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PRENATAL DIAGNOSIS OF HOMOZYGOUS α–THALASSEMIA 1 BY USING FETAL NUCLEATED RED BLOOD CELLS IN MATERNAL CIRCULATION

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This study examines the use of fetal Nucleated Red Blood Cells (NRBCs) in the diagnosis of homozygous α-thalassemia 1. This is the most common genetic disorder in Thailand. The 4 major thalassemic diseases are homozygous α-thalassemia 1, homozygous β-thalassemia, β-thalassemia in association with Hb E and Hb H diseases. Homozygous α-thalassemia 1 is the most severe form of thalassemia. Pregnant women with an affected child may counter certain complications such as toxemia of pregnancy. Prenatal diagnosis (PND) with selective abortion of the affected fetus is necessary. The procedures to obtain fetal cells for PND include fetal blood sampling by cordocentesis and chorionic villus sampling (CVS). These invasive procedures may create complications in both the fetus and pregnancy. Recent studies suggest analysis of NRBCs in the maternal circulation will prevent these complications. PND of homozygous α-thalassemia 1 using the fetal NRBCs is based on the immunological demonstration of the absence of normal α-globin chain in the fetal NRBCs with hydrops fetalis syndrome. The fetal NRBCs were isolated by density gradient centrifugation and enriched by immunomagnetic separation. The fetal NRBCs were further analyzed with fluorescent anti α-globin antibody.

Eleven high-risk pregnancies for Hb Bart's hydrops fetalis were studied using the fetal NRBCs in maternal blood. The blood was taken prior to CVS at 10-26 weeks of gestation. Eight out of eleven cases were non-hydrops fetuses, 3 showed the heterozygous α-thalassemia 1, 2 were Hb CS trait, 1 was heterozygous α-thalassemia 1 or Hb H disease and 2 were normal fetuses. Three cases were diagnosed to be Hb Bart's hydrops fetuses because no α-globin chain was detected in fetal NRBCs. All the results were confirmed with DNA analysis of chorionic villus. These results suggest that the immunofluorescent detection of fetal NRBCs enriched from maternal blood could be an accurate and sensitive method for screening of homozygous α-thalassemia 1. This procedure provides a noninvasive approach for PND of homozygous α-thalassemia 1.

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สายศิริ สีทองดี : การตรวจ homozygous α-thalassemia 1 เพื่อวินิจฉัยทารกในครรภ์ก่อนคลอดโดยศึกษาจากเซลล์เม็ดเลือดแดงอ่อนของลูกที่อยู่ในกระแสเลือดของแม่ (Prenatal diagnosis of homozygous α-thalassemia 1 by using the fetal nucleated red blood cell in maternal circulation) คณะกรรมการควบคุมวิทยานิพนธ์ : พัชรีย์ วิชยานุวัติ, พ.บ. , Ph.D., ปราณี ฟู่เจริญ, Ph.D., สุทัศน์ ฟู่เจริญ, พ.บ., 97 หน้า. ISBN 974-663-702-9

การศึกษานี้เป็นการใช้เซลล์เม็ดเลือดแดงอ่อนของลูกที่อยู่ในกระแสเลือดของแม่เพื่อวินิจฉัยโรค homozygous α-thalassemia 1 ของทารกในครรภ์ก่อนคลอด ธาลัสซีเมียเป็นโรคพันธุกรรมที่พบบ่อยที่สุดในประเทศไทย โรคธาลัสซีเมียที่ตรวจพบในคนไทยที่สำคัญมี 4 โรค ได้แก่ โรค homozygous α-thalassemia 1, homozygous β-thalassemia, β-thalassemia/Hemoglobin E และ โรค Hemoglobin H. Homozygous α-thalassemia 1 เป็นโรคธาลัสซีเมียที่รุนแรงที่สุด ในมารดาที่มีทารกเป็นโรคนี้มักจะมีอาการครรภ์เป็นพิษ การตรวจวินิจฉัยทารกก่อนคลอดเป็นสิ่งจำเป็น เทคนิคการตรวจวินิจฉัยทารกในครรภ์ที่ใช้กันอยู่ในปัจจุบัน คือการเจาะเลือดของทารกในครรภ์ และการเจาะชิ้นเนื้อรกออกมาเพื่อตรวจสอบ เทคนิคเหล่านี้มีความเสี่ยงสูงที่จะทำให้เกิดภาวะแทรกซ้อนต่างๆ ทั้งในทารกและแม่ ปัจจุบันนี้ได้มีการศึกษาเซลล์เม็ดเลือดแดงตัวอ่อนของลูกที่หลุดเข้ามาในกระแสเลือดของแม่ เพื่อที่จะสามารถให้การตรวจวินิจฉัยทารกในครรภ์ได้อย่างรวดเร็วและปลอดภัยในการศึกษานี้ได้ดัดแปลงวิธีการตรวจหา homozygous α-thalassemia 1 จากเซลล์เม็ดเลือดแดงตัวอ่อนของลูกที่หลุดเข้ามาในกระแสเลือดของแม่ โดยอาศัยความรู้พื้นฐานว่าในทารกปกติมีการสร้างสาย α-globin chain และในทารกที่เป็นโรคธาลัสซีเมียชนิด homozygous α-thalassemia 1 จะไม่มีการสร้างสาย α-globin chain ในเซลล์เม็ดเลือดแดงเลย เซลล์เม็ดเลือดแดงตัวอ่อนของลูกจะถูกแยกจากเลือดแม่โดยเทคนิค density gradient centrifugation และคัดแยกเซลล์เม็ดเลือดแดงตัวอ่อนของลูกโดยการใช้ immunomagnetic separation ที่มีแอนติบอดี้จำเพาะต่อ transferrin receptor จากนั้นตรวจหา homozygous α-thalassemia 1 ในเซลล์เม็ดเลือดแดงตัวอ่อนของลูกโดยการย้อม immunofluorescent ต่อ anti α-globin chain antibody

ได้ทำการศึกษาความแม่นยำของวิธีการตรวจนี้ในหญิงมีครรภ์ที่มีความเสี่ยงต่อการมีบุตรเป็น homozygous α-thalassemia 1 จำนวน 11 รายที่มีอายุครรภ์ระหว่าง 10-26 อาทิตย์ โดยเจาะเลือดหญิงมีครรภ์ก่อนทำการเจาะชิ้นเนื้อรกเพื่อใช้ในการยืนยันผลการตรวจ พบว่า สามารถตรวจพบทารกที่เป็น homozygous α-thalassemia 1 จำนวน 3 รายและทารกที่ไม่เป็น homozygous α-thalassemia 1 จำนวน 8 ราย ซึ่งผลการตรวจของชิ้นเนื้อรกโดยเทคนิค PCR พบว่า มีทารกที่เป็น homozygous α-thalassemia 1 จำนวน 3 ราย, 3 รายเป็น heterozygous α-thalassemia 1, 2 รายเป็น heterozygous Hemoglobin Constant Spring, อีก 1 รายเป็น heterozygous α-thalassemia 1หรือ โรค Hemoglobin H และ 2 รายเป็นทารกปกติ เทคนิคนี้มีประโยชน์สามารถใช้ตรวจวินิจฉัยทารกในครรภ์สำหรับคู่เสี่ยงต่อการมีบุตรเป็นโรค homozygous α-thalassemia 1 โดยไม่ต้องเจาะเลือดทารกหรือชิ้นเนื้อรก ทำให้ไม่เกิดความเสี่ยงต่อภาวะแทรกซ้อนของการทำหัตถการดังกล่าว

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CONTENTS

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF FIGURES (CONT.)

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LIST OF ABBREVIATION

α	alpha
ANC	antenatal care
β	beta
BSA	bovine serum albumin
CVS	chorionic villus sampling
CBC/Diff	complete blood cell counts plus white blood cell differential
δ	delta
DNA	deoxyribonucleic acid
ε	epsilon
EDTA	ethylenediamine tetraacetate
FISH	fluorescence *in situ* hybridization
FACS	fluorescent activated cell sorting
FITC	fluorescein isothiocyanate
γ	gamma
GPA	glycophorin A
g	gram
Hb	hemoglobin
HLA	human leukocyte antigen

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LIST OF ABBREVIATION (CONT.)

IFA	immunofluorescence assay
IgG	immunoglobulin G
μm	micrometer
MACS	magnetic activated cell sorting
MCV	mean corpuscular volume
ml	milliliter
mm	millimeter
M	molar
NRBCs	nucleated red blood cells
PCR	polymerase chain reaction
PND	prenatal diagnosis
PUBS	percutaneous umbilical cord blood sampling
RNA	ribonucleic acid
SBE	saline-boric acid-EDTA buffer
ψ	pseudo
θ	theta
WBC Diff	white blood cell differential
ζ	zeta

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CHAPTER I

INTRODUCTION

Thalassemia is a heterogenous group of genetic defects which results in defective globin synthesis. It is the most common genetic disorder in Thailand. The α–thalassemia, β-thalassemia, hemoglobin (Hb) E and Hb Constant Spring are prevalent. The gene frequencies of α–thalassemia reach 20-30% and β-thalassemia varies from 1-9% (1). The two major α–thalassemic diseases are Hb Bart's hydrops fetalis or homozygous α–thalassemia 1, and Hb H disease which occurs from the interaction between α–thalassemia 1 and α–thalassemia 2 or between α–thalassemia 1 and Hb Constant Spring. The interaction between β-thalassemia genes or β-thalassemia and Hb E genes leads to homozygous β-thalassemia and β-thalassemia/Hb E, which are major β-thalassemic syndromes in Thailand.

Hemoglobin is the major cytoplasmic protein produced in red cells. In adults; the predominant molecule consists of a tetramer of alpha and beta globin chains ($\alpha_2\beta_2$), and is known as hemoglobin A (Hb A). In the 5-35 weeks fetus, the predominant molecules consists of alpha and gamma globin chains ($\alpha_2\gamma_2$) and is known as hemoglobin F (Hb F). There is no α–globin production in Hb Bart's hydrops fetalis resulting in the most serious form of thalassemic disease. The fetus dies in utero or soon after birth because Hb Bart's does not release oxygen to the tissues. The affected fetuses are hydropic with severe growth retardation. Abnormal development of vital organs such as brain and lung

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contributes to the severe morbidity that makes the condition incompatible with life. Maternal complications such as toxemia of pregnancy have been observed in almost all pregnancy (2).

Although cure for some of these thalassemic diseases is possible by bone marrow transplantation, there are still technical problems and it is a very expensive treatment. Moreover, in Thailand, with a very high incidence of these abnormal genes, each year there are more than 10,000 thalasssemic births.

The best strategy for the prevention and control of this disease includes a combination of screening for thalassemia carriers and high-risk couples, a good genetic counseling and finally prenatal diagnosis (PND) with selective abortion of the affected fetus. The procedure of obtaining fetal cells for DNA diagnosis includes amniocentesis, fetal blood sampling and chorionic villus sampling (CVS) (3). These procedures are invasive for the fetus and may result in anomaly, infection or abortion. Leakage of fetal cells into the maternal circulation even in early pregnancy provides a noninvasive procedure for PND (4). Recovery of the fetal cells from maternal circulation avoids any fetal risks (5-7).

The three types of fetal cells that have been sought as the source of fetal DNA are lymphocytes, trophoblasts and nucleated red blood cells (NRBCs). Fetal NRBCs are a good target cell population for fetal DNA diagnosis because they are unlikely to circulate in the peripheral blood of a normal adult woman (8).

The fetal cells can be detected within the maternal circulation as early as the fifth week of gestation (9,10). Their sparse concentration in maternal blood (fetal/maternal

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ratios, $1/10^5$ to $1/10^9$) (11,12), prohibits direct analysis. To obtain quantities of fetal cells sufficient for analysis requires the use of enrichment techniques. Such techniques have involved either physical or immunochemical methods. Physical methods employ density gradient centrifugation and are based on the different sedimentation rates between nucleated cells (fetal NRBCs, trophoblasts and lymphocytes) and non-nucleated maternal cells.

Immunochemical separation techniques utilize antibodies directed against fetal NRBCs epitopes. These antibodies include; anti-glycophorin A (anti-GPA) (13), anti-transferrin receptor (anti-CD71) alone and in combination with anti-GPA monoclonal antibodies (13). Embryonic and fetal hemoglobins have also been used as the marker of fetal NRBCs (15).

In order to differentiate NRBCs of homozygous α–thalassemia 1 fetuses from those of heterozygous or normal fetuses and from maternal mononuclear cells, these cells were tested by immunofluorescence with specific monoclonal antisera against human α–globin and γ–globin chains. The indirect method with an affinity-purified goat anti-rabbit antiserum labeled with FITC was used for the immunofluorescence staining. The NRBCs of fetuses at risk for Hb Bart's hydrops fetalis syndrome are apparently absent of normal α–globin chains. This non-invasive procedure is very useful in prenatal diagnosis in the couple at risk of having lethal α–thalassemia disease i.e. Hb Bart's hydrops fetalis (homozygous α–thalassemia 1).

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Aims of the thesis

The objectives of this study are following:

1. To isolate the NRBCs by density gradient centrifugation using blood samples from the splenectomized β–thalassemia patient.
2. To detect the α–globin and γ-globin chains in the fetal NRBCs by the immunofluorescent technique. Fetal NRBCs were collected from the peripheral blood of pregnancies at risk for the β–thalassemia fetuses and their CVS cell washings (as positive control).
3. Enrichment of the fetal NRBCs by anti-transferrin receptors (CD71) antibody.
4. The detection of homozygous α–thalassemia 1 hydrops fetuses will be carried out in pregnant women at risk for Hb Bart's hydrops fetalis. The results are confirmed by PCR analysis of their chorionic villi.

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CHAPTER II

LITERATURE REVIEW

The thalassemias are a group of hereditary anemia characterized by decreased or absent synthesis of one of the globin subunits of the hemoglobin molecule. This is the most common genetic disorder in Thailand. Two major types are α-thalassemia and β-thalassemia. In addition, two abnormal hemoglobins (Hb), Hb E and Hb Constant Spring, which have the phenotypic expression of thalassemia, are also found in Thais.

Structure of human globin genes

Hemoglobin is a tetramer that consists of two α-like and two β-like globin subunits. The α-globin gene complex is clustered on the short arm of chromosome 16 in a 25-Kb region consisting of ζ, ψζ, ψα$_2$,ψα$_1$, α$_2$, α$_1$ and θ genes. The β–globin gene cluster is on the short arm of chromosome 11 in a 50 Kb region containing ε, ^{G}γ, ^{A}γ, ψβ, δ and β globin genes. The arrangement of human globin genes is shown in Figure 1 (16).

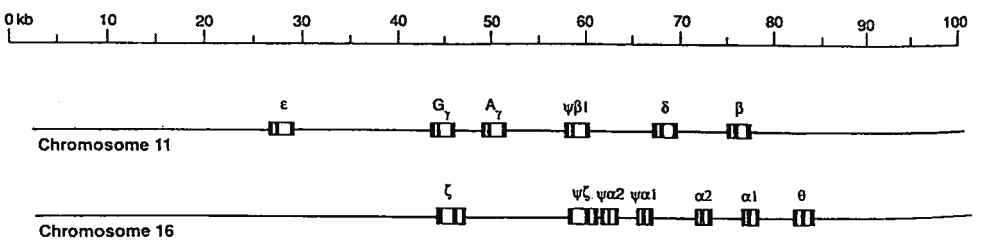

Figure 1 Organization of the human globin genes (from After Bunn HF, 1986).

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Regulation of human globin gene

Initiation of transcription occurs at the site on the DNA double helix corresponding to the 5' end of globin mRNA. After initiation of transcription, the 5' end of the nascent RNA molecule is modified by capping. The state site for RNA transcription on the gene is referred to as the "CAP" site. The poly A tract is added as a post translational modified A triplet that encodes for methionine (AUG) is the initiation codon. The initiation codon served to establish the reading frame of the mRNA. Each triplet codon is read sequentially to give a protein of correct sequence. Translation continues until a terminator codon (UGA, UAG or UAA) is encountered in the same reading frame as the initiator, AUG. Termination codons that are out of the reading frame have no effect on translation.

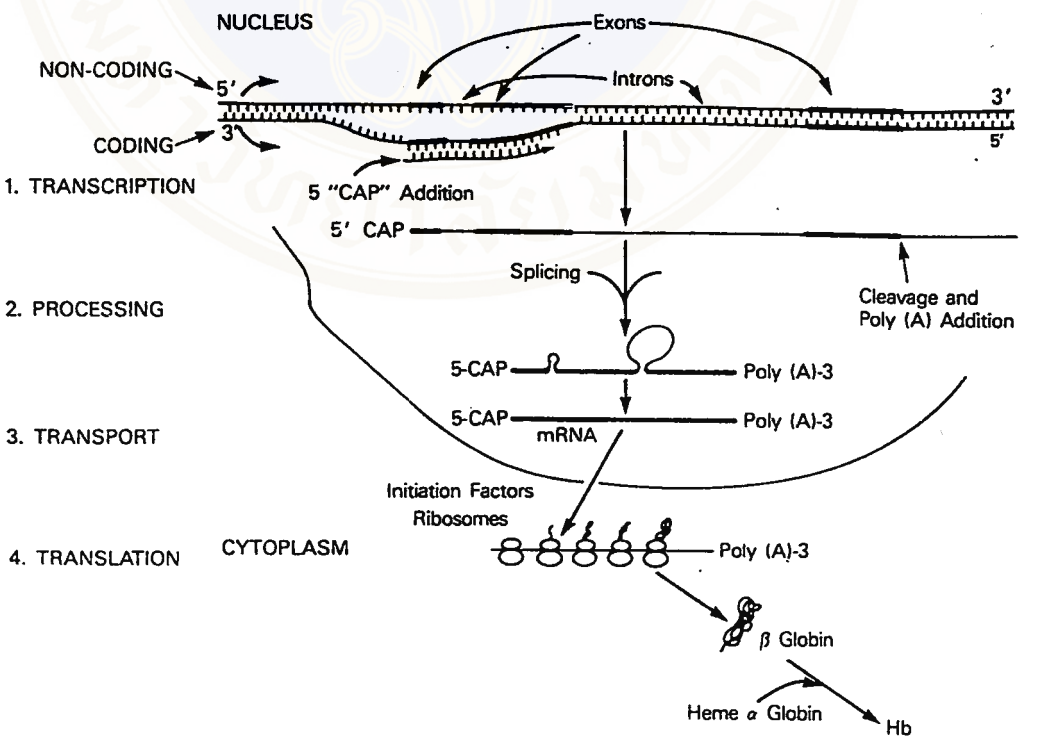

Figure 2 The expression of human globin gene (from AW Nienhuis, 1987).

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The way that the globin genes are regulated is of major relevance to an understanding of the pathogenesis of the thalassemias. In normal individuals, production of α– and non α–globin chains is equal. Gene deletions or point mutations in the nucleotide sequences necessary for transcription, RNA processing and translation are found to be the causes of thalassemic diseases.

Most of the DNA within cells that is not involved in gene transcription is package into a compact form that is inaccessible to transcription factors and RNA polymerase. Transcriptional activity is characterized by a major change in the structure of the chromatin surrounding a particular gene. Erythroid lineage-specific nuclease-hypersensitive sites are found at several locations in the β–globin gene cluster which vary during different stages of development. In fetal life these sites are associated with the promotor regions of all four globin genes, whereas in adult erythroid cells the sites associated with the γ–globin gene are absent. The methylation state of the genes plays an important role in their ability to be expressed. The changes in chromatin configuration around the globin genes at different stages of development are reflected by alterations in their methylation state.

Hemoglobin production during development

The switch from human embryonic to fetal hemoglobin formation coincides, at about 5 weeks of gestation, with a change in the site of hematopoiesis from the yolk sac islands in the embryo to the liver in the fetus (Figure 3) (17, 18).

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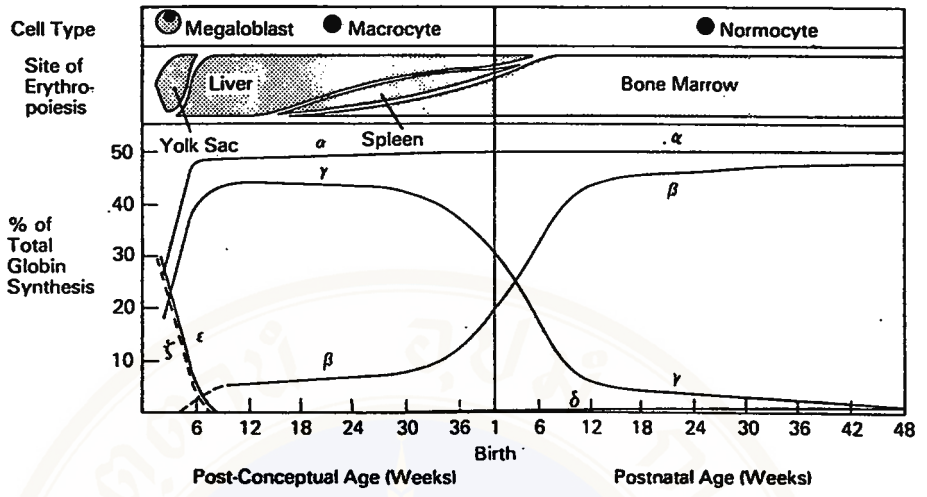

Figure 3 Changes in globin chain production and sites of hematopoiesis during the course of development (from Weatherall DJ, 1981).

