The isolation and cytogenetic analysis of fetal cells from maternal blood

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Abstract

This thesis describes an approach aimed at enriching and cytogenetically analysing fetal cells from maternal blood. It describes in detail the sequence from blood sampling and enrichment through to chromosome analysis of the enriched cells and the problems encountered at each stage. It also addresses the problems of cell deposition and the necessity of cell identification and demonstrates the inter-dependence of the individual techniques and how alteration of one of the stages had considerable effect on others. Throughout, therefore, an attempt has been made to develop an integrated enrichment and analysis procedure which, importantly, could be automated.

99.95 ± 0.07% of erythrocytes were removed by centrifugation through a Histopaque 1119 density gradient cushion with a recovery of 53.1 ± 26.9% of all the starting nucleated cells. 99.1 ± 0.4% of leukocytes of every type were removed using a cocktail of monoclonal antibodies conjugated to magnetic beads. Overall, it was estimated that 35% of fetal erythroblasts could be recovered. The cells were then lysed in suspension and deposited on to microscope slides for cytogenetic analysis.

In a prospective study involving fifteen women in early pregnancy, 15-20ml of blood was drawn from an antecubital fossa vein and subjected to the enrichment techniques described. Using either a Y-chromosome probe on its own or later X- and Y-probes simultaneously, the cells were then subjected to fluorescence in situ hybridisation. Gender prediction was 86.7% accurate (sensitivity 100%, specificity, 75.0%, $p < 0.004$). Furthermore, using this approach it was also possible to confirm a diagnosis of Down's syndrome which had been made by a conventional invasive technique one week previously.

This study showed that fetal cells, albeit in small numbers, can be isolated reliably from maternal blood in early pregnancy. Moreover, the simple procedures could be automated, a principle which is fundamental if maternal blood is to be used for prenatal diagnostic purposes.

Each step in the whole sequence continues to develop. An attempt has therefore been made to outline necessary future investigations and potential areas where enrichment and analysis might be improved. Furthermore, the implications and applications of such a technique in the field of antenatal diagnosis are discussed.

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I am indebted to Mrs. Patricia Eason for teaching me many basic laboratory techniques and to Lawrence Brett for his help with immunocytochemistry. My thanks to Mrs. Judy Fantes for teaching me the principles and practice of fluorescence in situ hybridisation and to Rob Speed for help with DNA preparation. I am indebted to Prof. Veronica van Heyningen for her attention to detail and extensive knowledge of molecular biology and related areas which has made possible many of the processes described in this thesis.

I must give much credit and thanks to Applied Imaging Corporation who funded Dr. Alison Hill and Mrs. Heather Finlayson to work with me during the later months of this project. It is fair to say that three pairs of hands have allowed the work to progress more than three times faster than mine alone.

I am also indebted to the obstetric staff at the Eastern General Hospital who have allowed me access to their patients and for their help and co-operation with this project.

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However, I am most indebted to Dr. Denis Rutovitz. The original ideas have evolved as experimentation has shown one path to be more promising than another. However, the basic concepts in the approach, originally proposed by Denis, have not altered.

Declaration

I, Dr. Mark R. Gaudoin, solemnly declare that the work contained in this thesis is my own unless otherwise stated and that it has not been submitted for any other degree qualification in Edinburgh or other seat of learning.

Mark R. Gaudoin (MB.Ch.B., MRCOG)

Medical career

I graduated from the University of Edinburgh, M.B.,Ch.B., in 1987.

I was a surgical house officer in Paediatric Surgery at the Western General Hospital, Edinburgh and medical house officer in General Medicine at the Borders General Hospital, Melrose. I then undertook a six month post in Paediatrics and Neonatology in the same hospital and spent six months as a General Practitioner trainee in Earlston, Berwickshire.

From 1989-90 I was a senior house officer (SHO) in Obstetrics and Gynaecology at the Western General Hospital, Edinburgh. From 1990-91 I was "Senior" SHO at the Eastern General Hospital, Edinburgh and from August 1991 to June 1994 I was a Medical Research Council Clinical Scientist in the MRC - Human Genetics Unit at the Western General Hospital, Edinburgh.

Since August 1994 I have been a Specialist Registrar in Obstetrics and Gynaecology in Glasgow and passed the MRCOG examination in May 1995.

I was awarded the Yorkhill Sick Children's Hospital Short Papers Prize in 1995 for presentation of work contained in this thesis.

Dedication

The work contained in this thesis is dedicated to my wife, Isobel, and to our children, Matthew and Anna.

It is not the job of obstetricians to direct, but to offer informed choice. This thesis is therefore also dedicated to those women, and their families, who have unexpectedly given birth to a child with Down's Syndrome. These children will be no less loved than others but it is hoped that one day greater choice will be available to all.

It is hoped that this thesis will contribute to the eventual development of a truly non-invasive, and yet definitive, antenatal diagnostic test available to all pregnant women.

Chapter 1. General introduction to prenatal screening and diagnosis

Around 8% of all pregnancies warrant some form of prenatal investigation (Brock, 1982). At present there are over 2500 indications for prenatal testing and an overall appreciation of the incidence and prevalence of congenital abnormalities and, in particular, chromosome abnormalities is necessary.

5.6/1000 live births are complicated by a chromosomal abnormality, accounting for 34% of all early mortalities. Sixty-four percent of these cases develop chronic problems requiring continuing medical and social support (Hook, 1992).

Fifteen percent of first trimester pregnancies abort of which 40% are karyotypically abnormal, a further 7% show developmental inconsistency (certain embryonic organ systems being more advanced than others) and another 7% demonstrate specific organ defects. Of the first trimester karyotypic abnormalities, 52% are autosomal trisomies, 19% are 45, XO, 16% are triploid and 6% are tetraploid. Twenty-six percent of spontaneous abortions in the second trimester are associated with at least one system defect though the absolute proportion of spontaneous abortions compared to ongoing pregnancies is less than 3% (Young, 1992).

Chromosomal abnormalities account for 6-7% of all major abnormalities at birth and single gene abnormalities account for a further 6-7%. Twenty-five to thirty percent of all perinatal loss (greater than 24 weeks' gestation to the end of the first week after birth), 27% of all infant mortality and 19% of all deaths of children aged 1-9 years is associated with congenital malformation (Young, 1992). Abnormalities involving chromosomes 21, 18, 13, X and Y constitute 95-98% of all chromosomal abnormalities at birth (Whiteman and Klinger, 1991; Hook, 1992). Trisomies involving chromosomes 21, 18 and 13, account for 26% (Hook, 1992). These proportions are rising as neonatal intensive care improves so that mortality previously associated with prematurity continues to fall.

Throughout this thesis particular reference is made to Down's syndrome (trisomy 21), the collection of symptoms and clinical signs associated with three copies of a specific region of chromosome 21. It is the most common cause of mental retardation in the UK with a birth prevalence of 1 in 800 live births. The characteristic clinical features were described by John Langdon Haydon Down in 1866. In 1959 Lejeune et al described the additional small acrocentric chromosome, now known to be an extra copy of chromosome 21. Ninety-two percent of Down's syndrome cases have 47 chromosomes with an extra chromosome 21 (usually of maternal origin), due to non-disjunction at first meiosis and 4-6% have 46 chromosomes but an extra copy of 21q is translocated on to another chromosome (often chromosome 13 or 14). Three percent are mosaics and tend to be clinically less severely affected (Mikkelson and Brøndum-Nielsen, 1992).

