CHAPTER IV

DISCUSSIONS

Hb Bart's hydrops fetalis syndromes are the most severe type of α-thalassemia syndrome caused by the deletion of all four α-globin genes. All of fetus with this genotype die just before birth or a few hours after birth (Weatherall & Clegg, 2001). Moreover, this phenomenon may also adversely affect the health of mothers during pregnancy. Mothers of hydrops usually suffer from several obstetric complications (Wanapirak *et al.*, 1998) and half of them are died from the complications in un-proper medical care (Chui & Waye, 1998). Couples of α-thalassemia 1 is the most common cause of Bart's hydrops fetalis (Chui & Waye, 1998). To control α-thalassemia disease, the α-thalassemia carriers are needed to be identified and counseled. The risk of obtaining baby with Bart's hydrops fetalis and mother un-safety have to be inform to the carriers (Tongsong *et al*., 2001).

Several methods have been developed for diagnosis thalassemia. These methods can be used effectively for identification of all types of thalassemia from disease to silent anemia carriers. According to their complexity and high cost, the available methods are not suitable for screening the α-thalassemia 1 carriers in the large population. Simple and rapid method, as immunological test, is the new trend for screening of α-thalassemia 1 carriers.

In any immunological test, specific antibodies are most important element. Monoclonal antibodies against hemoglobin variants and globin chains are crucial for development of immunodiagnostic kit for thalassemia. Monoclonal antibodies

directed against various hemoglobins and globin chains were generated by various researchers and used to develop a simple methods for diagnosis of thalassemia and hemoglobinopathies (Lafferty *et al*., 2000). Various monoclonal antibodies specific to human hemoglobin and globin chains have been generated. mAb specific for δ chain of Hb A_2 was produced and was later used for detection and quantification of Hb A_2 in adult, cord blood, and fetal hemolysates (Shyamala *et al*., 1991). mAb specific for β^E globin was used for detection of β^E thalassemia carriers (Makonkawkeyoon *et al.*, 2006). Monoclonal antibodies specific for other globin chain such as γ, ε, and ζ chains or Hb H have also been reported;(Luo *et al.*, 1988; Moscoso *et al.*, 1989; Shyamala *et al.*, 1992; Sugano *et al.*, 2000; Zhao *et al.*, 1988).

Previous studies have been reported that a minute amount of Hb Bart's (Makonkawkeyoon *et al.*, 1992; Wasi *et al.*, 1979) and embryonic ζ globin chains could be found in the hemolysate of α-thalassemia 1 carriers (Ausavarungnirun *et al*., 1998; Chui *et al*., 1986). The monoclonal antibodies which specifically bind to Hb Bart's or ζ globin chain, therefore, are very useful for development of immunological test for identification of α-thalassemia 1 carrier (Ma *et al*., 2002; Unitedbiotech, 2006). In this study, we aimed to produce mAbs against Hbs presented in the Bart's hydrops fetalis hemolysate. The generated monoclonal antibodies will be employed for development of immunodiagnostic kit for screening of α-thalassemia 1 carriers in the future.

In this study, firstly, we produced mAbs by using Bart's hydrops fetalis hemolysate as an immunogen. For this purpose, hemolysate of Bart's hydrops fetalis was prepared. Before using as an immunogen, the Hbs contained in the hemolysate were analyzed by cellulose acetate electrophoresis. Cellulose acetate electrophoresis