This switch in erythropoietic sites is accompanied by striking changes in the morphology of circulating red cells. Embryonic erythrocytes are very large nucleated cells (20-25 μm), while the erythrocytes of fetal liver origin are nucleated and morphologically similar to adult erythrocytes except for their larger sizes (10-15 μm) (19). The switch from ζ to α and from ε to β globin production begins very early in gestation, Hb F is readily detected in 5 weeks embryos (20, 21). These transitions are completed well before 10 weeks of gestation; by that time the embryonic hemoglobins are barely detectable in the fetal circulation (22).

The liver remains the predominant site of erythropoiesis in the fetus until about the twentieth week of gestation (Figure 3). Hematopoiesis subsequently occurs in the spleen and the bone marrow, and by the time of birth the bone marrow is the main hematopoietic organ (17). Shifts in the site of erythropoiesis coincide with changes in other morphological and biochemical characteristics (16).

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Maturation of the erythrocytic series

The erythroid precursor cell compartment includes cells contrary to the erythroid progenitor cells defined by morphologic criteria. The earliest morphologically recognizable erythroid cell is the proerythroblast, which after four to five mitotic divisions and serial morphologic changes gives rise to mature erythroid cells. Its progenies are basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatic erythroblasts, reticulocytes and finally erythrocytes (Figure 4, 23).

1. Proerythroblasts

This is a large cell (diameter 20-25 μm) with a regular oval or rounded shape. The nucleus is large (8/10 of the area of the cell), round or oval, centrally placed at a significant distance from the cell membrane with an ample zone of cytoplasm. The nuclear outline is clearly defined and the cytoplasm is intensely basophilic.

2. Basophilic erythroblasts

The cell size and nucleocytoplasmic ratio are reduced (16-18 μm). The nucleus is round, central, lacking in nucleoli and the chromatin is condensed into dark masses. The cytoplasm is intensely blue and appears more homogeneous because of the reduction in the clear area around the nucleus. There are not yet any traces of hemoglobin.

3. Polychromatophilic erythroblasts

The reduction in size (diameter12-15 μm) and nucleocytoplasmic ratio continues.

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The nucleus is smaller and darker but remains round, with the chromatin clumped into large very dark masses which are distinct and regularly distributed. In the more mature cells the clumps tend to be confluent, the nuclear membrane is very distinct and nucleoli are not apparent. The cytoplasm is violet or reddish.

4. Orthochromatic erythroblasts

The nucleus remains round, but is reduced in size (10-15 μm); the chromatin clumps are condensed into a single dark mass. The cytoplasm is greynish. The nucleus-to-cytoplasma ratio is low, and the nucleus is extremely concentrated and nonstructural. The cytoplasma is orthochromatic and not granular.

5. Reticulocytes

After the last cellular division, the inactive dense nucleus of the orthochromatic erythroblast moves to one cell side and is expelled surrounded by an attenuated cytoplasmic content. Extrudes nuclei are engulfed by marrow macrophages, and the resulting enucleated cells are called a reticulocyte. The life span of a reticulocyte confirms to the time between the moment of enucleated orthochromatic erythroblast to the moment of mature erythrocyte with undetectable cytoplasmic RNA.

6. Erythrocytes

Normal human red cells have a diameter of 7.5-8.7 μm, which decreases slightly with cell age. The normal resting shape of the erythrocyte is a biconcave disc. The normal

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erythrocyte stains reddish-brown in Wright-stained blood films and pink with Giemsa stain. The central one-third of the cell stains relatively pale compared with the periphery, reflecting its biconcave shape. The life span of an erythrocyte is 100-120 days, it spends most of its circulatory life within the capillarly channels of the microcirculation.

Figure 4 Maturation of the erythrocytic series (from Hynn BH, 1975).

Hydrops fetalis

Hydrops fetalis has been defined as a pathologic increase of interstitial and total fetal body water that usually appears primarily in soft fetal tissue and serous cavities (pleural, pericardial, intraperitoneal). Hydrops fetalis can be classified according to immune and nonimmune causes. The isoimmunization, i.e., fetomaternal blood group incompatibility is immune cause of hemolysis and anemia leading to hydrops fetalis.

Marked fetal anemia, particularly in association with abnormal hemoglobinopathies (most commonly α—thalassemia, gluocose-6-phosphate dehydrogenase deficiency), pure red cell aplasia or chronic fetal blood loss may be the nonimmune cause of hydrops. Hydrops fetalis occurs when there is alteration in hydrostatic pressure or colloid osmotic pressure leading to fluid collection in various fetal compartments. Hydrops can occur at

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any time during gestation. In the first trimester, hydrops is occasionally seen with spontaneous abortion. First and second-trimester hydrops is associated with fetal death. Polyhydramnios is frequently the presenting diagnosis in third-trimester hydrops.

The intrauterine diagnosis of nonimmune hydrops is easily made with diagnostic ultrasound. Fluid collections can be readily seen. Ascites and pleural effusion are seen as echo-free (dark) areas in the abdomen and pleural cavity of the fetus. Examination of the fetus can demonstrate marked skin edema. Pericardial effusion may be grossly detected. Polyhydramnios diagnosed is seen in 50-75% of the cases of nonimmune hydrops (24).

Alpha thalassemia

The α-thalassemia is characterized by the decreased or absent α-globin chain production. It occurs mainly from a large deletion in the α-globin gene cluster involving one or both of the linked α-globin genes. Less frequent α-thalassemia can occur from nondeletion defects such as point mutation or small deletion and insertion within either α_1 or α_2 globin gene (25). These mutations may affect splicing, polyadenylation, translation or post-translation stability of the α-globin product (26). The α-thalassemias are divided into two main classes; α-thalassemia 1 or α^0-thalassemia in which there is no α-globin gene expression, another is α-thalassemia 2 or α^+-thalassemia in which the output of α-globin gene is defective.

The cause of α-thalassemia 1 is due to gene deletions involving different lengths of the α-globin gene cluster that removes the two linked α-globin genes on the chromosome. The α-thalassemia 2 is due to either deletion of one α-globin gene or to mutations that

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partially or completely inactivate one of the linked pair of the α-globin genes (27, 28). The compound heterozygous state for α-thalassemia 1 and α-thalassemia 2 causes hemoglobin H (Hb H) disease. In nondeletion α-thalassemia more than 24 mutations have been detected. The most common nondeletion form is Hb Constant Spring (Hb CS). The interaction of Hb CS with deletional α-thalassemia 1 gene also causes Hb H disease (29, 30).

The most severe form of α-thalassemia is Hb Bart's hydrops fetalis. This condition results from a homozygosity for α-thalassemia 1 and is characterized by the deletion of all four α-globin genes (--/--) (31). The fetus can not produce Hb F ($\alpha_2\gamma_2$) and Hb A ($\alpha_2\beta_2$) leading to lethality, either in utero or soon after birth. The cause of death of the fetuses due to the physiological dysfunction of Hb Bart's which has high oxygen affinity (24). Residual expression of the ζ-globin gene results in synthesis of sufficient functional hemoglobin tetramers (Hb Portland ($\alpha_2\zeta_2$)) to carry fetus through mid to late gestation. Some infants are stillborn or expire within a few hours after birth. The predominant physical findings in the fetus are generalized and massing edema (hydrops), ascites, gross enlargement of the liver with a normal or slightly enlarged spleen and a larger friable placenta. The peripheral blood demonstrates severe erythroblastosis with reticulocytosis, target cell, hypochromic and fragmented cells. The mean corpuscular volume (MCV) is often very high due to the large number of circulation nucleated red cells in circulation.

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Beta thalassemia

The β-thalassemia is characterized by decreased or absent β-globin chain synthesis due to various abnormality of the β-globin gene. Point mutations and small deletions or insertions in the nucleotide sequence are mainly responsible for the molecular defect of β-thalassemia (32).

The cause of β^+-thalassemia is due to mutations affecting the promotor region of the gene or mRNA which reduce the level of functional mRNA, while β^0-thalassemia causes the production of nonfunctional mRNA or produces no mRNA (33). There are more than hundren mutations producing the β-thalassemia phenotypes (34). In Thailand, more than 20 mutations have been detected. The most common one is the 4-bp deletion in codons 41/42 which attains about 45% of β-thalassemia in Thailand (35).

The β-thalassemia can be classified into 3 groups: β-thalassemia trait or heterozygote, homozygous β-thalassemia and β-thalassemia in association with Hb E. A person who is β-thalassemia trait has no clinical abnormality. Homozygous β-thalassemia causes a severe disease known as thalassemia major or Cooley's anemia. The clinical manifestation develops in the first year of life. A severe depletion or absence in β-globin production results in chronic destruction of immature red blood cells in bone marrow and peripheral blood. These lead to a severe and chronic anemia and several other subsequent pathological changes. Regular blood transfusions are needed to reduce the degree of anemia. The interaction between β-thalassemia and Hb E results in a thalassemic intermedia but some may have a severe symptom which is similar to thalassemic major.

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Prenatal diagnosis of thalassemia

The best strategy for the prevention and control of thalassemia includes a combination of screening for thalassemia carriers and high-risk couples and offering a good genetic counseling, and finally prenatal diagnosis with selective abortion of the affected fetus. Advances in fetal sampling and in detecting mutant globin genes have provided the safe, accurate methodology required for prenatal diagnosis, which is available for pregnancies at risk for virtually all inherited disorder of hemoglobin production. The α–globin gene is critical in both fetal as well as adult life. Total loss of α–globin synthesis manifests in utero and results in Hb Bart's hydrops fetalis. Prenatal diagnosis of Hb Bart's hydrops fetalis allows early termination, which may decrease the risk of complications in the mother.

Fetal sampling methods

Prenatal diagnosis of thalassemia was first made in the 1970s by analysing the globin chain in fetal blood samples (36). Shortly, after the discovery that α-thalassemia results from deletions of the α-globin structural genes, DNA diagnosis was introduced (37, 38). Development of the DNA-based testing has led to improve fetal sampling techniques including amniocentesis, chorionic villus sampling (CVS), isolation of fetal cells from the maternal circulation and preimplantation diagnosis.

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1. Amniocentesis

Amniocentesis is used to obtain fetal cells for DNA diagnosis between 16 and 18 weeks of gestation. In current practice preamniocentesis counseling is provided; ultrasound is performed to assess the fetus, amniotic fluid and placenta. Transabdominal aspiration is performed with ultrasound guidance to obtain 20-30 ml amniotic fluid. Ultrasound assessment of bleeding and fetal viability is performed after the procedure (39). Five – ten μg genomic DNA for immediate DNA diagnosis may be obtained directly from amniocyte (40). A more effective means to obtain adequate DNA for testing is to amplify the DNA to be tested using the polymerase chain reaction (PCR) (41, 42). The tremendous sensitivity of PCR (43) allows successful genetic diagnosis with only nanogram quantities of DNA. Fetal mortality rates with amniocentesis are only 0.5% greater than in uninstrumented pregnancies. Maternal contamination of the samples is very rare; particularly when the first 1-ml of fluid is discarded. Maternal risks are amnionitis in 0.1% and vaginal spotting and amniotic fluid leakage in 2-3% (39).

2. Chorionic villus sampling

CVS can be used to obtain fetal DNA between 9 and 12 weeks of gestation, which permits earlier diagnosis and first trimester termination of pregnancy. Transcervical or transabdominal aspiration with ultrasonographic guidance is used to obtain cells of the chorion frondosum (44). CVS provides 5-25 mg tissue from which 5 μg DNA per mg is extracted (45). Experienced practitioners obtain the same low fetal wastage rate as with amniocentesis when transcervical or transabdominal approaches are used (45, 46).

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Transverse limb reduction defects that produce amputation-like anomalies occur in 6 per 10,000 CVS, only slightly higher than the uninstrumented rate (47). Because this risk is inversely related to gestational age, CVS should not be performed prior to nine weeks gestation (47). It is important to counsel parents that transverse limb reduction defects may occur even with later CVS (48) and to offer the alternative of amniocentesis later in gestation. There is a 7% chance of maternal bleeding with transabdominal CVS and a 20% chance with transvaginal CVS, neither of which are associated with maternal mortality or fetal loss (45, 48).

3. Fetal blood sampling

Fetal blood sampling is used to obtain fetal DNA between 18-20 weeks of gestation. The fetal blood was obtained by using a 20 gauge needle inserted into the umbilical cord under ultrasound guidance. Early (<2 weeks) fetal/neonatal losses occurred in 1.5 % of 5280 procedures performed at 17-39 weeks (49). Chorioamnionitis and premature rupture of membranes were the most common cause (57 %) with bleeding. Persistent bradycardia and thrombosis are the findings associated with the remainder of the fetal/neonatal losses. Spontaneous abortion or intrauterine death occurred 1.6 % (50). Despite improved methods of percutaneous umbilical cord blood sampling (PUBS), fetal loss remains 2% (51), and fetal blood sampling is seldom used (52).

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4. Preimplantation diagnosis

Preimplantation diagnosis promises a method of prenatal diagnosis that circumvents termination of pregnancy. The procedure for obtaining human embryos by *fertilization in vitro* involves using hormones to induce oocytes maturation, aspirating oocytes, fertilizing oocytes *in vitro*, and culturing embryos. A single blastomere is removed for PCR-based diagnosis and an unaffected embryo is implanted into the uterus a day after fertilization (53, 55). Highly successful delivery rates in women under age 40 years suggest that preimplantation diagnosis has promised for prenatal diagnosis.

5. Detection of fetal cells from maternal circulation

Testing of fetal cells from the maternal circulation would be the least invasive approach to prenatal diagnosis (54). Fetal cells have been isolated from the maternal circulation using flow cytometry, immunomagnetic antibody beads, magnetic activated cell sorting (MACS) and fluorescence *in situ* hybridization (FISH). The ability to isolate fetal normoblasts using antibodies against transferrin receptors and glycophorin A in flow cytometry (55) offers promise for non-invasive prenatal diagnosis.

Pattern of fetal cell traffic

Fetomaternal cell traffic in pathological conditions was first recognised in 1893, when Schmorl (56) identified trophoblast in the lung capillaries of 14 out of 17 women dying of eclampsia. In 1969 Walknowska *et al.* (57) identified male metaphases in lymphocytes cultured from cells isolated from the blood of healthy pregnant women.

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They were able to correlate the presence of these cells with the delivery of a male fetus in 19 out of 21 cases. This was the first demonstration indicating that fetal cells enter the maternal circulation in normal pregnancy and suggested that these cells had potential to be used for chromosome analysis. Subsequent work confirmed this study, with the presence of male metaphase in maternal blood reported by other workers (58).

Factors likely to influence the transfer of fetal cells into the maternal circulation include placentation, presence of a multiple gestation or a "vanishing twin", fetomaternal blood group incompatibilities, and maternal medical complications such as diabetes, bleeding, and preeclampsia (59). It appears that more fetal cells are detected in maternal blood when the fetus has a cytogenetic abnormality. Ultrastructural differences are present in the placentas of aneuploid fetuses (60, 61) and erythrocytes from some fetuses with aneuploidy are significantly larger than those of cytogenetically normal fetuses at the same stage of gestation (62).

Frequency of fetal cells in maternal blood

As the fetus and the maternal-placental interface grow, more fetal cells traverse the placental barrier. Estimates of the frequency of fetal cells in maternal blood vary, ranging from 1 in 10^5 to 1 in 10^9 (11, 63). Hamada *et al.* (64) used FISH on maternal blood to find evidence of cells bearing a Y chromosome. The frequency of cells with Y probe positivity increased as gestation progressed, from less than 1 per 10^5 in the first trimester to 1 per 10^4 at term.

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Types of fetal cells in maternal blood

Several fetal cell types have been reported to exist in the maternal circulation. These include fetal trophoblast cells, lymphocytes, granulocytes, erythroblasts (nucleated red blood cells) and platelets. Except for platelets (which lack genomic DNA), all of these cell types have been investigated as a source of fetal cells for non-invasive prenatal diagnosis.

1. Trophoblasts

Trophoblasts are the most obvious candidate cell, given their intimate relationship with the uterus. Syncytiotrophoblast buds and cytotrophoblasts are present in relatively high numbers in the maternal circulation early in pregnancy, the inevitable result of uterine invasion to establish the fetal circulation. However, the counterbalancing consensus is that these cells become trapped and sequestered by the lung at their first circulatory passage (65). Therefore, relatively few trophoblasts might be expected to be present in the peripheral venous circulation. Trophoblasts can be recovered from the inferior vene cava and uterine vein of women having eclampsia whereas peripheral blood failed to show trophoblasts (66, 67).

2. Lymphocytes

In the early 1970s many studies documented the presence of male metaphases from the peripheral blood cultures of women who were pregnant with male fetuses (68-70). Initial work suggested lymphocytes as a reasonable target cell for prenatal diagnosis (71,

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72), however, these cells may persist from one pregnancy to another, thus potentially affecting diagnosis accuracy (73). In addition, as lymphocytes are a common constituent of the adult circulation, the isolation of the fetal cells from the maternal cells is problematic.

3. Granulocytes

This cell type has received little attention. Fetal granulocytes have also been evaluated in a limited number of cases, but their presence in maternal circulation is uncertain. In 1975 Zilliacus *et al.* estimated that fetal granulocytes composed 0.02% to 0.04% of mononuclear cell samples taken from 19 pregnant women during their second and third trimester (74). More recently, using techniques of *in situ* hybridization, Wessman *et al.* demonstrated that 0.26% of maternal mononuclear cells hybridized to a Y chromosomal probe (75). These results were surprising, as the only enrichment technique used was density gradient centrifugation. These results indicated either a large number of fetal granulocytes were circulating within maternal blood or non-specific hybridization of the granulocytes to the DNA probe. Others have been unable to replicate this study.

4. Nucleated red blood cells

Fetal nucleated red blood cells (NRBCs) are the predominant nucleated cell type in the fetal circulation in the first trimester of pregnancy. If fetal cell trafficking occurs, they are likely to be the major cell type in the maternal circulation. It has been known for many years that fetal NRBCs cross into the maternal circulation, and the frequency of NRBCs

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in the fetus early in gestation is relatively high (76). The circulating NRBCs comprise about 0.5% of the red blood cells in a 19-week fetus (77). By contrast, NRBCs are rare in peripheral adult blood. The fetal NRBCs also fairly well differentiated and likely to have a limited life span in the maternal circulation.

Persistance of fetal cells after delivery

Disappearance of fetal cells from the maternal circulation after delivery is an important consideration because of the implications for subsequent pregnancies. Liou *et al.* investigated 28 women using the PCR for the Y chromosome, and followed them for 10 months after delivery (78). In 11 women fetal cells were detected four months after delivery but in one woman, the Y sequence was detectable 10 months after delivery. Bianchi *et al.* flow-sorted for lymphocytes in women who had had male offspring up to 27 years previously (73). They also demonstrated male DNA in two women who were pregnant with female fetuses, but previously had been delivered of male infants. Such persistence of fetal materials could certainly lead to misdiagnosis, but it also highlights another problem associated with non-invasive cell sorting: the difficulty in knowing which cell type is being isolated.

Enrichment of fetal cell from maternal blood

The frequency of fetal cells in the maternal blood is estimated at between 1 in 10^5 and 1 in 10^9 circulating cells (11, 63). The low fetal cell members necessitate removal of maternal cells, but each enrichment step risks the loss of fetal cells, potentially leaving too

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few for prenatal diagnosis. Consequently not all workers use enrichment techniques. Lo *et al.*, 1989 have reliably identified the Y chromosome in unsorted maternal blood using the PCR (81, 89). Similarly, Hamada *et al.*, 1995 studied unsorted maternal blood using FISH, correctly identifying 12/16 males with no false positives (90). With this approach there is no loss of fetal cells but Hamada commented that the procedure was time consuming, with 100,000 cells being manually assessed from each blood sample (90). They concluded that cell enrichment would be desirable to speed up processing.

