EDITORIAL

Fetal cells in maternal blood: current and future perspectives

Introduction
The goal to replace invasive prenatal diagnosis methods, with their inherent risks to the mother and fetus, by a non-invasive alternative has been around for a long time. One manner in which this aim could be accomplished is by the enrichment for fetal cells in the blood of pregnant women. Recently, a new-found optimism is to be felt among the researchers in this field, chiefly due to advances in genetic methods such as fluorescent in-situ hybridization (FISH) and especially single cell analysis using a combination of polymerase chain reaction (PCR) and micromanipulation. These practices are well established in reproductive medicine.

This brief overview of past and recent developments is intended to clarify the issues concerning current research and attempts to determine which innovations are most likely to influence future prospects.

Historically three different fetal cells have been the focus of research attention: trophoblasts, fetal leukocytes and fetal erythrocytes, and one of the issues which first had to be resolved was which of these fetal cells was the most promising candidate for further examination.

Trophoblasts
Despite the fact that trophoblasts were the first fetal cells found to traverse the placental barrier into the maternal circulation (Schmorl, 1893), research on this cell type has been hampered in several ways:

1. Trophoblast deportation does not appear to be a phenomenon common to all pregnancies (Sargent et al., 1994a).
2. Their large multinucleated nature means that these cells are invariably trapped in the lungs and rapidly cleared from the maternal circulation (Attwood and Park, 1960).
3. Furthermore, their heterogeneous genetic nature, arising on the one hand from their multinucleate status, but also due to the inherent mosaicism existing in the placenta (Henderson et al., 1996; Goldberg and Wohlferd, 1997), severely restricts the use of FISH as a means for genetic diagnosis.
4. The enrichment for these cells has often been hindered by the lack of specific antibodies (Covone et al., 1984, 1988; Bertero et al., 1988), a deficit which may be overcome by the use of the new markers HASH-2 (van Vught, 1997) and human placental lactogen hormone HPGL (Latham et al., 1996).

Nonetheless, some diagnostic success has been achieved by the use of this cell type, such as the correct determination of haemoglobinopathies by Hawes et al. (1994).

Leukocytes
In their 1969 landmark publication, Walknoska et al. (1969) used mitogen-stimulated lymphocytes to demonstrate conclusively the presence of fetal cells in the blood of pregnant women for the first time. This was done by showing that a Y chromosome could be detected in some of the lymphocytes isolated from pregnant women bearing male fetuses. These results were extensively corroborated either in an analogous manner or by investigating quinacrine-stained interphase nuclei for the presence of fluorescent Y chromosome signals (Schindler et al., 1971; Schröder and de la Chapelle, 1972). Two sets of observations during this period should have given some cause for caution: the persistently high number of female-bearing pregnancies which were found to contain Y chromosome positive cells (Schindler et al., 1971; Schröder and de la Chapelle, 1972; Grosset et al., 1974; Sargent et al., 1994b), and the possible longevity of fetal leukocytes in the maternal circulation (Schröder et al., 1974; Ciaranfi et al., 1977).

Fetal leukocytes were also the first cells to be successfully enriched from maternal blood by the use of fluorescent activated cell sorting (FACS) by the group of Herzenberg et al., (1979). A major drawback of this approach, which made use of differences in human leukocyte antigen (HLA) expression, is that it required the HLA type of the father, thereby raising the issue of paternity. Furthermore, these results were not always reproduced with the same degree of success, even when cells were sorted on the basis of several HLA differences and using monoclonal antibodies (Tharapel et al., 1993).

Despite these deficiencies, the precedent had been set: fetal cells did migrate into the peripheral blood of expectant women in normal pregnancies, from which they could be enriched for subsequent diagnostic purposes.

