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Original article

PRODUCTION OF A MOUSE HYBRIDOMA SECRETING MONOCLONAL ANTIBODY HIGHLY SPECIFIC TO β^E-GLOBIN CHAIN FOR THE DETECTION OF HEMOGLOBIN E IN SOUTHEAST ASIAN POPULATIONS

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Abstract Hemoglobin E (HbE) is an abnormal hemoglobin derived from a single nucleotide mutation at codon 26 (GAG –> AAG) of the beta globin gene causing change of amino acid from glutamic (GAG) to lysine (AAG). This hemoglobin is the most common hemoglobin variant in Southeast Asian populations. Hemoglobin E was isolated from heterozygous HbE by DEAE - Sephadex column chromatography. The globin chains were separated by CM - cellulose column chromatography in 8 M urea phosphate buffer. The isolated β^E- globin chain was characterized by isoelectric focusing electrophoresis (IEF). The β^E- globin chain was used to immunize Balb/c mice. The immunized mouse spleen cells were fused with mouse myeloma cells resulting in 3 hybridoma clones synthesizing and secreting monoclonal antibody (mAb) specific to β^E- globin chain after a total of 13 fusions. After characterization, one hybridoma clone secreted mAb highly specific to β^E- globin, but with very low reactivity to β^A-, γ-, δ-, and α-globin chains. The mAb comprised IgM with a kappa light chain.

The obtained hybridoma clone secreting mAb, with high differentiation power, differentiated β^E- globin from β^A- globin, which had only one amino acid difference at amino acid position 26 of the β - globin chain. The result encouraged further development in immunological diagnostic methods for the detection of HbE in general populations, with knowledge that the HbE carriers will enable the efficiency of lowering the coinheritance of HbE and β thalassemia disease. **Chiang Mai Med Bull 2006;45(1):1-10.**

Keywords: monoclonal antibody, HbE, β^E-globin chain

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Hemoglobin E is the most common hemoglobin variant, which occurs throughout Bangladesh, Myanmar, and other parts of Southeast Asia. It is also seen with increasing frequency in immigrants from Europe, North America, and Australia.[1,2] This abnormal hemoglobin exhibits a high incidence in the Thai population, with over 50% in the Northeast and 20-45% in other parts of the country.[3] Hemoglobin E is caused by a point mutation in the first exon of the β^E-globin gene by the substitution of a single nucleotide base from G to A at codon 26 (HbE : $\alpha_2 \beta_2^{E: 26\ GAG \rightarrow AAG}$). The point mutation alters the amino acid change from glutamic acid to lysine. This point mutation in the β-globin gene affects β^E-globin gene expression by creating an alternative mRNA splice site in exon 1 of the β^E-globin gene, resulting in unstable mRNA.[4] HbE heterozygotes are clinically normal, while HbE homozygotes have little or no anemia. However, coinheritance of HbE with β^o thalassemia may lead to severe HbE/β thalassemia, which is more common than homozygous β thalassemia in Thailand. HbE with β^o thalassemia is a blood disease causing severe clinical disorders, although there is a great variety of clinical expressions. In extremely severe cases without treatment, the disease is able to cause heart failure via the destruction of red blood cells. It can also cause severe enlargement of the spleen and liver, poor growth, and bone change.[2] Treatment of HbE/β thalassemia is serious and costly, therefore, married couples should be evaluated for their HbE and β thalassemia genes before deciding to have children.

Detection of HbE is commonly carried out by alkaline electrophoretic analysis. A simple method using 2, 6 dichlorophenolindophenol (DCIP) has also been used. Other sophisticated techniques such as high performance liquid chromatography (HPLC), isoelectric focusing electrophoresis (IEF), and allele specific polymerase chain reaction were also used for the detection of HbE. However, HbE cannot be distinguished from HbA_2, HbC and HbO Arab by alkaline electrophoresis, while the DCIP technique is not able to distinguish HbE from other unstable hemoglobins such as HbH. Sophisticated techniques are costly and require state-of-the-art equipment and expertise to use it. We have, therefore, generated a hybridoma clone secreting a highly specific monoclonal antibody to β^E-globin chain, in the hope that this monoclonal antibody might be useful for the detection of HbE in general populations.

