Short Communication

Analysis of Paternal Alleles in Nucleated Red Blood Cells Enriched from Maternal Blood

(nucleated red blood cells / CD71 / maternal blood / high-gradient magnetic cell separation / MACS / paternal allele / HLA / polymarker system)

I. HROMADNÍKOVÁ[1], N. BENDUKIDZE[2], M. MRŠTINOVÁ[3], E. IVAŠKOVÁ[2]

[1]2nd Clinic of Paediatrics and [3]Department of Obstetrics and Gynaecology, University Hospital Motol, Prague, Czech Republic

[2]Department of Immunogenetics, Institute for Clinical and Experimental Medicine, Prague, Czech Republic

Abstract. The purpose of our study was to identify paternal alleles in NRBC enriched from maternal peripheral blood for detection of the presence of foetal cells in the maternal circulation and to establish a reliable non-invasive method which should allow following genetic testing. For enrichment of foetal cells from peripheral maternal blood we combined Ficoll-Paque density gradient centrifugation and MACS. Maternal leukocytes were firstly depleted using anti-CD14 and anti-CD45 microbeads. NRBC were sorted from the $CD14^-/CD45^-$ fraction by positive selection using CD71 microbeads. Paternal alleles in the $CD14^-/CD45^-/CD71^+$ fraction were indicated by the PCR method using HLA (DRB1, DQB1, DQA1) and Polymarker System (LDLR, GYPA, HBGG, D7S8, GC) as genetic markers. Different paternal alleles of studied 8 loci were detected in 13 out of 19 samples of cells enriched from maternal peripheral blood between the 13th and 36th week of gestation. Our results demonstrate that foetal cells enriched from maternal peripheral blood may be used as a source of foetal DNA for prenatal diagnosis, paternity testing and other application.

Prenatal genetic diagnosis is now available for women at increased risk for carrying a foetus affected with a chromosomal aneuploidy or other genetically inherited diseases. However, all currently used invasive prenatal diagnosis methods like amniocentesis and chorionic villus sampling carry procedure-related risks to the mother and foetus. Therefore, there is a great effort to develop non-invasive techniques for prenatal diagnosis (Ganshirt et al., 1993, 1995; Holzgreve et al., 1994; Simpson and Elias, 1994; Simpson et al., 1995). The enrichment of foetal cells from maternal blood is an alternative promising source of foetal material for prenatal diagnosis (Herzenberg et al.,1979; Bianchi et al., 1990; Miltenyi et al., 1990). Different types of foetal cells have been found in low frequencies in maternal peripheral blood (Price et al., 1991), such as trophoblasts (Schmorl et al., 1905), nucleated red blood cells (NRBC) (Kleinhauer et al., 1957), lymphocytes (Walknowska et al., 1969) and granulocytes. NRBC are considered now as the main foetal cell candidate for diagnostic analysis. They are short-lived cells detectable early during pregnancy. They express several unique antigens such as the CD71 molecule (transferrin receptor), which makes it possible for them to be enriched from maternal blood (Jansen et al., 1999). Successful isolation of foetal cells from maternal blood depends upon identification of differences between foetal and maternal cell surface antigen expression on NRBC. A monoclonal antibody that binds only foetal cells has not yet been identified. The majority of these cells, even after enrichment, are of maternal origin (Steele et al., 1996; Bianchi, 1998; Hahn et al., 1998).

In this study we focused on the enrichment and detection of foetal erythroblasts circulating in maternal blood. The high-gradient magnetic cell separation (MACS) procedure was used for NRBC enrichment from peripheral blood mononuclear cells (Miltenyi et al., 1990). We combined depletion of maternal cells using anti-CD14 and anti-CD45 microbeads and positive selection using anti-CD71 microbeads for effective isolation of foetal cells (Busch et al., 1994). Paternal alleles in the sorted cells were identified by polymerase chain reaction (PCR) typing of HLA class II and Polymarker System.

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Corresponding author: Ilona Hromadníková, 2nd Clinic of Paediatrics, University Hospital Motol, V Úvalu 84, 150 06 Praha 5, Czech Republic. Tel.: +420 (2) 24432258; Fax: +420 (2) 24432220; e-mail: ilona.hromadnikova@lfmotol.cuni.cz.

Abbreviations: HLA – human leukocyte antigen, MACS – magnetic activated cell sorting, PCR – polymerase chain reaction, PEP – primer-extension preamplification.