is a method which can separate each type of hemoglobins from another on the cellulose acetate membrane according to their net charge and size (Rosenbaum, 1966). By this method, the prepared hemolysate was confirmed to contain almost Hb Bart's and a small amount of Hb Portland. The high proportion of Hb Bart's which constitutes more than 80% of total Hbs in the Hb Bart' hydrops fetalis hemolysate was as reported (Rifkind *et al.*, 1984). In principle, the prepared hemolysate could be used as immunogen and not necessary to be purified before immunization. Adjuvants are substances used for enhancing the immune response to the immunized antigens. The most commonly used adjuvants in animals are complete Freund's adjuvant (a water-in-oil emulsion in which contain killed *M. tuberulosis* bacteria) and incomplete Freund's adjuvant (omitting of the bacteria) (Goding, 1986). The mechanism of enhancing antibody responses of the adjuvants are complex (Freund, 1956). They probably involve in slow, prolonged release of antigen in a highly aggregated form for the immune cells. The pharmacologically active substance from the mycobacteria presented in the adjuvant, e.g. muramyl dipeptide, are also enhanced the immune responses (Chedid *et al.*, 1976). However, from the preliminary study of Assoc. Prof. Dr. Watchara Kasinrerk, mice immunized using Freund's adjuvant encountered fibroblast cells overgrow after cell fusion causing the loss of hybridoma cells (un-published observation). These may be the fact that hyper-irritation of adjuvant may induce the proliferation of fibroblast cells. The fibroblast cells are often contaminating during fusion procedure and usually resisted to HAT medium (Bone, 1995; Staines & Price, 2003). To avoid this problem, in our first experiment, mice were immunized with Bart's hydrops fetalis hemolysate in steriled PBS without adjuvant. After the third immunization, the specific antibody

response of the immunized mice was very low. This may be because of the absence of adjuvant. Without adjuvant the immunogens were eliminated quickly from the body and, therefore, induced low level of antibody response (Goding, 1986). Anyway, to obtain high antibody titer, we had continued immunizing the mice and monitoring their antibodies response at two weeks intervals. The specific antibody response in mouse "A" which was quite low in the first period of immunization, but after the 6^{th} immunization the antibody titer was increase to more than 1: 12,000. The antibodies responses in mouse "B" were slowly increased and rather stable at the last period of immunization. The different immune response between those two mice may be due to the individual genetics variation (Lavrovsky *et al.*, 1979). After 6^{th} immunizations, the antibodies titer of both mice was moderately, but it is enough for hybridoma production. Cell fusion between X63-Ag8.653 mouse myeloma cells and spleen cells of the immunized mice were performed by standard hybridoma technique (Kasinrerk *et al.*, 1998; Moonsom & Kasinrerk, 2000; Puttikhunt *et al.*, 2003). Over 500 hybridoma cells were obtained without fibroblasts overgrow as expected. This suggested that the immunization without Freund's adjuvant could reduce the fibroblast overgrow after cell fusion. We firstly screened the obtained clones by using Hb Bart's hydrops fetalis hemolysate as antigen. Only the clones which reacted to Hb Bart's hydrops fetalis hemolysate were selected and further subjected for the secondary screening. Because Hb Bart's and embryonic ζ globin chain are not present in normal cord blood and normal adult hemolysates (Chui *et al.*, 1989; Chui *et al.*, 1986; Luo *et al.*, 1988). Thus, the secondary screening was performed by using those two hemolysates to identify only the clones that produce mAb specific to Hb presented in the Bart's hydrops fetalis hemolysate. Cellulose acetate

electrophoresis was also used to confirm that normal cord blood and normal adult hemolysate used in the secondary screening for having their corresponding hemoglobins. The results showed that normal cord blood and normal adult hemolysates contained only their corresponding hemoglobins, i.e. Hb F, Hb A, Hb A_2 and a minute amount of enzyme carbonic anhydrase (Ingiulla & D'Asero, 1967; Krieg & Henry, 1967; Moini *et al.*, 2002; Rozman *et al.*, 1963), no Hb Bart's or Hb Portland were detected. These results indicated that the prepared hemolysates were suitable for using in the secondary screening. By the second screening, it was found that the generated mAbs were not only reacted to Bart' hydrops fetalis hemolysate, but also reacted to normal cord blood and normal adult hemolysates. These mAbs may react to the common epitope expressed in all types of hemoglobin. This may be due to the fact that the structures of all hemoglobin are similar. Hbs consist of four globin chains tretamer with different in few of amino acid sequences (Clarke & Higgins, 2000). Therefore, the chance to obtained mAb specific to epitope of Hb Bart's and ζ chain are rare. This may suggest that although an immunization without adjuvant can avoided the fibroblast overgrow, however, this method was inadequate to induce the specific antibody response to Hb Bart's that shares homology structure with other hemoglobins. By these fusions, the production of mAb specific against Hb Portland also did not achieve. This may be because of low proportion of Hb Portland presented in Hb Bart's hydrops fetalis hemolysate. Hb Portland constitutes only 10% of total hemoglobin in the Hb Bart's hydrops fetalis hemolysate (Rifkind *et al.*, 1984) which is not sufficient to induce the specific antibody response. Anyway, four clones of hybridoma, named HB1, HB2, HB3 and HB4, generated from these fusions were selected for further characterizations.

