 cells/well and 1 cell/well in 10% FCS-IMDM containing 10% BM condimed. The plate was incubated at 37°C, 5% CO_2 incubator for a week. After that, single clone was screened under inverted microscope.

The culture supernatant was harvested from well containing single clone and screened for their reactivity by indirect ELISA as described in 2.2.2

2.4 Determination of isotype of monoclonal antibodies

The isotype of interested mAbs were determined by isotyping-ELISA kit. 20 μg/ml of goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM at dilution 1:1,000 were coated into 96 well plates and incubated at 4^{o}C for overnight. After that the plate was 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. Then the plate was washed once with 0.05% Tween-PBS. After washing, 50 μl of culture supernatant from hybridoma clones were added and incubated at 37°C for 1 hr, the plate was washed with 0.05% Tween-PBS for 4 times and determined for antibody binding by adding 50 μl/well horseradish peroxidase conjugated with rabbit anti-mouse immunoglobulins at dilution 1:2,000 and incubated at 37^{o}C for 1 hr. After washing 4 times, 50 μl of TMB substrate was added and incubated in dark room temperature for 20 min. The reaction was stopped with adding 50 μl of 1N HCl and plate was then read by an ELISA reader at 450 nm.

2.5 Large scale production and purification of monoclonal antibodies

2.5.1 Production of ascitic fluid

Interested hybridoma clones including Thal GJA which produces anti-hemoglobin Bart's mAb and clone Thal N/B which produce anti-hemoglobin mAbs were selected for production of ascitic fluid. BALB/C mice were primed with 0.5 ml of pristane (2, 6, 10, 14-tetramethyl decanoic acid) by intraperitoneal injection. After seven days of injection, 1x10^{7} of hybridoma cells in 600 μl sterile PBS was inoculated into peritoneal cavity. Ascitic fluid was collected from the mice at 7-14 days later.

The ascitic fluid was centrifuged at 12,000 rpm, 4°C for 10 min**.** The clear ascitic fluid was collected and stored at -20°C until used. The obtained ascitic fluid was tested for the activity of mAbs by indirect ELISA as described in 2.2.2 before further purification.

2.5.2 Purification of Thal GJA and Thal N/B monoclonal antibodies by AKTA™ prime automated liquid chromatography system

To purify Thal GJA and Thal N/B mAbs which are IgG1 isotype, the ascitic fluid sample was diluted with 20 mM sodium phosphate buffer, pH 7.0 (binding buffer). Thereafter diluted sample was clarified by centrifuging at 14,000 rpm, 4°C for 10 min. For mAb purification by using AKTA™ prime, the HiTrap Protein G column was equilibrated with binding buffer. Then, clarified sample was applied into the equilibrated column. To remove unbound materials, column was washed by adding with 5-10 volumes of binding buffer until no protein was detected by UV absorbance at 280 nm. After that, the bound mAb was eluted by 0.1 M glycine-HCl pH 2.7 (elution buffer). The mAb fraction was collected, then, dialyzed against PBS for overnight. The concentration of purified mAb was determined by measuring the absorbance at wavelength of 280 nm. The specificity of purified mAbs was determined by indirect ELISA as was described previously in 2.2.2.

2.6 Determination of hemoglobin Bart's by sandwich ELISA

2.6.1 Labeling of Thal N/B with horseradish peroxidase (HRP)

In order to use Thal N/B anti-hemoglobins mAb as a secondary antibody in sandwich ELISA, mAb Thal N/B was conjugated with horseradish peroxidase (HRP). One milligram of HRP was reconstituted in 100 μl of water, then, enzyme solution

was added into 1 mg/ml of mAb Thal N/B in 0.2 M Carbonate-bicarbonate buffer, pH 9.4. The solution was mixed and incubated in dark at room temperature for 1 hr by gentle mixing. After that, 10 μl of 5.0 M sodium cyanoborohydride ($NaCNBH_3$) was added and let stand at room temperature for 15 min. The reaction was quenched at room temperature for 15 min by adding 20 μl of 3.0 M ethanolamine, pH 9.0. The concentration of HRP-conjugated Thal N/B was determined by measuring the absorbance at wavelength of 280 nm.