Materials and methods

Collection of blood and Preparation of hemoglobin solution.

Blood samples were obtained from volunteers, who showed hemoglobin typing of A/E by cellulose acetate electrophoresis with HbE over 10-35%. The genotype of β^E/β^A was confirmed by PCR with RFLP.[5] Hemolysate was prepared from EDTA blood of HbE/A by lysing RBC with distilled water. Hemoglobin solution was obtained after cell debris and lipid substances were removed by carbon tetrachloride.[6]

Cellulose acetate electrophoresis of hemoglobins.

Hemoglobin solution was subjected to cellulose acetate electrophoresis in alkaline buffer solution for hemoglobin typing. After the hemoglobin electrophoresis was over, the cellulose acetate strip was stained with Ponceau S solution. Analysis of hemoglobin typing was performed by measuring the migration distance of various hemoglobins and comparing it to known standard hemoglobins.[7]

Separation and quantitative determination of HbE.

Separation of HbE from hemoglobin solution was performed by DEAE-Sephadex column chromatography in Tris-HCl-KCN buffer.[8] The fraction containing hemoglobin E/A_2 was measured at 415 nm for optical density, then the percentage of HbE/A_2 was calculated.[9]

Separation and isolation of β^E-globin chain.

The fraction of HbE/A_2 from the DEAE-Sephadex column was treated with cold acid acetone to separate globins from hemoglobins, resulting heme in solution and globin chains in precipitate.[10] The globin chain fraction was separated further by a CM cellulose column in 8 M urea phosphate buffer to yield each individual globin chain.[11] The concentration of proteins was determined by Bradford's technique.[12]

Characterization of β^E-globin chain by isoelectric focusing electrophoresis (IEF)

The fraction of β^E-globin chain was characterized by IEF. A pH gradient of 4 to 10 was performed in gel by ampholytes, as indicated by the manufacturer (Bio-Rad). The fraction containing β^E-globin chain was analysed by running in IEF along with various standard globin chains and molecular weight markers. The IEF gel was stained with Coomassie blue in isopropanol-acetic acid and the electrophoretic staining bands were analysed for various globin chains.

Production of mouse hybridoma.

Generation of mouse hybridoma was based mainly on methods described by Goding.[13] Briefly, female Balb/c mice were immunized intraperitoneally with 100 mg of β^E-globin chain dispersed in 50 μL of complete Freund's adjuvant. The second, third and fourth doses of the same immunogen in incomplete Freund's adjuvant were injected every week after the first immunization. Sera were collected from the tail of mice for antibody determination. The immunized mouse spleen cells were fused with mouse myeloma cells (X63-Ag8.653) by polyethylene glycol (PEG). The hybridomas were selected and grew in HAT and HT media. Supernatant fluid from each hybridoma well was assayed for antibody to β^E-globin chain by ELISA. The selected hybridoma was cloned by limiting dilution at 0.5 cell per well through coculturing with mouse feeder cells.

Determination of monoclonal antibody against β^E-globin chain.

Mouse mAb against β^E-globin chain was determined by ELISA.[14] Microtiter plate was coated with β^E-globin chain. After the supernatant from the hybridoma well reacted with the coated antigen, the rabbit anti-mouse immunoglobulins labelled with HRP was added. The enzymatic activity of HRP was detected by OPD and stopped by sulfuric acid. Color intensity was read as O.D. by an ELISA reader.

Characterization of mouse monoclonal antibody.

Mouse mAb against β^E-globin chain was characterized for its specificity by reacting with β^A-, β^E-, γ-, δ- and α-globin chains using ELISA. Microtiter wells were coated with various types of globin chain. After the addition of mouse mAb, color reaction was developed as mentioned before. The isotype of the mouse monoclonal antibody was determined by using the mouse Isotype kit (Zymed).

Results

Separation and quantitative determination of HbE.