The question of fetal cell persistence
As male cells were continually detected with abnormally high frequency in pregnancies bearing female offspring (Schindler et al., 1971; Schröder and de la Chapelle, 1972; Grosset et al., 1974; Sargent et al., 1994b), the question was raised of how long fetal cells could persist in the maternal circulation. Early studies by Schröder et al. (1974), who examined mitogen stimulated leukocytes for the presence of Y chromatin, in women who had previously borne a son, determined that fetal leukocytes were detectable for a period of up to 1 year after birth. This was confirmed by Ciaranfi et al. (1977), who in some instances was still able to detect such cells 5 years postpartum. By performing Y chromosome specific PCR on peripheral blood mononuclear cells obtained from women who had previously given birth to a male, some groups (Hsieh
et al., 1993; Liu et al., 1994) were able to detect the presence of male fetal cells for periods of up to 8 months after birth. Under a similar premise, but by FACS enriching for T- and B-lymphocytes and haematopoietic progenitors, Bianchi et al. (1996a) were recently able to determine that fetal progenitor cells (CD34+38+) were still present 27 years postpartum. T-lymphocytes (CD3+4+5+) were found to persist, in one instance, for a period of 6 years after birth. These results imply that a short-lived cell, of limited replicating capacity, has to be chosen when considering diagnostic applications. A cell that fulfills several of these criteria is the fetal erythroblast, frequently termed nucleated red blood cells (NRBC).

Fetal erythrocytes

The development of a staining procedure specific for fetal haemoglobin by Kleihauer et al. (1957) originally demonstrated the presence of immature erythrocytes in pregnant women. These were more frequent under certain instances such as rhesus incompatibility, an observation confirmed by Clayton et al. (1964), who, however, also noted an increase in these cells following terminations, or amniocentesis. A drawback of these studies was the lack of independent confirmation that the immature erythrocytes detected were indeed of fetal origin. Since erythroblasts are nucleated, proof of fetal origin was later obtained by examining them for the presence of Y chromatin (Holzgreve et al., 1992). By flow sorting maternal blood cells on the basis of CD71 (transferrin receptor) expression, a molecule shown to be highly expressed on erythroblasts, Bianchi et al. (1990) were the first to enrich for nucleated erythrocytes containing fetal DNA. Using the same antigen, but by exploiting the then recent development of magnetic cell sorting (MACS) (Miltenyi et al., 1993), our group was similarly able to enrich for fetal NRBC (Gänshirt-Ahlert et al., 1992). The relative ease of these enrichment protocols and the first correct determination of fetal aneuploidy by Price et al. (1991) and subsequently by Bianchi et al. (1992) and our group (Gänshirt-Ahlert et al., 1993) have considerably enhanced the popularity of NRBC as the main fetal cell candidate for diagnostic analysis. The fact that they are among the first haemopoietic cells formed during fetal development and that they are detectable early during pregnancy (Gänshirt et al., 1994a) are further criteria in favour of their use.

The use of NRBC is, however, not without pitfalls, for it is probable that the majority of these cells, even after enrichment for fetal cells, are of maternal origin (Gänshirt et al., 1994a; Hamada et al., 1995; Slunga Tallberg et al., 1995, 1996; Holzgreve et al., 1998). This means that extreme caution has to be exercised to ensure that the cells used as the basis of a diagnosis are indeed fetal, and therefore considerable issues remain to be resolved regarding the optimizing of enrichment, fetal cell recognition and diagnostic efficiency.

Current and new modes of enrichment

Since fetal cells in maternal blood are rare, being present in the order of 1 in 10^5 to 10^7 or less early in gestation (<10 weeks) (Gänshirt-Ahlert et al., 1990; Hamada et al., 1993, 1995; Bianchi et al., 1997a; Sohda et al., 1997) when diagnosis is most desirable, their enrichment clearly presents a considerable challenge. Currently, two methods seem to predominate, FACS (Bianchi et al., 1990, 1993, 1996; Simpson et al., 1995; Zheng et al., 1995; Lewis et al., 1996) and MACS (Gänshirt-Ahlert et al., 1992, 1993, 1994b; Campagnoli et al., 1997; Valerio et al., 1997). The argument in favour of FACS is that purer populations of a defined phenotype can be achieved. In this manner, fetal erythrocytes are not only selected on the crude basis of CD71 expression, but can also be selected simultaneously for the presence of a nucleus and fetal haemoglobin (Bianchi et al., 1993; Zheng et al., 1995) or other erythrocytic markers such as glycophorin A (Bigbee and Grant, 1994; Simpson et al., 1995; Lewis et al., 1996). Purity can further be enhanced by the depletion of maternal cells and fetal lymphocytes with anti-CD45 (Simpson et al., 1995; Bianchi et al., 1996b; Lewis et al., 1996). In this manner a relatively small pool of cells is attained, containing a high percentage of fetal NRBC.