Enrichment can be used to positively select the target cells, or to deplete contaminating cells. Various methods of enrichment exist: density gradients, red cell lysis, fluorescent activated cell sorting, and magnetic activated cell sorting.

1. Fluorescent activated cell sorting (FACS)

Fluorescent activated cell sorting (FACS) or flow cytometry is used for positive selection following depletion of contaminating cells. The sample for sorting is incubated with a fluorescent labeled antibody specific for the target cell. The fluorescent activated cell sorting scan identifies the cells labeled with the antibody which are then collected into a tube or onto a slide for PCR (73) or FISH (91).

Unfortunately, this method of cell isolation requires considerable expertise, is time consuming and the expense of the equipment also limits its application on a wide scale. The main advantage is the high purity of target cells sorted. This makes assessment, particularly by FISH, easier and more accurate. In a recent study, DeMaria et al.,1995

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used an anti-Hb F antibody to flow sort maternal blood samples and obtained fetal cell purities of 59-73% in a model system (92).

2. Magnetic activated cell sorting (MACS)

Magnetic activated cell sorting (MACS) is the most widely used method of fetal cell isolation and can positively or negatively select cells. There are several magnetic activated cell sorting systems, all are cheaper, quicker, and require less expertise to perform than fluorescent activated cell sorting. All utilize metallic beads labeled with an antibody specific for the target cell. This is incubated with the sample and the cell/antibody/bead complex isolated by placing on a magnet. If the antibody used isolates the cells of interest, it is termed positive selection (86). The fluid is removed leaving the target cell antibody bead complex. If the antibody isolates contaminating cells, it is termed negative selection (93-94), and the cell antibody bead complexes sorted by the magnet are discarded, the remainder being the cells of interest. The magnetic sorting systems have been used successfully to sort fetal cells, both trophoblasts (95) and nucleated red blood cells (15, 96), with recovery rates of 60-80% (15, 94).

Other methods of fetal cell enrichment are less specific than fluorescent or magnetic activated cell sorting, aiming to remove contaminating cells.

3. Ammonium chloride buffer

Ammonium chloride buffer causes hypotonic lysis of mature red cells, leaving only the nucleated cells which can be further sorted using FACS or MACS (97).

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4. Density centrifugation

Density centrifugation exploits the differing density of the nucleated cells likely to be present. It can be used to remove all mature red cells alone, or many of the contaminating maternal nucleated cells using single, double or triple gradients. Centrifugation can enrich fetal cells by 1000-fold, but recent evidence suggested the techniques may lose 80% to 95% of fetal cells (98). The widespread use of density gradients makes this a cause for concern but further confirmation is required.

5. Cell culture

In 1996, Alter (99) proposed the use of cell culture exploiting the differing sensitivity of fetal and maternal red cells to erythropoietin in culture. The technique was successfully tested in a model system, confirming that there is a growth differrence in favor of neonatal cells of up to tenfold. Lo *et al.*, 1996 used a similar method in a study on five maternal blood samples where the fetus was known to be male (100). Using the PCR they identified the Y chromosome in all five cases after seven days of culture and calculated a fetal cell enrichment of between 1/400 and 1/9500.

A drawback was the length of time taken for the culture: up to 14 days. Valerio *et al.*, 1996 (99) utilized a magnetic cell sorting method to separate fetal erythroid progenitor cells from maternal blood and cultured them for 10-12 days. Cell colonies were harvested and subjected either to PCR for the Y chromosome or FISH for the Y chromosome or for chromosome 21. In all 10 cases studied they successfully developed fetal clones in culture and in all eight euploid pregnancies the fetal sex was correctly identified by PCR. Two

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pregnancies were known cases of trisomy 21 and in both trisomic cells were successfully identified in culture using FISH. These cell culture techniques require further study to determine optimal culture techniques and whether cells can be cultured reliably in every case.

6. Charge flow separation

In 1996, Wachtel *et al.* reported an alternative approach using charge flow separation, a technique that requires no antibody for cell selection (100). It exploits differing surface charge densities of cells to sort them directly into collecting tubes. They identified 8/12 male fetuses by FISH with one false positive and one false negative. In addition, they successfully cultured erythroid cells isolated by charge flow separation and used the colonies to identify Y-specific DNA by PCR in four out of six males. Further study is required to assess this method.

Identification of fetal cells in maternal blood

1. Fetal karyotypes from non-sorted maternal blood

Recovering fetal cells from maternal blood was first reported in 1969 by Walknowska *et al.*, who found XY metaphases in maternal blood of pregnant women carrying male fetuses (71). In 1971 de Grouchy and Trubuchet also reported male metaphases from pregnant women carrying male fetuses and other groups followed (69-70, 79). Relatively high proportions of fetal cells to maternal cells were claimed, approximately 0.1-0.3%. There are several problems in studying fetal karyotypes. First,

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not all individuals carrying male fetuses showed XY metaphases, perhaps fetal cells being rare events in maternal blood. Second, XY metaphases were also present in some women carrying female fetuses, perhaps clones of fetal cells being established in the mother's bone marrow during prior pregnancies.

2. Flow sorting from maternal blood

Herzenberg *et al.*, 1979 and Iverson *et al.*, 1981 applied flow sorting technology to isolate fetal cells from maternal blood (72, 80). They exploited human leukocyte antigen (HLA) differences between mother and fetus. In eight cases Y-chromatin positive cells, identified as lymphocytes, were successfully isolated. Unfortunately, the using of HLA type is limited as it requires prior knowledge of the fetal type and can only be certainly identified after delivery or by invasive testing.

3. Polymerase chain reaction to verify fetal origin

The polymerase chain reaction (PCR) provided sensitive methods for the identification of fetal cells isolated from the maternal blood. In 1989 Lo *et al.* were the first to identify the Y chromosome in the peripheral blood of pregnant women (81). Using the PCR for a Y chromosome-specific sequence, they correctly identified male pregnancies in 12 women. There were no false positives. Subsequently, several groups have isolated fetal cells from maternal blood (14, 82-84).

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4. Fluorescent *in situ* hybridization

While the polymerase chain reaction is suitable for the identification of fetal sex for X-linked disorders, the identification of aneuploidies generally requires the visualization of the chromosome numbers. Fluorescent *in situ* hybridization (FISH) offers this possibility by using chromosome specific labeled probes which bind to regions of the target chromosome. In 1993 Simpson and Elias (4) detected the fetal aneuploid cells ranged from 0% to 74%. They successfully diagnosed fetal Klinefelter syndrome (47, XXY), trisomy 18, and trisomy 21. Other groups have also diagnosed fetal trisomies18 and 21 (85-86) and 47, XYY (87). In another case, FISH using a Y chromosome probe and fetal hemoglobin staining demonstrated a large fetomaternal hemorrhage after a minor automobile accident (88).

Study of fetal nucleated red blood cells

Fetal nucleated red blood cells (NRBCs) have been the most commonly studied cell type. It has been known for many years that fetal NRBCs cross into the maternal circulation (77), and the frequency of NRBCs in the fetus early in gestation is relatively high (78). These cells are also fairly well differentiated and likely to have a limited life span in the maternal circulation.

Many studies have exploited fetal NRBCs, but all have been hampered by the lack of a specific antibody. There are three antibodies directed against fetal NRBCs epitope. These antibodies include anti-glycophorin A (anti-GPA), anti-transferrin receptor (anti-CD71) and anti-thrombospondin receptor (anti-CD36). The majority of work has utilized

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anti-CD71, an antibody directed against the transferrin receptor which is present on all cells actively incorporating iron (55, 82, 85, 101,102). Other cell types in the circulation, such as activated lymphocytes and monocytes, have this receptor but anti-CD71 provides a reasonable level of enrichment once such maternal cells have been removed. By contrast, glycophorin A (GPA), a major sialoglycoprotein of the erythroid cell membrane, arises later in the erythroid lineage and is absent in lymphocytes. Anti-CD36 is an antibody recognized the thrombospondin receptor, which is expressed during the colony-forming unit erythroid stage of erythroid development (103).

Anti-CD71 alone or combination with anti-GPA monoclonal antibody, anti-CD36 alone or combination with anti-GPA monoclonal antibody have been used to increase the specificity of recovery. Unfortunately, recent evidence showed that anti-GPA caused agglutination of the target red cells preventing efficient sorting (104). In the search for more specific fetal cell markers, Zheng et al., 1993 used an antibody to fetal hemoglobin (Hb F), conjugated with fluorescent markers, to identify fetal NRBCs (15). This should be followed with negative depletion of maternal cells by magnetic activated cell sorting (MACS). Using FISH for the X and Y chromosomes, they were able to identify fetal sex from Hb F positive cells despite the presence of excess maternal cells.

A major advantage of this was to allow the differentiation of female fetal cells from maternal cells. Unfortunately, Hb F is not fetal-specific and can occur in small numbers in the adult circulation, for example in thalassemia and sickle cell disease. In particular, it may be increased in pregnancy itself limiting the usefulness of the Hb F antibody (105). Embryonic hemoglobin has been suggested as a more specific fetal cell marker than Hb F,

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but as production ceases early in fetal life, this may limit its usefulness in a clinical setting. The search continues for a truly fetal specific marker for NRBCs.

The potential role of non-invasive cell sorting, whether as a screening or diagnostic test, remains to be established. It may be that as a screening test it will allow more accurate targeting of patients and decrease the number of invasive tests performed. As fetal cells are present from early in pregnancy, repeated maternal blood samples at weekly intervals could be performed, giving several chances for diagnosis. However, if by the end of the first trimester no result was obtained, there would still be the option of assessment by chorionic villus sampling. Certainly as a diagnostic test, non-invasive cell sorting would need to be as accurate as currently available tests, which is the major challenge.

Immunofluorescence assays

Immunofluorescence assay (IFA) uses fluorescent probes to label antigen-antibody reactions. The use of fluorescent compounds in this manner was initiated in 1941 by Coons et al., who labeled an antibody with a blue fluorescent compound, then used this as a reagent to detect the presence of the corresponding antigen in the tissue (106).

The phenomenon known as fluorescence is the ability of certain molecules or compounds (fluorochromes or fluorophores) to absorb energy, usually from an incident light source, and convert that energy into photons of light of a different, characteristic wavelength within approximately 10^{-8} seconds. Most fluorescent compounds are organic molecules and each has a characteristic optimal absorption range and quantum efficiency yield. Fluorescein is the most frequently used probe in the clinical laboratory because of

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its inherent characteristics, which include a high fluorescence intensity, good photostability, high quantum efficiency yield and an emission wavelength that is usually distinguishable from the background.

Immunofluorecence assays may be categorized as homogeneous or heterogeneous. Heterogeneous assays include a step for separation of labeled from unlabeled reactants. They may also be referred to as "solid-phase" reactions, as they are commonly performed on a bead, in plastic tubes or on a microscope slides. Homogenous assays, by contrast, do not require a separation step and are carried out in solution. These are called "fluid-phase" reactions.

1. Direct solid-phase IFA

This was used to identify an unknown antigen that can be attached to a soid phase. The antigen is reacted with a known labeled antibody and nonreacting molecules are washed away. The solid phase is observed for the attached fluorescence which would indicate a reaction between the known antibody and the fixed antigen (Figure 5).

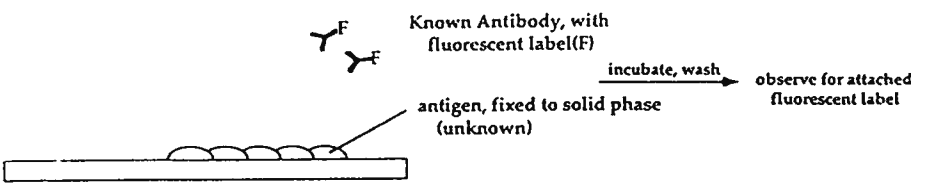

Figure 5 Heterogeneous IFA: direct solid-phase IFA (from Miller, 1991).

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2. Indirect solid-phase IFA

This technique is used to search for the presence of antibody with a chosen specificity in a patient's serum. A known antigen is attached to the solid phase and reacted with patient serum. A fluorochrome-labeled antihuman immunoglobulin is subsequently incubated with the remaining reactants. Observation of fluorescein indicates that antibody specific for the antigen used was present in the patient sample. Indirect solid phase IFA may also be used to detect cellular antigens by incubation of test cells with an antibody specific for the marker to be identified, followed by addition of a fluorochrome-labeled anti-immunoglobulin (Figure 6).

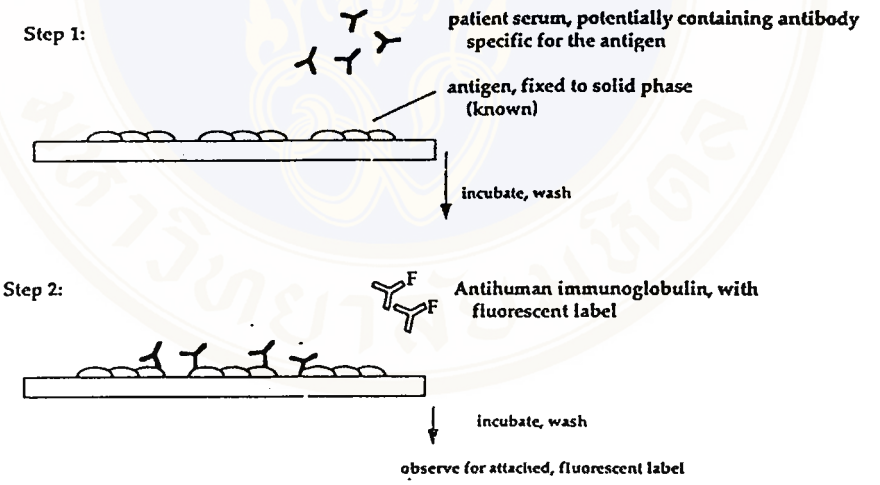

Figure 6 Heterogeneous IFA: indirect solid-phase IFA (from Miller, 1991)

3. Competitive binding IFA

In competitive binding IFA, labeled and unlabeled antibody are allowed to compete for a limited number of antigen sites fixed to a solid phase (Figure 7). After incubation and washing, the reaction is observed for fluorescence. With this technique, an inverse

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relationship occurs between the presence of patient antibody and the fluorescence observed, i.e. patient antibody, if present, interferes with the ability of the labeled reagent to bind to the antigen.

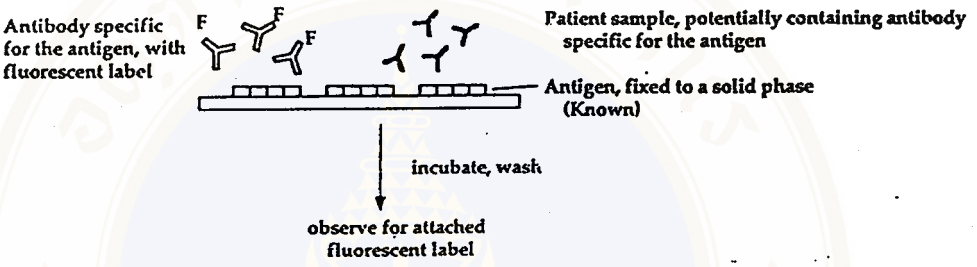

Figure 7 Heterogeneous IFA: competitive binding IFA (from Miller, 1991)

4. Sandwich IFA

In sandwich assays, known antibody is usually attached to the solid phase. Subsequently, the sample which is being tested for antigen is added. After incubation and washing, a fluorechrome-labeled antibody is added followed by an additional incubation and wash. If fluorescence is detected in this method, it is indication of the presence of antigen in the sample (Figure 8).

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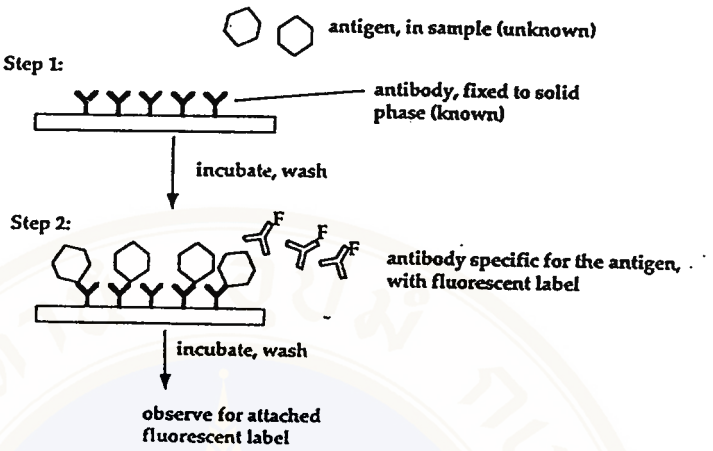

Figure 8 Heterogeneous IFA: sandwich IFA (from Miller, 1991)

5. Homogeneous IFA

Homogeneous, or fluid-phase, immunofluorescence assays do not require a separation procedure before observing results. They are rapid and simple assays although historically somewhat limited in application. In most instances, homogeneous assays involve competitive binding of reactions and can only be applied to reactions where binding of antibody to labeled antigen causes some change in the fluorescent label, rotational freedom, polarity or dielectric strength. Figure 9 shows a direct homogeneous IFA.

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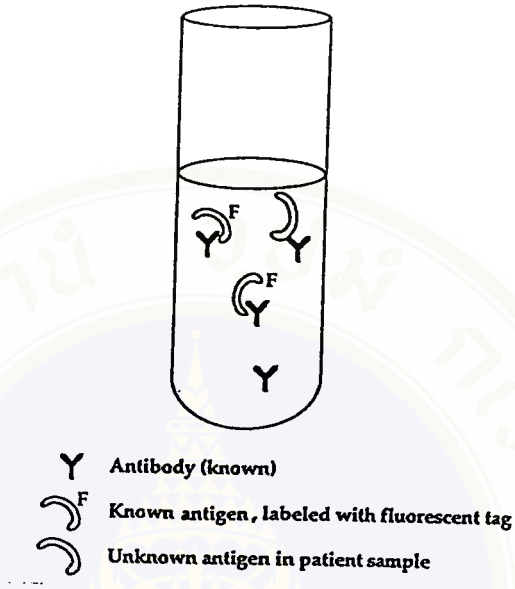

Figure 9 Homogeneous IFA (prototype) (from Miller, 1991)

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CHAPTER III

MATERIALS

1. Specimens

1.1 Establishment for appropriate condition of density gradient for NRBCs isolation

A 10-ml of peripheral blood was collected from a splenectomized β-thalassemia patient into a vacutainer containing EDTA anticoagulant. This specimen was used to establish the density gradient separation for fetal NRBCs because it had increased number of NRBCs.

1.2 Assessment of the indirect immunofluorescence staining and enrichment of fetal NRBCs

Ten-ml of blood was withdrawn from 18 subjects, mostly 7-21 weeks pregnancy, who were under chorionic villi biopsy for prenatal diagnosis of β-thalassemia disease. These specimens were used to establish immunomagnetic bead enrichment and immunocytochemical staining of fetal NRBCs. Their CVS cell washings were also smeared and used as controls for immunocytochemical staining.

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1.3 Detection of Hb Bart's hydrops fetuses from maternal blood

Venous blood samples (5-10 ml) were collected from 11 pregnant women between 10-26 weeks of gestation, who are at increased risk of Bart's hydrops fetalis. The age of the pregnant women at sampling ranged between 17 and 34 years. Two of them were in their first pregnancies at risk for Hb Bart's hydrops fetuses and nine in subsequent pregnancies with previous hydrops fetuses.

Nine blood samples were taken prior to chorionic villus sampling (CVS) or fetal blood sampling performed for prenatal diagnosis of Hb Bart's hydrops fetalis. Two samples were taken immediately prior to the termination of pregnancy because of late gestation and hydrops fetus could be diagnosed by ultrasonography. The fetal NRBCs were isolated by density gradient and enriched by immunomagnetic beads (CD71). The fetal NRBCs were then used to diagnose for Hb Bart's hydrops fetalis by immunostaining with anti α-globin antibody.

Chorionic villi sampling was carried out by the transabdominal route and the tissues were collected in the culture medium. A mixture of maternal and fetal red cells was recovered from the washings of the biopsy material. Fetal NRBCs from CVS cell washings were used as the controls for immunostaining of fetal NRBCs obtained from the maternal circulation.

The gestational age at sampling was calculated as week started from the first day of the last menstruation and confirmed by fetal ultrasonography. All subjects were taken with the consent form from the antenatal care (ANC), Department of Obstetrics and Gynecology, Siriraj Hospital, Mahidol University.