Hemoglobin E was obtained from a heterozygous HbE (β^E/β^A) subject, as shown in Fig. 1. The HbE was separated from other hemoglobins by DEAE-Sephadex column chromatography. Three peaks were revealed by the DEAE-Sephadex column, as shown in Fig. 2A, while Fig. 2B shows the normal hemoglobin solution chromatogram from the DEAE-Sephadex column, which illustrated the majority of HbA and a very small amount of HbA_2. When the chromatogram in Fig. 2A was superimposed to Figure 2B to yield Fig. 2C, it revealed that peak 1, 2, and 3, should be HbE or HbA_2, HbA, and HbF, respectively. When peaks 1, 2 and 3 were analysed by cellulose acetate electrophoresis along with standard HbA_2, HbE, HbF and HbA, it revealed that peak 1, 2 and 3 was, HbE/HbA_2, HbA and HbF, respectively, as shown in the electrophoretogram of Fig. 3.

Separation, isolation and characterization of β^E-globin chain.

After peak 1 from the DEAE-Sephadex column was treated with cold acid acetone to separate globins from hemoglobins, the globin chains were separated by a CM cellulose column, as shown in Fig. 4. The major peak revealed from the CM cellulose column was β^E-globin chain. It was shown by cellulose acetate electrophoresis that the isolated hemoglobin was HbE or HbA_2 (see Figure 3). The β^E-globin chain was isolated from HbE by the CM cellulose column (see Fig.4). The isolated β^E-globin chain was characterized by IEF along with various standard globin chains. The IEF electrophoretogram revealed that the isolated β^E-globin chain had the isoelectric point (pI) of 7.45, as shown in Fig. 5.

Production of mouse hybridoma and monoclonal anti-β^E globin chain

Fusion of cells between β^E-globin chain immunized mouse spleen cells, and mouse my-

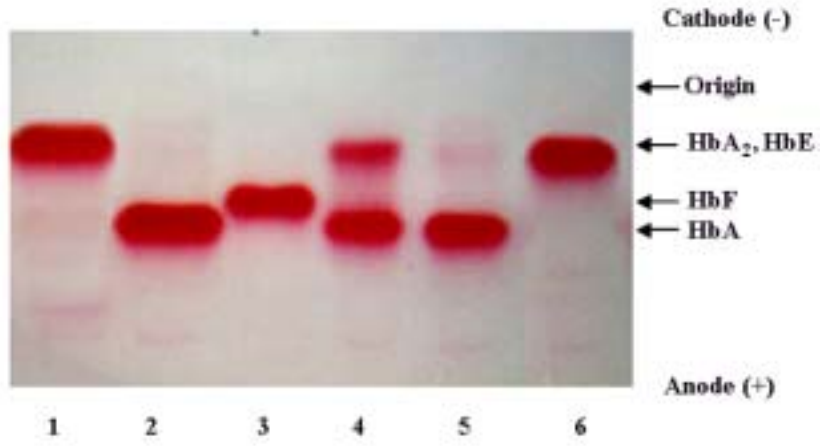

Figure 1. Electrophoretogram of hemoglobins from normal adult and heterozygous HbE hemolysates.
Lane 1, 2, 3, 6 : Standard hemoglobin E, A, F, or A_2, respectively.
Lane 4 : Hemolysate from heterozygous HbE.
Lane 5 : Hemolysate from normal adults.

