Journal of Reproduction Engineering
[JRE] Vol., 3, No.1, 139~145, 2000

The use of live nucleated red blood cell from the pregnant mother's peripheral blood for the prenatal diagnosis

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Abstract

Nucleated red blood cells (NRBC) from pregnant mother's peripheral blood were enriched by percoll gradient and anti-CD71 magnetic cell sorting (MACS). The NRBC could be identified by Nomarskii microscope without staining the cell. The live single NRBC was picked up and its DNA were analyzed by PCR and restriction enzyme digestion. Among 15 NRBCs from the peripheral blood of male-fetus-carrying pregnant mother, 5 NRBCs were successfully analyzed and one NRBC was found male. This proves the possibility to identify live NRBC, and their use for the prenatal diagnosis. This also shows that only 20% of the total NRBC found in the peripheral blood of pregnant mother are fetus origin.

Keywords: Prenatal diagnosis, Nucleated red blood cell, Live NRBC.

Introduction

It has been expected for about a decade that the fetal cells in the maternal peripheral blood can be selected and used for the prenatal diagnosis by analyzing the DNA in the cell. Different approaches has been taken for this goal. Our approach is the following. First, single nucleated red blood cell(NRBC) is selected from maternal peripheral blood and its DNA is analyzed by PCR. This approach has been initiated by Takabayashi et al (1) and followed by Sekizawa et al (2,3,4,5) and others(6,7). The method has a difficulty in performing PCR on stained NRBC, at

The patient's blood used in this report was obtained with an informed consent and with an approval of I.R.B. of Juntendo Urayasu Hospital.

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Jun. 2, 2000(Received), Aug. 20(Accepted).

least, in our hands. Therefore, we have tried to identify NRBC among non-stained cells and performed PCR on these live cells. We have succeeded in identifying live NRBC under Nomarski microscope and obtained PCR products from a single live cell with a good efficiency for restriction enzyme analysis.

Materials and Methods

Enrichment for the nucleated red blood cells

5ml to 10ml of pregnant women's peripheral blood was diluted two-fold with 0.9% (1.5mM) NaCl solution and gently layered at the top of a step gradient of 3ml each of percoll layer at the density of 1.090 and 1.065 in 15ml Falcon plastic tube according to Takabayashi et al (1). After spinning ar about 800g (3,000rpm) for 30min. at room temperature, the slightly colored portion between two layers of density 1.090 and 1.065 was withdrawn by a pasteur pipette. The solution was filtered through 5ml syringe clogged with cotton which was washed with 0.9% NaCl first. The filtrate was centrifuged ar 1,200 rpm for 10min after 10ml of 0.9% NaCl solution was added. The precipitate was mixed with 2ml of cold phosphate buffered saline (PBS), tapped and centrifuged again. 80~160ul of cold PBS was added to the precipitate. It was suspended gently and kept on ice. To this cold sample tube 20~40ul of anti CD71 microbeads (Miltenyi Biotec GmbH, Germany) was added and incubated at 12C for 15min. The sample was filtered and the cells which stuck to the anti CD71 antibody were separated by MS column according to the manufacture's specification. The final sample was kept at 4C.

Identification of the nucleated red blood cells under Nomarski microscope

The enriched NRBC solution was appropriately diluted and 10ul~20ul was placed In a 35mm diameter polystyrene dish (Iwaki Glass, Japan), overlayered with mineral oil (Squibb, NJ, USA) and the NRBC was identified under inverted Nomarskii microscope (Olympus,Japan) as a cell with a nucleus-like material and somewhat red colored cytoplasm (figure1,2,3,and 4). Identification of NRBC was originally done by picking up each live cell, which was stained in isolation by May-Giemsa method and the final stained cell was identified as NRBC or lymphocyte or other nucleated cell morphologically.