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2. Equipments

- ADVIA^{TM} 120 Hematology System, Bayer, USA
- invert mixer, Ames, USA
- centrifuge, Centurion, UK
- cytocentrifuge, Iris, USA
- Dynal MPC magnet, Dynal, Norway
- freezer (-20^{o}C), Puff, France
- fluorescent microscope, Zeiss, Germany
- microscope, Olympus, Japan
- Neubeaur hematocytometer, Blau brand, Germany
- pH meter, Radiometer, Denmark
- pipetman, Gilson, France
- refrigerated centrifuge, Himac, Japan
- refrigerator, Whilpool, USA
- shaking waterbath, Julabo, Germany
- vortex, Scientifics industries, USA

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3. Chemicals

Chemical	Chemical formula	Company	Country
Acetone	C_3H_6O	Merck	Germany
Boric acid	H_3BO_3	Merck	Germany
Bovine serum albumin (BSA)	-	Sigma	USA
Dipotassioum hydrogen phosphate	K_2HPO_4	Fluka	Germany
Disodium-ethylenediamine tetraacetate dihydrate (Na_2EDTA)	-	Merck	Germany
Diazabicyclo-octane (DABCO)	-		
Glycerol	-		
Histopaque-1119, 1083, 1077	-	Sigma	USA
Non-fat dried milk	-	Carnation	USA
Methanol	CH_3OH	Merck	Germany
Sodium azide	NaN_3	Fluka	Germany
Sodium chloride	NaCl	Fluka	Germany
Sodium hydroxide	NaOH	BHD	England
Hydroxymethyl amino-methane (Trizma base)	-	Sigma	USA
Tween 20			

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4. Monoclonal antibodies

4.1 Dyna M-450 CD71(transferrin receptor) antibody, Dynal, Norway

4.2 Rabbit anti α- globin chain antibody, From Prof. L.F. Bernini, Netherlands

4.3 Rabbit anti γ-globin chain antibody, From Prof. L.F. Bernini, Netherlands

4.4 Goat anti-rabbit IgG-FITC antibody, Immunotech, France

5. Miscellaneous

- Conical tube (15 ml), Sarstedt, Germany
- Cover slip (22 x 22 cm), Menzel-glaser, Germany
- EDTA blood vacutainer, Venoject, USA
- Falcon tube (5 ml), Falcon, Germany
- Film (400), Kadak, Germany
- Glass slide, Sail brand, China
- Nail enamel, Thailand
- Moist chamber, Thailand
- Pasteur pipette (9 inches), Corning, USA
- Pipette tips (for P20, P100, P200), Treff, Switzerland
- Pipette tips (for P1000), Scientific, USA
- Plastic microcentrifuge tube (1.5 ml), Scientifics, USA
- Rubber, Thailand

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6. Reagents

6.1 Reagents for mononuclear cells isolation

- Histopaque-1119, Histopaque-1083, Histopaque-1077, Sigma, USA
- SBE buffer pH 8.0: 0.1 M NaCl, 0.1 M H_3BO_3, 0.01 M Na_2EDTA, and 0.025 M NaOH.
- PBS buffer pH 7.2: 0.129 M NaCl, 0.020M K_2HPO_4, 0.005 M Na_2EDTA.
- PBS with 5%BSA: 5 g of BSA was dissolved in 100 ml of PBS.

6.2 Reagents for fetal NRBCs enrichment

- Dynal M-450 CD71 (transferrin receptor), Dynal, Norway
- Washing buffer: 1% BSA, 0.02% NaN_3, PBS pH 7.2.

6.3 Reagents for slide preparation and cell counting

- Trypan Blue dye
- Wright's stain

6.4 Reagents for immunocytochemical staining

- Blocking solution: 1% BSA, 10% non-fat dried milk in PBS pH 7.2.
- 5% Tween 20: 5 µl of Tween 20 in 95 µl of PBS pH 7.2
- Cocktail of primary antibody:

anti α- or γ- globin chain antibody	100 µl
blocking solution	45 µl
5% Tween 20	5 µl

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- Cocktail of secondary antibody:

goat anti-rabbit IgG-FITC antibody	10 µl
blocking solution	135 µl
5% Tween 20	5 µl

- Antifading medium: dissolve10 ml of 1 M Tris-Cl pH 8.0 and 2.3 g of DABCO in 90 ml of glycerol.

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METHODS

After blood collection, the specimens were processed within 24 hours. The blood was diluted 1:2 with saline-boric acid-EDTA (SBE) buffer and overlayered onto the density gradients. After the density gradient separations, each of the cell layers was injected into the ADVIA^{TM} 120 hematology System to evaluate the numbers of NRBCs.

1. Mononuclear cells isolation

1.1 Discontinuous double density gradient

A double density gradient was set up (107) by pipetting 3 ml of Histopaque-1119 to a 15-ml centrifuge tube and carefully overlayered with 3 ml of Histopaque-1077 and 6 ml of the diluted blood using a Pasteur pipette. The tube was put to centrifuge at 600 *g* for 30 minutes at room temperature. After centrifugation, three distinct cell layers were observed (Figure 10). Layer 1 (plasma-Histopaque-1077 interface) consisted of mononuclear cells and platelets, layer 2 (Histopaque-1077-1119 interface) consisted of polymorphonuclear cells and fetal NRBCs and layer 3 (pellet) consisted of erythrocytes.

Cells from the upper interface and the 1119/1077 interface were removed, placed in separate tubes, washed twice in PBS at 400 *g* for 10 minutes and resuspended in PBS with 5% bovine serum albumin (BSA).

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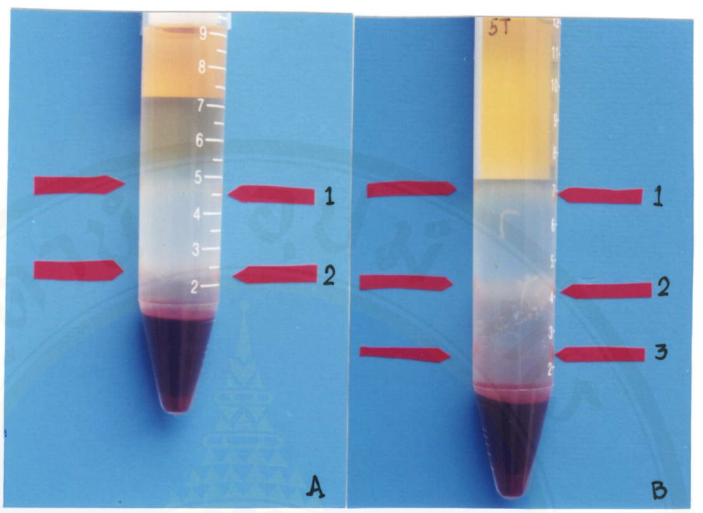

Figure 10 A: double density gradient centrifugation with Histopaque-1077 and 1119
B: triple density gradient centrifugation with Histopaque-1077, 1107 and 1119

1.2 Discontinuous triple density gradient

A triple gradient was set up (108) by pipetting 2.5 ml of Histopaque-1119 to a 15-ml centrifuge tube, carefully adding 2.5 ml of Histopaque-1107 (2 parts Histopaque-1119 and 1 part of Histopaque-1083) and 2.5 ml of Histopaque-1077 was finally overlayered. Six ml of diluted blood was carefully added using a Pasteur pipette and the tube was centrifuged at 600 *g* for 30 minutes at room temperature. After centrifugation, four distinct layers were visible. The first layer contained mononuclear cells. The second layer contained predominantly fetal NRBCs. The third layer contained polymorphonuclear cells and the fourth layer contained erythrocytes. Cells were removed from each of the four resulting interfaces (figure 10) and washed as previously described for the double density gradient protocol.

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2. Cells counting

After the density gradient separation, cells counts were carried out using an improved Neubauer hemocytometer (109). It determined the percentage of viable (intact) cells in the preparation using the dye exclusion method. The hemocytometer is a modified microscope slide that bears two polished surfaces each of which displays a precisely ruled, sub-divided grid (Figure 11). The grid consists of nine primary squares, each measuring 1 mm on a side (area 1 mm^2) and limited by three closely spaced lines. These triple lines are used to determine if cells lie within or outside the grid. Each of the primary squares is further divided to help direct the line of sight during counting. The plane of the grid rest 0.1 mm below two ridges that support a sturdy coverslip.

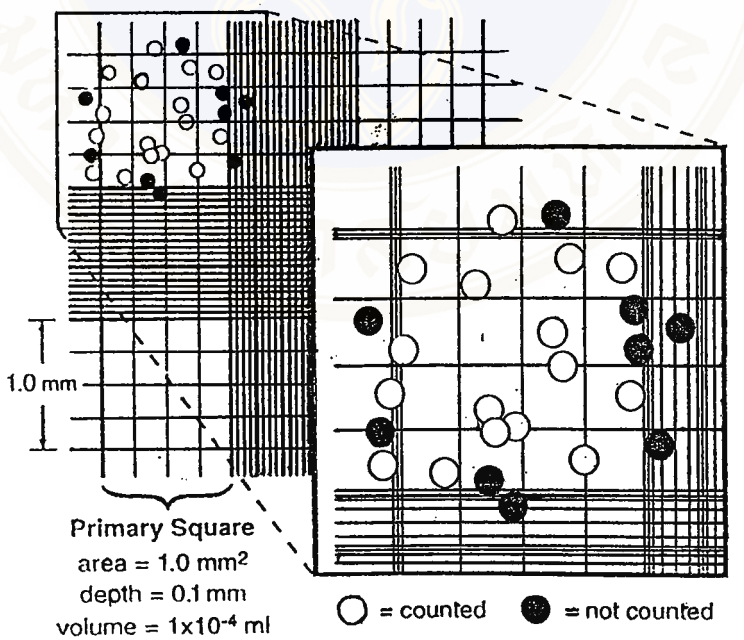

Figure 11 Grid pattern of improved Neubauer ruled hemocytometer. Inset shows cells (enlarged for clarity) distributed over a primary square. Cells that are within, or that touch, the left or top boundary are counted, while those that touch, or are outside, the lower or right boundary are not counted (from Davis JM, 1994).

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When the haemocytometer is properly loaded, the volume of cell suspension that will occupy one primary square is 0.1 mm^3 (1.0 mm^2x 0.1 mm) or 1.0 x 10^{-4} ml. The counting of the cells should follow a specific pattern as shown in Figure 11. The procedure for counting must follow a sequence so that cells touching the lines on two sides of each square are counted as within the square and cells touching the other two sides are outside the square. In this way, every cell is assigned to one square and no cell is counted twice.

Dye exclusion involves mixing an aliquot of blood cells from each interface in an equal volume of Trypan blue dye that is visible when Trypan blue dye leaks into cells that have damaged plasma membranes. When counting cell suspensions, it is convenient to use a phase-contrast microscope and so distinguish viable (phase-bright) from dead (phase-dark) cells, as an adjacent to dye exclusion. By counting total cells and stained (damaged) cells, one can calculate the percent viability. A dilution factor [(volume of sample + volume of diluent) / volume of sample] is needed if the suspension was diluted with buffer, or with a dye used to perform a viable cell count.

Calculation of the number of viable cells:

Number viable cells per ml = number of viable cells counted x dilution factor x 10^4

3. Enrichment of fetal nucleated red blood cells from maternal cells

As pregnancy advanced there would be an increase in the number of circulating NRBCs reflecting maternal erythropoiesis and fetomaternal transfusion. Transferrin receptor (CD71) is an antigen expressed by cells in the early and mid erythropoietic

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lineage, and also by activated lymphocytes and activated monocytes. CD71 positive selection can be utilized to enrich the fetal NRBCs from maternal blood. The separation was achieved using an immunomagnetic technique. A monoclonal mouse antibody against human transferrin receptor was bound to magnetic microspheres conjugated with sheep antimouse antibody. These immunomagnetic beads bound directly to transferrin receptors on the NRBCs, which could then easily be separated from nonreactive cells by a magnet. These fetal NRBCs can be identified by immunofluorescent method with anti α–globin or γ-globin antibodies.

To enrich the fetal NRBCs by positive selection with the immunomagnetic bead, Dynabeads M-450 CD71 which contains antibody against transferrin receptor was used (110). The anti-CD71 monoclonal antibody coated microbeads bound to fetal NRBCs and the cells were placed to a magnet. Unbound cells suspension were removed by the pipet and the bound cells were detached from the beads.

After calculation the number of cells in the suspension, cells were resuspended at a concentration of $1x10^7$ cells/ml in PBS containing 5% BSA. The Dynabeads M-450 CD71 were provided from the bottle at a concentration of 4×10^8 beads per ml and used with a ratio of 4 beads per cell. Approximately 10 μl of beads was added per 10^6 cells. According to the manufacturer's instruction, magnetic beads were washed three times in PBS containing 0.5% BSA and then resuspended in their original volume in PBS with 0.5% BSA (111). After pipetting from the bottle, the Dynabeads were kept at 4 °C. Appropriate amounts of cells and beads were incubated for 10 minutes at 4 °C and then separated with a Dynal MPC-6 magnet for 10 minutes. The unbound cell suspension (CD

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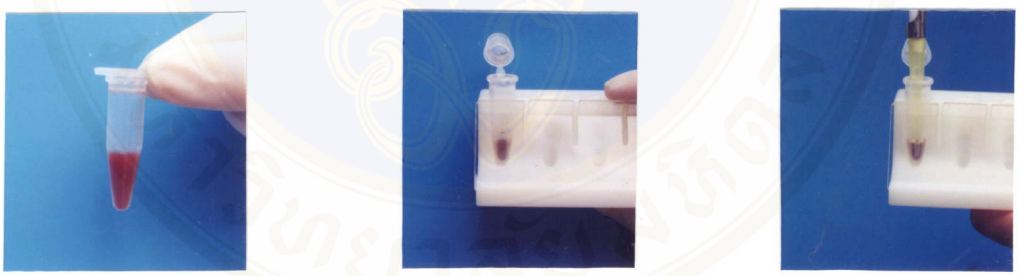
71^- cells) was carefully pipetted into a clean tube. Cells and beads in the tube were then resuspended in PBS with 0.5% BSA and placed against the magnet for another 10 minutes to ensure that all remaining unbound cell suspension was removed from the sample. Finally, the enriched cell suspension bound to the beads ($CD71^+$ cells) was resuspended in a new tube and was pipetted up and down for several times to detach the magnetic beads from the positive cells (fetal NRBCs). Figure 12 showed the positive selection with immunomagnetic beads.



A B C

Figure 12 Fetal NRBCs enrichment by positive selection. A: cells and Dynabeads M-450 CD71 suspension, B: CD71 positive cells were isolated from the negative cells by Dynal MPC-6 magnet, and C: CD71 negative cells were removed from the positive cells.

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4. Preparation of chorionic villi washings

Since the fetal NRBCs are known to be present in the washings of CVS, this material was used to optimize the conditions for immunostaining. The chorionic villi washings were filtered through the plastic wool to get rid of the chorionic villus residual. The cells were washed three times for 10 minutes at room temperature and centrifuged at 400 *g*. The cells from chorionic villi washings were resuspended in PBS containing 5% BSA.

5. Slide preparation

The isolated cells from the gradient interface, the CD71 positive cells, and chorionic villi washings were plated onto each slide by cytocentrifugation at 700 rpm for 4 minutes and air-dried. Under a light microscope, the nucleated red blood cells morphologically differentiated from the mature red blood cells and lymphocytes.

6. Wright's staining

After slide preparation, Wright's stain was applied to cover the smear and incubated for 3-5 minutes. The same volume of water was applied to the slide and mixed well. The slide was incubated for 2-3 minutes, rinsed the slide under gently running tap water and air-dried (112).

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7. Immunocytochemical staining

The indirect fluorescent immunostaining was set up (113). An anti-γ globin chain antibody was established to identify the fetal cells and an anti-α globin chain antibody was used to diagnose the homozygous α-thalassemia 1 fetuses. These antibodies were developed with the goat anti-rabbit IgG labeled with FITC.

Before staining, the smeared slides were fixed, 10 minutes each in methanol and acetone (both at -20° C), whereafter they were rinsed in PBS for 5 minutes. Slides were then pre-incubated for 1 hour in a blocking solution to prevent non-specific staining.

For staining of the hemoglobin, the slides were incubated under the cover glass in the moist chamber for 1 hour with a cocktail of rabbit anti α-globin chain antibody or anti-γ globin chain antibody in 5% Tween 20 and blocking solution. The cover glass was discarded and the slide was washed in PBS for 5 minutes. The anti α-globin chain antibody or anti γ-globin chain antibody was developed with a cocktail of goat anti-rabbit IgG-FITC antibody in 5% Tween 20 and blocking solution for 1 hour. The slides were washed in PBS for 5 minutes and rinsed in distilled water for 3 minutes. They were then allowed to dry at room temperature in the dark place and antifading medium was added to prevent fluorescent fading.

8. Morphologic analysis by a fluorescent microscope

After immunostaining, the fetal NRBCs were analyzed using a Zeiss fluorescent microscope. The fetal NRBCs were determined by (1) presence of homogenous and

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intensely fluorescent anti γ -globin staining, and (2) characteristic morphology such as the large nucleated cells, 10-15 μm, with the low nucleus-to-cytoplasm ratio. Although lymphocytes also have a round nucleus and can be mistaken for fetal NRBCs, in our experience, their nuclei are large and occupy almost all space of cytoplasm which do not appear fluorescent with the anti-γ globin chain antibody.

9. Detection of homozygous α-thalassemia 1

To differentiate the fetal NRBCs for homozygous α-thalassemia 1 from those of heterozygous or normal fetuses and from maternal cells, these cells were stained by immunofluorescence with specific monoclonal antisera against human α–globin chains (anti α-globin antibody). The fetal NRBCs that reacted with the anti α-globin antibody included red cells of heterozygous carriers of α-thalassemia 1 or normal fetuses. The fetal NRBCs that did not react with the anti α-globin antibody were classified as homozygous α-thalassemia 1.

10. Principles of peroxcytogram analysis by ADVIA™ 120 Hematology System

The ADVIA™ 120 Hematology System is a fully automated diagnostic instrument with a throughput of 120 samples per hour. The ADVIA™ 120 Hematology System White Blood Cell Differential (WBC Diff) methods, consisting of both the peroxidase method and the basophil/lobularity method, are intended to quantitatively measure of the

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following WBC hematological parameters: neutrophils, lymphocytes, monocytes, eosinophils, large unstained cells, and basophils in percentage of WBC and absolute count.

The peroxidase method was based on the principle of differential cellular staining. Leukocytes are classified by the characteristic properties exhibited by cell specific constituents when the cells are treated with cytochemical stains. The enzyme peroxidase is present and active in several leukocyte types. Normal neutrophils and eosinophils posses significant levels of peroxidase activity, with enzyme activity corresponding to cell maturation.

The peroxidase cytochemical reaction consists of 2 steps. In the first step, the sample is diluted with ADVIA™ 120 PEROX 1 reagent. Surfactants and thermal stress cause lysis of the red blood cells. Formaldehyde in reagent 1 fixes the white blood cells. During the second step, ADVIA™ 120 PEROX 2 and 3 reagents are added to the peroxidase reaction chamber. The 4-chloro-1-napthol in reagent 2 and the hydrogen peroxidase in reagent 3 stain the sites of peroxidase activity in the granules of neutrophils, eosinophils, and monocytes. Lymphocytes, basophils and large unstained cells contain no granules with peroxidase enzyme activity.

A constant volume of the cell suspension from the Perox reaction chamber passes through the flow cell. The two fluids flow as independent, concentric streams (no mixing), with the ADVIA™ 120 PEROX SHEATH stream encasing the sample stream. The absorbance and the forward light-scattering signatures of each blood cell are measured. The optical signals are converted to electrical pulses by photodiodes. After processing, the

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information is displayed in two histograms (figure 13). The Perox Y histogram contains the forward-scattering data (cell size). The Perox X histogram contains the absorbtion data (peroxidase staining). The two histograms are combined to form the Perox cytogram from which cells are identified and counted.

White blood cells are identified based on size and different intensity of peroxidase reaction (staining). Neutrophils, eosinophils, and monocytes are stained based on their levels of peroxidase activity. Since lymphocytes, basophils, and large unstained cells contain no peroxidase, these cell types remain unstained.