The first characterization using three different hemolysates, Bart's hydrops fetalis, normal cord blood and normal adult hemolysates, the strong reactivity of all selected mAbs with all antigens used was obtained. This suggested that the epitopes recognized by mAb HB1, HB2, HB3 and HB4 are present in all types of Hbs in the hemolysates. The mAbs were further characterized with several of purified hemoglobins. Various Hbs including Hb A, Hb A_2, Hb E and Hb F were purified from the hemolysate by DEAE-Sepharose anion-exchange column chromatography. The principle of this method is that the positive charge on DEAE Sepharose resins can bind to negative charge of Hbs (Bernini, 1969; Dozy *et al.*, 1968). The changing of pH gradient by using the eluting buffer (THK buffer pH 6.5) caused the separation of different Hb according to their net charge. Using this method, hemoglobins can be separated from each other except for the fraction of Hb E that still contain Hb A_2, as Hb E has an equal or nearly equal isoelectric point to Hb A_2 (Huisman & Dozy, 1961). The cellulose acetate electrophoresis technique was confirmed that each isolated Hbs was pure and no contamination with other hemoglobin, except Hb E as was mentioned. This suggested that DEAE-Sepharose anion-exchange column chromatography can be used to separated an individual hemoglobin from the hemolysate and suitable for using as an antigens for characterization of mAbs. After characterization, all four mAbs clones shown strong reactivity with all purified Hbs tested. Moreover, we found that mAb HB2 also cross reacted with bovine serum albumin. All of these results suggested that mAbs HB1, HB2, HB3, and HB4 were recognized a common epitope presented in all types of hemoglobin and the epitope that recognized by mAb HB2 was share the homology with an epitope on BSA. Beside of this, the animals hemoglobins were also use to characterize the specificity

of all four mAbs. This information is of interest, because mAbs specifically react to human hemoglobin, but not to animal Hbs, can be use in criminal sciences such as detection of human blood in the witness objects (Team, 2005). After characterized with various animal hemolysates, unfortunately, all mAbs were found to react all animal hemoglobin tested. However, differently reactivity to different of animal hemolysates, especially mAb HB1, was observed. This may be due to the copy number of the epitopes that recognized by mAbs are unequal between species to species.

Although the generated mAbs are not specific to Hb presented in the Bart's hydrops fetallis hemolysate as was purposed, these mAbs might be useful. They can be used as a tool for development of test kit for occult blood in stool for diagnosis colon cancer (Nagata & Tanaka, 1992; Ozturk & Cirakoglu, 2001).

The immunoglobulin isotyping was shown that all obtained mAbs were IgM isotype. This may be according to the immunization without adjuvant that induces lower immune response. IgM is the first immunoglobin class that produced after the immune cells had exposed to the antigens (Goding, 1986). The affinity and specificity of IgM immunoglobulin is lower than IgG that produced at the last period of hyper-immunization. In this study, the immunization method might not suitable for induction of potent immune response and consequently the antibodies responses are almost reacted to the common epitope of hemoglobin rather than that specific to Hb Bart's.