The incidence of Down's syndrome, Edward's syndrome (trisomy 18), and Patau's syndrome (trisomy 13) rise with advancing maternal age. All three conditions are over-represented at term, especially trisomy 21, because of the lower attrition rate when compared to other autosomal aneuploidies. At around 10 weeks' gestation 26.1% of all autosomal abnormalities are trisomy 21; at 17 weeks' gestation it is 40.6% and at term it is 78% (Hook, 1992). Only 30% of Down's syndrome cases abort after the mid-second trimester and it represents only 2.3% of all spontaneous abortions. Hence, Down's syndrome contributes enormously to the overall prevalence rates of the clinically serious chromosomal abnormalities.

If a woman was older than thirty years old when she had a Down's syndrome child, the recurrence rate is 0.76%, similar to the expected overall risk of 0.72%. If she was younger than thirty it is 0.68% whilst the expected risk would be only 0.27% for age-matched controls (Wald and Cuckle, 1992). This probably reflects an inherent predisposition to non-disjunction or gonadal mosaicism particularly as half of the abnormalities in recurrent trisomy cases are different from the index case (Mikkelson and Brøndum-Nielsen, 1992).

Goldstein (1989) estimated that the cost of looking after a Down's syndrome adolescent at home was US$24,213/ year compared to US$3,718 for an able-bodied adolescent. The cost of institutionalisation was US$42,728/ year. Hence, it is of major economic importance whilst the social costs are incalculable.

With this background, it is easy to appreciate the importance of antenatal diagnosis, the impact it might have on abortion rates, especially late and distressing pregnancy losses, and the effect on perinatal and childhood morbidity and mortality.

A screening programme detects a (predisposition to a) particular condition in people who are generally considered to be free of the condition. Importantly, screening embodies the principles of giving informed choice and autonomy to the widest possible range of the population. These principles are no different for prenatal diagnosis.

Hence, prenatal testing should include as many women as possible, providing reassurance and reducing the anxiety levels associated with reproduction. It allows counselling so that parents realise they are not alone and gives them the opportunity to decide whether or not they want to continue with the pregnancy (Luck, 1992). Furthermore, if the couple wish the pregnancy to continue, the optimal mode, place and timing of delivery and subsequent management of affected infants may be ensured (Gaudoin, 1993a).

Unfortunately, for those involved in prenatal diagnosis programmes there is the danger of being misinterpreted as trying to create a eugenic society. This might in turn be used as a way to divert money away from disability schemes and/or deride the value of specific groups in human society. Every effort must be made to emphasise that this is not the case. Accepted, affected children born to informed parents is counted as a benefit in a prenatal screening programme and not as a cost (Modell, 1992).

The ideal screening test is simple, safe and effective, highly sensitive and highly specific, with a high positive predictive value. Furthermore it must be repeatable and relatively

inexpensive. Current definitive antenatal diagnostic techniques for fetal aneuploidy fulfil many of these criteria but involve some risk to the pregnancy. As such they are only offered to certain women.

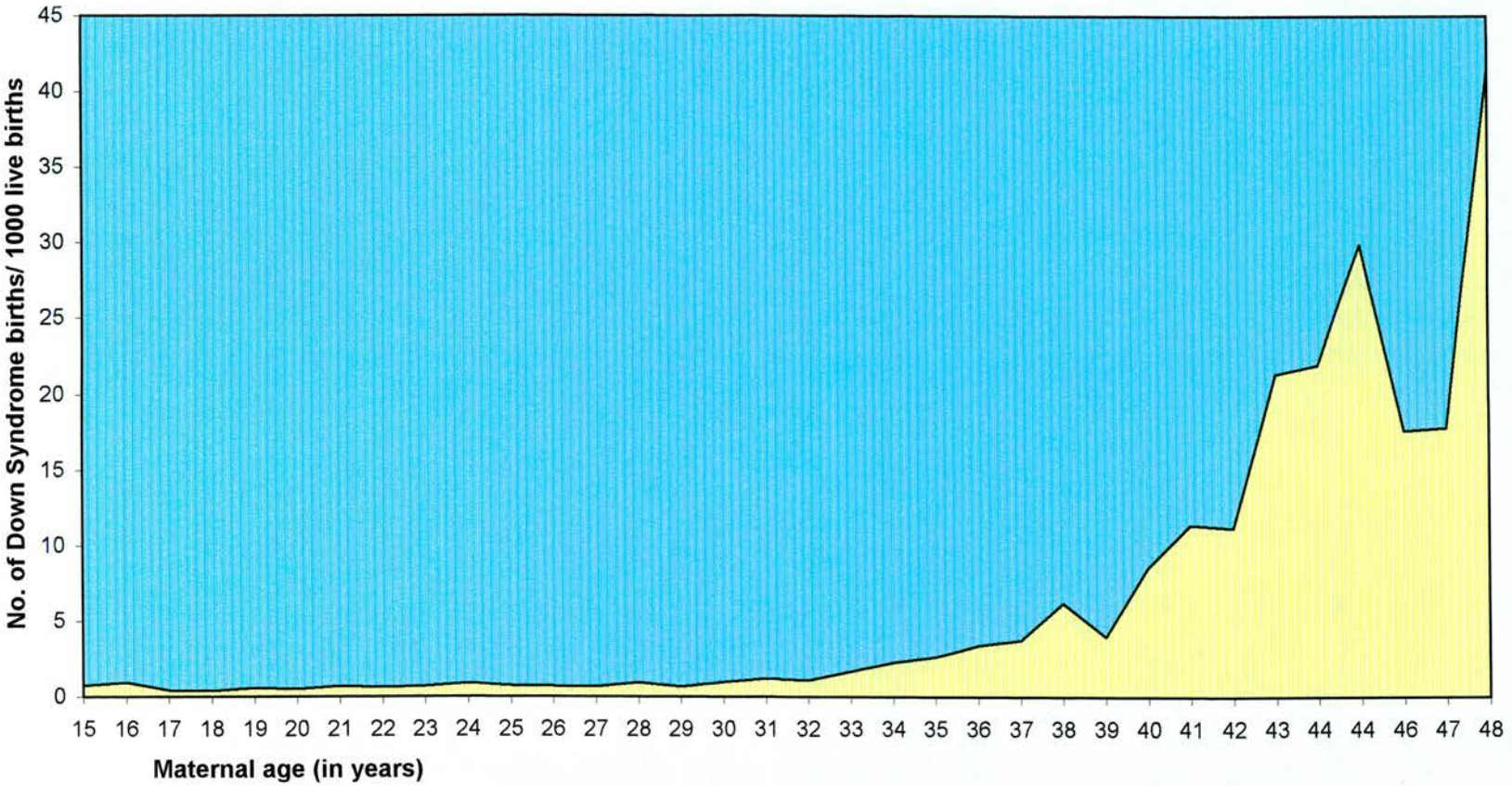
The chance of fetal aneuploidy, especially trisomy 21, but also trisomy 13 and trisomy 18, rises with increasing maternal age, particularly after 35 years old (Ferguson-Smith and Yates, 1984, figure 1.1). Because of this, and because the risk of iatrogenic miscarriage is similar to the chance of carrying an abnormal fetus, 35 years and above has been the traditional threshold to offer definitive testing. However, since most pregnant women are less than 35 years old, the area under the curve and to the right of the bold vertical line in figure 1.2 shows that only 30% of Down's syndrome cases are actually born to women over this traditional age threshold (Staples et al, 1991, figure 1.2). Therefore, only 30% might be detected if age is the sole screening criterion. Maternal serum biochemical screening has improved detection rates but the diagnosis is still dependent on invasive testing thereafter and, in the absence of definitive testing, many of the other common aneuploidies are missed.