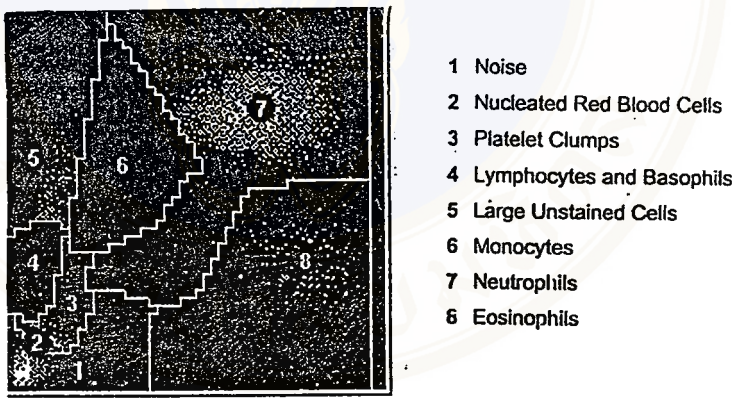

Figure 13 The Perox cytogram of ADVIA^{TM} 120 Hematology System White blood Cell Differential methods. Each gate number represents the cell types of white blood cells. Nucleated red blood cells (NRBCs) are located at the gate number 2.

If abnormal cells are present, their sizes and peroxidase activity determine their location on the Perox cytogram. Nucleated red blood cells (NRBCs) appear in the NRBC cluster that is located between the noise and lymphocyte areas (Figure 13). Perox cytogram shows the NRBCs in three severity levels:

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+	% NRBC	=	2.0% to 4.9%
++	%NRBC	=	5.0% to 10.0%
+++	%NRBC	>	10.0%

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CHAPTER IV

RESULTS

1. Isolation of nucleated red blood cells (NRBCs) by density gradient centrifugation

In homozygous β-thalassemia, there is a profound deficiency of the β-globin production such that little or no Hb A is present in erythroid cells. The excess α-globin chains precipitate within the developing erythroid progenitors in the bone marrow as well as within peripheral red blood cells. These precipitates damage the cell membrane leading to premature intramarrow red cell destruction. This ineffective erythropoiesis leads to extramedullary hematopoiesis in a vain attempt to compensate for inadequate Hb A production. The peripheral blood smear displays marked microcytosis and hypochromasia as well as gross variation in shape and fragmentation of red cells and often the presence of nucleated red cells, especially after splenectomy (114, 115). Peripheral blood withdrawn from a splenectomized β-thalassemia patient was used to establish the density gradient centrifugation for the isolation of NRBCs.

Two density gradients were set up using the same starting sample, a double density gradient (Histopaque-1119 and-1077) and a triple density gradient (Histopaque-1119, -1107, and-1077). After centrifugation, cells were collected from each of the different resulting interfaces. Each of cell interfaces was analysed on the ADVIA™

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120 Hematology System White Blood Cell method using the Perox cytogram. Cytospin slides from the gradient interfaces were also stained with Wright's stain and examined under the light microscope (x 1000 magnification).

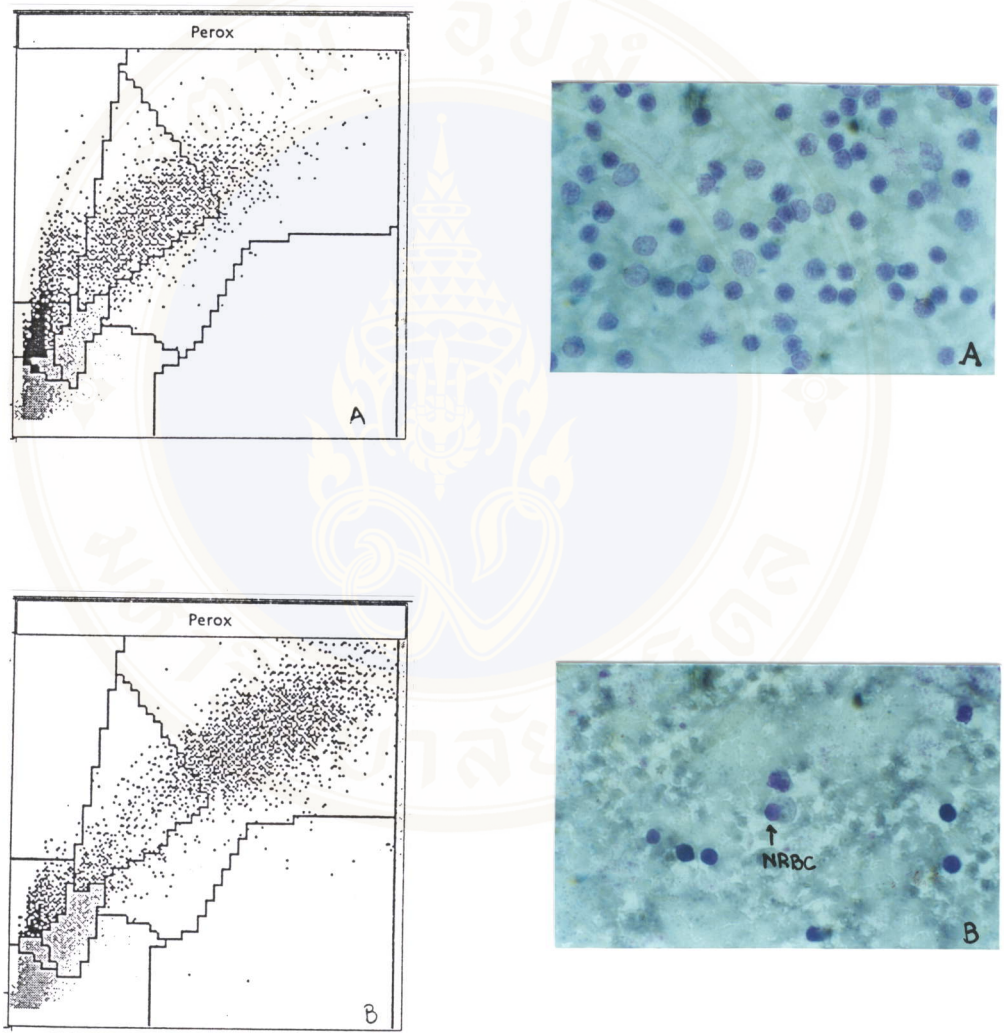

Figure 14 The morphologic pictures and Perox cytograms of double density gradient centrifugation from a splenectomized β-thalassemia patient: (A) plasma/Histopaque-1077 interface, (B) Histopaque-1077/1119 interface.

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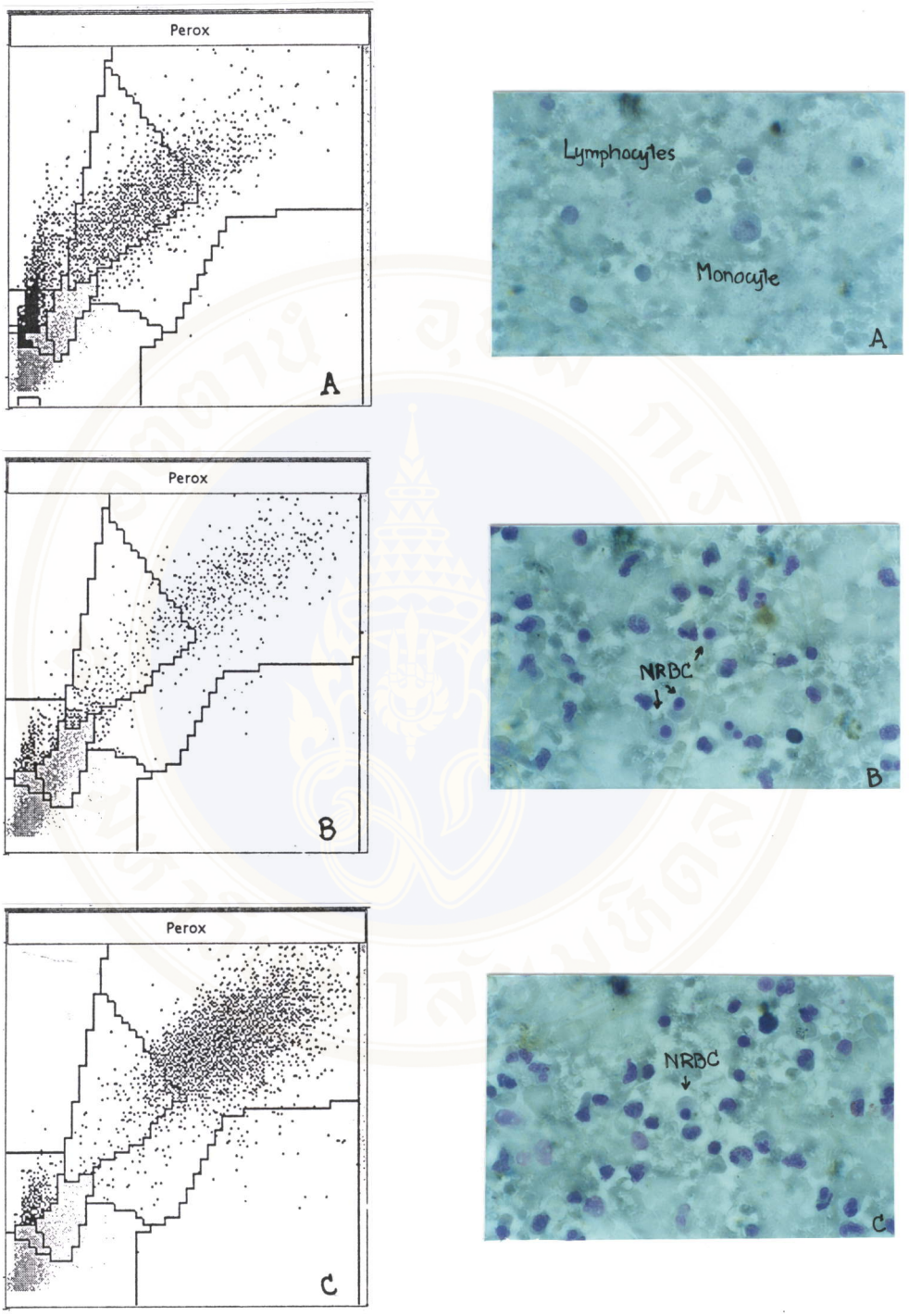

Figure 15 The morphologic pictures and Perox cytograms of triple density gradient centrifugation from a splenectomized β-thalassemia patient: (A) plasma/Histopaque-1077 interface, (B) Histopaque-1077/1107 interface and (C) Histopaque-1107/1119 interface.

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Table 1 Comparison of the complete blood cell counts plus white blood cell differential counts (CBC/Diff) between double and triple density gradient of splenectomized β–thalassemia patient analyzed by ADVIA^{TM} 120 Hematology System

CBC/Diff	Double gradient		Triple density gradient		
	H-1077	H-1119	H-1077	H-1107	H-1119
RBC (x10^6 cells/μl)	0.06	0.20	0.05	0.08	0.12
WBC (x10^3 cells/μl)	16.94	5.76	18.57	1.41	5.37
% Lymphocyte	77.4	41.2	77.6	39.5	27.8
% Monocyte	9.5	9.0	9.9	21.1	4.6
% Neutrophil	4.0	48.9	3.9	37.8	66.6
% Eosinophil	0.0	0.3	0.0	0.5	0.8
% Basophil	0.3	0.2	0.2	0.6	0.2
NRBCs	++	+++	++	+++	++

The perox cytograms of the two different gradients were compared using the same starting sample in Figures 14 and 15, and the completed blood counts plus white cell differential counts (CBC/Diff) values are demonstrated in Table 1. The result showed that the NRBCs were distributed in every cell layers. In the double density gradient, the upper cell layer (H-1077) was expected to contain mononuclear white cells, the lower cell layer was expected to contain NRBCs and granulocytes (107). In this experiment more NRBCs were in the lower layer which has higher density. In the triple density gradient, the upper cell layer (H-1077) was expected to contain mononuclear cells, the cell layer below (H-1107) was expected to contain NRBCs, and the third cell layer from the top (H-1119) was expected to contain granulocytes (108). The result showed that most NRBCs were in the middle layer.

Although each cell layer obtained after the gradient separation contained a large proportion of mature red cells, a major enrichment of NRBCs (of the order of 1000-fold)

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occurred at this step. The greatest proportion of NRBCs to white cells was in the lower layer of the double gradient and the middle layer of the triple gradient. However, there were significant numbers of NRBCs in other cell layers. Since the H-1107 layer in the triple density gradient gave the highest yield of NRBCs with the lowest number of white cells. The triple density gradient centrifugation will be used in this experiment.

2. Assessment of the indirect immunofluorescence staining of fetal NRBCs

A specific approach to identify cells containing HbF is to use immunocytochemical staining with a monoclonal antibody specific for the γ-globin chains of fetal hemoglobin (HbF). Antibodies directed against fetal hemoglobin appear to be among the most fetal specific agents currently available to identify fetal red cells, although there is evidence that pregnancy stimulates synthesis of small amounts of Hb F in the adult red cells (104).

We have developed an indirect immunocytochemical detection using a combination of a rabbit monoclonal antibody specific for the γ-globin chain and a goat anti-rabbit IgG conjugated FITC. The method was optimized for the specificity of γ-globin staining by using a washing of CVS samples (known to have fetal NRBCs) from 18 pregnant women (7-25 weeks' gestation) at risk for β–thalassemia fetuses. Maternal blood samples (5-10 ml)of the same individuals were also collected before invasive CVS procedure.

Fetal NRBCs were isolated from maternal blood samples using the triple density gradient followed by positive selection using anti-CD71 antibody (see below). The cells from chorionic villi washing and CD71-positive cells were spun on the slides, stained

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with a rabbit anti γ-globin antibody and developed with a goat anti-rabbit IgG-FITC. The fetal NRBCs were identified using a Zeiss fluorescent microscope and a 40x objective. The fetal NRBCs were determined from the morphology and the presence of homogenous and intensely fluorescent anti γ-globin antibody staining.

For CVS cell washings, γ-globin positive NRBCs were predominantly seen whereas the fluorescent positive NRBCs were rarely found in peripheral blood samples of the pregnant women (Figure 16 and 17). Mature erythrocytes containing Hb F also showed green fluorescence.

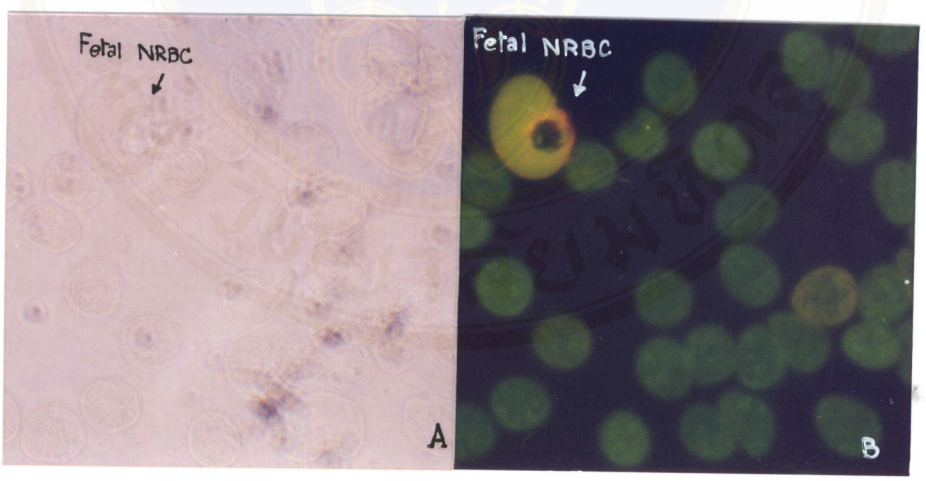

Figure 16 Immunocytochemically stained for γ-globin chains in chorionic villi washings : phase contrast (A) and fluorescent-FITC (B) (magnification x 1,000).

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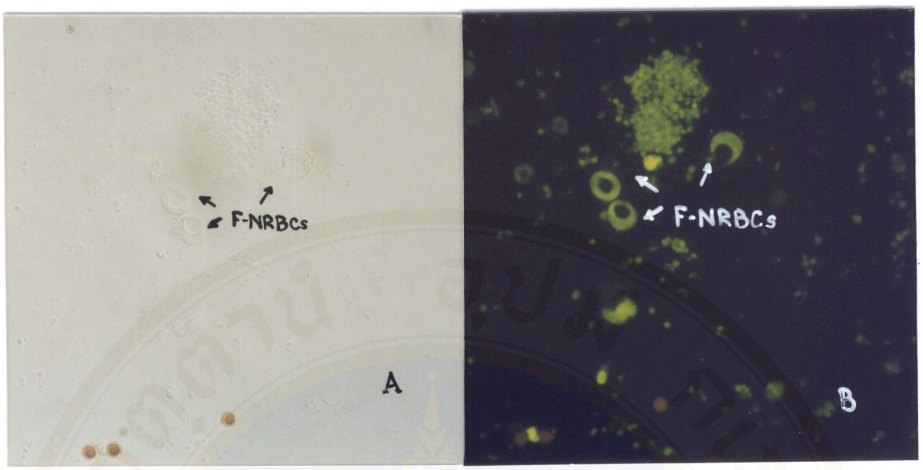

Figure 17 Fetal NRBCs from maternal blood (14 weeks of gestation) stained with anti γ-globin antibody : phase contrast (A) and fluorescent-FITC (B) (magnification x 400).

3. Enrichment of fetal NRBCs by immunomagnetic beads

Before CVS biopsy, peripheral blood from 18 pregnant women were sorted for fetal NRBCs by using the preenrichment step with a triple density gradient and a positive selection for fetal NRBCs with anti-transferrin receptor monoclonal antibody (CD71) linked to magnetic beads. The intensely fluorescent cytoplasmic anti γ-globin staining can be used as a marker to identify fetal cells on the microscope slides.

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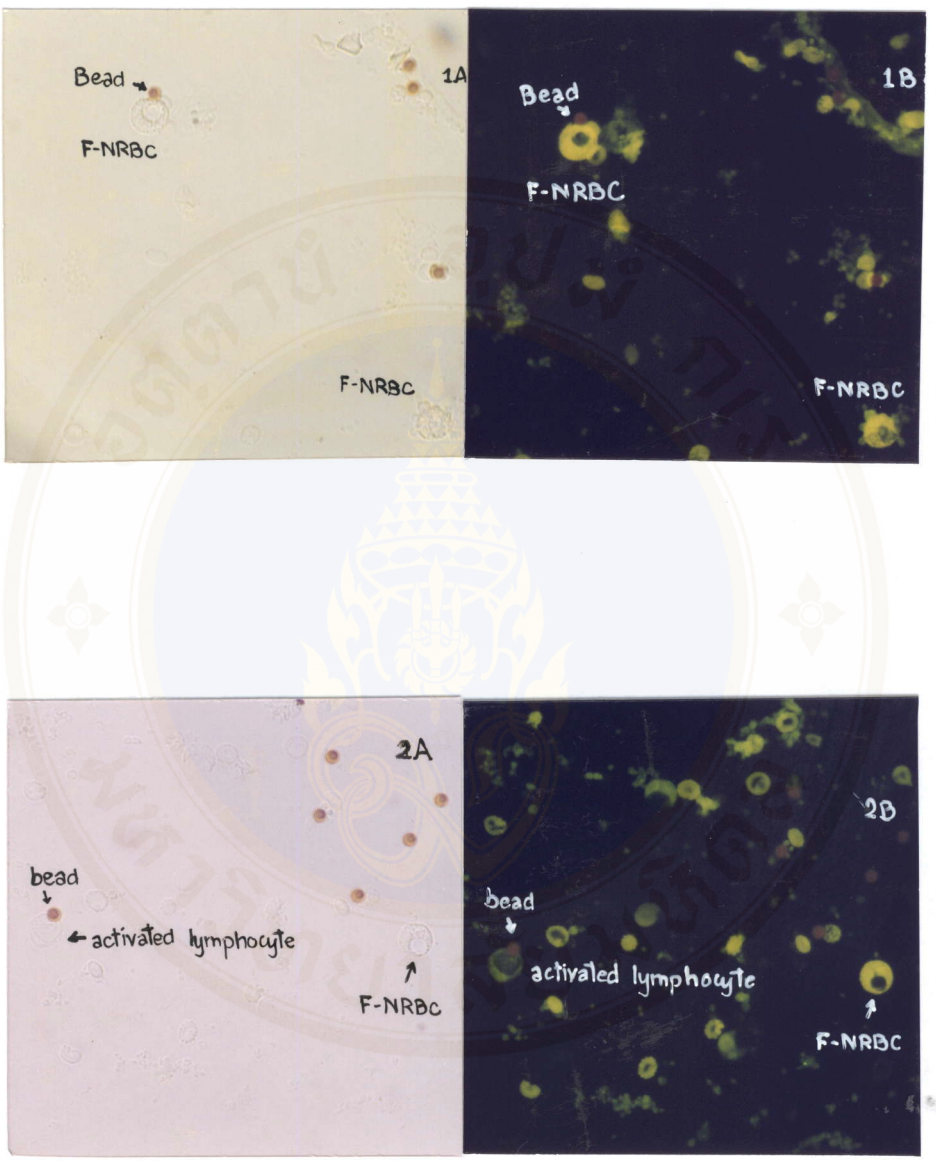

Figure 18 The phase contrast (A) and fluorescent FITC anti γ-globin (B) and CD71-positive cells enriched from pregnant blood with at risk for β–thalassemia disease. Fetal NRBCs attached to immunomagnetic bead CD71 stained positive with anti γ-globin antibody (1) and activated lymphocyte attached to CD71 but stained negative with anti γ–globin antibody (2).

