Fetal cells in cervical mucus and maternal blood

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Research in developing effective and accurate methods for non-invasive prenatal diagnosis has focused on two main techniques: the retrieval of trophoblast cells from the cervix and the enrichment of fetal erythroblasts from the blood of pregnant women. The isolation of fetal cells by both approaches has permitted the identification of fetal aneuploidies by the use of fluorescence in-situ hybridization (FISH) with appropriate probes, as well as fetal single gene disorders by polymerase chain reaction (PCR). In the latter instance, it has been shown that in order to attain the high degree of specificity required for prenatal diagnosis, it is necessary to analyse single fetal cells isolated by micromanipulation. This practice has permitted the successful characterization of fetal rhesus status, haemoglobinopathies, Duchenne’s muscular dystrophy and spinal muscular atrophy, amongst others.

Further developments include investigations into whether the diagnostic potential of fetal cells retrieved by either method can be expanded by the possible culturing of such cells, as well as the possibility of performing successive rounds of FISH and PCR by the recycling of isolated fetal cells.

A novel observation that our group has made is that the traffic of fetal cells is enhanced in pregnancies affected by the pregnancy related disorder, pre-eclampsia. Our subsequent investigations have shown that this elevation in fetal cell traffic may serve as an early marker for those pregnancies at risk for this disorder.

A very recent exciting discovery has been that free extracellular fetal DNA can be detected in the plasma and serum of pregnant women, which may permit the rapid and accurate detection of uniquely fetal loci, such as the fetal rhesus D gene in rhesus D negative pregnant women.

Key words: non-invasive; prenatal diagnosis; trophoblast; erythroblast; FISH; PCR.

Health care in the Western world is currently having to face a dramatic change in population demographics. With regard to the field of obstetrics and gynaecology the effects are most notably pronounced in the facts that women are generally having fewer children and are bearing these at more advanced maternal ages. This places physicians and genetic counsellors in the dilemma that these patients have a higher risk of bearing a fetus with a chromosomal abnormality, and yet since it is likely to be their only child they are reluctant to subject it to the risk of an invasive prenatal diagnostic procedure such as amniocentesis or chorionic villus sampling (CVS). Furthermore, current trends indicating a decrease in fertility have made it necessary for more
couples to require some form of reproductive assistance. These couples are especially unwilling to undergo any risk associated with the loss of their long desired offspring. Although recent advances have increased the accuracy of non-invasive screening procedures for the detection of fetal chromosomal anomalies by combining ultrasound with serum analyte screening, these approaches still have a false positive rate approaching 5%. Hence, a large number of unnecessary invasive procedures are still being performed, which places an unnecessary psychological stress on the couples involved, as well as straining already stretched health resource budgets.

An issue frequently not considered is that current non-invasive methods are incapable of detecting fetal Mendelian disorders. These needs have fuelled the search for non-invasive, and hence, risk-free methods of prenatal diagnosis that will permit the detection of both fetal chromosomal aberrations and single gene disorders. To achieve this task two main avenues have emerged:

1. The isolation of fetal cells from the blood of pregnant women.
2. The isolation of trophoblast cells from the cervix.

Both of these approaches rely on early reports that indicated their feasibility, and yet despite decades of intense research, neither is available yet for routine analysis. Research in this field has, however, not been held up by these apparent setbacks. On the contrary, recent reports indicate that many of the pitfalls are in the process of being overcome, and that even if this technology may not be introduced in the immediate future, there is no doubt that it will shape the future of prenatal diagnosis.

**FETAL CELLS IN CERVICAL MUCUS: A SIMPLE ALTERNATIVE TO AMNIOCENTESIS?**

In 1971, Shettles published a short intriguing note in Nature, in which he suggested that trophoblastic cells shed from the placenta into the cervical canal could be retrieved and used for prenatal sex determination. By examining mucus smears for transcervical cells (TCC) that had been collected using a cotton swab from the mid-cervical canal of pregnant women for Y chromosome positive cells, he was able to correctly determine the fetal sex in 10 out of 18 instances investigated.

