Isolation of Fetal Nucleated Red Blood Cell from Maternal Blood using Immunomagnetic Beads for Prenatal Diagnosis

T. Pongsritasana[1], S. Wongratanacheewin[2], V. Prasertcharoensuk[1] and R.W. Sermswan[3]

SUMMARY The immunomagnetic beads method for isolation of fetal nucleated red blood cells (FNRBCs) from peripheral blood of 78 pregnant women for prenatal diagnosis was developed. The study subjects were classified into 8-10 and 11-14 weeks of gestation (n = 39 each). Peripheral blood cells were divided into two for the FNRBCs isolation using two protocols, one with anti-CD45 depletion followed by anti-CD71 and anti-GPA monoclonal antibodies and another without CD45 depletion. The use of CD45 depletion gave a slightly higher number of sorted cells but not significantly different ($p > 0.05$). The percentage of $CD71^+$ and GPA^+ cells obtained from 8-10 weeks and 11-14 weeks of gestation was not different ($p > 0.05$). The sensitivity in determining the sorted FNRBCs for male fetal sex by PCR using 8-10 and 11-14 weeks of gestation was generally 50 and 69%, respectively. The method so developed is simple and cost effective and may thus be applied for prenatal diagnosis.

Fetal cells isolated from maternal blood represent a good source of fetal chromosome or DNA obtained non-invasively by maternal venipuncture that would avoid the complication and disadvantage of amniocentesis and chorionic villus sampling (CVS) which currently are the gold standard samples for prenatal diagnosis.[1-3] The possible fetal cell types that can be isolated from maternal blood included trophoblasts, lymphocytes and nucleated red blood cells. Nucleated red blood cells (NRBC) are the best candidate to be used in prenatal diagnosis. Most NRBCs occur in maternal blood during the first trimester.[4,5] They represent a small number in the adult blood circulation with a short life span. They have transferrin and glycophorin A receptors on the membrane and produce zeta and gamma fetal hemoglobin which have the potential to be used as identification markers. Fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS), immunomagnetic beads, immunoaffinity columns and charge flow separation have been used for cell separation.[1,6-9] The immunomagnetic bead method is an easy method and inexpensive as large equipment is not needed. The fetal cell yield and purity obtained from this technique were higher than MACS but lower than FACS.[6] In this communication, we isolated fetal nucleated red blood cells from maternal blood using immunomagnetic beads in order, in the future, to apply the method for prenatal diagnosis.

From the [1]Department of Obstetric and Gynecology, [2]Department of Microbiology, [3]Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
Corespondence: Rasana Sermswan
E-mail: rasana@kku.ac.th

MATERIALS AND METHODS

Blood samples

Fifteen milliliters of peripheral blood samples were collected from two groups of 39 pregnant women each of either 8-10 or 11-14 weeks of gestation. The blood was taken when they received antenatal care at Srinagarind Hospital, Khon Kaen University, Thailand, after informed consent was obtained. The gestational age was established on the basis of the first day of the last menstrual period. The anticoagulant used was 0.5 ml of 0.2 M EDTA. Blood samples from three non-pregnant and three male volunteers were also collected to be used as negative and positive control for Y-chromosome detection. Two milliliters of cord blood samples were collected from two normal babies at birth for fetal hemoglobin-staining as positive controls.

Mononuclear cells preparation

The mononuclear cells were prepared by methods of Zheng *et al*.[10] and Mavrou *et al*.[11] with some modification. The blood samples were gently diluted in equal volume of phosphate buffered saline, pH 7.4 (PBS). Five milliliters of diluted blood were overlaid on the surface of 3 ml Ficoll-Hypaque gradient (Pharmacia Biotech, Sweden) and centrifuged at 300 x *g* for 30 minutes. The peripheral blood mononuclear cells (PBMC) were collected from the fluid-cell interphase, washed twice in 5 ml of PBS and re-suspended in 1 ml of 2% BSA in PBS. The number of PBMCs was enumerated by a hemocytometer.