The possibility of isolating and analysing fetal cells from maternal blood as a means of prenatal aneuploidy detection is, therefore, a very attractive concept. It could be used as a screening procedure, available to all pregnant women and not just selected groups. Using modern molecular biological techniques potentially any case of Down's syndrome and many of the other common fetal aneuploidies could be detected. Initially putative diagnoses would need to be confirmed by established diagnostic techniques but ultimately the need for confirmatory invasive methods may be obviated and counselling of the couple performed on the basis of the blood result alone.

This thesis reviews the currently available diagnostic tests and their limitations and much of the published literature on isolating fetal cells from maternal blood. It describes the work carried out by myself and others at the MRC-Human Genetics Unit, enriching for fetal cells

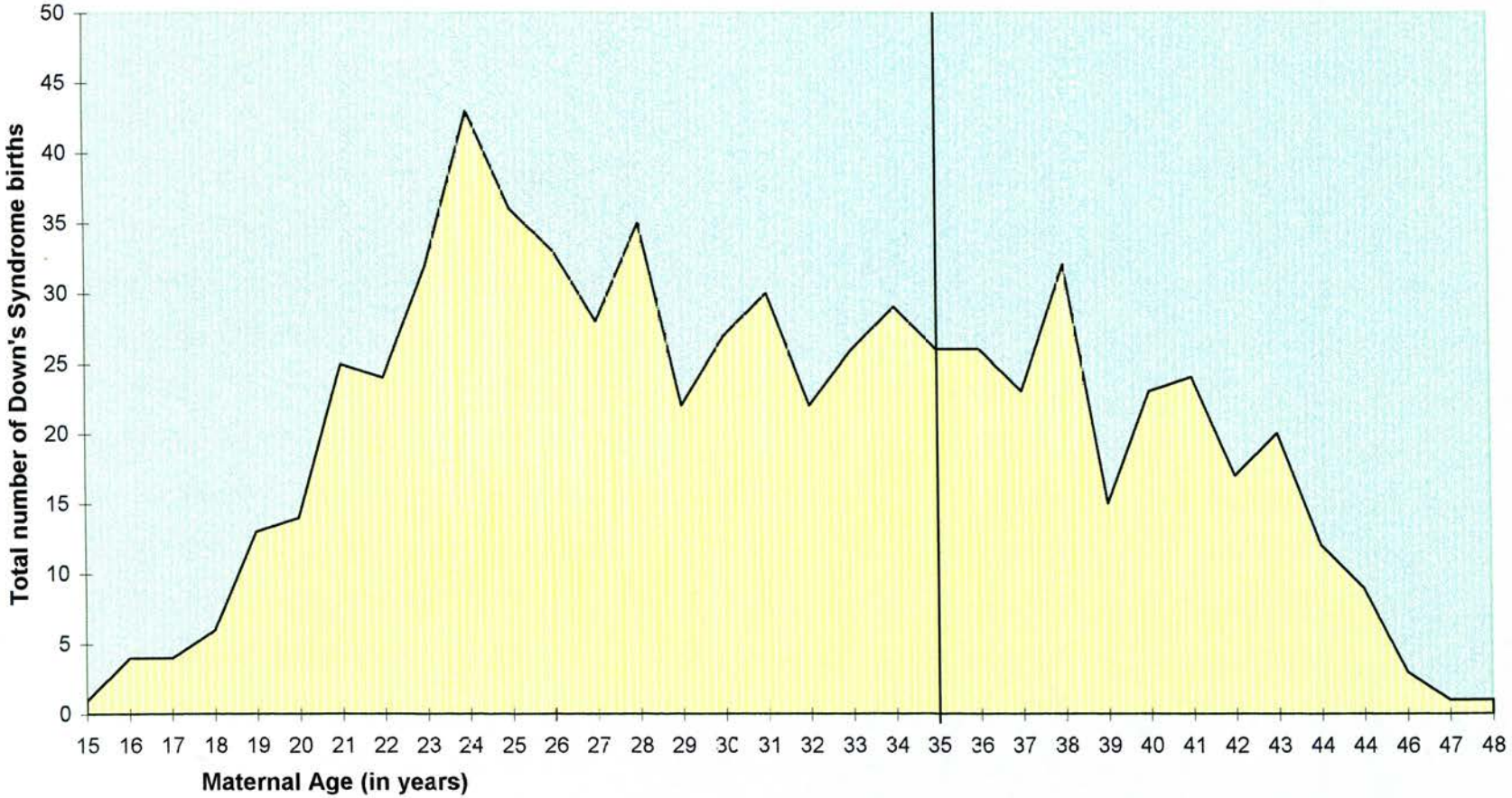
and subsequently performing limited cytogenetic analyses on these cells. The rationale for each stage, the results and the problems incurred are explained.

Figure 1.1 Rates of Down's Syndrome related to Maternal Age



From Ferguson-Smith and Yates, 1984. Note the steep rise in the rates of Down's syndrome with increasing maternal age.

Figure 1.2. Number of Down's Syndrome births in S. Australia 1960-1989 related to Maternal Age



From Staples et al, 1991. Note that the majority of births actually occur to women less than 35 years old.

Chapter 2. Current antenatal diagnostic procedures

2.1 Introduction

The aims and benefits of prenatal screening have been described in Chapter 1 and at present there are over two thousand specific indications to offer prenatal diagnosis. Principally these are to search for chromosome abnormalities, inborn errors of metabolism (to demonstrate enzyme activity or protein deficiency), measurement of metabolites in amniotic fluid or to culture infective agents.

As antenatal screening and diagnosis has developed, the tendency has been to earlier and/ or less invasive methods. The isolation of fetal cells from maternal blood is the natural progression in this field and to understand it's need it is necessary to appreciate the development and limitations of currently available techniques. There are clearly vast numbers of published studies in this field which, out of necessity, compels selection and inclusion of some whilst omitting others. The techniques can be divided into non-invasive (effectively only ultrasound scanning) and invasive, which involve some risk to the pregnancy. Although not diagnostic, an overview of maternal serum biochemical screening for Down's syndrome is given and, for completeness, a number of novel tests are also reviewed.

2.2 Amniocentesis

The cells found in amniotic fluid are shed from a variety of tissues, notably the fetal skin, gastrointestinal, genito-urinary and respiratory tract, the amniotic membranes and the umbilical cord. However, only 20% of the cells are viable and can be cultured. Amniocentesis involves needle aspiration of amniotic fluid from the uterine cavity. Ultrasound visualises a pool of amniotic fluid and the placenta to avoid placental perforation. Maternal blood contamination does not affect cell cultures but it does increase the chance of confusing results as maternal leukocytes are cultured as well.