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Figure 18 demonstrates phase contrast and immunofluorescence of FITC anti γ-globin positive cells attached to the CD 71 immunomagnetic beads. However, not only NRBCs (Figure 18.1 A, B) but also some activated lymphocytes (Figure 18.2 A, B) may attach to anti-CD71 antibody. By observing the morphology and the homogenous intensely fluorescent anti γ-globin staining of NRBCs (Figure 18.1 B), these cells can be separated from lymphocytes which stained negative (Figure 18.2 B).

4. Detection of homozygous α–thalassemia 1 in fetal NRBCs from maternal blood samples

Noninvasive prenatal diagnosis for homozygous α–thalassemia 1 (Hb Bart's hydrops fetalis) in eleven high-risk pregnancies, between the 10th and 26thweek of gestation, were studied. Immunofluorescence with specific monoclonal antisera against human α-globin chains was used in order to differentiate fetal NRBCs of homozygous α–thalassemia 1 fetuses from those of heterozygous or normal fetuses, and from maternal red blood cells. The affected fetus was diagnosed based on the immunological demonstration of the apparent absence of normal α–globin chains in the fetal NRBCs. At the same time immunostaining with anti α–globin antibody was also performed with CVS cell washings and analysis of genomic DNA extracted from chorionic villi was implemented by phenol-chloroform extraction. α–Thalassemia 1 gene was detected by the gap PCR technique (120). The latter two specimens served as positive control for the noninvasive prenatal diagnosis. Clinical data regarding the cases studied are summarized

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in Table 2. Results of prenatal diagnosis for homozygous α–thalassemia 1 (Hb Bart's hydrops fetalis) in eleven high-risk pregnancies, between the 10th and 26th week of gestation, are summarized in Table 3.

Table 2 Clinical data of 11 pregnant women at risk for homozygous α–thalassemia 1 (10) and Hb H disease (1) fetuses.

Case No.	Age (yrs)	Obstetrics history	Gestational age (weeks)	Phenotype	
				subject	husband
1	26	$G_3 P_0 A_2$	13	α–thalassemia 1 trait	Hb E/α–thalassemia 1
2	29	$G_3 P_2 A_0$	12	Hb E/α–thalassemia 1	α–thalassemia 1 trait
3	28	$G_1 P_0 A_0$	17	α–thalassemia 1 trait	α–thalassemia 1 trait
4	26	$G_3 P_1 A_1$	10	α–thalassemia 1 trait	α–thalassemia 1 trait
5	34	$G_3 P_2 A_0$	17	α–thalassemia 1 trait	Hb CS trait
6	30	$G_2 P_1 A_0$	24	α–thalassemia 1 trait	Hb E/α–thalassemia 1
7	34	$G_5 P_1 A_3$	10	α–thalassemia 1 trait	Hb E/α–thalassemia 1
8	26	$G_2 P_1 A_0$	11	α–thalassemia 1 trait	α–thalassemia 1 trait
9	19	$G_1 P_0 A_0$	14	α–thalassemia 1 trait	Hb H disease
10	22	$G_1 P_0 A_0$	26	α–thalassemia 1 trait	α–thalassemia 1 trait
11	17	$G_2 P_1 A_0$	12	α–thalassemia 1 trait	Hb H disease

G = gestation

P = pregnancy

A = abortion

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Table 3 Comparison of the results of prenatal diagnosis performed by immunofluorescent staining with anti α-globin antibody and DNA analysis (* DNA analysis for Hb CS and α-thalassemia 2 was not carried out).

Case	Gestational age (weeks)	anti α -globin staining of fetal NRBCs	DNA analysis by PCR from chorionic villi
1	13	positive	α–thalassemia 1 trait
2	12	positive	normal
3	17	positive	α–thalassemia 1 trait
4	10	negative	Hb Bart's hydrops
5	17	positive	Hb CS trait
6	24	negative	Hb Bart's hydrops
7	10	positive	α–thalassemia 1 trait
8	11	positive	normal
9	14	positive	α–thalassemia 2 trait or Hb CS trait*
10	26	negative	Hb Bart's hydrops
11	12	positive	α–thalassemia 1 trait or Hb H disease*

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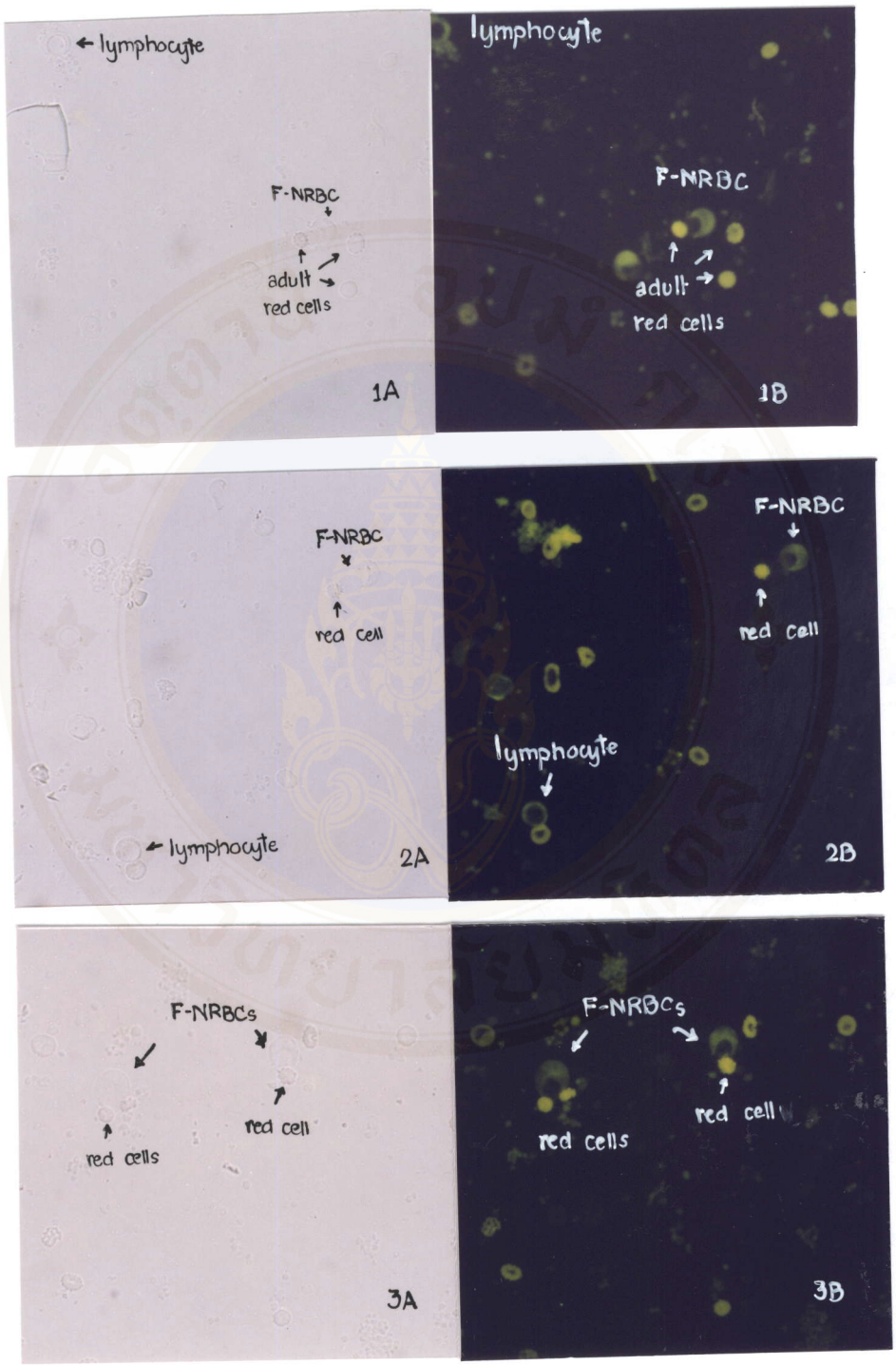

Figure 19 Noninvasive prenatal diagnosis for 3 Hb Bart's hydrops fetuses. Immunofluorescence stained for anti-α globin chain in fetal NRBCs from pregnant blood at risk for Hb Bart's hydrops fetalis. The cells containing α-globin chains show the green fluorescence and the cells deficient of α-globin chains show the negative. (1A-3A): phase contrast and (1B-3B) : immunofluorescent staining. The results were from case number 4, 6 and 10 respectively.

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In three cases (Table 3), all the fetal NRBCs present in the smears did not react with the anti-α polypeptide antiserum. The fetal NRBCs are easily detectable and distinguished from the maternal lymphocytes because of their size and morphology (Figure 19). These three fetuses were also diagnosed as homozygous α-thalassemia 1 or Hb Bart's hydrops fetalis by DNA analysis.

In eight cases (Table 3), all the fetal NRBCs present in the smears reacted with the anti-α polypeptide antiserum. The positive control cells from chorionic villi washings as well as the maternal red blood cells stained with fluorescence. These eight fetuses were classified as heterozygous carriers for α-thalassemia 1 or normal. The DNA analysis confirmed the results obtained with the immunological procedure, three were diagnosed as heterozygous α-thalassemia 1, one as Hb H disease or heterozygous α-thalassemia 1, two as heterozygous α-thalassemia 2 or Hb CS and two as normal fetuses.

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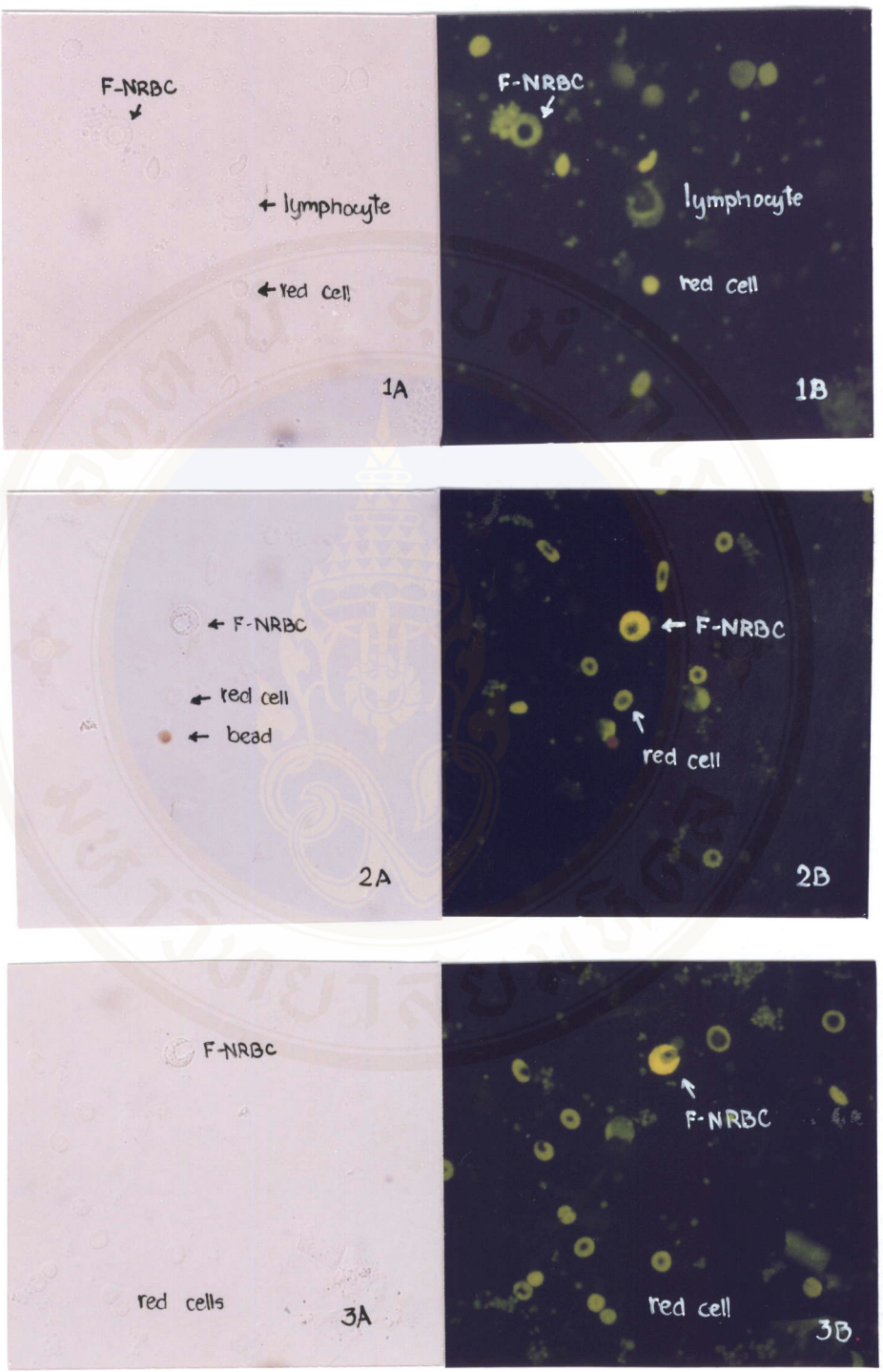

Figure 20 Noninvasive prenatal diagnosis for Hb Bart's hydrops fetalis. Fetal NRBCs were stained with anti α-globin antibody. Fetal NRBCs containing α-globin chain show the green fluorescencence (1B-3B) and (1A-3A) are phase contrast. These results were from cases 1, 3 and 7 respectively. They were later proof by DNA analysis to be heterozygous α–thalassemia 1.

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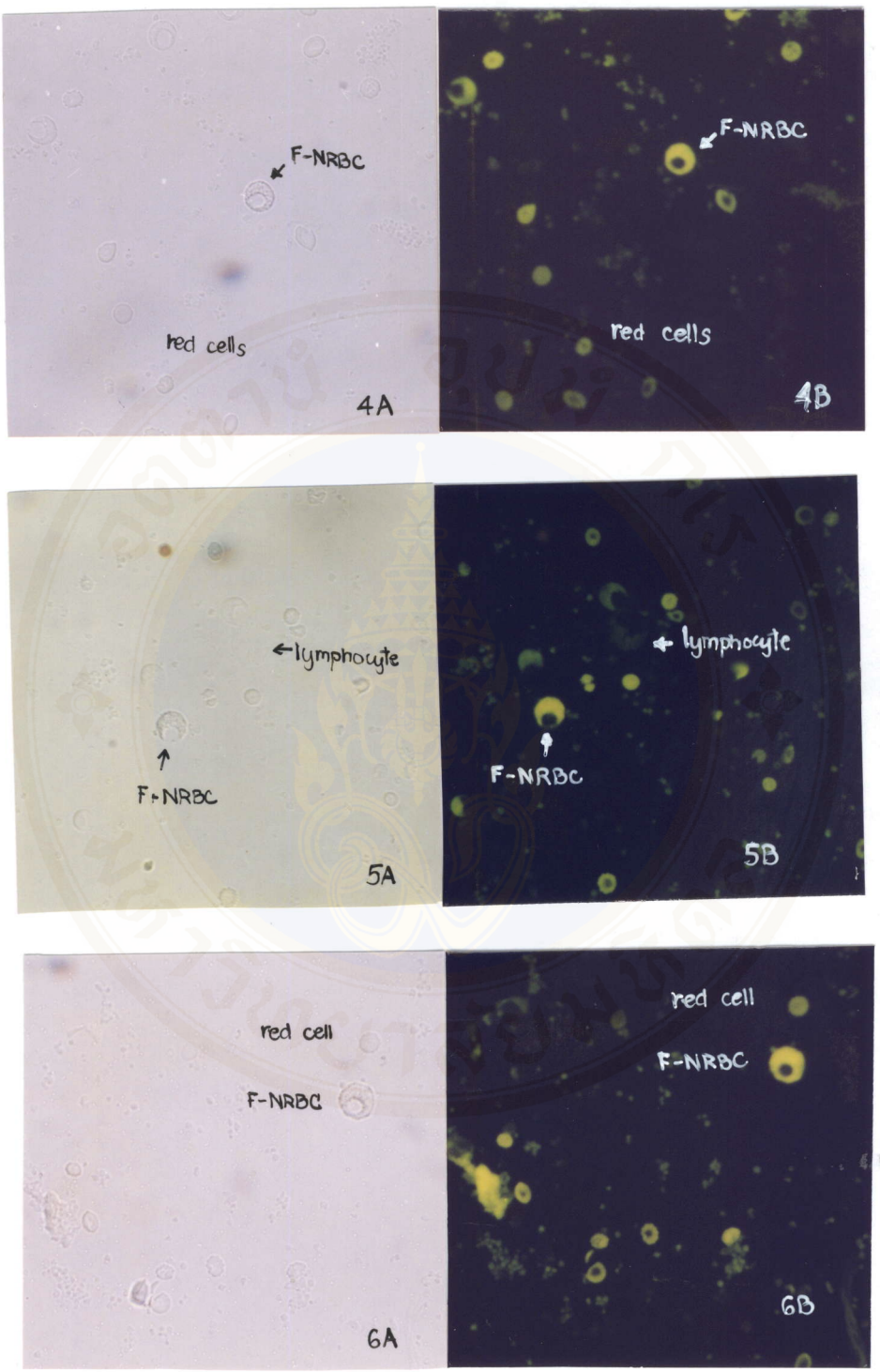

Figure 21 Noninvasive prenatal diagnosis for Hb Bart's hydrops fetalis. Fetal NRBCs were stained with anti α-globin antibody. Fetal NRBCs containing α-globin chain showed the green fluorescencence (4B-6B) and (4A-6A) are phase contrast. Case 11 (4AB) was heterozygous α–thalassemia 1 or Hb H disease. Case 5 (5AB) was Hb CS trait and case 9 was heterozygous α–thalassemia 2 or Hb CS trait.

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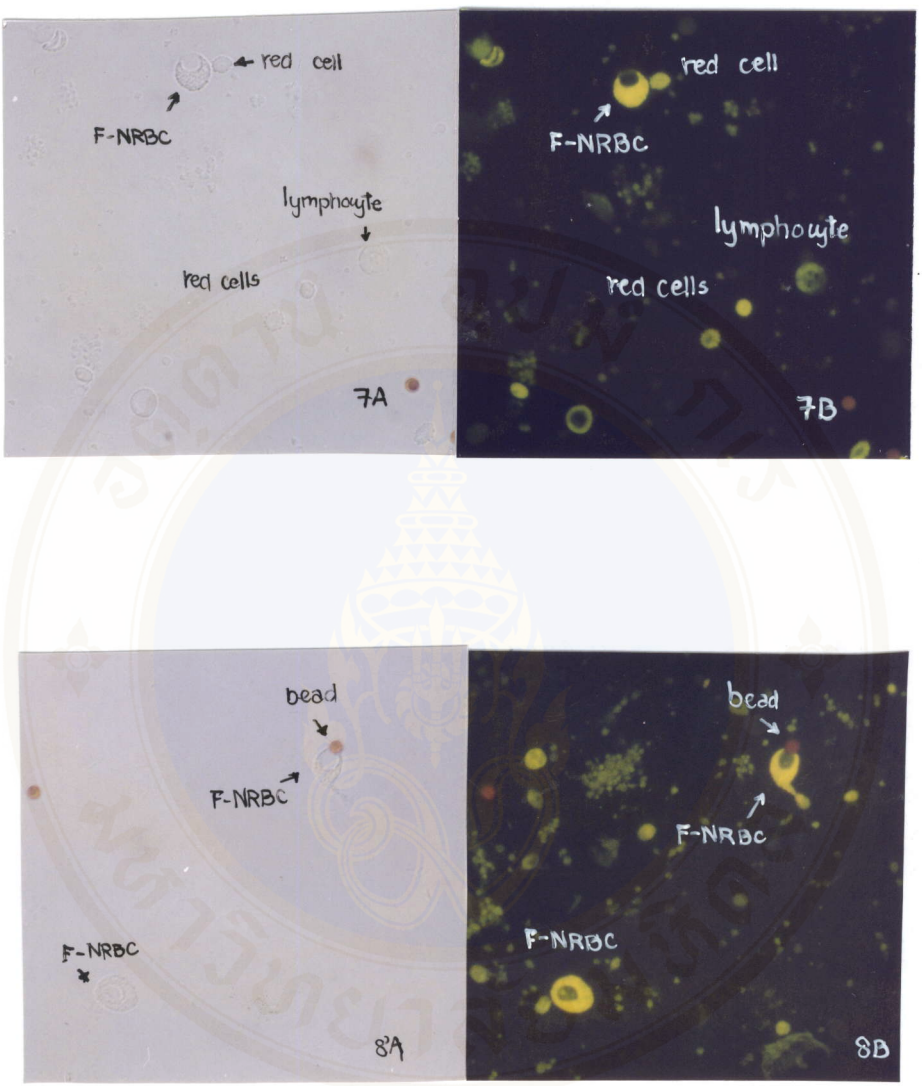

Figure 22 Noninvasive prenatal diagnosis for Hb Bart's hydrops fetalis. Fetal NRBCs were stained with anti α-globin antibody. Fetal NRBCs containing α-globin chain show the green fluorescencence (7B-8B) and (7A-8A) are phase contrast. These results were normal fetuses (case 2 and 8 respectively).