The first clear report confirming this pioneering observation was published by Rhine et al. in 1975, in that by using an analogous approach they were able to correctly predict the fetal sex in 31 out of 36 cases examined. Furthermore, all 18 of the pregnancies bearing male fetuses were correctly identified as such. This report was soon followed by ones from China, indicating that this procedure could be used for fetal sexing, which drew an appropriate response from Shettles in a letter to the American Journal of Obstetrics and Gynecology in which he stated that his intention had never been to introduce a test that facilitated the selective abortion of a particular fetal sex.

In the following years four modes have evolved for the retrieval of cervical trophoblast cells:

1. Aspiration.
2. Cytobrush or cotton wool swabs.
3. Endocervical lavage.
4. Intrauterine lavage.

Convinced that large numbers of trophoblasts were deposited behind the cervical mucus plug, Rhine and colleagues attempted to retrieve more of these cells by performing an endocervical lavage. For this process they developed an antenatal cell
extractor (ACE). In essence this instrument consisted of a 15 cm long plastic tube to which a 5 ml syringe was attached and a plunger, which facilitated an opening and closing of the anterior end of the tube. By opening the tube, sterile saline solution could be flushed into the cervical canal and withdrawn by manipulating the syringe. By subsequently closing the tube, it was hoped that the fetal cells that had become dislodged by flushing would be retained inside the tube and could then be safely expelled following extraction from the cervix. By these means it appeared that they were able to obtain sufficient fetal cells for cell culture and subsequent karyotyping. These results could, however, not be confirmed by Goldberg et al\textsuperscript{5}, who used an almost identical instrument for the TCC sample extraction.

In the early 1980s Liu and associates examined the efficacy of blind transcervical aspiration as a means of collecting fetal cells exfoliated into the cervical mucus.\textsuperscript{6} Trophoblasts, identified histologically, were found in 33\% of the aspirated samples. The amount of tissue retrieved generally permitted the extraction of 5 mg of DNA. Tissue culture was possible in ten of the samples. No attempt was made to determine any fetal genetic characteristics e.g. sex. A problem with these and other studies at that time was that they were performed without the aid of ultrasound guidance, which could increase the risk of the procedure.

The advent of the polymerase chain reaction (PCR) revolutionized molecular diagnostics, and it was using this technology that Griffith-Jones and colleagues examined trophoblast cells either retrieved with cotton wool swabs or by flushing of the lower uterine cavity with 5 ml saline.\textsuperscript{7} Ultrasound was employed for guidance and to confirm fetal well-being. In the experiments using cotton wool swabs, samples were taken from 26 women, between 8 and 13 weeks pregnant. In only one of the nine pregnancies bearing a male fetus was no Y chromosome-specific amplification obtained. Several false positives were, however, obtained, which could be attributed to residual sperm from prior sexual intercourse. In all of the seven samples obtained by lavage, syncytial clumps that stained positive with trophoblast-specific antibodies could be identified. Since these fetal TCC samples were contaminated by maternal cells, the authors suggested that by using fetal-specific antibodies and magnetic cell sorting, pure populations of fetal cells could be obtained. This would, however, in part defeat the object of having a simple and rapid diagnostic test.

**DETECTION OF FETAL ANEUPLOIDIES AND SINGLE GENE DISORDERS**

Probably the strongest evidence for the use of transcervical trophoblast cells, following this initial exploratory phase, has come from the collaboration between Adinolfi and Rodeck. In 1993 this team reported the first prenatal diagnosis of a fetus with trisomy by the use of appropriate FISH (fluorescence in situ hybridization) probes on trophoblast cells that had been obtained by transcervical flushing.\textsuperscript{8} Three fluorescent signals, indicative of trisomy 18, were detected in 26\% of the cells collected by transcervical flushing. Furthermore, fetal sex could be determined correctly in seven out of seven pregnancies bearing a male fetus by the use of a PRINS (primed in situ labelling) test for the Y chromosome. False positive signals were however detected in two out of four pregnancies with a female fetus.