PCR analysis on the isolated single nucleated red blood cell

The NRBC was picked up by micro-manipulator and denatured by alkali (2.5ul of 0.2M NaOH for 10min at room temperature) and then neutrarized with tris-HCl (2.5ul of 0.9M tris-HCl/0.2M HCl). This sample was treated as 5ul in volume and used for the whole genome amplification by PEP (primer-extension preamplification) (8,9). Then the nested PCR for ZFX and ZFY gene was done according to Chong et al (10) as the 1st and 2nd step PCR and finally the 3rd step

PCR was done by the following primer pair .CAGCAAGGCAGAG AAGGCCATTGAA-3' and ACTCCACACAAATATGAGGAAAGTT. The final PCR products were analyzed by restriction digestion enzyme HaeIII (10).

Result

(1) Identification of NRBC among live cells under Nomarski microscope

At the beginning each morphologically different, nucleated cell was picked up and stained in isolation on a slide glass and from the stained cell morphology the type of the original live cell was assumed. The live NRBC was a cell similar to the lymphocyte with a bumpy nucleus but somewhat red colored in the cytoplasm which is more confined in the round area, like a red blood cell(RBC). These examples are shown in the figures 1~4. The NRBC from the cord blood(Figure 1) can be more easily identified than the one from the peripheral blood of a pregnant mother (Figure 2~4). Some of the NRBC thus far identified was prooven to be the fetal origin (Result section 2).