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The gestational age, volume of blood withdrawn and the amount of enriched fetal NRBCs obtained from 11 pregnant women at risk for Hb Bart's hydrops fetalis (10) and Hb H disease (1) fetuses are summarized in Table 4.

Table 4 The amount of fetal NRBCs in pregnant blood at risk for Hb Bart's hydrops fetalis obtained from each individual.

Case	Gestational age (weeks)	Blood withdrawn (ml)	Fetal NRBCs (cells)	Fetal NRBCs (cells/ml)
1	13	10	10	1.0
2	12	8	8	1.0
3	17	8	9	1.1
4	10	10	9	0.9
5	17	7	8	1.1
6	24	6	7	1.2
7	10	5	5	1.0
8	11	5	5	1.0
9	14	10	11	1.1
10	26	7	9	1.3
11	12	9	9	1.0

The number of detected fetal NRBCs from 5-10 ml of pregnant blood ranged from 5 to 11 cells with the average number of fetal cell of 8.36 cells per 8 ml of pregnant blood (Table 4).

Figure 23 demonstrates the amount of fetal NRBCs obtained from 11 pregnancies at risk for Hb Bart's hydrops fetalis in relation to volume of blood collected between the 10^{th} and 26^{th} week of gestation.

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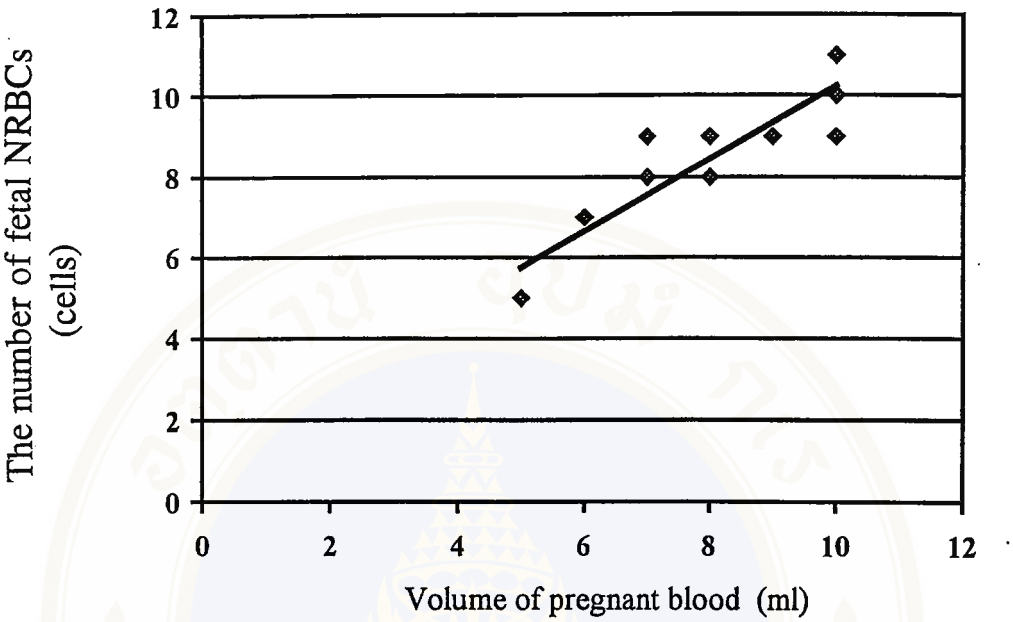

Figure 23 The number of fetal NRBCs in relation to volume of pregnant blood between the 10th and 26th gestational week at risk for Hb Bart's hydrops fetalis.

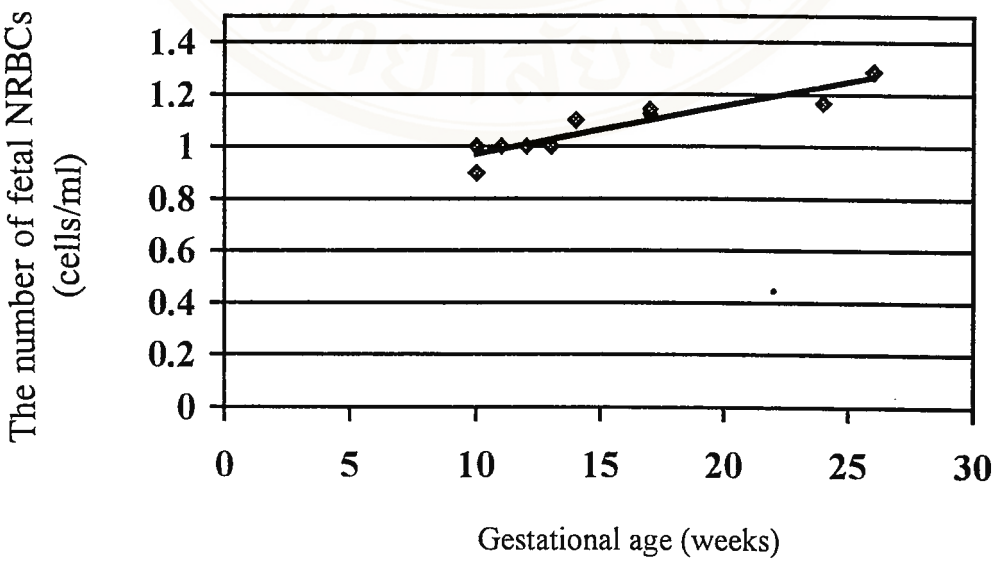

Figure 24 The number of fetal NRBCs in relation to different gestational ages (10th to 26th weeks of gestation) from 11 pregnant women at risk for Hb Bart's hydrops fetalis.

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Figure 24 demonstrates the number of fetal NRBCs per ml of pregnant blood obtained from 11 pregnant blood samples at risk for Hb Bart's hydrops fetalis in relation to different gestational ages (10^{th} and 26^{th} week of gestation). The frequency of fetal NRBCs had a tendency to increase as the pregnancy advanced. The number of fetal NRBCs is low at the beginning of pregnancy and increases at the second trimester.

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CHAPTER V

DISCUSSION

Couples with α–thalassemia 1 encounter a 25 percent risk of having fetuses with homozygous α-thalassemia 1, which results from deletion of all four α-globin genes. Since the α-globin-dependent fetal hemoglobin is the major hemoglobin of the fetus from 8 weeks of gestation (121), affected fetuses develop anemia early in the first trimester. The hemoglobin of the affected fetus consisted primarily of Hb Bart's (γ_4) and Hb Portland ($\zeta_2\gamma_2$) giving rise to Hb Bart's hydrops fetalis. The disease is not treatable. Affected fetuses are either stillborn or die soon after birth. Obstetric complications in women carrying an affected pregnancy are common. Prenatal diagnosis is thus allowed early pregnancy termination. This is currently performed by DNA analysis of chorionic villi or amniocytes obtained by chorionic villus sampling or amniocentesis. However, there is a procedure-related miscarriage risk depending on the technique employed and the experience of the operator. In order to minimize these risks, a non-invasive method of fetal cell isolation from maternal blood samples has been developed.

Three kinds of fetal cells have been separated from maternal blood: lymphocytes, trophoblasts and NRBCs. The NRBCs are the most potential cells for noninvasive prenatal diagnosis as their frequency in the fetus is relatively high and rare in adult. These cells are also fairly well differentiated and likely to have a limit life span in the maternal

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circulation. However, one of the main obstacles for non-invasive prenatal diagnosis using maternal blood is the rarity of fetal cells in maternal blood. Therefore, enrichment of fetal NRBCs is a prerequisite for most prenatal diagnosis.

To develop a reproducible isolation procedure for fetal NRBCs in maternal blood, we have systematically examined several key variables including double and triple density gradient centrifugations first described by Baht *et al.* (108). In addition, we established the fetal NRBCs enrichment technique using magnetic activated cell sorting (MACS) according to the method first described by Ganshirt-Ahlert *et al.* (86) with modification. They have used MACS to enrich CD71-positive cells from maternal blood after depletion of dense maternal leukocytes with a three-step density gradient. In this study a splenectomized β-thalassemia blood sample was used to establish the system as this condition contains comparatively more nucleated red cells than the maternal blood.

The density gradient centrifugation is an important step in the enrichment procedure that provides a 1000-fold increase of the NRBCs concentration. Although the mononuclear cells, especially lymphocytes, can be clearly separated from the double or triple density gradients (Table 1), there is a serious risk of losses of nucleated red cells if we only select the fraction of the gradient which is expected to contain the highest proportion of these cells (H-1119 in double and H-1107 in triple density gradient, respectively). Since a small number of fetal NRBCs will be found in the maternal circulation and none of them should be lost by the enrichment method. In the pregnant blood that contains only low fetal NRBCs, one step gradient with H-1077 may be enough to enrich the fetal NRBCs in the mononuclear cells layer. These fetal NRBCs will be

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further separated from the mononuclear cells by immunomagnetic beads selection and their morphological differences. This will save the cost of cell saparation and reduce the loss of fetal NRBCs.

After the isolation of fetal NRBCs from the maternal blood, we determined the presence of fetal hemoglobin in the sorted NRBCs by using fluorescent immunostaining. The specificity of anti γ-globin antibody on Hb F-containing cells was first tested with CVS cell washings. Clear γ-globin positive cells were detected in CVS cell washings as well as in maternal blood samples. Additionally, some maternal granulocytes bind non-specifically with the anti γ-globin antibody. This was due to the inadequate fixation of the hemoglobin within the cell, which allowed for leakege of hemoglobin out of the NRBCs into other cell types, such as granulocytes and lymphocytes (122). These contaminating cells are easily distinguished from the true anti γ-globin positive fetal NRBCs by microscopic examination.

We have shown that fetal NRBCs can be further enriched from the peripheral blood of pregnant women by immunomagnetic positive selection. Transferrin receptor (CD71) is expressed by cells incorporating iron. Besides developing erythrocytes, the cells including monocytes and activated lymphocytes also contain this receptor. Therefore, the use of transferrin receptor antigen alone is inadequate for the isolation of fetal cells. Bianchi et al. (122) began to enrich the fetal cells not only on the basis of CD71, but also for either glycophorin A (GPA) or thrombospondin receptor (CD36). In their most recent work, this group first uses negative sorting for CD45, a step that depletes lymphocytes, followed by positive sorting (123). In this study we have enriched the proportion of fetal

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NRBCs by CD71 but as expected the sorted cell population contains certain numbers of white cells.

Additionally, we found residual magnetic bead debris accumulated on the sorted nucleated cells, which led to an uninterpretable result. However, this problem was overcome when the bead detachment technique was changed. The beads were detached by pipetting up and down for several times instead of incubation at 37 ^{0}C overnight.

Irrespective of origin, fetal NRBCs in maternal blood are known to be very rare. Bianchi *et al.* has estimated by PCR that a 20-ml sample of maternal blood contains only some 0-20 fetal cells (1:10^{6} cells) (118). Sekikawa *et al* identified 1-7 fetal NRBCs (average 3.2 cells) from 7 ml of maternal blood (119). Cheung *et al.* retrieved pure fetal cells by using CD71 antibody and staining for embryonic hemoglobin. They identified 7-22 fetal cells from 16-18 ml of maternal blood (8). In this study, we start to identify fetal NRBCs when the gestational age was 10 weeks. The number of detected fetal NRBCs ranged from 5 to 11 cells from 5-10 ml of maternal blood (Table 4). The higher volume of pregnant blood was collected, the more fetal NRBCs were obtained (Figure 23). Our data also showed that the number of fetal NRBCs had a tendency to increase as the pregnancy advanced (Figure 24).

Since fetuses affected by homozygous α–thalassemia 1 have lost the function of α-globin synthesis, we examined the apparent or absence of normal α-globin chains in fetal NRBCs from maternal blood. Homozygous α–thalassemia 1 could be clearly distinguished from heterozygous state and normal subjects with the absence of α–globin chain. Fetuses with heterozygous α–thalassemia 1, Hb H disease, heterozygous Hb CS

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and normal α–globin genotype will have the positive results because all produce the α–globin chains. It is unable to distinguish these genotypes by immunocytochemistry and DNA analysis will be required.

In this series, all of the 11 fetuses were diagnosed from maternal peripheral blood. Nine pregnant women were previously found to carry hydrops fetuses caused by homozygous α–thalassemia 1 (patients 1, 2, 4, 5, 6, 7, 8 and 11). The remaining three pregnancies (patients 3, 9 and 10) were primigravidas and their hematological data indicated that three couples were heterozygous α–thalassemia 1.

After maternal blood collection, CVS was performed and DNA was analyzed by PCR technique for prenatal diagnosis of Hb Bart's hydrops fetalis. The results were comparable to the anti α–globin immunofluorescent staining method. This suggests that an accurate diagnosis for homozygous α–thalassemia 1 can be obtained by identification of fetal NRBCs in maternal blood. No fetal loss or complications occurred in our series.

Our data shows the feasibility of prenatal diagnosis of homozygous α–thalassemia 1 using fetal NRBCs from maternal blood of the risk couples. The method we have developed enabled us to avoid the invasive procedure such as CVS, amniocentesis and fetal blood sampling and can be repeated many times. Thus, this strategy should make feasible rapid screening for prenatal diagnosis of homozygous α–thalassemia 1 in population with a high frequency of the Southeast Asian type of α–thalassemia 1. Additional experience with more cases, this procedure could well be applied to diagnose other hemoglobinopathies and other genetic disorders.

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CHAPTER VI

CONCLUSION

1. The density gradient separation and enrichment technique with immunomagnetic beads specific for transferrin receptors were sufficient to isolate fetal NRBCs from maternal blood.

2. With the use of specific antibodies against the human α–globin polypeptide, it is possible to show the deficiency of α–globin in the erythroid cells of fetuses with the Hb Bart's hydrops fetalis syndrome.

3. Lymphocytes had a similar morphology to fetal NRBCs and had a negative staining to anti α–globin antibody. It may be, sometimes, difficult to differentiate these maternal lymphocytes from fetal NRBCs of the affected Hb Bart's hydrops fetalis. In this event, the uncertain cells should be scraped by manipulator and analyzed α–thalassemia 1 gene by DNA technique.

4. This qualitative test is unable to distinguish normal fetuses from those who are heterozygous α–thalassemia 1 or α–thalassemia 2 or Hb H disease.

5. This method is useful for diagnosis of homozygous α–thalassemia 1 in the couple at risk of having severe α–thalassemia 1 disease which had high frequency in Thailand.

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REFERENCES

1. Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia. Hemoglobin 1987;11:65-88.
2. Fucharoen S, Winichagoon P.Thalassemia in Southeast Asia: Problems and strategy for prevention and control. Southeast Asean J Trop Med Public Health 1992;23:647-55.
3. Evans MI, Drugan A, Koppitch FC, Zador IE, Sack AJ, Sokol RJ. Genetic diagnosis in the first trimester: the norm for the 1990s. Am J Obstet Gynecol 1989;160:1332-39.
4. Thomas MR, Tutschek B, Frost A, Rodeck CH, Yazdani N, Craft I, Williamson R. The time of appearance and disappearance of fetal DNA from the maternal circulation. Prenat Diagn 1995;15:641-46.
5. Simpson JL, Elias S. Isolating fetal cells from maternal blood: Advances in prenatal doiagnosis through molecular technology. J Am Med Assoc 1993;270:2357-61.
6. Adinolfi M, Non- or minimally invasive prenatal diagnostic tests on maternal blood samples or transcervical cells. Prenat Diagn 1995;15:889-96.
7. Bianchi D. Prenatal diagnosis by analysis of fetal cells in maternal blood. J Pediatr 1995;127:847-56.
8. Cheung MC, Goldberg JD, Kan YW. Prenatal diagnosis of sickle cell anemia and thalassemia by analysis of fetal cells in maternal blood. Nature Genetics 1996;14:264-68.

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9. Thomas MR, Williamson R, Craft I. Y-chromosome sequence DNA amplified from peripheral blood of women in early pregnancy. Lancet 1994;343:413-14.

10. Ganshirt D, Garritsen H, Miny P. Fetal cells in maternal circulation throughout gestation. Lancet 1994;343:1038-39.

11. Price JO, Elias S, Wachtel SS. Prenatal diagnosis using the fetal cells isolated from maternal blood by multiparameter flow cytometry. Am J Obstet Gynecol 1991;165:1731-37.

12. Ganshirt-Ahlert D, Basak N, Aldyhli K. Fetal DNA in uterine vein blood. Obstet Gynecol 1992;80:1-3.

13. Bianchi DW, Zickwolf GK, Yin MC. Erythroid-specific antibodies enhance detection of fetal nucleated erythrocytes in maternal blood. Prenat Diagn 1993;13:293-300.

14. Bianchi DW, Flint AF, Pizzimenti MF. Isolation of fetal DNA from nucleated erythrocytes in maternal blood. Proc Natl Acad Sci USA 1990;87:3279-83.

15. Zheng Y, Carter NP, Price CM. Prenatal diagnosis from maternal blood: Simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell. J Med Genet 1993;30:1051-56.

16. Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW. The molecular basis of blood diseases. Saunders company, Philadelphia, 1987.

17. Wintrobe MM. Clinical hematology. Philadelphia, Lea and Febiger, 1981.

18. Wintrobe MM, Shumacker HB. Comparison of hematopoiesis in the fetus and during recovery from pernicious anemia. J Clin Invest 1935;14:837-52.

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19. Beutler E, Lichtman MA, Coller BS, Kipps TJ. Williams Hematology, 5th edition, McGraw-Hill, New York, 1995.

20. Huehns ER, Dance n, Beaven GH, Keil JV, Hecht F, Motulsky AG. Human embryonic hemoglobins. Nature 1964;201:1095-97.

21. Hecht F, Motulsky AG, Lemire RJ, Shepard TE. Predominance of hemoglobin Gower 1 in early human embryonic development. Science 1966;152:91-95.

22. Gale RE, Clegg JB, Huehns ER. Human embryonic hemoglobins Gower 1 and Gower 2. Nature 1979;280:162-64.

23. E Beutler, MA Lichtman, BS Coller, TJ Kipps, Wiiliam Hematology, International edition, Mc Graw-Hill, New York, 1995.

24. Arnold L Medearis. Nonimmune hydrops fetalis In Management of Common Problems in Obstetrics and Gynecology, edited by Daniel R Mishell Jr, Paul F Brenner, 3rd edn, page 189-92. Blackwell Scientific Publications, Boston, 1994.

25. Winichagoon P, Thonglairuam, Fucharoen S, Tanphaichitr VS, Wasi P. α-Thalassemia in Thaialnd. Hemoglobin 1988;12:485-98.

26. Weatherall DJ, Higgs DR, Clegg JB, Hill AS, Nicholls R. Heterogenicity and origins of the α–thalassemia In Thalassemia: Pathophiusiology and Management, Part A, edited by S Fucharoen, PJ Rowley, NW Paul, page 3-14, Alan R. Liss, Inc., New York, 1988.

27. Higgs DR, α-Thalassemia. Baillier's Clinical Haematology 1993;6:117-50.

28. Leihber SA. α-Thalassemia. Hemoglobin 1989;13:685-731.

Copyright by Mahidol University

29. Liu TC, Choiu SS, Lin SF, Chen IP, Tseng WP, Chen PH, Cheng JG. Molecular basis and hematological characterization of Hb H disease in Southeast Asia. Am J Hematol 1994;45:293-97.

30. Fucharoen S, Winichagoon P, Pootrakul P, Piankijagum A, Wasi P. Differences between two type of the Hb H disease, α-thalassemia1/α-thalassemia 2 and α–thalassemia 1/ Hb Constant Spring. In Thalassemia: Pathophysiology and Management, Part A, edited by S. Fucharoen, PJ Rowley and NW Paul, page 309-15, Alan R. Liss, Inc., New York, 1988.