Since sampling was once again performed on a patient about to undergo a termination of pregnancy, a critical issue that still needed to be addressed was, what was the relative risk of this sampling technique to both mother and fetus, in terms of infection, fetal damage and possible abortion.
Having proven that they could detect gross fetal chromosomal abnormalities, in their next study they examined whether they could detect more detailed fetal genetic characteristics using PCR. For this purpose they used the rhesus D gene system. Although rhesus D incompatibility and the associated haemolytic disease of the fetus and new born (HDN) is of clinical relevance in obstetrics, it is also most suitable as a model for the establishment of prenatal diagnostic tests. The relative ease of this PCR test for the determination of the fetal rhesus D genotype lies in the fact that the rhesus D gene is absent in rhesus d (D negative) individuals. This means that when examining the fetus of a rhesus d pregnant woman, any contaminating maternal tissue is usually of little or no consequence.

In Adinolfi et al’s study TCC samples were taken from 12 rhesus D pregnant women, six being collected by aspiration of cervical mucus prior to CVS, and the other six by lavage immediately prior to termination of pregnancy. Two out of three aspirated samples, and three out of four lavage samples correctly identified rhesus D positive fetuses as such. This discordance was ascribed to there being too few fetal cells in some of the TCC samples. On the other hand, fetal sex could be accurately determined in the lavage samples by performing PCR for the Y chromosome. Furthermore, in four of these six samples, informative highly polymorphic microsatellite markers, also termed short tandem repeats (STRs) for chromosome 21 could be detected using fluorescent PCR. This latter PCR analysis formed the basis for examining such TCC samples for the prenatal detection of fetuses with trisomy 21.

Encouraged by these results the authors next set out to examine whether fetal haemoglobinopathies could be detected using TCC aspirates taken before CVS from six couples at risk for either thalassaemia or sickle cell disease. Unlike the situation when testing for rhesus D, where the gene of interest was absent from the maternal genome, for autosomal recessive disorders, to be affected the fetus needs to inherit a mutant allele from each parent. Hence, here a clear distinction had to be made between maternal and fetal tissue, since contamination by maternal cells would give a false representation of the fetal genotype. In order to achieve this, the TCC sample was mechanically dispersed in phosphate buffered saline and clumps of cells having syncytial or trophoblastic morphological characteristics were separated under an inverted microscope. DNA was then extracted from these isolated clumps and examined using PCR for the presence of the relevant mutant globin alleles. Up to seven clumps were investigated per case. The results from this study, although encouraging, did show the inherent difficulties associated with the PCR based analysis of tissues that are potentially contaminated from other sources. In this manner, the correct fetal genotype could be derived in three out of the six case studies. Incorrect genotypes, however, were determined with an alarming frequency (five out of six). Since it is unlikely that any clump of cells will ever be entirely free of any maternal contaminants, the only way to resolve this issue would be to examine single trophoblast cells that have been positively identified as such.