The advantages of MACS, especially the MiniMacs (Miltenyi, Bergisch Gladbach, Germany) system, from our experience lie in its ease, speed of operation and relatively low cost. The disadvantage is that selection is based only on one criterion. This can be circumvented to some extent, either by the selective enrichment for NRBC by step Ficoll gradients (Gänshirt-Ahlert et al., 1993), or by CD45-mediated depletion of maternal cells (Zheng et al., 1993; Andrews et al., 1995). Nevertheless, a great number of maternal cells are co-purified by this procedure, making it difficult to identify the few enriched fetal cells. To increase the efficiency of this system, the rapid automated detection of these few fetal cells is a prerequisite. By both methods (FACS and MACS), generally fewer than 20 fetal cells are obtained from a 20 ml maternal blood sample (Bianchi et al., 1996b). Other methods which have been used for the enrichment of fetal cells include avidin–biotin columns (Hall et al., 1994), magnetic ferro-fluids (Steele et al., 1996), new density gradients (Sitar et al., 1997) as well as the possibility of increasing the yield of fetal erythroblasts by cell culturing (Lo et al., 1994; Valerio et al., 1996).

Discontinuous gradients, originally pioneered by the group of Takabayashi (1995), have also recently been used successfully. In this method, NRBC are enriched by separating a 2 ml maternal venous blood sample over a discontinuous Percoll gradient, after which the NRBC are identified morphologically in Pappenheim- or Giemsa-stained slide preparations. An average of 4.1 NRBC (range 1–22) were found per sample analysed, from which the fetal sex could be accurately determined in 10 out of 11 samples. NRBC were found as early as during the eighth week of gestation. The enrichment of very high numbers of NRBC has also been achieved by charge flow separation (CFS) (Wachtel et al., 1996), where several thousand (average = 6910) NRBC were electrophoretically enriched from a 20 ml maternal venous blood sample, from which both fetal sex and ploidy could be accurately determined. These results, however should be viewed with some caution, as several other studies have indicated that the minority of NRBC are of fetal origin (Gänshirt et al., 1994a; Slunga Tallberg et al., 1995, 1996; Holzgreve et al., in press). Thus,
it is not only sufficient to have enriched for morphologically identified NRBC, one also needs to be sure that the cells examined are fetal. This can be achieved, to some extent, by the use of specific markers specific for fetal cells, such as fetal haemoglobin.

**New antibodies and markers**

To enhance the enrichment of fetal NRBC several workers have sought for more specific antibodies or biochemical markers. Biomarkers include 2,3-biphosphoglycerate (BPG) (von Koskul and Gahmberg, 1995) and thymidine kinase (TK) (Hengstschlager and Bernaschek, 1997), both of which rely on chemical assays for analysis. BPG is proposed to identify fetal haemoglobin by exposing the fetal haem iron to oxidation in a subsequent peroxidase reaction, thereby forming a coloured substrate associated complex. By the use of fluorescent thymidine analogues, the high levels of TK enzyme levels expressed by fetal cells can be exploited to separate them from adult cells by FACS, since such enzyme activity is virtually absent in adult cells. The specificity of these methods remains to be established, before they are likely to find wide appeal.

Several new candidates have been described in the hunt for an optimal fetal cell antibody, including the anti-erythroblast HAE9 (Savion et al., 1997) and those developed by Genzyme Corporation (FB3-2, 2-6B/6 and H3-3) (Zheng et al., 1997). While the former has specifically been shown to stain samples from pregnant women and has not yet been tested for the actual enrichment for fetal erythroblasts, promising results have been obtained with FB3-2 by virtue of its high expression on fetal NRBC and its negligible presence on their maternal counterparts. The efficacy of these reagents has not yet been assessed by MACS. Possible use of the erythropoietin receptor has also been explored (Valerio et al., 1997), as this should be highly expressed on erythroblasts. Currently, however, most researchers still either use anti-CD71 or anti-gamma globin antibodies for the enrichment of NRBC.

**Automated scanning**

The general inefficiency of fetal erythroblast enrichment necessitates an automated detection system to alleviate technician fatigue incurred in the scoring and analytical process. A prerequisite for such a process is the reliable identification of fetal NRBC. An approach pioneered by Zheng et al. (1993) and Ferguson Smith et al. (1994), which we have also used, is to stain those NRBC which have not yet been tested for the actual enrichment for fetal erythroblasts, promising results have been obtained with FB3-2 by virtue of its high expression on fetal NRBC and its negligible presence on their maternal counterparts. The efficacy of these reagents has not yet been assessed by MACS. Possible use of the erythropoietin receptor has also been explored (Valerio et al., 1997), as this should be highly expressed on erythroblasts. Currently, however, most researchers still either use anti-CD71 or anti-gamma globin antibodies for the enrichment of NRBC.