Fetal nucleated red blood cell sorting by immunomagnetic beads

The FNRBCs were sorted according to the method described by Bianchi *et al*.[6] with some modification. The experiment was done using two protocols as follows:

Fetal nucleated red blood cells sorting with CD45 depletion

One half of purified mononuclear cells from 15 ml blood in a 1.5 ml microcentrifuge tube was incubated with 20 μl of monoclonal antibodies specific for the leukocyte common antigen (CD 45) (DAKO, Denmark) on ice for 30 minutes. The cells were collected by centrifugation at 300 x *g* for 5 minutes and washed twice with 1% BSA in PBS to remove unbound antibodies and re-suspended in 1% BSA in PBS. A 25 μl aliquot of washed immunomagnetic beads (Dynabeads M-450 polystyrene coated with monoclonal rat anti-mouse IgG1, Norway) was added and incubated at 4°C for 30 minutes with mixing every 5 minutes. The tube of suspension was placed in a single tube magnet stand (Dynal Magnetic Particle Concentrator, Dynal MPC®) for 1 minute. The unbound cells were removed to a new tube containing 10 μl of anti-CD71 monoclonal antibody specific to the transferrin receptor (DAKO, Denmark) and 10 μl of anti-GPA monoclonal antibody specific to glycophorin A (DAKO, Denmark). The suspension was incubated on ice for 30 minutes, centrifuged at 300 x *g* for 5 minutes to obtain the cells and washed twice using 1% BSA in PBS. The cells were again incubated with Dynabeads at 4°C for 30 minutes with gentle mixing every 5 minutes. The bound cells were separated using MPC and washed twice with 1% BSA in PBS. The sorted FNRBCs were re-suspended in 25 μl of PBS and stored at 4°C until used.

Fetal nucleated red blood cells sorting without CD45 depletion

The other half of the purified mononuclear cells was processed as described but the anti-CD45 monoclonal antibody sorting step was omitted.

DNA extraction

DNA extraction from isolated fetal NRBCs was done by using a Wizard® genomic DNA purification kit (Promega, USA) and 0.5 μg of DNA was used for sex identification by PCR using X and Y specific primers.

Evaluation of CD71 and glycophorin A positive cells by FACS

Ten microliters of sorted FNRBCs suspension were stained with 10 μl of anti-CD71 monoclonal antibody conjugated with fluorescence isothiocyanate, FITC (DAKO, Denmark) and 10 μl

of anti-GPA monoclonal antibody conjugated with R-phycoerythrin, RPE (DAKO, Denmark) for 30 minutes at 4°C in the dark. The cells were washed twice and re-suspended in 1 % BSA in PBS. About 5,000 cells were counted by flow cytometry (FACS Vantage, Becton Dickinson, USA) and evaluated for the CD71 and GPA positive cells.

Immunocytochemical staining

The thin smear of sorted FNRBCs on a glass slide was stained by a procedure modified from Zheng *et al.*[10] The slide was fixed in 2 % formaldehyde in PBS at room temperature for 1 minute. It was then rinsed twice in Tris buffered saline, pH 7.6 (TBS) for 2 minutes and incubated in TBS containing 10 % normal goat serum (DAKO, Denmark) in a moist chamber at room temperature for 15 minutes. Thirty microliters of 1:80 dilution of sheep anti-fetal hemoglobin (Cortex Biochem, USA) in normal goat serum were added and incubated at room temperature for 60 minutes in a moist chamber. After washing the slides in TBS, thirty microliters of a 1:60 dilution of rabbit anti-sheep IgG alkaline phosphatase (Zymed Laboratories, USA) in TBS containing 10% normal rat serum (DAKO, Denmark) was added. The reaction was allowed to occur at room temperature for 60 minutes in a moist chamber, and then washed with TBS. Finally, 5 drops of freshly prepared alkaline phosphatase Vector® blue substrate (Vector Laboratories, USA) were added to each slide and incubated in the dark for 20-30 minutes. The slides were washed in a TBS bath for 15 minutes, air-dried, mounted with mounting medium and examined by color development under a light microscope.

PCR development for sex determination

PCR was used to evaluate the sorted fetal NRBCs. The fetal sex was detected by using Y-chromosome specific primers:Y1; (5' ATG ATA GAA CGG AAA TAT G 3'),Y2; (5' AGT AGA ATG CAA AGG GCT CC 3') and X-chromosome specific primers: X1; (5' AAT CAT CAA ATG GAG ATT TG 3'), X2; (5' GTT CAG CTC TGT GAG TGA AA 3').[12-14] One hundred microliters of PCR mixture consisted of 1x PCR buffer (with 1.5 mM $MgCl_2$), 3.5 mM $MgCl_2$, 50 μM of each dNTPs, 0.4 nM of each primer either for X or Y, 2 U *Taq* DNA polymerase, sterile distilled water and 1 μl of 0.5 μg DNA template. The control DNA was obtained from normal females and males. The PCR was performed for 35 cycles of denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute and extension at 72°C for 2 minutes with one last step of extension for 5 minutes at 72°C in a Perkin-Elmer DNA Thermal Cycler 480. The amplified products (15 μl) were analyzed by 2% agarose gel electrophoresis in 1x TBE buffer at a 6-volts/cm constant voltage for 1.5 hours. The gel was stained in ethidium bromide (0.5 mg/ml) and visualized and recorded by VDS Immage Master® gel document (Phamacia Biotech, Sweden). The sex determination in each sample was confirmed by neonatal sex examination.