**TCC SAMPLING: WHICH IS THE BEST ROUTE?**

As described above, four major options exist for the retrieval of TCC samples: aspiration, cytobrush, endocervical lavage and intrauterine lavage. Cotton wool swabs had been shown to be ill-suited for this task since the retrieved cells remained trapped in the cotton fibres. Since no clear studies have previously been undertaken to indicate which of these four methods was best suited for TCC sampling and which was associated with the lowest risk for mother and fetus, considerable debate has existed as to which option was optimal. This issue was probably best addressed by Rodeck and colleagues.
in a study where they examined the efficacy of all four methods for the retrieval of TCC samples. In that study, TCC samples obtained from 59 patients examined by intrauterine lavage or aspiration, 22 examined by endocervical lavage or cytobrush and 130 examined solely by aspiration were compared. Perhaps as an indication of the potential invasiveness of the other methods, and hence associated risk to the fetus, only the latter 130 aspirated samples were obtained from ongoing pregnancies whereas all the other samples were taken prior to termination of pregnancy. Cells of trophoblast origin were initially identified by immunohistochemistry and cellular morphology and subsequently by genetic analysis using either FISH or PCR.\textsuperscript{13} Their data showed that fetal cells could be found in samples obtained by all four methods, with a tendency to obtain better samples by lavage, whereas aspirated sampling improved with increased gestational age. In general, better results were obtained using PCR analysis compared to FISH. No adverse affects were recorded in the 130 patients in whom TCC samples had been obtained by aspiration when compared to a matched control cohort. This indicates that this method may be safe enough for routine screening applications. Fetal cells were, however, not obtained in all 100\% of the cases in any of the sampling methods. This suggests that considerable improvements will have to be made to the actual sampling techniques, in particular for aspirated samples where cervical mucus consistency was determined to be an important factor, to ensure that an adequate number of fetal cells are retrieved for the subsequent analysis. In a separate study performed by Falcinelli and colleagues a comparison of TCC sampling by either cytobrush or lavage showed no clear advantage of the latter over the former.\textsuperscript{14} This implies that the less invasive and lower risk cytobrush method may establish itself as the method of choice, since this is the only method that has been performed on ongoing pregnancies with no apparent deleterious effects to mother and fetus. This is in strong contrast to reports from Taiwan where TCC sampling by uterine lavage is used for fetal sexing, itself a questionable practice, and has been shown to be associated with gross fetal malformations.\textsuperscript{15}

**TCC Sampling: Future Directions**

Independent studies in the past decade have indicated that fetal cells can be detected in TCC samples obtained using various approaches. As a considerable conflict of opinion exists amongst the researchers in the field as to which method is the most optimal for the retrieval of fetal TCCS and as to which method poses the least risk to mother and fetus, there is a real need for a large multicentre study. As is discussed below, such an approach has been conducted under the auspices of the NIH to evaluate the optimal manner of enriching and analysing fetal cells in maternal blood.

A feature that has emerged from these studies, and indeed those of fetal cells from maternal blood, is that pure preparations of fetal cells are rarely, if ever, obtained. To circumvent this problem it is necessary to analyse single fetal cells isolated by micromanipulation. One advantage of using trophoblast cells from TCC samples is that they can be readily identified by their morphology and immunohistochemistry using appropriate antibodies.\textsuperscript{13,16,17} With regard to the PCR analysis, a large amount of the technology has been transferred from the field of pre-implantation genetic diagnosis, and has successfully been applied by Sherlock and colleagues for the analysis of several fetal loci including haemoglobinopathies, microsatellites and sex.\textsuperscript{18,19}

A noteworthy implication of the various studies examining TCC samples is that the exfoliation of fetal cells into the cervix may not be a common event. This also raises the issue of what physiological events lead to the shedding of fetal cells into the cervix. Since the extraplacental villi are covered by the decidua capsularis, it is unlikely that
they are simply shed into the uterine cavity, but rather that the invasive nature of the trophoblast leads to their migration through the decidua capsularis.\(^\text{13}\) This feature could also account for the viable and healthy nature of the cells obtained, which is so high that it even permits culturing.

**FETAL CELLS IN MATERNAL BLOOD: HISTORICAL OVERVIEW**

Researchers interested in fetal cells in maternal blood are quick to point out that the first report of such an instance was made more than a century ago by Schmorl, who found trophoblasts in the lungs of women who had succumbed to eclampsia.\(^\text{20}\) Although trophoblasts should be among the first fetal cells to enter the maternal circulation during pregnancy they have generally been determined to be an inadequate candidate since trophoblast deportation does not appear to be a feature common to all pregnancies.\(^\text{16,21}\) A further complication with this cell type has been that most efforts to enrich for trophoblast cells, even when using purported specific antibodies, have failed.\(^\text{22}\) In recent years there has, however, been a renewed interest in this cell type, using a variety of different approaches and antibodies including anti-Hash-2, anti-placental lactogen hormone or the trophoblast-specific antibodies LK26 or 340.\(^\text{17,23-25}\)