2.6.2 Determination of HRP-conjugated Thal N/B anti-hemoglobin mAb activity

To determine HRP-conjugated Thal N/B anti-hemoglobin mAb activity, indirect ELISA was carried out. 50 μl of 50 μg/ml of Hb Bart's hydrops fetalis hemolysate in coating buffer was coated into 96-well plate at 4°C for overnight. The plate was washed four times with 0.05% Tween-PBS to remove unbound Hb Bart's. Then, 96-well plate was blocked with 2% skimmed milk in PBS at 37° C for 1 hr. After washing, 50 μl of HRP-labeled Thal N/B at various concentrations were added into each well and incubated at 37° C for 1 hr. The plate was washed four times with 0.05% Tween-PBS. The color was developed by adding TMB color substrate. After 5 min, 50 μl of 1N HCl was added to stop the reaction. The absorbance was measured by ELISA reader at 450 nm.

2.6.3 Optimization of Sandwich ELISA for detection of hemoglobin Bart's in blood sample hemolysate

To set up the sandwich ELISA for detection of hemoglobin Bart's in blood sample hemolysate, the optimal concentration of Thal GJA anti-Bart's mAb (which was used as a capture antibody) and HRP-conjugated Thal N/B anti-hemoglobin mAb

(which was used as a secondary antibody) were titrated. The concentration of mAb Thal GJA vary between 100-400 μg/ml was coated into 96-well plate. The plate was incubated at 4°C for overnight. After washing with Tween-PBS for 4 times, 60 μl of 2% skimmed milk-PBS was added and incubated at 37°C for 1 hr. After washing 1 time, various concentrations of Hb Bart's hydrops fetalis hemolysate, normal cord blood hemolysate or normal blood hemolysate were added into each well and incubated at 37°C for 1 hr. After that, plate was washed 4 times, then HRP-conjugated Thal N/B at various concentrations were added into each well. One hour after incubation at 37°C, the plate was washed 4 times to remove un-reacted HRP-conjugate. Then, 50 μl of TMB color substrate was added into each well and incubated in dark at room temperature for 20 minute. The reaction was stopped by adding 50 μl of 1N HCl. Then, the color reaction was measured by ELISA reader at 450 nm.

2.6.4 Determination of specificity and sensitivity the developed sandwich ELISA

The specificity and sensitivity of the developed sandwich ELISA were determined. ELISA plate was coated with 50 μl of 100 μg/ml Thal GJA mAb. After incubation, the plate was washed with Tween-PBS for 4 times. Plate was blocked with 2% skimmed milk. Then, purified form of Hb Bart's, Hb F, Hb A, Hb A_2 and Hb E at various concentrations were applied and incubated at 37°C for 1 hr. After washing step, 50 μl of 1.25 μg/ml of HRP-conjugated mAb Thal N/B was added into each well and incubated at 37°C for 1 hr. After that, 50 μl of TMB substrate was added into each well and incubated in dark room temperature for 20 minute. The

reaction was stopped by adding 50 μl of 1N HCl and the absorbance was determined by ELISA reader at 490 nm.

2.7 Determination of hemoglobin Bart's in hemolysate by sandwich ELISA

2.7.1 Blood samples

The 91 blood samples were deliberated selected from the Clinical Microscopy Unit, Department of Central Laboratory, Maharaj Nakorn Chiang Mai Hospital. Automated complete blood analysis (Sysmex KX-21 N), one-tube osmotic fragility test (OF test) and Hb H inclusion body test were carried out in all these blood samples. Based on these preliminary hematological and thalassemia screening data, two group of blood samples were obtained including thalassemia group and control group. The thalassemia group comprised these having the mean corpuscular volume (MCV) of less than and equal to 80 fL and / or positive OF test. The control group was comprised of those blood samples with MCV of more than 80 fL and / or negative OF test. The blood samples in thalassemia group were further examined for the presence of Hb H inclusion body (modified brilliant cresyl blue) (Hartwell *et al*., 2005) to rule out the α-thalassemia 1 heterozygote. Hb identification was performed in all 91 blood samples by the weak-cation exchange high performance liquid chromatography (HPLC) (Primus variant system 99 (PVS 99)) to detect heterozygous status for β-hemogobinopathy.