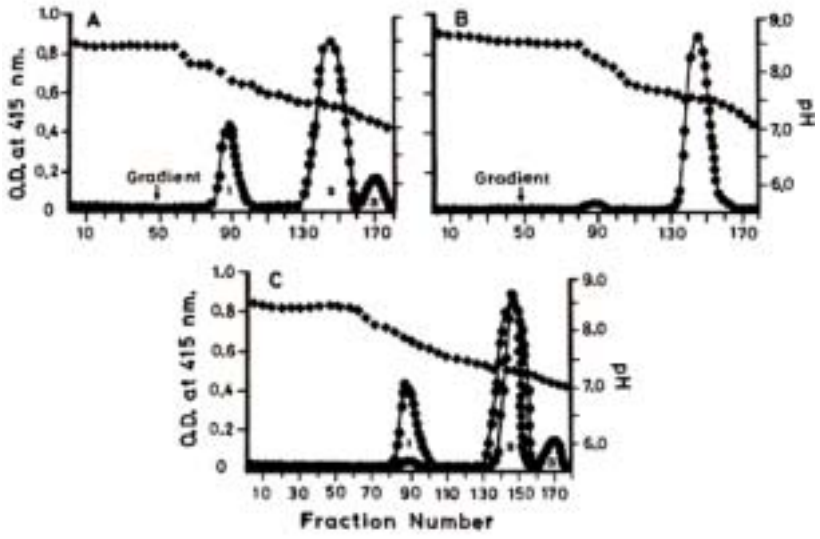

Figure 2. Separation and isolation of hemoglobin E by DEAE-Sephadex column chromatography. The chromatographic separation of heterozygous HbE hemolysate is shown in Figure 2A, while the normal blood hemolysate is shown in Figure 2B. The chromatogram of Figure 2A is superimposed on to Figure 2B to yield Figure 2C. This revealed that peak 1, 2 and 3 was HbE/HbA_2, HbA, and HbF, respectively.

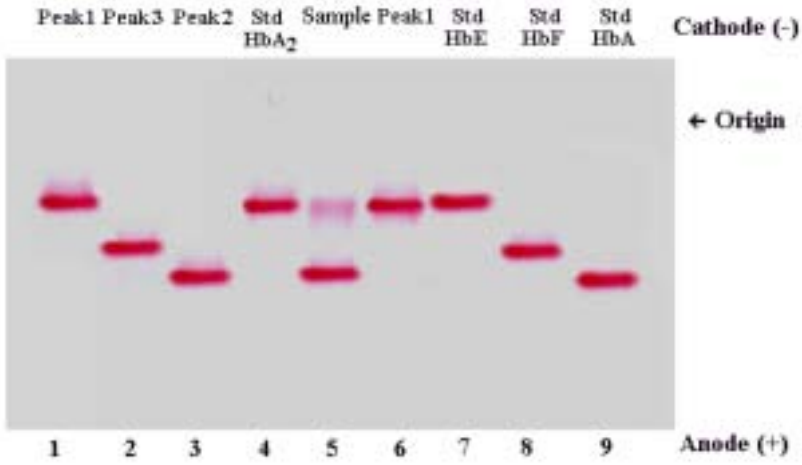

Figure 3. Cellulose acetate electrophoretic analysis of the isolated HbE/HbA_2 (peak 1 of Figure 2A). Peak 1 from chromatographic separation of heterozygous HbE was analysed by cellulose acetate electrophoresis.
Lane 1, 6 : Peak 1 eluate, Lane 2: Peak 3 eluate, Lane 3: Peak 2 eluate, Lane 4: Standard HbA_2, Lane 5: Hemolysate of heterozygous HbE, Lane 7, 8, 9 : Standard HbE, HbF, HbA, respectively.

eloma cells using PEG as a fusing agent, was tried to generate hybridoma secreting mAb against β^E-globin chain. Thirteen attempts at cell fusion were made before successfully obtaining 3 hybridoma clones secreting monoclonal antibodies specific to β^E-globin chain, as shown in Fig. 6A. The hybridoma secreting highly specific monoclonal antibody to β^E-globin chain was recloned and its specificity was re-evaluated. The mAb had highly specific activity to β^E-globin chain, as shown in Fig. 6B. The isotype of the mAb was IgM with κ light chain (data not shown).

Discussion

Hemoglobin variants, in which there is a structural alteration in one of the globin chains, is one group of hemoglobin genetic disorders. As of early 1998, 750 hemoglobin variants had been described,[15] 90% of which were single amino acid substitutions in the α, β, γ or δ chains. The remaining 10% were abnormal hemoglobins with two substitutions in the same globin chain, deletions or insertions, N-terminal or C-terminal elongations, or hybrid globins. The majority (~75%) of structural variants described were due to mutations in the α or β chains of HbA.[2] Hemoglobin E is probably the most common hemoglobin variant in the world's population. The β-chain codon 26 Glu−>Lys mutation (GAG−>AAG) partially activates a cryptic splice site towards the 3' end of exon 1 of the β-globin gene, resulting in a proportion of abnormally spliced mRNA.[4] Therefore, in HbE, less β^E-globin is synthesized and a mild thalassemia phenotype results.