The failure to determine the true fetal karyotype using amniocentesis is rare because all the different cultured cell types arise from the inner cell mass which reflects the fetal karyotype more accurately than trophoblast cells. Overall only 0.6% of all amniocenteses require a further invasive procedure for cytogenetic clarification (MacLachlan, 1992). The fetal loss rate up to 28 weeks' gestation, and corrected for spontaneous background losses, is about 1% (Smidt-Jensen et al, 1992). Major and minor fetal trauma has been documented though never if ultrasound has been used and chorio-amnionitis is rare. Chronic amniotic fluid leakage increases the risk of premature labour, respiratory distress syndrome and pneumonia at birth but other abnormalities, such as neurodevelopmental delay, are no different from age-matched controls. Despite placental location there may be transplacental haemorrhage following amniocentesis, necessitating prophylactic anti-D immunoglobulin for Rhesus- negative women (Bowman and Pollock, 1985).

The major drawback of amniocentesis is the late stage of pregnancy, usually 16-19 weeks' gestation, at which the procedure is performed so it is well into the second trimester before karyotypic abnormalities are detected. The fetus is well formed, the woman is experiencing fetal movements and she is obviously pregnant to those around her. Data suggest that such women, years after termination of pregnancy, experience greater pangs of guilt than those who undergo termination of pregnancy for fetal abnormality at earlier gestations (MacKenzie, 1992).

Early amniocentesis is performed at 10-11 weeks' gestation but to confer advantage over chorionic villus sampling (CVS) it must be as safe or safer and give as fast and reliable results so that the time for the less traumatic procedure of suction termination of pregnancy has not passed. Unfortunately the estimated amniotic fluid volume at 10 weeks' gestation is only 30ml, at 13 weeks' only 70ml, rising to 200ml at 16 weeks' gestation.

Kennerknecht et al (1992) performed amniocentesis on 100 women immediately prior to first trimester termination of pregnancy. Tenting of the amniotic membranes, so blocking the lumen of the needle limited the amount of fluid which could be drawn off and at lesser

gestations, longer culture times were required, presumably because there were fewer cells and culture conditions were suboptimal. The success rate in terms of definitive cytogenetic results was only 40-50% when the procedure was performed at less than 9 weeks' gestation, 80-90% at 9-11 weeks' and 100% at twelve weeks' or more. They concluded that early amniocentesis is feasible but, because of the prolonged culture times and high cytogenetic failure rates, it offered no great advantage over second trimester amniocentesis.

In a much larger study of 1375 patients, Lockwood and Neu (1993) estimated that although the mean turnaround time (the time from procedure to cytogenetic results being available to the clinician) was greater for early amniocentesis, results were available 3.5 weeks before results from the standard procedure. However, 94% of their samples were greater than 12 weeks' gestation and they had an overall culture failure rate of 1.4% compared with their failure rate of less than 0.5% for the standard procedure.

In a controlled study comparing early amniocentesis with CVS and controls (no prenatal investigations), infants in the early amniocentesis group had significantly more respiratory problems than the other two groups (Greenough et al, 1997). In the Canadian Early and Mid-trimester Amniocentesis Trial (1998) 1916 women underwent amniocentesis before 13 weeks' gestation compared to 1775 women who underwent amniocentesis after 15 weeks' gestation. There were more cases of postprocedural amniotic-fluid leakage ($p < 0.001$), greater fetal loss ($p < 0.02$) and more cases of talipes equinovarus ($p = 0.0001$) in the early amniocentesis group.

Termination of pregnancy is psychologically and physically less traumatic in the first trimester. However, given that many of the cases in the initial reports were performed in the early second trimester, with results available at a variable period after the procedure, it suggests that there is no great advantage conferred by these relatively late "early" amniocenteses. Furthermore, the questions raised about the immediate and long term safety of early amniocentesis requires urgent investigation in a properly controlled manner.

2.3 Chorionic villus sampling

The chorion frondosum contains the mitotically active villus cells and becomes the definitive placenta. Chorionic villus sampling (CVS) involves biopsy and subsequent cytogenetic analysis of this tissue.

In 1968 Hahnemann and Mohr successfully cultured these cells and although many groups persevered over the next few years with sampling techniques and cytogenetic analysis it received scant attention as the problems associated with amniocentesis were being overcome. However, interest in first trimester prenatal diagnosis was rekindled in the early 1980's largely as a result of improved culturing from tiny amounts of tissue and the development of quality ultrasound imaging which allowed reliable and accurate biopsy.

The Canadian collaborative group (1989) reported that CVS had a higher frequency of abnormal cytogenetic results compared to amniocentesis (3.8% compared to 1.9%). The difference in abnormality rates in this multicentre study to some extent reflected the earlier gestational age of sampling at CVS as many of these pregnancies might have gone on to abort spontaneously but there was also a significantly higher false positive rate. Overall 9.9% of women undergoing CVS required further invasive testing. Cultures failed in 1.5% of CVS samples compared with only 0.1% of amniocentesis samples whilst maternal cell contamination was also more common in CVS samples because maternal cells (the decidua) are intimately associated with the villi. Furthermore, 1.6% of CVS samples demonstrated confined placental mosaicism (CPM, or pseudomosaicism). Mosaicism implies the presence of two or more distinct cell lines containing different karyotypes in the same individual. Confined placental mosaicism is restricted to placental elements with complete absence in the fetus and reflects the complexity of early placental development (Kalousek et al, 1987). Once established the aberrant cell line has a similar rate of proliferation and maintenance to that of a normal cell line. Level 1 and level 2 mosaicism is seldom confirmed in the fetus and even level 3 mosaicism (multiple cells with the same cytogenetic abnormality in multiple flasks) may not be truly representative of the fetal karyotype (Silverman and Wapner, 1992).

The large European collaborative study on mosaicism at CVS, which involved 11,855 cases, found that only 5.2% of villus biopsies complicated by mosaicism were confirmed by amniocentesis. Verjeslev and Mikkelson (1989) reported that the most common autosome mosaics involved chromosomes 3, 7, 8, 13, 15, 18, 20 and 21 but 45, XO was the most frequently observed mosaic cell line. Table 2.1 shows that a mosaic diagnosed at CVS involving one of the common trisomies is not necessarily reliable and certainly warrants further testing (usually amniocentesis).

Table 2.1. Analysis of chromosome mosaics at CVS (Verjeslev and Mikkelson, 1989)

Mosaic at CVS	Number	Normal fetal karyotype	Mosaic Confirmed	Not reported
Chromosome 21	8	3 (38%)	4 (50%)	1 (12%)
Chromosome 18	10	6 (60%)	1 (10%)	3 (30%)
Chromosome 13	9	4 (44%)	4 (44%)	1 (11%)

Chorionic villus sampling is an invasive procedure, carrying a risk to the pregnancy. Jackson et al (1992) reported that the fetal loss rate for transabdominal- or transcervical-CVS was similar at approximately 2.4%. Smidt-Jensen et al (1992) reported that post-procedure unintentional fetal loss (up to 28 weeks') was 7.63% for transcervical CVS and 2.34% for transabdominal CVS which was similar to amniocentesis. They concluded that transabdominal CVS was the diagnostic technique of choice because of the success rate, it's safety and the earlier gestation at which it could be performed (compared to amniocentesis). It is certainly a more dignified approach than the transcervical technique and, for obstetricians, an easier one to learn as they are usually already familiar with transabdominal amniocentesis.