To induce the specific antibody response to Hb Bart's in the future, an adjuvant should be used although it may induce the fibroblast overgrow after cell fusion.

The ζ chain is one of the globin chains of Hb Portland which contained only 10% in Bart' hydrops fetalis hemolysate (Rifkind *et al.*, 1984). In order to produce mAb to ζ globin chain, we therefore decided not to use Hb Bart's hydrops fetalis hemolysate as immunogen, but used purified Hb Portland, in stead. To purify Hb Portland, cellulose acetate electrophoresis technique was applied. Using this electrophoresis, Hb Bart's was migrated faster than Hb Portland. On the membrane, the separated Hb Portland was cut out and eluted. After the purification process, the purity of the obtained Hb Portland was confirmed to be rather high by cellulose acetate electrophoresis suggesting it was suitable to use as an immunogen. Because of low antibody response obtained in the mice immunized without adjuvant, Hb Portland immunizations were done using Freund's adjuvant. As predicted, after three immunizations, the antibody response of the immunized mouse was very high and suitably for hybridoma production. After fusion, more than 300 clones of hybridoma were obtained with some fibroblast overgrowing. As the Hb Portland contained ζ and γ globin chain (Williams *et al.*, 1991), to identify hybridomas produce anti-ζ antibodies purified Hb Portland and purified Hb F were used as antigen in ELISA screening method. This strategy can exclude the clones that produced the anti-hemoglobin antibody or antibody against γ globin chain which contained in both Hb Portland ($\zeta_2\gamma_2$) and Hb F ($\alpha_2\gamma_2$). After screening, five clones of the generated hybridomas demonstrated to produce mAbs specific to Hb Portland, but do not reacted with Hb F. Unfortunately, two of them were died due to yeast contamination and other two clones stop produced antibody. These may be due to the genetically unstable of the hybridoma cells (Goding, 1986). However, a mAb named Thal-PL1 was established in this study. After characterizations with different hemolysates,

hemoglobin and various animal hemolysate, the results were confirmed that mAb Thal-PL1 was specifically reacted to purified Hb Portland. Moreover, mAb Thal-PL1 was not reacted with any types of animal hemoglobins. The immunoglobulin isotype of the mAb Thal-PL1 was IgG1. This confirmed that the immunization with adjuvant could induce the hyper-immune responses and consequently production of the specific antibodies.

Knowing of the concentrations of mAbs is very useful for control the quantities of mAbs used in any experiments. Large amount of mAb Thal-PL1 was prepared in ascites. The mAbs were then purified from the ascites by affinity chromatography. The concentration of purified mAbs was measured. SDS-PAGE was used to determine the purity of both mAbs after purification and confirmed its purity.

To study that mAb Thal PL1 react to the whole molecule of Hb Portland or the ζ globin chains, Western blotting analysis was carried out. Hemolysates including Bart's hydrops fetalis, purified Hb A_2 and normal adult hemolysate were used as the source of ζ, $^{A}\gamma$, $^{G}\gamma$, δ, β and α globin chains (Alter *et al.*, 1980). The globin chains were dissocated from hemoglobins by urea acid buffer and separated from each other according to their net charge on the TAU-PAGE (Efremov *et al.*, 1981). The order of globin chain migration from anode to cathode was ζ, $^{A}\gamma$, δ, $^{G}\gamma$, β and α, respectively (Alter *et al.*, 1980). Coomassive blue stain was confirmed that all of the globin chains were completely separated from each other. By Western blotting, the mAb Thal-PL1 was demonstrated to recognize the linear epitope of ζ globin chain.

In this study, the mAb specific to ζ globin chain and mAbs specific to hemoglobin were produced. The generated anti-hemoglobin mAbs may be useful in development of immunological test for occult blood diagnostic in colon cancer.

The high specificity against ζ globin chain of mAb Thal-PL1 has potential to be use for mass screening of α thalassemia 1 carrier to reduce the incident of Bart's hydrops fetalis cases in the future.