The results of these studies have not always been convincing, with many being characterized by the failure to detect fetal DNA in the enriched fractions.\(^\text{24}\) However, Lim and colleagues have recently reported the successful enrichment of both fetal erythroblasts and trophoblast cells using a magnetic colloid system.\(^\text{25}\)

Since fetal lymphocytes have been shown to persist for long periods postpartum, they have not been pursued actively any more, since there was always the danger that cells from a previous pregnancy could be retrieved for analysis.\(^\text{26,27}\)

It is for these reasons that most researchers in this field have decided to focus their attention on fetal erythroblasts, also termed nucleated red blood cells (NRBCs).\(^\text{28}\) The advantage of these cells is that they are particularly abundant in the fetal circulation early in gestation and are rare in the normal adult periphery. Furthermore, since they have a very short life-span of approximately 90 days, there is no danger of obtaining cells from previous pregnancies. Fetal erythroblasts can be tentatively identified by the expression of embryonic and fetal haemoglobins, which together with the high levels of expression of the transferrin receptor (CD71:TfR), the blood group antigen glycophorin A (GPA) and potential fetal-specific antigens, such as the HAE9 antigen have been employed for the enrichment and isolation of these rare cells.\(^\text{29}\)

Two methods predominate for the enrichment of these cells: fluorescent activated cell sorting (FACS) and magnetic cell sorting (MACS).\(^\text{30}\) While FACS was first used by Herzenberg and colleagues for the enrichment of fetal lymphocytes from maternal blood using HLA disparities between mother and fetus,\(^\text{31}\) this technology has been used extensively by the groups of Bianchi and Elias who used either CD71, glycophorin A or the gamma globin molecule to target erythroblast selection.\(^\text{32,33}\) The use of MACS for the enrichment of fetal erythroblasts from maternal blood was pioneered by our group.\(^\text{34}\) By using these two technologies the detection of fetal aneuploidies using fetal erythroblasts enriched from pregnant women was reported in the early 1990s.\(^\text{35-37}\)

**THE NICHD NIFTY STUDY AND THE DETECTION OF FETAL ANEUPLOIDIES**

These encouraging results prompted the NICHD (National Institute for Child Health and Development) to initiate a large scale study, the aim of which was to examine the
feasibility of using fetal cells from maternal blood for the detection of fetal aneuploidies.\textsuperscript{38} In this so-called NIFTY Trial (National Institute for Child Health and Development Fetal Cell Isolation Study), some 3000 women at risk of bearing an aneuploid fetus will be recruited, i.e. maternal age of over 35, abnormal sonographical or serum screening result, or previous instance of a fetal aneuploidy.

The enrichment procedures used by the four laboratories involved include two different FACS protocols\textsuperscript{33,39} and two forms of magnetic separation, a magnetic colloid system\textsuperscript{40} and the traditional miniMacs columns.\textsuperscript{34} Preliminary indications are that fetal aneuploidies can be detected by these means with specificities that are superior to current non-invasive methods.\textsuperscript{41} These studies have also shown that fetal cells are easier to detect in pregnancies bearing an aneuploid fetus than in normal pregnancies.\textsuperscript{42} This is probably a reflection upon previous observations, which have indicated that fetal cell numbers are elevated in aneuploid pregnancies.\textsuperscript{35,37,43} To obtain the maximum amount of information from the few isolated fetal cells multicolour-FISH procedures have been developed to permit the identification of the most common aneuploidies (X, Y, 13, 18 and 21) simultaneously in a single cell.\textsuperscript{44} Another alternative is the sequential hybridization of the chromosomes of interest as pioneered by the laboratories of Ferguson Smith and Bianchi.\textsuperscript{45,46} A further approach to gain the maximum amount of information from the few enriched cells is a procedure that has been termed fetal cell recycling, whereby the fetal cells are first analysed by FISH to determine sex and ploidy, and then subsequently by PCR following micro-manipulation.\textsuperscript{47}