Amniotic membrane rupture and chorio-amnionitis are rare following CVS. Maternal serum α-fetoprotein rises post-CVS, implying that fetomaternal haemorrhage has occurred and anti-D immunoglobulin is indicated in rhesus-negative women. However, this iatrogenic

rise has usually fallen to normal by 16-18 weeks' gestation and this biochemical marker can still be used to screen for neural tube defects.

The association between CVS and the rare oromandibular-limb hypogenesis (OLH) syndrome (with characteristic hypoglossia and hypodactyli) and transverse limb reduction defects remains controversial. The incidence of OLH syndrome is 1 in 175,000 live births. Experimental evidence suggests that it is due to a vascular insult resulting in incomplete distal perfusion of an appendage, in early pregnancy. Firth et al (1991) reported five cases in 289 CVS procedures (or 1 in 58) between 56 and 66 days' gestation but Jackson et al (1991) found no such association. Miny et al (1991), in the same correspondence columns, reported an association between the severity of the lesion and the gestational age at CVS. They recommended that CVS should be performed only after 11 weeks' gestation. Firth et al (1994), in a follow-up analysis of their original paper reported 75 cases of limb reduction defects associated with CVS and also suggested the same inverse association with gestational age. Although the standard deviation was large, more severe lesions were associated with earlier sampling. From our personal data in the West of Scotland, there were 78,866 births and 47 reported LRDs though none was associated with CVS (Gaudoin and Mackenzie, 1997). Furthermore, in the World Health Organisation CVS Registry there were 48 cases in 80,051 births following CVS, an incidence very similar to our rate in the untested population. However, until this question is resolved it seems prudent to perform CVS as late as is possible whilst still obtaining results within the first trimester.

One possible solution to sampling as late in the first trimester as possible might be to use fluorescence in situ hybridisation with chromosome-specific probes. This will be discussed in Chapter 7.

Chorionic villus sampling is a valuable tool in the obstetrician's repertoire as it enables diagnoses to be made reliably in the first trimester. However, in contemplating invasive testing, the advantage of earlier diagnosis must be weighed against the slightly higher unintentional pregnancy loss when compared to second trimester amniocentesis, the

possibility of false positive diagnoses and the possibility of oromandibular-limb hypogenesis syndrome.

2.4 Cordocentesis (fetal blood sampling)

Cordocentesis involves needling the umbilical cord under ultrasound visualisation. Less than 17 weeks' gestation it is technically difficult with an associated 5% fetal loss rate though this improves as pregnancy advances (Orlandi et al, 1990). From 17 to 39 weeks' gestation overall losses, usually within 2 weeks of the procedure, are around 1.1%. The loss rate is greater with inadvertent arterial puncture and after intravascular transfusion (Weiner et al, 1991). Losses are dependent on the experience of the operator and the indication i.e. increasing if the fetus is already compromised and after intravascular transfusion (Nicolini and Rodeck, 1992).

Cordocentesis for rapid fetal karyotyping has been superseded largely by the increasing use of amniocentesis or CVS in later pregnancy but it may still be indicated in cases of known morphological abnormality or intrauterine growth restriction where genetic abnormality is suspected (Nicolaides et al, 1992). It is also indicated in circumstances such as fetal hydrops though these instances are rare.

It has provided enormous insight into fetal physiology and pathophysiology but, ironically, it has to some extent been a victim of its own success. Assessment of fetal acid-base status may still be an indication but the values of the various biochemical parameters measured are similar in those cases which survive compared with those that die in the perinatal period. Furthermore, the information provided by cordocentesis has validated non-invasive methods of fetal evaluation, such as the fetal biophysical profile and Doppler velocimetry, which have been shown to correlate equally well with adverse fetal outcome.

Therefore, because of the inherent dangers, the limited indications and the availability of other technologies, the role of cordocentesis as a diagnostic technique is diminishing (Fisk and Bower, 1993).

2.5 Diagnostic Ultrasound Scanning

Until the advent of ultrasound scanning (USS) the uterine contents hid most of its secrets. Ultrasound relies on the pulse-echo principle: a transducer emits a short pulse of ultrasound and the time taken for the echo to return to the same transducer is analysed. Depending on the tissue, the reflected signal is altered and a composite image of the object can be built up. The state-of-the-art machines give excellent images but are expensive and require highly trained operators. Importantly, fetal internal organ systems, such as the urinary tract, can be imaged.

Although acoustic energy passing through tissues is attenuated and dissipated as heat, to date there have been no confirmed reports of fetal damage. However, Newnham et al (1993) reported that infants who were intensively monitored by ultrasound in utero weighed 25g less than the control group. Furthermore, there were significantly more babies born in this group who weighed below both the 10^{th} and 3^{rd} percentiles.

In general, screening tests are not diagnostic but USS is peculiar as diagnoses of structural fetal anomalies are possible. If congenital malformations can be detected and the pregnancy terminated, one would expect the perinatal mortality rate (PNMR) to fall. However, studies on the effect of routine second trimester USS on the PNMR are conflicting. In a large study of almost 9000 patients, Chitty et al (1991) detected 40.9% of all structural abnormalities and, more importantly, 74.4% of all abnormalities were detected in those patients undergoing USS. In their study the PNMR was significantly lower in this group as termination of pregnancy was offered and performed in the majority. However, if aneuploidies are considered specifically, in the USS group only 3 of 23 cases were detected. Sixteen were undetected because there were no obvious anatomical markers and three with anatomical markers were not identified. Three with anatomical markers were detected but two refused karyotyping and in the other the association between fetal abnormality and an abnormal karyotype was not made. There were also two false positive diagnoses (a possible

tracheo-oesophageal fistula and another with possible cystic adenomatoid lung malformation).

In a similar sized study involving an unselected population in the mid-second trimester, the incidence of anomalies was 1.9% (including minor renal tract anomalies) and 85% of these were detected (Luck, 1992). There were two false positive results (specificity, 99.9%) but many abnormalities went undetected. Specifically, 64% of cardiac anomalies were missed as were 60% of diaphragmatic hernias and 40% of skeletal abnormalities. Cardiac and skeletal abnormalities in particular may be associated with fetal aneuploidy and USS alone would not have detected many cases.

Even in a highly selected group with a known structural abnormality, if there was one anatomical abnormality only 2% of cases had a chromosomal abnormality. In the presence of multiple abnormalities 29% showed a chromosomal abnormality (Nicolaides et al, 1992). Trisomy 18 is usually the most easily identified because of characteristic skeletal abnormalities. However, only 33-44% of Down's syndrome cases have a significant anatomical abnormality and these are less easily identified because the defects (particularly short bones and cardiac anomalies) are more subtle and interpretation that much more difficult (Nyberg et al, 1990). In their highly selected patients, Nicolaides et al (1992) detected respectively only 57.2%, 54.2% and 71.0% of trisomy 21, trisomy 18 and trisomy 13 cases less than 24 weeks' gestation.