2.7.2 Identification of α-thalassemia by PCR analysis

To determine blood samples into α-thalassemia, PCR was performed. Identification of α-thalassemia 1 (SEA type) was examined by Gap-PCR. The 3.7 kb and 4.2 kb deletion α-thalassemia 2 were also examined by PCR method.

2.7.2.1 DNA preparation

The genomic DNA was isolated from peripheral blood mononuclear cells using standard phenol-chloroform technique as described follows. EDTA-blood samples were centrifuged at 2000g, for 20 min. After plasma layer was discarded, 1 ml of buffy coat containing mononuclear cells was carefully aspirated and transferred to a new tube. Then, 3 ml of 0.5% NP-40 (non-ionic detergent) was added and vigorously mixed. The solution was centrifuged at 2000g for 5 min then, supernatant was discarded. After that the solution were mixed with 750 μl of lysis buffer, 40 μl of 10% SDS and 100 μl of 5 mg/ml proteinase K. The mixture was incubated in 37°C water bath for overnight. After cell lysis, 200 μl of 5X ANE buffer, 500 μl of saturated phenol and 500 μl of chloroform were added. The solution was mixed by shaking end-to-end and then spun at 2000g 10 min. The lower layer containing organic solution was discarded and phenol/chloroform extraction was repeated until the interface was cleared. Then, upper layer of solution was carefully aspirated into a new tube. The DNA was precipitated by adding 100 μl of 3M NaOAc and 2.5 ml of cold absolute ethanol. Then, DNA was centrifuged and washed with cold 70% ethanol. After that, TE buffer was added to dissolve 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:

$$1\ OD_{260}/ml = 50\ \mu g/ml \text{ genomic DNA}$$

$$\text{Concentration of DNA} = O.D._{260} \times 50\ \mu g/ml \text{ genomic DNA} \times \text{dilution factor}$$

2.7.2.2 Detection of α-thalassemia 1 (SEA type) by PCR analysis (Kitsirisakul *et al*., 1996)

The Southeast Asian (SEA) type of α-thalassemia 1 was identified by the Gap-PCR technique. The principle of this technique was that normal α-globin gene fragment and α-thalassemia 1 (SEA type) were detected using three primers, P1, P2 and P3, as shown in Figure 2.1. Primer P1 (5'GCG ATC TGG GCT CTG TGT TCT-3'), P2 (5'-GTT CCC TGA GCC CCG ACA CG-3') and P3 (5'-GCC TTG AAC TCC TGG ACT TAA-3') were used in this study. The PCR products for normal α-globin gene and α-thalassemia 1 (SEA type) were 314 bp and 188 bp, respectively. The fragment with 314 bp was generated by two primers (P1+P2) and used as internal control of this amplification system as shown in Figure 2.1.

The Gap-PCR reaction was performed as was shown in Table 2.1. The PCR was run at 40 cyclers by using DNA engine Thermal cycler. The amplification was done at 95°C for 1 min (denaturation step), 58°C for 1 min (annealing step), and 72°C for 1 min (extension step) as was shown in Table 2.2. For PCR fragment analysis, 5 μl of PCR product was mixed with 6X loading dye and run on a 2.5% agarose gel in 0.5X TBE buffer. The PCR product was visualized after staining with ethidium bromide by UV transilluminator.