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for the detection of the common aneuploidies, X, Y, 13, 18 and 21.

**The NICHD NIFTY trial and the need for multicolour FISH**

Currently two trials are being conducted to test the diagnostic feasibility of using fetal cells enriched from maternal blood for the detection of aneuploidies: the National Institute of Child Health and Human Development Fetal Cell Isolation Study, the so-called NIFTY trial (Holzgreve et al., 1994; De la Cruz et al., 1995) and a second trial sponsored by Applied Imaging, which makes exclusive use of their proprietary imaging analysis equipment (Golbus, 1997). After having sponsored an initial study (1987–1994) (Simpson et al., 1993, 1995), the NIHCD initiated a phase II clinical investigation (1994–1997) to assess the diagnostic efficacy of maternal blood-derived fetal cells in detecting fetal aneuploidies. For this study, a cohort of at least 3000 women considered to be at risk for bearing an aneuploid fetus (>35 years, indicative serum screening or sonographic results) and who are about to undergo an invasive diagnostic procedure are being recruited. The experimental results obtained from the non-invasive procedure regarding fetal sex and ploidy will then be compared to the full karyotype obtained from the invasive procedure, the traditional gold standard. In all instances, informed consent is required with the understanding that no experimental results will be made available to the participants. An additional aspect of this trial is a psycho-social study investigating issues such as social acceptance and possible coercion of pregnant women into undergoing a prenatal diagnostic examination. While the results of this trial are still to be evaluated, the experience gathered so far indicates the need for a multicolour FISH methodology that would routinely allow for the simultaneous screening of the common aneuploidies (X, Y, 13, 18 and 21). The recent release of such reagents by Vysis is a step in the right direction.

**The use of polymerase chain reaction**

The advent of the PCR initially proved to be a valuable tool in providing further evidence in support of the existence of fetal cells in maternal blood, which had been a matter of considerable dispute (Holzgreve et al., 1992). This was first achieved when Lo et al. (1989, 1993a) were able directly to determine the sex of the fetus by performing a Y chromosome specific PCR analysis on a non-enriched blood sample taken from pregnant women, thereby proving the existence of fetal genetic material (nucleated cells) in the maternal circulation.

Soon thereafter it was shown that such an allele specific amplification approach, i.e. where the gene of interest is present in the fetal genome but absent in the maternal one, can also be used diagnostically, and researchers were able to examine for the presence of a paternally inherited haemoglobinopathy (Camaschella et al., 1990) and determine the fetal RhD status (Lo et al., 1993b).

Another approach had to be taken, however, when attempting to examine the fetal genotype for genes carried by both parents, for example single gene disorders such as sickle cell anaemia, thalassaemia, cystic fibrosis and Duchenne’s muscular dystrophy. Researchers sought to overcome this obstacle by isolating single fetal cells by micromanipulation under a microscope and then performing PCR, a practice similar to the examination of preimplantation embryos (Handyside and Delhanty, 1997).

The first prenatal diagnosis of a single gene disorder, in this instance Duchenne’s muscular dystrophy, by this means from singly manipulated NRBC that had been identified solely on the basis of morphology, was achieved by Sekizawa et al. (1996a). By analogous means they were subsequently able to determine fetal RhD status (Sekizawa et al., 1996b).

This report was soon followed by that from the laboratory of Y.W.Kan (Cheung et al., 1997), which focused on haemoglobinopathies, using multiple singly manipulated fetal NRBC identified by anti-fetal haemoglobin staining. Several such cells were pooled in this examination to circumvent the problem of allele drop-out, a phenomenon which frequently occurs when using single or low template copies for PCR. This, however, raises the problem of maternal contamination and consequent false diagnosis (Holzgreve et al., 1990). Apart from the questionable practice of using morphologically determined NRBC, where the majority are of maternal origin, the group of Sekizawa set the standard, in that several PCR examinations were performed on the genetic material obtained from a single cell — a feature made possible by whole genome random primer extension preamplification (PEP) (Zhang et al., 1992). In this manner, they were able not only to examine the gene of interest, but also to determine whether it was of fetal origin, using the inherent differences in the ZFX/ZFY loci. A more elegant manner to achieve this aim would be the use of microsatellite or short tandem repeat (STR) markers, as have been used by the group of Clausssen (von Eggeling et al., 1997) and which have recently been shown to be effective in the forensic analysis of single cell material (Findlay et al., 1997).