In 1984 a report by the Royal College of Obstetricians and Gynaecologists concluded that routine USS for fetal abnormality was cost effective and this was endorsed by the Northern Regional Survey Steering Group (1992). It concluded that nearly half the total decline in PNMR over the period 1982-1990 was due to the increased antenatal detection, and subsequent termination, of pregnancies involving fetal anomalies. However, the multicentre RADIUS study (Routine Antenatal Diagnostic Imaging with Ultrasound), involving over 15,000 patients found no significant differences between the study and control groups in adverse outcome measurements (death or periventricular haemorrhage) whilst the rates of

preterm delivery and intrauterine growth restriction were very similar. Furthermore, only 17% of fetal anomalies were detected before 24 weeks' gestation (Ewigman et al, 1993).

A number of groups have reported the potential of fetal nuchal translucency (NT) as a first trimester screening test for fetal chromosomal abnormalities, particularly Down's syndrome. The actual pathophysiology remains undetermined but screening for this "marker" is extremely appealing. Pandya et al (1995) reported a prospective study of NT screening based at two district general hospitals and a co-ordinating tertiary referral centre. Scans were performed by trained ultrasonographers but importantly, implementation of the programme did not require an increase in staff or the purchase of extra (expensive) machines. Scans were performed at 10-13 weeks' gestation on 1763 fetuses (1673 singleton and 45 twin pregnancies) and the maximum subcutaneous nuchal translucency was measured. Nuchal translucency thickness increased with advancing gestation and overall 3.6% (63) demonstrated an NT thickness of greater than 2.5mm. Sixty-two women were offered CVS and 49 accepted. Of the 49 who underwent CVS, 45 showed a normal karyotype whilst 4 were abnormal: 3 cases of Down's syndrome and 1 case of Turner's syndrome (45, XO). The other 13 cases which did not have CVS performed went on to deliver phenotypically normal infants. Fifty-five women who had a negative first trimester screening ultrasound underwent definitive antenatal diagnostic testing (usually by amniocentesis), because of advanced maternal age or increased risk based on maternal serum biochemical screening. Of these, there was one case of trisomy 21 in a 42 year old woman. The authors found that there was a slight, but statistically insignificant, rise in fetal karyotyping rate over the study period.

If we consider only Down's syndrome, of 1763 fetuses there were 4 cases. Three might have been detected by increased NT thickness (sensitivity, 75%). Similarly, of the 63 positive scans, 3 were true positives (positive predictive value, 4.8%; false positive rate, 3.4%). Likewise, the specificity was 96.6% (1699/1759) with a false negative rate of 25% (1/4). Given that screening for Down's syndrome would involve large numbers of women, the numbers involved in this study are relatively small. That said, the data compare very

favourably with detection rates from maternal serum biochemical screening (chapter 2.8) which also requires invasive testing to confirm the suspicion of fetal aneuploidy. Furthermore, biochemical assays for Down's syndrome are not as reliable for twin pregnancies compared with singleton pregnancies.

Pandya et al (1995) reported that many older women "were reassured by the decreased risk for trisomies associated with nuchal translucency < 2.5mm and decided against invasive testing". However, until larger datasets have been evaluated, we must exercise caution in prematurely adopting such enthusiasm or, in the long term, we risk jeopardising the implementation of what is an extremely promising development in the field (Gaudoin and Mackenzie, 1997). Nevertheless, in time it may be possible to combine NT thickness with other screening modalities, such as biochemical assays, to improve the predictive value compared with each test being used alone.

Ultrasound machines and clinical expertise is costly. The structural lesion may be present at the time of the scan but the ultrasonographic signs may only develop later e.g. duodenal atresia. Moreover, the subsequent prognosis in some situations, such as choroid plexus cysts, can be uncertain and this presents a dilemma to the counsellor. Enormous anxiety can be generated with false positive diagnoses and, disastrously, even termination of pregnancy may occur. There are technical problems such as obesity and many karyotypic abnormalities have subtle or no anatomical markers so that false negative results inevitably occur. Diagnostic ultrasound is non-invasive and, as far as is known, harmless to the fetus. However, detection of fetal anomalies, particularly the early diagnosis of aneuploidies, is not as yet very reliable and although a normal USS is reassuring, it is clearly not infallible.

2.6 Coelocentesis

During the first 12 weeks' of intrauterine life the amniotic sac is surrounded by coelomic fluid in the extra-embryonic coelomic cavity, a derivative of the extra-embryonic mesoderm. Jurkovic et al (1993) reported sampling and cytogenetically analysing the cells from this bright yellow fluid. Sampling was performed at 6-12 weeks' gestation, immediately prior to termination of pregnancy and amniocentesis was performed simultaneously. The procedure was not associated with alteration in fetal heart rate and there was no evidence of fetal haemorrhage into the coelomic cavity based on ultrasound visualisation. Unfortunately, attempts at culturing coelomic fluid and obtaining metaphase preparations were wholly unsuccessful and only half of the amniotic fluid samples were successful. However, fluorescence in situ hybridisation and polymerase chain reaction successfully predicted fetal sex with 100% sensitivity and specificity. They suggested that, as the amniotic membranes remain intact, the risk of fetal trauma was minimal. Furthermore, because the coelomic cells are derived from the extra-embryonic mesoderm there may also be fewer problems with pseudomosaicism compared to CVS. Moreover, as it does not involve direct trauma to the definitive placenta, there is possibly less likelihood of limb reduction defects.

Findlay et al (1996) reported their experience with a fluorescent PCR technique to provide results within five hours of coelocentesis, including DNA fingerprinting to exclude maternal contamination. Although all sampling was performed prior to first trimester termination, gender prediction was very accurate ($p < 0.0004$) though the false positive rate was 20%. Makrydimas et al (1997) performed coelocentesis on four women at 7-9 weeks' gestation, prior to termination of pregnancy (for non-medical reasons). The four couples were known to be at risk of having a baby with β-thalassaemia and DNA analysis in their blood identified their individual mutations. Allele-specific polymerase chain reaction identified the mutations in the DNA extracted from the coelomic cells and placental tissue. Three fetuses were found to be carriers of either the paternal or maternal mutation, while one was found to be affected by β-thalassaemia.

Although the safety of this procedure is, as yet, undetermined, it offers an earlier and potentially safer method of antenatal diagnosis than present first trimester techniques.

2.7 Cervical sump sampling

Shettles (1971) postulated that the villi of the chorion laeve simply exfoliated in early pregnancy. He sampled the endocervical mucus, subjected the cells to quinacrine mustard staining and accurately predicted fetal gender in 100% of cases ($p<0.001$). Rhine et al (1975) blindly sampled the cells from behind the cervical mucous plug, the cervical sump and, using Y-body fluorescence predicted 18 males and 18 females. All 18 male predictions were correct though there were 5 false negatives i.e. 5 males in the group of predicted females. Five of the samples were in the first trimester and six in the second and for these trimesters gender prediction accuracy was not statistically significant. However, because most were performed in the third trimester, the overall results were highly significant ($p < 0.001$).