In our experience we have found that male fetal cells could be detected with sensitivities and specificities approaching 60–80\%.\textsuperscript{48} A factor influencing the specificity of this analysis in a significant manner was how many fetal cells were detected, since specificities of close to 95% could be attained if three or more XY positive cells were found. This result is indicative of the power of this system, since no other non-invasive method is able to determine specific fetal chromosomes with such accuracy. It does, however, also indicate that three or more cells are required for an accurate diagnosis. As such these results do parallel our observations made with the analysis of single fetal cells by PCR, where four or more individual fetal cells would have to be analysed in order to obtain a diagnostic accuracy of close to 100\%.\textsuperscript{49}

An integral part of the NIFTY study is an evaluation of the psychosocial response of pregnant women to the introduction of a new non-invasive method for prenatal diagnosis. This is especially aimed at clarifying whether pregnant women would feel coerced to undergo such an examination. Preliminary data from this study have indicated that there is an overwhelmingly favourable response amongst pregnant women who have an elevated risk for bearing an aneuploid fetus for the introduction of such a non-invasive diagnostic test.\textsuperscript{50} Our own extensions of this study have shown that this favourable response is shared by the majority of pregnant women, even if this test could not cover the entire spectrum of chromosomal abnormalities. This desire was especially high in the group that had sought reproductive assistance, and in those women who were currently pregnant with their first child. Very few of the women interviewed felt that they would be coerced into undergoing such a test. An interesting aspect of this study was that while women would generally not question results indicating that the fetus was normal, they would invariably opt for an 'invasive' second opinion, to confirm that a fetal anomaly was present in those cases where an abnormality was detected using fetal cells.
THE USE OF SINGLE CELL PCR FOR THE DETECTION OF SINGLE GENE DISORDERS AND TO DETERMINE WHAT PROPORTION OF ERYTHROBLASTS ARE FETAL

Following the demonstration by Takabayashi that single erythroblasts could be retrieved by micromanipulation\(^1\), others\(^2,3\) have demonstrated the feasibility of using single fetal erythroblasts for the prenatal diagnosis of inherited single gene disorders. In these studies, erythroblasts were identified morphologically or judged to be fetal by staining with an anti-zeta globin antibody.

In our examination of this approach, we have used highly polymorphic microsatellites or short tandem repeats (STRs) to be able to identify cells as being either fetal or maternal.\(^4\) These examinations showed that 4–5 fetal cells would have to be individually analysed in order to attain those levels of diagnostic accuracy required for prenatal diagnosis.\(^4,5\)

Since during pregnancy both fetal and maternal erythroblasts are present in the maternal circulation a considerable debate has existed as to what the relative proportion of these two pools was.\(^5,5^5-5^8\) This problem had only been addressed inadequately to date because most studies used only a single locus as being indicative of fetal origin. To address this question we used single cell PCR on isolated fetal cells to examine both the fetal rhesus D status and sex in pregnancies where the mother is rhesus D negative.\(^5^9\)

Thus, by using two fetal loci that were absent from the maternal genome, namely the SRY locus on the Y chromosome and the rhesus D gene, we should be able to accurately discern between fetal and maternal cells. For this study we examined 19 cases. Erythroblasts were successfully recovered in 14 instances, from which single erythroblasts identified solely by morphology were micromanipulated and individually examined using a multiplex PCR reaction. This reaction allows the simultaneous detection of the SRY locus, the rhesus D gene and the \(\beta\)-globin gene. The latter gene sequence was used as a control to ensure that a cell had been transferred to the reaction vessel and that the PCR amplification had functioned. This study showed that approximately half of the erythroblasts examined were of fetal origin. Furthermore, we were able to correctly determine the fetal genotype for both loci in all 14 cases analysed. This study currently represents the largest non-invasive analysis of fetal loci and illustrates the potential inherent in this system. It also shows that there may be instances in which no fetal cells are recovered (five out of 19).