Figure1. NRBC from cord blood

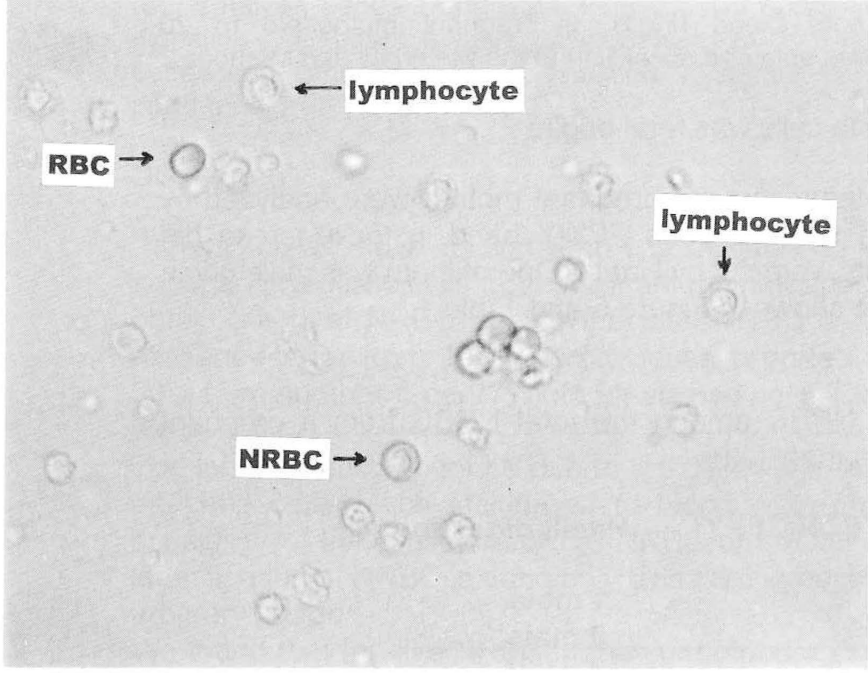


Figure2. NRBC from peripheral blood

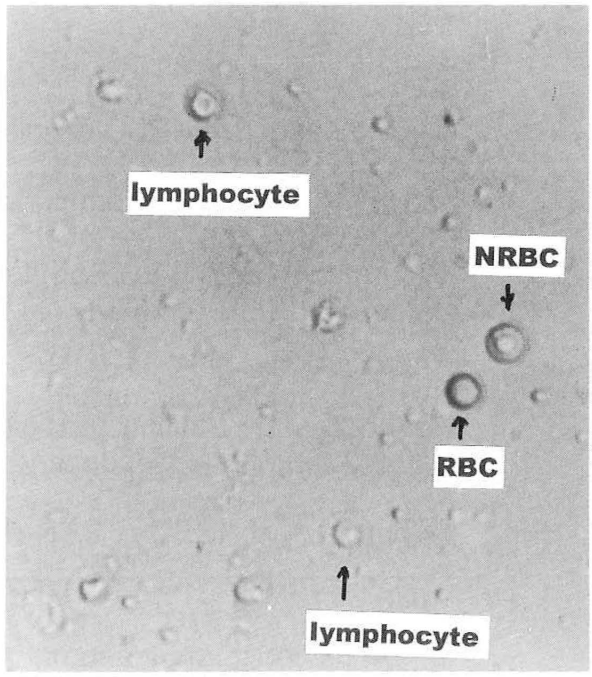


Figure3. NRBC from peripheral blood

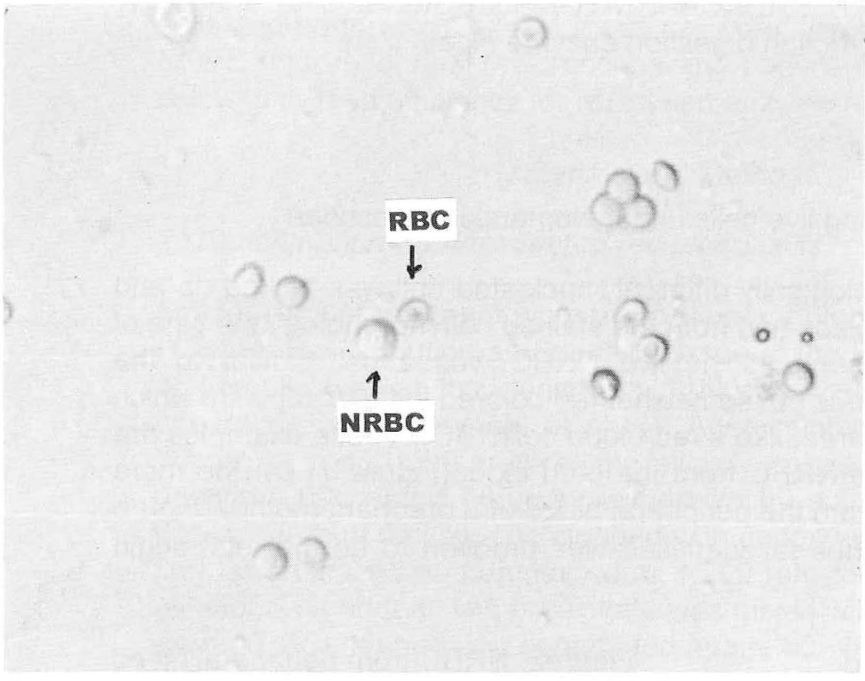
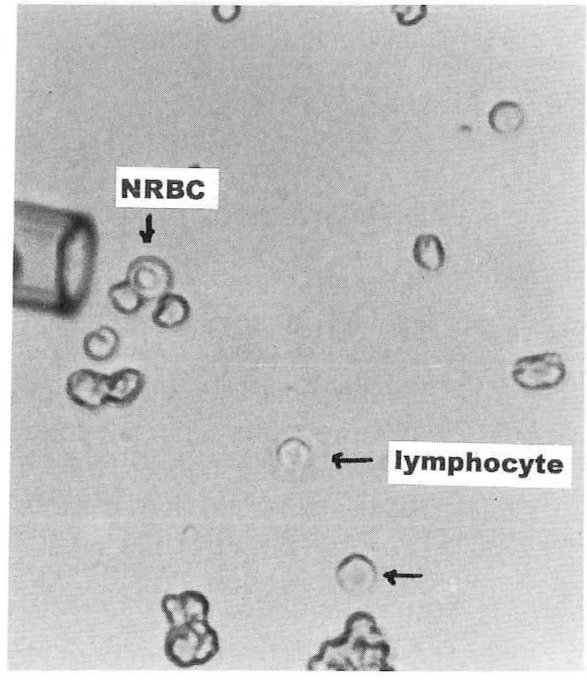


Figure4. NRBC from peripheral blood



The blood samples were from cord blood (Fig1) or pregnant mother's(8 to 20 weeks)peripheral blood and NRBC was enriched according to the Materials and Methods.