Similar work was performed by Griffiths-Jones et al (1992) taking samples from the lower uterine pole, prior to first trimester termination of pregnancy, using their Transcervical Cell Retrieval technique (TRACER). The cells were identified as syncitiotrophoblasts both morphologically and immunologically. Using PCR with a Y-chromosome repeat sequence they compared the result of the retrieved cells to that of the abortus. They correctly identified 8 of the 9 male fetuses whilst in another Y-positive sample culture of tissue from the abortus failed. However, the false positive rate was 6/16 though these were from vaginal and endocervical swabs rather than from the lower uterine pole. Overall their results were not significant and they suggested that contamination due to residual sperm from recent intercourse or contamination from a male technician were possible reasons for the high false positive rate. Bulmer et al (1995) also had variable recovery results when comparing different sampling techniques. They too identified trophoblast cells immunologically but found considerable antigenic heterogeneity in the cell populations, leading them to conclude

that a panel of mAbs directed against various villus and extravillus trophoblast populations would be necessary to improve the predictive value of the subsequent cytogenetic analyses.

Adinolfi et al (1993) flushed the lower uterine pole with sterile saline prior to first trimester termination of pregnancy. In an attempt to avoid the exquisite sensitivity and hence potential drawback of polymerase chain reaction, they subjected the recovered cells to fluorescence in situ hybridisation (FISH) and primed in situ DNA synthesis (PRINS) using Y-chromosome-specific sequences. In their study, gender prediction accuracy was 90.1% ($p<0.006$). However, the proportion of fetal-specific cells was very small and the range very large (2-33%), and there was considerable overlap between the percentage of cells bearing a Y-signal in male and female pregnancies. One case involved a trisomy 18 fetus that had been diagnosed previously by transcervical CVS and termination of pregnancy was about to be performed. The recovered cells were subjected to FISH using a chromosome 18-specific probe and 65% of cells appeared to be trisomic. Although encouraging, the proportion of fetal cells was much greater than with the other samples suggesting that the numbers may have been increased iatrogenically by the initial diagnostic technique (Gaudoin, 1993b).

Disappointingly, Bahado-Singh et al (1995) could only recover trophoblast cells in 50% of cases and cultures were successful in only 20%. Similarly, Adinolfi et al (1995) had variable results using chromosome-21-specific small tandem repeat sequences. Using this approach, Overton et al (1996) reported that although gender prediction accuracy was statistically significant using PCR or FISH, the high false positive rates, particularly in the PCR group, rendered cervical sump sampling unreliable for prenatal diagnosis.

More recently, Sherlock et al (1997) obtained first and second trimester samples prior to termination of pregnancy, morphologically identified these cells as trophoblasts, and correctly identified a variety of aneuploidies using FISH. Chang et al (1997) immunologically identified trophoblast cells in 12 of 14 cases and subjected these to FISH analysis with 100% gender prediction accuracy. Using a chromosome 13/21 α-satellite probe they also correctly identified a trisomy 21 conceptus. In the two remaining cases, FISH failed to identify a male

conceptus thus emphasising the importance of positive identification of fetal cells prior to cytogenetic analysis, in order to avoid a false-negative results. Importantly, some of the pregnancies were allowed to continue and, to date, no maternal or fetal complications have been reported. Encouragingly, Falcinelli et al (1998) reported that gestation did not influence transcervical cell recovery.

A potential criticism of these studies is that they were performed without the aid of ultrasound to determine the placental position and in many cases, particularly in early pregnancy, the placenta might have been biopsied directly. Furthermore, a recurring point is that positive identification prior to cytogenetic analysis is an absolute necessity to improve the predictive value of the test. However, the limited data on pregnancies which were allowed to continue suggests that further investigation and longitudinal studies of pregnancies should be encouraged as it may prove safer than the obvious comparative technique, chorionic villus sampling.

2.8 Maternal serum biochemical screening for Down's syndrome

Approximately 5% of pregnant women are more than 35 years old but give birth to only 30% of Down's syndrome babies. Therefore, maternal age alone is an inadequate indicator of trisomy risk (chapter 1). Furthermore, in effect, only 15% of Down's syndrome cases are detected as fewer than half these women undergo a definitive antenatal diagnostic procedure (Wald and Cuckle, 1987).

Although not a diagnostic test, maternal serum screening has contributed significantly to the antenatal detection of Down's syndrome and, therefore, discussion of antenatal diagnosis is incomplete without it's inclusion. Moreover, using appropriate algorithms, it may be used to screen for other aneuploidies as well.

In 1983 Davenport and Macri reported an association between low maternal serum α-fetoprotein (MSAFP) and late abortion. A year later Merkatz et al (1984) reported an association between very low MSAFP and fetal trisomy. They noted an undetectable

MSAFP in a woman who subsequently delivered a trisomy 18 fetus and retrospectively analysed 41 cases of autosomal trisomy from which serum was still available. In those cases MSAFP was significantly lower than that of 3862 controls. In their study, if only the autosomal trisomies were considered, then 17% had values less than 0.25 x MoM (multiples of the median), compared with 5.3% of the normal population and 28% had a MSAFP less than 0.4 x MoM, compared with 11.3% of the normal population. If MSAFP was used as the sole indicator for amniocentesis, 11% of the pregnant population would require amniocentesis to detect a similar proportion of aneuploid pregnancies as screening based on age alone and the authors suggested that a further discriminating marker was necessary.

The principles and application of maternal serum biochemical screening are excellently summarised by Reynolds and Penney (1989). For any variable parameter, there is a continuum of measurements which follow a Gaussian distribution. Provided the Gaussian distributions for a given parameter are known for both unaffected and Down's syndrome pregnancies, then the chance of a Down's syndrome-associated pregnancy is the ratio of the heights of the Gaussian distribution for affected and unaffected pregnancies. The screen need not be limited to biochemical data but may include other parameters such as maternal age and nuchal thickness. Using matrix algebra, any number of variables can determine the risk of a pregnancy being affected. In devising such algorithms (or "decision trees"), so called "decision limits" are set. For example, if the risk of a woman carrying a Down's syndrome fetus is 1 in 250, then she is offered amniocentesis. If the risk is less than this, say 1 in 300, then she is not. The limitations to the complexity of these algorithms are the inevitable trade-off in sensitivity, specificity and predictive values of the test and hence an acceptable balance must be struck.

A number of biochemical factors are altered in pregnancies bearing Down's syndrome fetuses. Because the cell cycle of trisomy 21 cases is longer than for normal karyotypes, they are physiologically different so that, for instance with trisomic fetuses, mean corpuscular volume is increased relative to gestational age (Sipes et al, 1991). Aneuploid fetal and

placental development is different from euploid pregnancies and one might therefore expect that the pattern of production of placental proteins is altered as well. However, why this difference exists remains undetermined.

In Down's syndrome, the geometric mean level of human chorionic gonadotrophin (hCG) is raised whilst AFP and unconjugated oestriol (uE_3) are decreased (Wald and Cuckle, 1992). Wald et al (1988) predicted that screening based on Age + hCG + AFP + uE_3 could detect 60% of Down's syndrome cases if 4.7% of pregnant women underwent amniocentesis. In their prospective study involving over 12,000 patients, Wald et al (1992) reported that 48% of Down's syndrome cases were detected with a 4.1% false positive rate, and 43 amniocenteses were performed to detect one case. Although not as good as their original prediction, it still represented considerable improvement when compared to age-based screening. They calculated that the cost of detecting a case of Down's syndrome was £38,000, considerably less than the life-time cost of care of a Down's syndrome patient.