We have been able to extend these experiences by examining whether other fetal genes could also be detected in a similar manner, by investigating the use of fetal cells for the diagnosis of \(\beta\)-thalassaemia.\(^6^0\) Here, fetal erythroblasts identified by immunohistochemistry for zeta globin were observed in four out of the six cases examined. By using single cell PCR they were indeed determined to be of fetal origin, since in all these cases the fetal \(\beta\)-globin genotype was correctly determined. By analogy to our other experiences, we again observed in this study that at least four fetal cells have to be analysed in order to obtain a highly accurate result.\(^4,5\)

CURRENT STRATEGIES TO IMPROVE THE RECOVERY AND IDENTIFICATION OF FETAL ERYTHROBLASTS FROM MATERNAL BLOOD

Since as few as 20 fetal cells may be present in the maternal blood sample drawn\(^6^1\), it is a foregone conclusion that a loss of these rare cells should be avoided at all costs. As such, a lot of effort has been spent on optimizing the recovery of these rare cells.
With regard to this aspect, we have recently shown that better recoveries could be achieved by using simpler high density Ficoll or Percoll gradients or different antibodies such as anti-glycophorin A (GPA). By switching to these reagents we have been able to achieve a three- to fivefold higher yield than by using our previous protocols. This has also meant that we have been able to reduce the volume of maternal blood required from 40 ml to 16 ml.

In a similar manner, altering the protocols for enrichment using FACS, by such means as intracytoplasmic fetal haemoglobin staining or new more efficient staining and storing protocols has led to significantly improved yields.

Another possible alternative that has recently been described is that of charge flow separation, which is reported to result in phenomenal recoveries of erythroblasts. Unfortunately these results have not been reproduced by any independent researchers. Other gradient dependent enrichment techniques that have been explored include those described by Oosterwijk et al as well as step Percoll gradients.

A problem with all of these procedures is that none result in a pure preparation of fetal cells. Indeed in most instances the majority of recovered cells are still of maternal origin, with as few as 1 in 100 to 1 in 1000 of the recovered cells actually being fetal. For this reason several groups have tried to obtain fetal cell specific markers that should permit the automatic detection of the few fetal cells in the enriched preparations. Most of these experiments have focused on the use of anti-fetal (HbF) or embryonic-haemoglobin (HbE) antibodies. The detection of such labelled fetal erythroblasts could then be accomplished by either using laser scanning systems, fluorescence correlation spectroscopic microscopy or digital recognition equipped microscopes. Such an approach has been tested by the group of Jan Hoovers in association with Applied Imaging. In their system, enriched erythroblasts were identified by automated scanning for cells that had been positively labelled for gamma globin. To establish whether these were maternal or fetal, they were then subsequently examined using FISH for the X and Y chromosomes. This study showed that such an approach could be used to detect rare cells, and that the fetal cells could then be determined with a high degree of accuracy. A problem, however, was that many of the gamma globin positive erythroblasts were found to be of maternal origin. Furthermore, fetal cells were only detected in maternal blood samples taken following CVS, which is known to lead to feto-maternal transfusion, which implies that other more efficient enrichment procedures should be used. One approach for identifying the fetal cells more specifically may be to use the method described by Mesker and colleagues in which fetal erythroblasts were reproducibly identified immunohistochemically by a combination of anti-zeta and anti-epsilon antibodies.

**CULTURE OF FETAL ERYTHROBLASTS ENRICHED FROM MATERNAL BLOOD**

One of the most serious limitations when dealing with isolated fetal erythroblasts is the small number of recovered cells. Furthermore, since these cells are not actively dividing it is not possible to obtain information regarding the entire karyotype.