A recent interesting and novel observation was that made by Lo et al. (1997), where fetal DNA was found to exist freely in maternal serum or plasma, and could be used for correct fetal sexing. Provided it is reproducible and sufficiently sensitive, this method will be particularly attractive for the rapid and simple assessment of fetal RhD status. This novel discovery does, however, bear a considerable ethical burden, because it makes it fetal sex determinable from the waste products of routine diagnostics. This development is of concern when regarding countries in which female fetuses are already subject to discriminatory practices.

**Biological aspects**

Although numerous physical and financial resources have been spent on exploiting the diagnostic potential of fetal cells in maternal blood, comparatively little attention has until recently been focused on why human fetal cells should undertake this voyage into the maternal circulation, when in the mouse it appears to be a very rare event (Bonney and Matzinger, 1997). This is especially salient in view of the reported long persistence of fetal progenitors and their possible involvement in autoimmune diseases, such as scleroderma as orally reported by the groups of B.Smith (Philadelphia) and D.Bianchi (Boston).
(cited by Goldberg, 1997) and very recently reported by Nelson et al. (1998). The biological variability of pregnancy is also worthy of closer investigation. This applies both to the reports that fetal cells appear to be more frequent in aneuploid pregnancies (Bianchi et al., 1997), an observation we have also previously made (Günschert-Ahlert, 1994b) that these fetal cells express greater densities of markers such as CD71 (Thiligathan et al., 1995) or FB3-2 and H3-3 (Zheng et al., 1997). This implies that aneuploid fetal cells are easier to enrich for, thereby loading the system in the favour of their detection, which is certainly good news for future diagnostic evaluations.

A feature we have recently addressed is that of altered fetal–maternal trafficking in pre-eclampsia, suggested by the historic observations of Schmorl (1893) and Clayton et al. (1964). To date no direct proof has existed that the cells observed were indeed of fetal origin. By the exclusive use of male pregnancies, we determined that significantly more fetal cells traversed the placental barrier in pre-eclamptic patients (9/1000) than in the control group (2/1000). We furthermore observed a large increase in the number of NRBC (38 versus seven) in these patients, most of which were of maternal origin (Holzgreve et al., 1998).

It is unlikely that the perturbation in feto-maternal traffic we have observed is causal to pre-eclampsia, but rather that it is a consequence of the placental alterations peculiar to this disorder, whereby the failure of the syncytiotrophoblast to invade the maternal spiral arterioles leads to conditions of hypoxia (Redman, 1997). This could explain the rise in NRBC, as an increase in their production would be an attempt to alleviate such a stress. The fact that we observed the existence of a strikingly linear correlation between the fetal and maternal NRBC populations implies that the same factor may be responsible for production of both pools.

**Future prospects**

Although great strides have been made in the enrichment and identification of fetal cells from maternal blood, only by ensuring the rapid application of both molecular genetic and technological advances, such as new modes of enrichment, multicolour FISH or multiplex PCR, automatic detection and image analysis, will the full potential of this risk-free method of prenatal diagnosis be harvested.

Looking into the future the question can be raised based on the results described here whether this non-invasive method will ever become available as a clinical routine diagnostic test and whether it could have the potential to fully replace invasive methods. From our point of view this is unlikely, because another advance in prenatal medicine such as chorionic villous sampling with its obvious advantage of an earlier diagnosis has not replaced amniocentesis due to inherent disadvantages of the earlier gestational age (higher spontaneous abortion rate independent from the procedure-related risk). Because fetal cells isolated from the blood of pregnant women currently cannot be cultivated efficiently, only interphase analysis by FISH and PCR is possible which, according to our assessment, would only cover ~70% of all the chromosomal abnormalities detected in our prenatal diagnosis programme. So the woman in counselling would get the option of either having the most frequent fetal aneuploidies detected non-invasively or the full cytogenetic evaluation from metaphase analysis after a conventional invasive procedure (Holzgreve, 1997).

It is interesting to observe similarities of this field with the development of preimplantation genetics where the combination of molecular genetic techniques such as FISH and PCR with micromanipulation and steady improvements in the various steps and skills involved have also finally made this long sought-after method of prenatal diagnosis possible.

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**References**


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