Statistical analysis

Descriptive statistics and t-test analyzed data by SPSS program.

RESULTS

Number of sorted FNRBCs

The means ± SD of maternal age and gestational age of group 1 were 26.85 ± 5.21 years (19-39 years) and 8.76 ± 0.81 weeks. The means of maternal and gestational age of group 2 were 25.90 ± 5.08 years (16-35 years) and 12.46 ± 1.17 weeks, respectively. The means of mononuclear cell numbers in groups 1 and 2 were 23.73 x 10^6 cells and 24.12 x 10^6 cells, respectively. The mean ± SD value of half of the purified PBMCs that were sorted by the two protocols and the mean ± SD value of sorted FNRBCs in each group are summarized in Table 1.

CD71 and glycophorin A positive cells by FACS

The mean ± SD values of percentages of sorted cells by FACS that were positive to both CD71 and GPA with and without CD45 depletion in 8-10 weeks are 0.74 ± 0.58% and 0.88 ± 0.65%. The mean ± SD values for the percentage of sorted cells with and without CD45 depletion in 11-14 weeks are 0.68 ± 0.51% and 0.76 ± 0.70%.

Evaluation of isolated FNRBCs

The isolated FNRBCs were confirmed by fetal hemoglobin (Hb F) immunocytochemical staining

as shown in Fig. 1. Staining of isolated FNRBCs from 78 blood samples showed only 12.8% HbF positive.

The specific X and Y chromosome primers gave PCR amplification products of 130 and 170 bp respectively. The sensitivity of detection of X is 20 pg and Y chromosome is 30 pg. The results of PCR amplified products from isolated cell samples are shown in Fig. 2. Those sample where followed-up was not available and abortion cases were not analyzed. The sensitivity of fetal sex diagnosis in the 8-10 weeks group is 71 % (20/28) and in the 11-14 weeks group is 86 % (31/36) (Table 2).

DISCUSSION

Many methods have been attempted to enrich a small amount of FNRBCs from maternal peripheral blood for a non-invasive prenatal diagnosis.[1,2,6,15] A simple and reliable method with cost effective is still needed to be developed for both high risk and general population in developing country.

The experiments performed here compared the sorted-cell method using transferrin and glycophorin-A receptors with and without anti-CD45 (leukocyte specific antigen) depletion between two groups of patients of different gestational ages using immunomagnetic beads. The average number of sorted cells using CD45 or non-CD45 depletion in each gestational age group was not significantly different ($p > 0.05$). The CD45 depletion gave a slightly higher number of cells than the non-CD45 depletion group but was not statistically different ($p > 0.05$).

The number of sorted NRBCs in our study was in the same range as reported by Bianchi *et al.*[6] (2.3×10^2 to 6.85×10^5 cells/ml) using FACS and Ganshirt-ahlert *et al.*[1] (5 to10 x 10^5 cells/ml) using MACS. The number of sorted NRBCs in 11-14 weeks was not significantly higher than 8-10 week.

Table 1 The mean ± SD value of a purified PBMC and the mean ± SD value of sorted cells in each gestational age group

Gestational age (weeks)	**Half of PBMC from 15 ml blood** (x 10^6 cells)	**Method I: with CD45 sorted cells** (x 10^5 cells)	**Method II: Non-CD45 sorted cells** (x 10^5 cells)
8-10	11.86 ± 3.24	2.84 ± 2.78 (450-850,000)	2.45 ± 2.33 (5,250-930,000)
11-14	12.06 ± 3.03	3.51 ± 2.80 (320-905,000)	3.06 ± 4.02 (4,200-1,660,000)

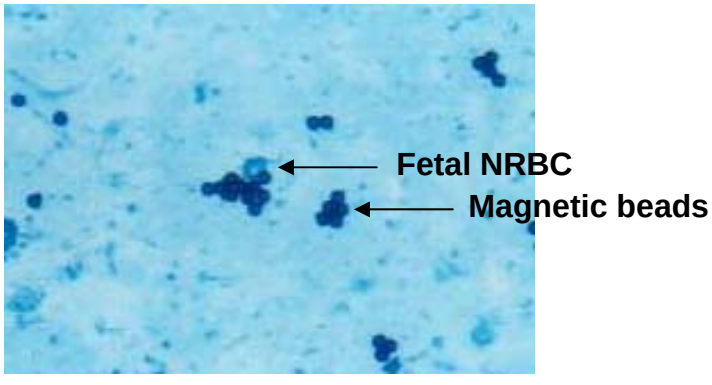

Fig. 1 Sorted fetal nucleated red blood cells from maternal blood stained with anti-fetal hemoglobin combination with Vector® blue stain. Arrows indicate a FNRBC and magnetic beads.