Hemoglobin E heterozygotes, with approximate 30% HbE, are clinically normal and have only minor hematological changes. Homozygotes have a very mild anemia, and their hematological changes are similar to those of heterozygous β thalassemia. However, a compound heterozygous state between HbE and β thalassemia gives rise to serious clinical

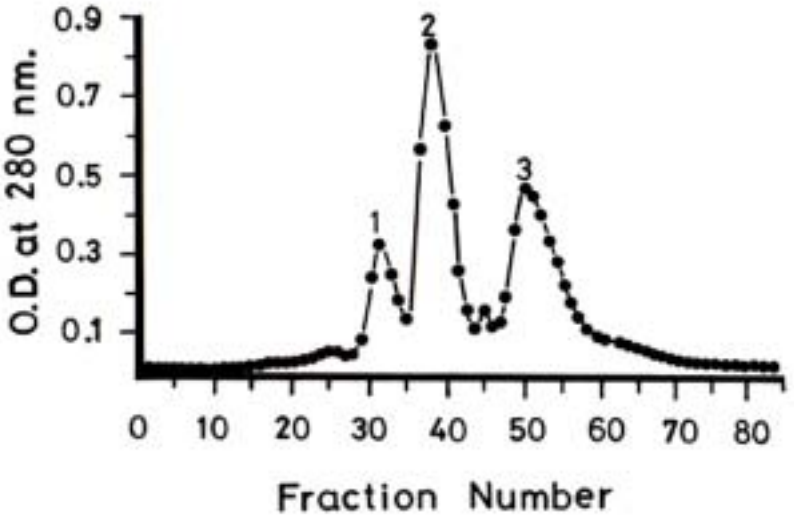

Figure 4. Isolation of globin chains by CM cellulose column chromatography. Globin chains from HbE/HbA_2 (peak 1 of Figure 2A) were separated by CM cellulose column chromatography yielding 3 protein peaks. Protein 1, 2 and 3 was δ-, β^E-, and α-globin chain, respectively.

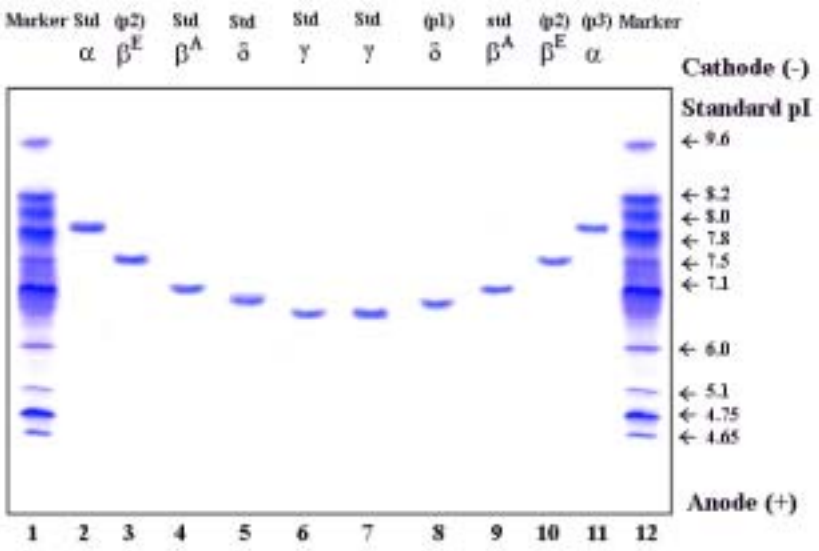

Figure 5. Analysis of the isolated β^E-globin chain by isoelectric focusing electrophoresis.
Lane 1, 12: Standard pI of polypeptide markers, Lane 2: Standard α-globin chain (pI = 7.83), Lane 3, 10 : Protein 2 eluate, Lane 4, 9: Standard β^A-globin chain (pI = 7.20), Lane 5: Standard δ-globin chain (pI = 6.88), Lane 6, 7: Standard γ-globin chain (pI = 6.90), Lane 8: Protein 1 eluate, Lane 11: Protein 3 eluate.