(2) One out of five NRBC candidate cells was fetal origin.

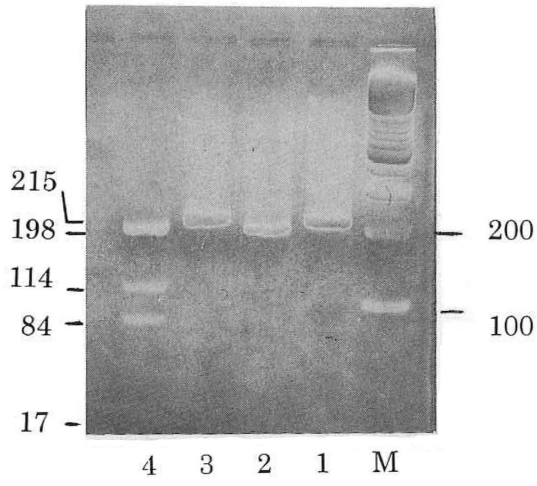
15 NRBC candidates from male-fetus-carrying pregnant mother were analyzed by PEP+PCR(ZFX/Y). Five of them showed good ZFX/Y band in the agarose gel electrophoresis. The PCR products were cut by HaeIII. One of them was male origin and the rest were female origin as shown in Figure 5 and Table 1.

Table 1. The fraction of male NRBC among the total NRBC from a pregnant mother's peripheral blood carrying male fetus

patient	# of NRBC	PEP → PCR(ZFXY)	HaeIII digestion
#46	8	2	1 male
#49	7	3	0 male
Total	15	5	1 male

From two pregnant mother carrying male fetus, total 15 NRBC were isolated and amplified by PEP and nested PCR (up to the 3rd step for ZFXY gene) as described in the Materials and Method and the final product was analyzed by HaeIII restriction enzyme.

Figure5. HaeIII digestion of PCR^{123} (ZFXY product)



Two different single live NRBC was picked up from patient #46 (lanes 1 and 2 are sample NRBC No.1 and lanes 3 and 4 are sample NRBC No.2) and ZFXY gene was amplified by PEP→PCR^{123} followed by HaeIII restriction enzyme digestion. Lane M is molecular size (bp) marker. Lanes 1 and 3 are PCR product before digestion. Lanes 2 and 4 are after digestion. 3% agarose gel(Nusieve:Sekem=2:1 by weight) electrophoresis in 0.5xTBE buffer and ethidium bromide staining (0.5ug/ml in the electrophoresis buffer) was performed.

Discussion

Thus far we have shown that it is possible to identify the live NRBC among enriched live blood cells from pregnant mother's peripheral blood. The frequency to obtain the fetal NRBC among the total NRBC is about one in five or 20%. This efficiency is far from 100% as previously reported (2,3,4,5) or much smaller than 50% from another report(7) both for stained cells. This point should be more closely examined.

The low efficiency might be due to the difficulty in identifying the live NRBC among enriched cells which include white blood cells and red blood cells, and to the possibility of the presence of the NRBC of mother's origin. In this experiment the identification of NRBC is done only by naked eyes, which might very well present an arbitrary factor.

We found that the live NRBC in the cord blood and in the peripheral blood showed somewhat different color (figure 1).

The technique to identify the live NRBC under Nomarski microscope, to pick up by micro-manipulator and analyze its DNA is more laborious and difficult than to deal with stained NRBC.

Therefore it is desirable to develop a stable technique to handle stained NRBC. Previous reports showed that the efficiency to obtain PCR results from the stained NRBC was 50~100%(2,3,4,5) which was very high compared to our experience. We have established the efficiency to obtain PCR results from live lymphocytes as almost 100% for a few genes (in preparation) and from stained lymphocytes as 25~60% (in preparation). We assume the efficiency from live or stained NRBC might be much less.

Acknowledgment

We thank Dr.Katsuhiko Hayashi for his initial help in the micromanipulation and Mr.Hiromichi Kondo for his technical help in the PCR.

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