To overcome these obstacles some researchers have attempted to culture enriched fetal cells. These attempts have largely been unsuccessful. This is probably due to the few fetal cells being out-competed by the abundance of maternal cells present in the culture inoculum. To redress this situation new cytokines in combination with better culture conditions have been examined to determine whether they will favour the selective proliferation of fetal progenitor cells over maternal ones.
Our own examinations, carried out on early fetal progenitor cells, have demonstrated that these cells display a much higher basal proliferative capacity than comparable mature progenitor cells and that this can be enhanced by the addition of particular cytokine cocktails such as flt-3 ligand and thrombopoietin. These studies have indicated that considerable experimentation lies ahead to determine the optimal culture conditions. Since any form of culture requires a period of time the idea of a quick-screening test is somewhat compromised.

Larger cell numbers will, however, facilitate easier PCR analyses, since the problem of allele drop out (ADO) is no longer a concern. Also, the genome of pools of cells is much more amenable to whole genome amplification methods, thereby generating sufficient DNA for dozens, if not hundreds, of analyses. In addition modern molecular genetic techniques can be applied to these cells, e.g. comparative genome hybridization (CGH) or multicolour spectral karyotyping (SKY) to obtain further karyotypic information.

**FETAL CELL TRAFFIC IS ELEVATED IN PRE-ECLAMPSIA**

A fortuitous finding we have made during our routine examinations is that the levels of erythroblasts are significantly elevated in pregnancies affected by pre-eclampsia. In order to determine the origin of these erythroblasts we have conducted a case-controlled study in which we only used pregnancies bearing male fetuses. Here, by using FISH we were able to show that a large proportion of these erythroblasts were fetal. Our current focus has been to determine whether this elevation occurs earlier in the pregnancy and, if so, whether our finding can serve as a prognostic marker for those at risk for pre-eclampsia. Recent data from our laboratory show that this is indeed the case and that the number of erythroblasts in maternal blood is significantly elevated in those pregnancies that subsequently develop pre-eclampsia (W. Holzgreve et al., unpublished results).

A further interesting facet of these studies has been the observation made by Lo and colleagues concerning the levels of free fetal DNA in maternal plasma. This was initially demonstrated using PCR for Y chromosome specific sequences in pregnancies bearing male fetuses. Since both maternal and fetal DNA are present in maternal plasma, only those fetal loci that are absent from the maternal genome can be detected by this technique. Therefore, it was by these means that the fetal rhesus D gene could be reliably detected in pregnancies at risk for rhesus D iso-immunization. These assays did however seem to offer the greatest degree of sensitivity in plasma samples taken during the second trimester. Our own experience has shown that multiple fetal loci can be reliably determined by these means. For this purpose we used the multiplex PCR assay described above for the analysis of single fetal cells. Our examination showed that both
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fetal sex and rhesus D status could be correctly determined in 100% of the 22 samples analysed. Currently a serious drawback of this test is that no positive control exists to indicate that fetal DNA was indeed present in the plasma sample and that it was correctly amplified. As such, no protective strategies can be developed against obtaining false positive results.

In order to quantify the amount of fetal DNA present in maternal plasma, Lo and colleagues developed a real time quantitative PCR assay. Using this they were able to show that in normal pregnancies almost 5% of the total plasma DNA was fetal. This technology has proven to be most useful and interesting, for in this way it has been shown that the amounts of fetal DNA are elevated not only in pregnancies affected by pre-eclampsia, but also in those complicated by the onset of preterm labour. Furthermore, significant elevations have been noted in pregnancies bearing trisomy 21 fetuses, which opens up the prospect for a new screening assay.

CONCLUSION

Although none of the technologies discussed are currently available for routine prenatal diagnostic purposes, there is no doubt that these different technologies have progressed enormously in the past few years. In this manner, recent applications of single cell PCR on enriched fetal cells for the determination of fetal genetic loci show great promise. Furthermore, new developments, such as the detection of fetal circulatory DNA in maternal plasma have also opened up new and exciting prospects for the examination of fetal loci. In addition, new developments, such as the observation that fetal cell traffic or the levels of fetal circulatory DNA are elevated in certain pregnancy related disorders such as pre-eclampsia or in aneuploid fetuses, serve to extend the diagnostic scope of these methodologies.

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