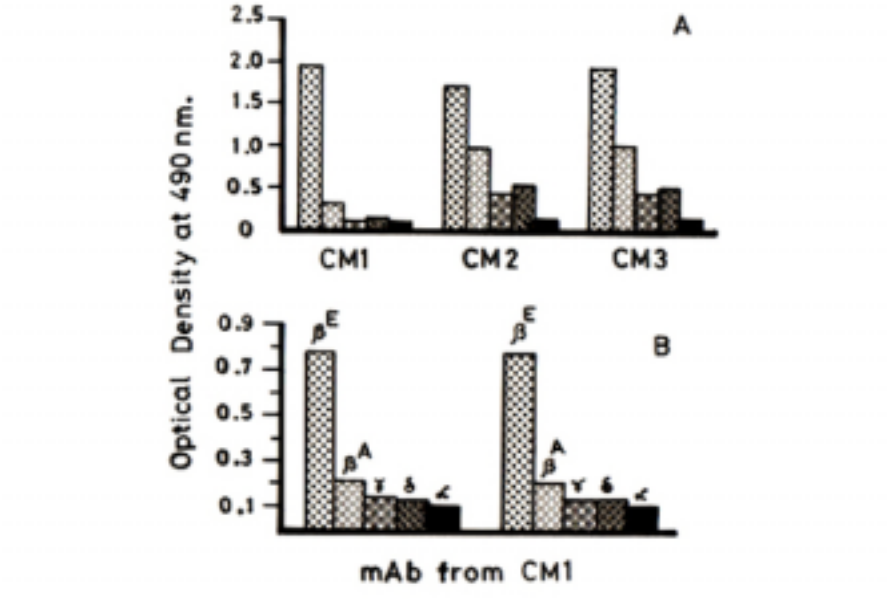

Figure 6. Evaluation of the mouse monoclonal antibody to β^E-globin chain. The mouse mAb to β^E-globin chain was evaluated for its specificity by reacting with β^E-, β^A-, γ-, δ-, and α-globin chains. Figure 6A shows the immunological activities of all 3 hybridomas obtained, while Figure 6B shown the CM1 hybridoma secreting mAb with the highest specificity to β^E-globin chain (duplicated experiments).

disease,[2] with a phenotype ranging from mild anemia to the most severe form of β thalassemia major. The interaction of HbE with β^o thalassemia alleles produces a profound degree of anemia that is observed in many compound heterozygotes.

Due to the clinical significance of compound heterozygous HbE and β thalassemia, methods for detection and quantitation of HbE have been available. Simple methods are starch gel or cellulose acetate electrophoresis. However, both techniques are not able to distinguish HbE, HbA_2 HbO Arab and HbC (also see Fig. 3). Therefore, globin chain electrophoresis in 6 M urea is frequently employed for a more definitive identification. Hemoglobin E can also be distinguished from HbC, HbA_2 and HbO Arab by IEF or high performance liquid chromatography (HPLC).[16,17] Separation of globin chains by IEF and HPLC is expensive and requires sophisticated equipment and expertise to use it. Therefore, a simple and less expensive technique was necessary for HbE determination, and so the antigen-antibody reaction principle seems appropriate.

A report of six mAbs specific to human globin chains was produced using hybridoma technology: mAbs 16-2 and 37-8 were β chain specific, mAb 31-2 recognized an epitope common to both α and β subunits, mAb 30-3 was reactive to epitopes in the $\alpha_2\beta_2$ tetramers, mAb 45-1 recognized an epitope common to β and γ subunits and mAb 51-7 was γ chain specific. These mAbs against various globin chains would be useful probes for studying hemoglobin expression and qualitative or quantitative determination of various types of hemoglobins.[18] A murine mAb specific to the δ chain of HbA_2, which did not react with α, β and γ chains, was also generated.[19]

In this study, we were successful in generating 3 hybridoma clones producing mAb against β^E-globin chain. One hybridoma clone (CM1) secreted monoclonal antibody highly specific to β^E-globin chain with very low reactivity to β^A-, γ-, δ-, and α-globin chains. This mAb was highly specific to β^E-globin chain because it gave strong reactivity to β^E-globin chain, but low reactivity to β^A-globin chain. The only difference between β^E- and β^A-globin chains is the amino acid at position 26. The β^A-globin chain is a normal β-globin chain containing glutamic acid (GAG) at position 26. However, the β^E-globin chain has lysine (AAG) instead of glutamic acid, due to point mutation of G→A (GAG → AAG) at this position. Therefore, our mAb (CM1) had high discriminating power by its ability to differentiate β^E- from β^A-globin chain. There are two more types of hemoglobin variants with a point mutation at the beta globin chain, changing from a glutamic acid to a lysine at position 6 in HbC and 121 in HbO Arab. Therefore, it would be interesting to evaluate the specificity of our mAb to the β^C and β^{O-Arab} globin chains when both hemoglobin variants are available for evaluation. In this study cell-fusion was performed 13 times before only one hybridoma producing highly specific mAb to β^E-globin chain was obtained. We are now in the process of developing an immunological assay for qualitative and quantitative determination of HbE using the mouse mAb specific to β^E -globin chain. Knowing who the HbE carriers are in the population may enable us to decrease the coinheritance of HbE and β thalassemia

disease.