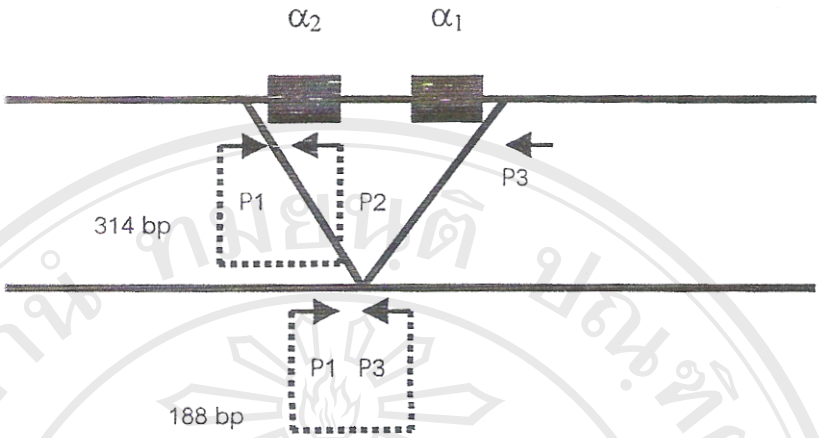

Figure 2.1 Demonstration of diagram normal α-globin-like-gene cluster and α-thalassemia 1 (SEA). The positions of primer P1 and P2 used to amplify the normal area; P1 and P3 used to amplify the breakpoint are shown.

Table 2.1 The reaction mixture of Gap-PCR for detection of α-thalassemia 1 SEA type

Reaction mixture	**1 tube (μl)**
10X Buffer	2.5
25 mM $MgCl_2$	1.5
1 mM dNTPs	2.5
Primer 1 (3pmol/μl)	7
Primer 2 (3pmol/μl)	3
Primer 3 (3pmol/μl)	3
Glyceral	1.8
Taq DNA polymerase (5 U/μl)	0.25
Distilled water	2.45
Genomic DNA	500 ng
Total volume	24

Table 2.2 The PCR cycling condition for α-thalassemia 1 (SEA) using the Thermal cycler

X1	X40	X1
95°C, 5min	95°C, 1 min	95°C, 1 min
58°C, 1min	58°C, 1 min	58°C, 1 min
72°C, 1min	72°C, 1min	72°C, 7 min

2.7.2.3 Detection of α-thalassemia 2 by PCR amplification (Fucharoen *et al*., 2002)

To detect α-thalassemia 2 with 3.7 kb and 4.2 kb deletions by PCR, three pairs of primer were designed and synthesized. As shown in Figure 2.2, Primer A (5'-CCCAGAGCCAGGTTTGTTTATCTG-3') and B (5'- GAGGCCCAAGGGGCAAG AAGCAT-3') were used to amplify a 1779 bp specific fragment for the 3.7 kb deletion. Primer C (5' GCTAGAGCATTGGTGGTCATGCC-3') and D (5'TTCTG ACTCTGCCCACAGCCTGA-3') were used for detection of the 4.2 kb deletion with fragment of 1529 bp. The αG1 (5'-GCTGACCTCCAAATACCGT-3') and αG11 (5'-CTCTGCCTCCTTGTTTAAAA-3') primer were designed for amplification of 1395 bp DNA fragment from α-globin gene region without deletion. Thus this fragment can be used as internal control. As shown in Table 2.3 and 2.4, the reaction mixture was performed using the MJ Thermal cycler. The PCR amplification protocol to detect α-thalassemia 2 with 3.7 kb and 4.2 kb deletions was shown in Table 2.5. Amplified DNA was analyzed by electrophoresis on 1.5% agarose gel in 0.5X TBE. The PCR products were visualized after staining with ethidium bromide by UV transilluminator.