Material and Methods

Enrichment of NRBC

NRBC were enriched from 20 ml of peripheral blood (EDTA) between the 13th and 36th week of gestation. The mononuclear cells were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation (400 *g* for 40 min at 20°C). To deplete maternal leukocytes, peripheral blood mononuclear cells (1×10^7) were simultaneously incubated with anti-CD14 (20 l) and anti-CD45 (20 l) microbeads in PBS containing 0.5% bovine serum albumin (BSA), 2 mM EDTA and 0.01% sodium azide (NAN_3) at 6°C for 15 min. The labelled cells were washed in PBS and applied to the MACS-BS column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The CD14/CD45 negative fraction was stained with anti-CD71 microbeads (20 µl) in 80 µl PBS/BSA/NAN_3 at 6°C for 15 min. The cells were then washed and sorted using MiniMACS. The number of viable cells was evaluated at every enrichment step by a Neubauer counter chamber. Dead cells were excluded by trypan blue staining.

Extraction of DNA and PCR amplification

Genomic DNA from adult blood (19 mothers and 19 fathers) was extracted by standard protein salting-out procedure. QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany) was used for DNA purification from 19 NRBC samples. DNA concentration was measured at 260 nm using a spectrophotometer DU 640B (Beckman Inc., Fullerton, CA). Generic human leukocyte antigen (HLA) class II – loci DRB1 and DQB1 – genotyping was performed by PCR at following reverse hybridization with sequence-specific oligonucleotide probes (SSOP) using Automated Inno-LiPA system (Innogenetics Inc., Gent, Belgium). Results were interpreted by the LiPA Expert software routinely used for HLA typing analysis in immunogenetic laboratories. The AmpliType PM+DQA1 PCR Amplification and Typing Kit (Perkin Elmer, Wellesley, MA) was used for simultaneous amplification of Polymarker System (PM) including six genetic loci: HLA DQA1, low-density lipoprotein receptor (LDLR), glycophorin A (GYPA), haemoglobin G gammaglobin (HBGG), microsatellite D7S8 and group-specific component – vitamin D-binding protein (GC). This kit also uses reverse dot-blot typing technologies. In 7 cases with very low concentration of DNA, primer-extension preamplification (PEP) was done before specific amplification. We used Operon RAPD 10-mer Kit A, containing randomly selected (non-specific) 10-base oligonucleotide primers. For DNA preamplification we used conditions recommended by Williams et al. (1990). Amplification products were analysed by electrophoresis.

Results and Discussion

NRBC enriched from maternal blood have recently been considered as the main foetal cell candidate for prenatal diagnostic analysis. But even after the enrichment, the majority of enriched NRBC are of maternal origin (Steele et al., 1996; Bianchi, 1998; Hahn et al., 1998). Successful detection of foetal cells depends upon the identification of differences between foetal and maternal cells as well as upon using sensitive molecular genetic methods that allow detection of low frequencies of foetal alleles (Price et al., 1991). In this study we investigated the possibilities of the MACS method to enrich nucleated red blood cells from maternal peripheral blood using a monoclonal antibody against the transferrin receptor (CD71) conjugated with superparamagmetic microbeads (Miltenyi et al., 1990; Ganshirt-Ahlert et al., 1992). For more effective isolation of foetal cells we firstly depleted CD45$^+$ and CD14$^+$ cells from maternal peripheral blood mononuclear cell fraction isolated via Ficoll-Paque density gradient centrifugation (Busch et al., 1994).

Subsequently, paternally inherited alleles were analysed using PCR amplification and typing AmpliType PM+DQA1 as well as HLA-DRB1 and HLA-DQB1 LIPA commercial kits. The AmpliType PM+DQA1 kit simultaneously co-amplifies 6 distinct markers of low polymorphic loci including HLA DQA1, LDLR, glycophorin A (GYPA), haemoglobin G gammaglobin (HBGG), D7S8 and group-specific component (GC).

In this study different paternal alleles of the above-mentioned 8 loci in the CD14$^-$/CD45$^-$/CD71$^+$ fraction were indicated in 13 cases out of 19 studied (Table 1).

One representative sample is presented in Fig. 1 and Fig. 2, which demonstrate that paternal alleles could be detected in the NRBC fraction enriched from maternal circulation.

Results of the enriched NRBC typing showed two mother's alleles and one father's allele. The foetus inherited paternal LDLR B allele, GYPA B allele, HBGG A allele, D7S8 B allele and DQA1*0501 (4.1) allele and did not inherit the paternal DQA1*0602/0401

Table 1. PCR detection of PM and HLA in nucleated red blood cells enriched from maternal peripheral blood

Name, locus	Chromosome localization	Number of alleles	Number of detections
LDLR	19p13.3	2	4
GYPA	4q28 – q31	2	3
HBGG	11p15.5	3	1
D7S8	7q22 – q31.1	2	3
GC	4q11 – q13	3	1
HLA DQA1	6p21.3	7	4
HLA DRB1	6p21.3	183	5
HLA DQB1	6p21.3	30	1