31. Winichagoon P, Higgs DR, Goodbourn SEY, Clegg JB, Wasi P. The molecular basis of α-thalassemia in Thailand. The EMBO Journal 1984;3:1813-18.

32. Neinhuis AW, Anagnou NP, Ley TJ. Advance in Thalassemia research. Blood 1984;63:738-58.

33. Orkin SH, Antonarakis SRE, Kazazian HH. Polymorphism and molecular pathology of the human β–globin gene. Prog Hematol 1983;13:49-73.

34. Baysal E, Carver MFH. The β and δ–thalassemia respiratiry, 8 th edition, Hemoglobin 1995;19:213-36.

35. Fucharoen S, Winichagoon P,. Hemoglobinopathy in Southeast Asia: Molecular biology and clinical medicine. Hemoglobin 1997;21:299-319.

36. Kan YW, Golbus MS, Klein P, Dozy AM. Successful application of prenatal diagniosis in a pregnancy at risk for homozygous β–thalassemia. New Engl J Med 1975;292:1096-99.

Copyright by Mahidol University

37. Ottolenghi S, Lanyon WG, Paul J, Williamson R. The severe form of α-thalassemia is cuased by a haemoglobin gene deletion. Nature 1974;251:389-91.

38. Taylor JM, Dozy A, Kan YW, Lie-Injo LE. Genetic lesion in homozygous α-thalassemia (hydrops fetalis). Nature 1974;251:392-93.

39. Verp MS, Simpson JL. In Human prental diagnosis, 2 nd edition, edited by K. Filkins, JF Russo, page 305, Marcel Dekker, Inc., New York, 1990.

40. Robinson A, Henry GA. Prenatal diagnosis by amniocentesis. Ann Rev Med 1985;36:13-26.

41. Mullis KB, Faloona FA. Methods Enzymol 1987;155:335-370.

42. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of β–globin genome sequences and restriction site analysis for diagnostic of sickle cell anemia. Science 1985;230:1350-52.

43. Li H, Gyllensten UB, Cui X, Saiki RK, Erlich HA, Arnheim N. Amplification and analysis of DNA sequences in single human sperm and diploid cells. Nature 1988;335:414-18.

44. Kazy Z, Rosovsky IS, Bakharev VA. Prenat Diagn 1982;2:39-42.

45. Jackson LG, Wapner RJ, Grebner EE, Barr MA, Davis GH. In Human prenatal diagnosis, 2 nd edition, eduted by K. Fillins, Russo, page 37, Marcel Dekker, Inc., New York, 1990.

46. Jackson LG, Zachary JM, Fowler SE, Desnick RJ, Golbus MS, Ledbetter DH, Mahoney MJ, Pregament E, Simpson JL, Black S, Wapner RJ and The US NICHHD Chrionic-Viluus Sampling and Amniocentesis Study Group. A randomized

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comparison of transcervical and transabdominal chorionic-villus sampling. N Engl J Med 1992;327:594-98.

47. Risk of Evaluation of CVS, Copenhagen, Denmark: World Health Organization/European Reginal Office, 1992.
48. Jackson LG, Wapner RJ, Barr Ma. The Lancet 1986;1:674.
49. Daffos F, Capella-Pavlosky M, Forestier F. Fetal blood sampling via the umbilical cord using a needle guided by ultrasound. Report of 66 cases. Prenat Diagn 1983;3:271-77.
50. Daffos F. Fetal blood sampling. In:Harrison MR, Golbus MS, Filly RA 9eds) The unborn patient. WB Saunders, Philadelphia, 1990, page 75-81.
51. D'Alton ME, DeCherney AH. Prenatal diagnosis. N Engl J Med 1993;328:114-20.
52. Goldberg JD, Ahtsaklis AJ. In Human prenatal diagnosis, 2 nd edition, edited by K. Filkins, JF Russo, page 389, Marcel Dekker, Inc., New York, 1990.
53. Varawalla NY, Dokrast A, Old JM, Sargent IL, Barlow DH. An approach to preimplantation diagnosis of β–thalassemia. Prenat Diagn 1991;11:775-85.
54. Holzgreve W, Garritsen HSP, Ganshirt-Alhert D,. Fetal cells in maternal circulation. J Reprod Med 1992;27:410-12.
55. Price JO, Elias S, Wachtel SS, Klinger K, Dockter M, Tharapel A, Schulman LP. Phillips OP, Meyers CM, Shook D. Am J Obstet Gynecol 1991;165:1731-37.
56. Schmorl G. *Pathologiisch-Anatomische Untersuchungen Uber Publeraleklampsie*. Leipzig:Vogel,1983.

Copyright by Mahidol University

57. Schindler AM, Graf E, Martin-du-Pan R. Prenatal diagnosis of fetal lymphocytes in maternal blood. Obstet Gynecol 1972;40:340-46.

58. Grossett L, Barelet V, Odartchenko N. Antenatal fetal sex determination from maternal blood during pregnancy. Am J Obstet Gynecol 1974;120:60-63.

59. Bianchi DW. Prenatal diagnosis by analysis of fetal cells in maternal blood. The Journal of Pediatrics 1995;127:847-56.

60. Rochelson B, Kaplan C, Guzman E, Arato M, Hanzen K, Trunca C. A quantitative analysis of placental vasculature in the third-trimester fetus with autosomal trisomy. Obstet Gynecol 1990;75:59-63.

61. Kuhlmann RS, Werner AL, Abramowicz J, Warsof SL, Arrington J, Levy DL. Placental histology in fetuses between 18 and 23 weeks' gestation with abnormal karyotype. Am J Obstet Gynecol 1990;163:1264-70.

62. Sipes SL, Weiner CP, Wenstrom KD, Williamson RA, Grant SS. The association between fetal karyotypee and mean corpuscular volume. Am J Obstet Gynecol 1991;165:1371-76.

63. Ganshirt-AhlertD, Pohlschmidt M, Gal A, Miny P, Horst J, Holzreve W. Raio of fetal to maternal DNA is less than 1 in 5000 at different gestational ages in maternal blood. Clin Genet 1990;38:38-43.

64. Hamada H, Ariami T, Kubo T, Hamaguchi H, Iwasaki H. Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. Hum Genet 1993;91:427-32.

Copyright by Mahidol University

65. Benirschke K. Anatomical relationship between fetus and mother. In: Simpson JL, Elias S, (Eds). Fetal cells in maternal blood: Prospects for noninvasive prenatal diagnosis, Ann NY Acad Sci 1994;731:9-20.

66. Douglas GW, Thomas L, Carr M, Cullen NM, Morris R. Trophoblast in the circulating blood during pregnancy. Am J Obstet Gynecol 1959;78:960-73.

67. Holzgreve W, Garritsen HSP, Ganshirt-Ahlert D. Fetal cells in the maternal circulation. J Reprod Med 1992;37:410-18.

68. Schindler AM, Graf E, Martin-Du-Pan R. Prenatal diagnosis of fetal lymphocytes in the maternal blood. Obstet Gynecol 1972;40:340-46.

69. Takahara H, Kadotani I, Kusumi K, Makino S. Some critical aspects of prenatal diagnosis of sex in leukocyte cultures from pregnant women. Proc Jpn Acad 1972;48:603-07.

70. Whang-Peng J, Jeffin JS, Harris C, Lee E, Stites J. The transplacental passage of fetal leukocytes into the maternal blood. Proc Soc Exp Biol Med 1973;142:50-53.

71. Walknowska J, Conte FA, Grumback MM. Practical and theoretical implications of fetal/maternal lymphocyte transfer. Lancet 1969;1:1119-22.

72. Herzenberg LA,. Fetal cells in the blood of pregnant women: Detection and enrichment by fluorescence-activated cell sorting. Proc Natl Acad Sci USA 1979;76:1453-55.

73. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria M. Male fetal progenitor cells persist in maternal blodd for as long as 27 years postpartum. Proc Natl Acd Sci USA 1996;93:705-08.

Copyright by Mahidol University

74. Zilliacus R, de la Chapelle A, Schroder J, TillikanenA, Kohne E, Kleihauer E. Transplacental passage foetal blood cells. Scand J Haematol 1975a;15:333-38.

75. Wessman M, Ylinen K, Knuutila S. Fetal granulocytes in maternal venous blood detected by *in situ* hybridization. Prenat Diang 1992;12:993-1000.

76. Clayton EM, Felclhaus MT, Whitacre FE, Fetal erythrocytes in the maternal circulation of pregnant women. Obstet Gynecol 1964;23:915-19.

77. Thomas DB, Yoffey JM. Human foetal haematopoiesis I. The cellular composition of foetal blood. Br J Haematol 1962;8:29.-95.

78. Liou J-D, Hsieh TT, Pao CC. Presence of cells of fetal origin in maternal circulation of pregnant women. Ann NY Acad Sci 1994;731:237-45.

79. Schindler AM, Graf E, Martin-Du-Pan R. Prenatal diagnosis of fetal lymphocytes in the maternal blood. Obstet Gynecol 1972;40:340-46.

80. Iverson GM, Bianchi DW, Cann HM, Herzenberg LA. Detection and isolation of fetal cells from maternal blood using the fluorescence-activated cell sorter (FACS). Prenat diagn 1981;1:61-73.

81. Lo Y-MD, Wainscoat JS, Gilmer MDG, Patel P, Sampietro M, Fleming KA. Prenatal sex determination by DNA amplification from maternal peripheral blood. Lancet 1989;2:1363-65.

82. Durrant LG, McDowall KM, Holmes RA, Liu DTY. Screening of monoclonal antibodies recognizing oncofetal antigens for isolation of trophoblasts from maternal blood for prenatal diagnosis. Prenat diagn 1994;14:131-40.

Copyright by Mahidol University

83. Mueller UW, Hawes CS, Wright AE. Isolation of fetal trophoblast cells from peripheral blood of pregnant women. Lancet 1990;336:197-200.

84. Elias S, Price J, Dockter M, Wachtel S, Tharapel A, Simpson JL. First trimester prenatal diagnosis of trisomy 21 in fetal cells from maternal blood. Lancet 1992;340:1033.

85. Bianchi DW, Mahr A, Zickwolf GK, Houseal TW, Flint AF, Klinger KW. Detection of fetal cells with 47, XY, +21 karyotype in maternal peripheral blood. Hum Genet 1992;90:368-70.

86. Ganshirt-Ahlert D, Borjesson-Stoll R, Burschyk M. Detection of fetal trisomies 21 and 18 from maternal blood using triple density gradient and magnetic cell sorting. Am J Reprod Immunol 1993;30:194-201.

87. Cacheux V, Milesi-Fliet C, Tachdijian G. Detection of 47, XYY trophoblast fetal cells in maternal blood by fluorescence in situ hybridization after using immunomagnetic lymphocyte depletion and flow cytometry sorting. Fetal Diang Ther 1992;7:190-94.

88. Zheng YL, Craigo SD, Price CM, Bianchi DW. Demonstration of spontaneously dividing male fetal cells in maternal blood by negative magnetic cell sorting and FISH. Prenat Diang 1995;15:573-78.

89. Lo YM, Patel P, Sampietro M, Gillmer MDG, Fleming KA, Wainscoat JS. Detection of single-copy sequence from maternal bloo. Lancet 1990;335:1463-64.

90. Hamada H, Arinami T, Sohida S, Hamaguchi H, Kubo T. Midtrimester fetal sex determination from maternal peripheral blood by fluorescent *in situ* hybridization without enrichment of fetal cells. Prenat Diang 1995;15:78-81.

Copyright by Mahidol University

91. Zheng YL, DeMaria M, Zhen D, Vadnais TJ, Bianchi DW. Flow sorting of fetal erythroblasts using intracytoplasmic anti-fetal haemoglobin: preliminary observations on maternal samples. Prenat Diagn 1995;15:897-905.

92. Andrews K, Wienberg J, Ferguson-Smith MA, Rubinsztein DC. Enrichment of fetal nucleated cells from maternal blood: model system using cord blood. Prenat Diagn 1995;15:913-19.

93. Lewis DE, Schober W, Murrell S. Rare event selection of fetal nucleated erhthrocytes in maternal blood by flow cytometry. Flow cytometry 1996;23:218-27.

94. Durrant LG, Martin WL, McDowell KM, Liu DTY. Isolation of fetal trophoblasts and nucleated erythrocytes from the peripheral blood of pregnant women for prenatal diagnosis of fetal aneuploidies. Ear Hum Devl 1996;47(suppl):79-83.

95. Johansen M, Knight M, Maher EJ, Smithe K, Sargent IL, An investigation of methods for enriching trophoblast from amternal blood. Prenat Diagn 1995;15:921-31.

96. Huber K, Wolf H, van Lindern M. Development of a rapid means of estimating the hemoglobin F content of candidate fetal cells isolated from maternal blood using HPLC. Prenat Diagn 1996;16:1011-19.

97. Alter BP. Biology of erythropoiesis. Ann NY Acad Sci 1994;731:36-47.

98. Lo YMD, Morey AL, Wainscoat JS, Fleming KA. Culture of fetal erythroid cells from maternal peripheral blood. Lancet 1994;344:264-65.

99. Valerio D, Aiello R, Altieri V. Culture of fetal erhthroid progenitor cells from maternal blood for non-invasive prenatal genetic diagnosis. Prenat Diagn 1996;16:1073-82.

Copyright by Mahidol University

100. Wachtel SS, Sammons D, Manley M. Fetal cells in maternal blood: recovery by charge flow separation. Hum Genet 1996;98:162-66.

101. Ganshirt-Ahlert D, Burschyk M, Garritsen HSP. Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. Am J Obstet Gynecol 1992;166:1350-55.

102. Wachtel SS, Elias S, Price J. Fetal cells in the maternal circulation: isolation by multiparameter flow cytometry and confirmation by PCR. Hum Reprod 1991;6:1466-69.

103. Edelman P, Vinci C, Villeval JL. A monoclonal antibody against an erythrocyte ontogenic antigen identifies fetal and adult erythroid progenetors. Blood 1986;67:58-63.

104. Simpson JL, Lewis DE, Bischoff FZ, Elias S. Isolating fetal nucleated red blood cells from maternal blood: The Baylor experience-1995. Prenat Diagn 1995;15:907-12.

105. Pembrey ME, Weatherall DJ, Clegg JB. Maternal synthesis of haemoglobin F in pregnancy. Lancet 1973;1:1350-54.

106. Miller EE, Ludka HR, Peacock, Tomar RH. Manual of laboratory immunology. 2nd ed., Lea & Febiger, Philadelphia, USA, 1991.

107. Zheng YL, Carter NP, Price CM, Colman SM, Milton PJ, Hackett GA, Greaves MF, Ferguson-Smith MA. Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting. J med Genet 1993;30:1051-56.

Copyright by Mahidol University

108. Baht NM, Bieber MM, Teng NNH. One-step enrichment of nucleated red blood cells-a potencial application in prenatal diagnosis. J Immunol Methods 1993; 158:277-80.

109. Devis JM. Basic cell culture: a practical approach, IRL press, USA, 1994.

110. Bianchi DW, Klinger KW, Vadnais TJ. Development of a model system to compare cell separation methods for the isolation of fetal cells from maternal blood. Prenat Diagn 1996;16:289-98.

111. Dynal company, Cell separation and protein purification: Technical handbook, Second edition, Norwey, 1996.

112. Brown BA. Hematology principles and procedure, 5th ed., Lea & Febiger, Philadelphia, USA, 1988.

113. Bernini LF, Kanhai HHH, Losekoot M, Giordano P, Harteveld L. Prenatal diagnosis of homozygous α^0–thalassemia by an immunological method. Ann NY Acad Sci 1994;731:193-96.

114. Orkin SH. Disorders of hemoglobin synthesis: the thalassemia. In: The molecular basis of blood diseasea, edited by Stamatoyannopoulcs G, Nienhuis AW, Leder P, Malerus PW, page 106-107, Suander company, Philadelphia, 1987.

115. Weatherall DJ. The thalassemias. In: William Hematology, edited by Ernest, Marshall, Barry, Thomas. Page 581-615, McGraw-Hill Inc, USA, 1995.

116. Simpson JL, Elias S. Fetal cells in maternal blood-overview and historical perspective. Ann NY Acad Sci 1994;731:1-8.

Copyright by Mahidol University

117. Bianchi DW, Klinger KW. Prenatal diagnosis through the analysis of fetal cells in the maternal circulation. In: Milunsky A (Ed.). Genetic Disorders and the Fetus, 3 rd edn, Baltimore: John HopskinUniversity Press 1992;759-70.

118. Bianchi DW, Shuber AP, DeMaria M, Fougner AC, Klinger KW. Fetal cells in maternal blood: determination of purity and yield by quantitative polymerase chain reaction. Am J Obstete Gynecol 1994;171:922-26.

119. Sekikawa A, Kimura T, Sasaki M, Nakamura S, Kobayashi R, Sato T. Prenatal diagnosis od Duchenne muscular dystrophy using a single fetal nucleated erythrocytes in maternal blood. Neurology 1996;46:1350-53.

120. Winichagoon P, Fucharoen S, Kanokpongsakdi S, Fukumaki Y. Detection of α–thalassemia-1 (Southeast Asian Type) and its application for prenatal diagnosis. Clin Genet 1995;47:318-20.

121. Bianchi DW. Clinical trials and experience: Boston. In:Simpson Jl (Eds) Fetal cells in Maternal Blood:Prospects for Noninvasive Prenatal Diagnosis, New York: New York Academmic of Science 1994;731:1-10.

122. DeMaria MA, Zheng YL, Zhen D, Weinschenk NH, Vadnais TJ, Bianchi DW. Improved fetal nucleated erythrocytes sorting purity using intracellular antifetal hemoglobin and Hoechst 33342. Cytometry 1996;25:37-45.

123. Hann JM. Development of blood in the fetus. In:Hann IM, Gibson BES, Letsky EA. Fetal and Neonatal Haematology. London: Bailliere Tindall, page 1-28, 1991.

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APPENDIX I

Summary of the hematological data and phenotype of 10 pregnant women at risk for homozygous α–thalassemia 1 (Hb Bart's hydrops fetalis) and one at risk for Hb H disease.

Case	Age	Hb	MCV	Hb type	Phenotype	Couple's phenotype
1	26	11.2	68	A_2A	α-thal1trait	HbE/α-thal1trait
2	29			EA	HbE/α-thal1trait	α-thal1trait
3	28	10.6	65	A_2A	α-thal1trait	α-thal1trait
4	26	11.6	62	A_2A	α-thal1trait	α-thal1trait
5	34	10.8	65	A_2A	α-thal1trait	Hb CS trait
6	30	10.9	71	A_2A	α-thal1trait	HbE/α-thal1trait
7	34	11.3	72	A_2A	α-thal1trait	HbE/α-thal1trait
8	26	10.6	75	A_2A	α-thal1trait	α-thal1trait
9	19	10.8	66	A_2A	α-thal1trait	Hb H disease
10	22	10.1	67.5	EA	α-thal1trait	α-thal1trait
11	17	11.8	63.4	A_2A	α-thal1trait	Hb H disease

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APPENDIX II

Summary of hematological data and phenotype of 18 pregnant women at risk for β–thalassemia disease.

Case	Age	Hb	MCV	Hb Type	Phenotype	Couple's phenotype
1	36	12.6	80	EA	Hb E trait	β-thal trait
2	30	11.1	71	A_2A	β-thal trait	homozygous β-thal
3	28	10.9		A_2A	β-thal trait	β-thal trait
4	26	10	77	A_2A	β-thal trait	β-thal trait
5	21	11.2		A_2A	β-thal trait	homozygous β-thal
6	25		57.1	EA	Hb E/ α -thal 1	Hb CS trait
7	29		84	EA	Hb E trait	β-thal trait
8	29	12	70	EE	homozygous HbE	β-thal trait
9	23	9.9	64	A_2A	β-thal trait	β-thal trait
10	37	10	65	A_2A	β-thal trait	β-thal trait
11	33	11.7	76.3	EA	Hb E trait	β-thal trait
12	35	10.6	63	A_2A	β-thal trait	homozygous HbE
13	33	9.6	61	A_2A	β-thal trait	HbE trait

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Case	Age	Hb	MCV	Hb type	Phenotype	Couple's phenotype
14	31	9.6	69	EA	Hb E trait	β-thal trait
15	35		73	A_2A	β-thal trait	β-thal trait
16	36	6	78	EF	β-thal/Hb E	Hb E trait
17	24		59.1	EE	homozygous HbE	β-thal trait
18	36	12.4	75	EA	HbE trait	β-thal trait

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44727