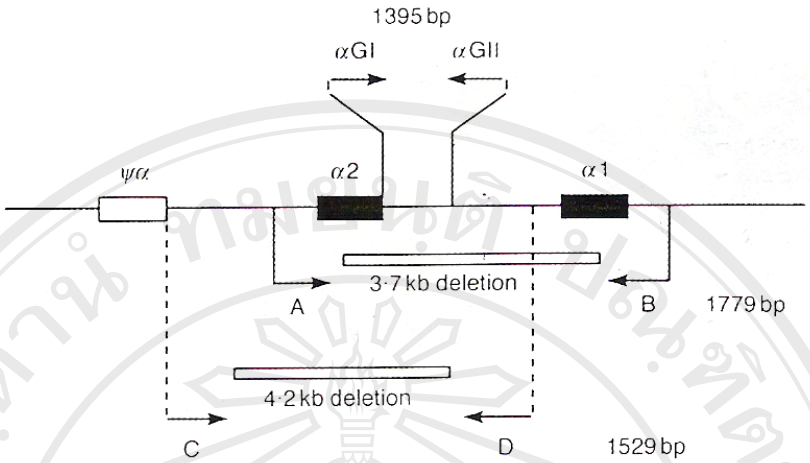

Figure 2.2 Demonstration of 3 pairs of primer amplify PCR product with 1779, 1529 and 1395.

Table 2.3 The reaction mixture of PCR amplification for detection of α-thalassemia 2 with 3.7 and 4.2 kb

Reaction mixture	**1 tube (μl)**
10X Buffer with $(NH_4)_2SO_4$	2.5
25 mM $MgCl_2$	2.5
1 mM dNTPs	5.0
α 3.7A or 4.2 C (15 pmol/μl)	1
α 3.7B or 4.2 D (15 pmol/μl)	1
10% dimethyl sulfoxide	2.5
Taq DNA polymerase (5 U/μl)	0.3
Distilled water	8.2
Genomic DNA	200 ng
Total	23

Table 2.4 The reaction mixture of PCR amplification for detection of internal control

Reaction mixture	**1 tube (μl)**
10X Buffer with $(NH_4)_2SO_4$	2.5
25 mM $MgCl_2$	2.5
1 mM dNTPs	5.0
α G 1 (15 pmol/μl)	1
α G 11 (15 pmol/μl)	1
10% dimethyl sulfoxide	2.5
Taq DNA polymerase (5 U/μl)	0.3
Distilled water	8.2
Genomic DNA	200 ng
Total	23

Table 2.5 The PCR cycling condition for α-thalassemia 2 (3.7 and 4.2 kb deletions) using the Thermal cycler

X1	X10	X10
94°C, 5 min	94°C, 1.30 min	94°C, 1.30 min
94°C, 1.30 min	60°C, 1.30 min	60°C, 1.30 min
60°C, 1.30 min	68°C, 2.20 min	68°C, 2.20 min
68°C, 2 min		

2.7.3 Detection of Hb Bart's in blood samples by the developed sandwich ELISA

2.7.3.1 Blood sample preparation

Blood samples were centrifuged at 2000g, 4°C for 10 min, then plasma was discarded. Red blood cells were washed three times in normal saline. Packed red cells were lysed by adding 2 volume of distilled water and 1 volume of toluene, and then vigorously mixed. The solution was then centrifuged at 2000g, 4°C for 30 min. The toluene layer containing stroma cells was discarded. The clear hemolysate solution was aspirated and kept at -20°C until used.

2.7.3.2 Detection Hb Bart's in blood sample by sandwich ELISA

Fifty microlitres of 100 μg/ml Thal GJA anti-Bart's mAb in coating buffer was coated into 96-well plate and incubated at 4°C for overnight. The plate was washed four times with 0.05% Tween-PBS, then 60 μl of 2% skimmed milk-PBS PBS was added into each well. After incubation, 50 μl of hemolysate samples at dilution 1:30 and 1:60 or 0.0049-10.0 μg/ml of purified Hb Bart's were added and incubated at 37°C for 1 hr. Fifity microlitres of 1.25 μg/ml HRP-conjugated Thal N/B was added in each well after washing step. Then, 50 μl of TMB substrate was added and incubated in dark for 15 min. The reaction was stopped by adding 50 μl of 1N HCl. The absorbance was determined by ELISA reader at 450 nm.