However, the gap between our two gestational age groups may not large enough to see the difference. Even though we cannot obtain an exact gestational age but rather in range as it depends largely on when pregnant women start to receive antenatal care in our hospital. However, for the future application, the optimum gestational week to perform a reliable non-invasive prenatal diagnosis was suggested to be 15 weeks.[16]

Evaluation of sorted cells from immunomagnetic beads by FACS indicated a large number of lymphocytes among CD71 and GPA positive NRBCs fraction. It also indicated a variation of CD71 and GPA positive cells in each case that affect the sensitivity of detection (data not shown). The frequency of fetal cells in maternal mononuclear cells evaluated by FACS here is 1.7 to 2.2 x 10^{-6} which is in the range estimated by Wachtel *et al*.[17] (10^{-5} to 10^{7}). The fetal cells in maternal blood was found to be increased in the second trimesters and may be increased in women carrying aneuploid fetuses.[16,17] The sorted NRBCs in our experiment from 15 ml blood not only divided by half for two comparison experiments but a small volume from each case was also used to stain with anti-fetal hemoglobin and FACS analysis. A small number of fetal cells were detected in 12.8 % (10/78) of cases.

Table 2 Sensitivity of fetal sex diagnosis by PCR

Gestational age (weeks)	Sensitivity of male fetus diagnosis		Sensitivity of female fetus diagnosis		Total sensitivity	
	Number	%	Number	%	Number	%
8-10	5/10	50	15/18	83	20/28	71
11-14	11/16	69	20/20	100	31/36	86
Total	16/26	61	35/38	92	51/64	80

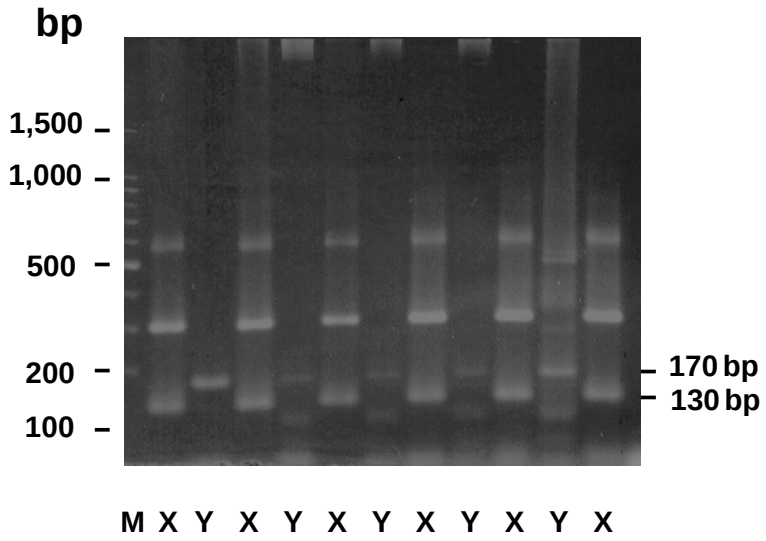

Fig. 2 Ethidium bromide staining of a 2% agarose gel showing the amplified DNA products from sorted cell samples. Lane M, DNA sizes markers, lane 1, control X-chromosome and lane 2, and control Y chromosome. The X and Y labels indicate the size of bands positive for X (130 bp) and Y (170 bp). Lanes 3-4 and 5-6 were from the same blood samples which both indicated as male fetus.

The whole amount of sorted NRBCs from 15 ml blood together with a triple density gradient for cell separation[1,15] may lead to a more efficient purification of FNRBCs. The sensitivity of detection of a male fetus is 69% in the 11-14 weeks of gestational age group which is quite low. We evaluated further only 3 cases with the non-depleted method using whole sorted NRBCs from 15 ml blood and found one to be male of which already confirmed after birth (data not shown).

The presence of maternal NRBCs in sorted cells does interfered with sorting. Direct staining without using a counter stain for the nuclei[18,19] or a concentration step[10,21,22] was simpler and cost effective but resulted in low sensitivity of detection for confirmation in the study. The limitation of techniques in sorting or isolation of the very small number of fetal cell in maternal blood for prenatal diagnosis may not be overcome easily. Nevertheless, its cost effective with no requirement of any special equipment still marks its need for risk population in the poor countries. In the future, detection of fetal DNA in maternal blood [23,24] is a better choice. However, it needs sometime and further development to be suitable and cost effective to be applied in developing countries.

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