Acknowledgments

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การสร้างไฮบริโดม่าหนูที่สามารถหลั่งโมโนโคลนอลแอนติบอดีที่จำเพาะต่อสายเบต้าอีโกลบินเพื่อใช้คัดกรองฮีโมโกลบินอีในประชากร

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บทคัดย่อ ฮีโมโกลบินอี (HbE) เป็นฮีโมโกลบินผิดปกติที่เกิดจากการมีมิวเตชั่นของเบต้าฮีโมโกลบินยีนส์ที่ตำแหน่ง 26 (GAG-->AAG) ทำให้กรดอะมิโนเปลี่ยนจากกลูตามิกไปเป็นไลซีน เป็นฮีโมโกลบินผิดปกติที่พบมากที่สุดในประชากรเอเชียอาคเนย์ คณะวิจัยได้ทำการแยกฮีโมโกลบินอีจากเฮทเทอโรซัยกัส HbE โดยวิธี DEAE sephadex column chromatography ทำการแยกสายโกลบินอีออกจาก HbE โดยวิธี CM-cellulose column chromatography ใน 8 โมลาร์ยูเรีย ทำการวิเคราะห์คุณสมบัติของสายโกลบินอีที่แยกได้บริสุทธิ์ด้วยวิธี isoelectric focusing electrophoresis (IEF) นำสายโกลบินอีไปฉีดหนู Balb/c เพื่อกระตุ้นให้สร้างแอนติบอดี นำเซลล์ม้ามหนู Balb/c นั้นมาทำการฟิวส์เซลล์กับเซลล์มะเร็งหนู (myeloma cells) หลังจากทำการฟิวส์เซลล์ถึง 13 ครั้ง ตรวจพบมีไฮบริไดม่า 3 โคลนที่สร้าง และหลั่งโมโนโคลนอลแอนติบอดี (monoclonal antibody; mAb) ที่มีความจำเพาะต่อสายโกลบินอี แต่มีเพียงหนึ่งโคลนที่สร้าง mAb ที่มีความจำเพาะสูงกับสายโกลบินอี โดยทำปฏิกิริยาน้อยมากกับสายเบต้าโกลบินเอ โกลบินแกมม่า โกลบินเดลต้าและโกลบินอัลฟา เมื่อตรวจหา isotypes ของ mAb ที่ได้ พบว่าเป็นชนิด IgM ที่มี light chain เป็นชนิด kappa

การที่ได้ hybridoma ที่สามารถสร้างและหลั่ง mAb ที่มีความจำเพาะสูงมากจนสามารถแยกความแตกต่างของสายเบต้าโกลบินอีออกจากสายเบต้าโกลบินเอ ซึ่งมีความแตกต่างกันเพียงหนึ่งกรดอะมิโนที่ตำแหน่ง 26 ของสายเบต้าโกลบินจากกรดอะมิโนทั้งหมด 146 ตัวได้ น่าจะนำมาใช้เป็นประโยชน์ได้อย่างดี โดยนำเอา mAb ที่ได้ไปพัฒนาวิธีการตรวจทางอิมมูโน เพื่อใช้ตรวจกรองพาหะฮีโมโกลบินอีในประชากรได้ โดยวิธีไม่ยุ่งยากเช่นในอดีต อันจะเป็นทางหนึ่งในการลด HbE ไปปฏิสัมพันธ์กับเบต้าธาลาสซีเมีย เป็น β-Thalassemia/HbE ลงได้ **เชียงใหม่เวชสาร 2549;45(1):1-10.**

คำสำคัญ: โมโนโคลนอลแอนติบอดี ฮีโมโกลบิน เบต้าอีโกลบินเชน