Zeitune et al (1991) in a retrospective study attempted to identify trisomy 21, 13 and 18 simultaneously. They analysed 113,000 unaffected and 142 trisomy pregnancies and found a significantly reduced MSAFP. When this was combined with maternal age they would have detected 37% of affected pregnancies with a 6.6% false positive rate. However, closer analysis suggests that they would have detected only 12% of trisomies in the 25-29year age group compared with 71% in the 35-37year age group. In common with many algorithms, detection is weighted in favour of older women (Gaudoin, 1993c). Although the authors developed an algorithm which encompassed the three common autosomal trisomies simultaneously, it was not much better than using maternal age alone.

Palomaki et al (1992) in a prospective multivariate analysis, again using the combination of hCG, AFP and uE_3, specifically addressed the detection of trisomy 18 (Edward's syndrome) which is associated with reduced levels of all three biochemical parameters. Although 25% of Edward's syndrome show elevated AFP levels (due to a co-existing neural tube or ventral abdominal wall defect), and these were missed by their algorithm, they

should be detected by standard AFP protocols and detailed ultrasound scanning. They correctly detected 6 cases (85%) but, because of the small numbers in the study population, the 95% confidence interval for detection was 40-95%. They only required to perform 14 amniocenteses for every diagnosis, though again the 95% confidence limits were very broad (7-41 amniocenteses).

Accurate gestational dating by ultrasound can improve detection rates by almost 10% and a number of other biochemical markers might in the future be included in analyses (Wald and Cuckle, 1992). Maternal serum pregnancy-specific b-1 glycoprotein (SP1), human placental lactogen, progesterone and thyroid antibody levels are all raised in Down's syndrome pregnancies. Urea-resistant neutrophil alkaline phosphatase is raised and is an extremely effective marker for Down's syndrome. Unfortunately, it cannot yet be incorporated into clinical practice as it is labour intensive and currently requires subjective interpretation of neutrophil stippling (Wald and Cuckle, 1992).

Inhibin is a heterodimer with an α- and a β-subunit. The β-subunit may be either a βA- or a βB-subunit, (inhibin A and inhibin B, respectively). Aitken et al (1996) in a case-controlled study retrospectively evaluated an algorithm incorporating maternal age, AFP, intact HCG or free βHCG (FβHCG) and inhibin A from 528 pregnant women in the West of Scotland. Of these, 58 were Down's syndrome pregnancies, 32 were trisomy 18 samples and 438 were control samples. There was no difference between affected and unaffected pregnancies in the first trimester but after 13 weeks' gestation inhibin A levels in affected pregnancies were significantly greater. They estimated that they might detect 75% of Down's syndrome cases (60-87% confidence interval) whilst maintaining a 5% amniocentesis rate. This promising data will require large prospective studies to determine if inhibin A is as promising as this retrospective data suggests. If so, it will be a substantial step forward in second trimester prenatal serum screening.

Another approach is first trimester aneuploidy identification by biochemical markers that would allow confirmation by CVS and subsequent first trimester termination of pregnancy.

Kratzer et al (1991) designed an algorithm incorporating first trimester hCG, αhCG and progesterone. They developed an 'aneuploidy index' and estimated that they could have detected 9 of 17 Down's syndrome cases (53%) with only a 4.5% false positive rate. Unfortunately all similar studies are too small and a large prospective study is required.

Unfortunately, in the United Kingdom, particularly in the older pregnant population, invasive methods remain the first-line screening test (Mutton et al, 1993). However, using maternal serum screening in the second trimester, it might be possible to detect around 50-70% of Down's syndrome cases and it is clear that there is potential for improvement. The best combination of biochemical parameters has yet to be defined but inhibin A is a promising development. At present, screening is most discriminatory in the mid-second trimester but in time it may be possible to incorporate physical parameters, such as nuchal thickness, as well.

69% health boards now offer some kind of biochemical screening programme but problems remain. All have experienced difficulty in counselling and educating patients (and care-givers themselves) and, in particular, conveying the message that a negative result does not necessarily mean that the woman is not carrying a Down's syndrome baby. Furthermore, and not least, the biochemical suggestion of fetal aneuploidy requires an invasive procedure thereafter to confirm or refute the possible diagnosis.

2.9 Discussion

It is clear that each antenatal diagnostic method has its benefits and limitations. None is infallible and for definitive diagnoses, an invasive procedure is necessary which inevitably carries a risk to the pregnancy.

However, as the field has developed, there has been a trend to earlier and/ or less invasive methods. In particular, ultrasound in the mid-second trimester can detect many structural anomalies but is poor at detecting Down's syndrome. First trimester ultrasound has enormous potential and is being investigated further. Maternal serum screening has

improved the detection rate of Down's syndrome, particularly in women less than 35 years old. Unfortunately biochemical screening is not diagnostic and many invasive procedures are still required to detect an affected pregnancy.

With this background knowledge it is easy to appreciate why the detection and cytogenetic analysis of fetal cells from maternal blood is such an appealing concept.

Chapter 3. Isolation of Fetal Cells from Maternal Blood

3.1 Introduction

In 1893 Schmorl, described multinucleated syncitiotrophoblast cells in the pulmonary vasculature of women who had died of eclampsia but it was only in 1969 that Walknowska et al proposed that fetal cells isolated from maternal blood might be used for prenatal diagnostic purposes. Such a suggestion, however, was only possible because of recent developments and better understanding of cell biology. In particular, it was possible to culture human leukocytes and arrest mitosis in metaphase and Levan (1956) demonstrated that human diploid cells possessed 46 chromosomes. Three years later Lejeune et al (1959) showed that Down's syndrome was due to an extra small acrocentric chromosome (now called chromosome 21).

Since Walknowska's original idea, enthusiasm for the possibility of cytogenetically analysing fetal cells from maternal blood has waxed and waned as encouraging results have been tempered by unlikely reports. However, in recent years intense interest has once more been rekindled, largely because of further scientific progress. Fluorescence activated cell sorting and, more recently, immunomagnetics have furthered cell enrichment possibilities whilst fluorescence in situ hybridisation and polymerase chain reaction have made possible more detailed genomic analysis. The principles and application of these techniques are explained at this juncture as constant referral is made to them in much of the following text.

3.1.1 Fluorescence activated cell sorting

The use and capabilities of fluorescence activated cell sorting (FACS or flow sorting), are excellently summarised by Parks and Herzenberg (1982), the latter of whom is one of the pioneers in this field.

Cells are labelled with a monoclonal antibody (mAb) with an attached fluorescent marker. As the cells pass across a laser beam the fluorescent molecules are excited and the emitted light is collected by a photomultiplier system and converted into an electrical signal. When a

cell is detected which fulfils the necessary conditions in light scatter and fluorescence, droplets containing that cell are electrically charged. The droplets pass through an electric field which draws charged droplets out of the stream and these are collected as sorted samples. FACS can be applied to any cell which can be obtained in single-cell suspension and can use any fluorescent label capable of being excited by laser light. Moreover, different mAbs reacting to different antigens on the same cell may be used simultaneously. Each type of mAb may be labelled with a different fluorochrome so that cells may be sorted with greater discrimination. In addition, cells can be differentiated by their light scattering properties based on their size and cytoplasmic characteristics. Such multi-parameter flow sorting allows highly specific subsets of cells to be collected whilst other cells, which do not fulfil the necessary criteria, are said to be gated out. In this way FACS has allowed rare cells to be separated accurately from cells of no interest.

The sensitivity of the technique is dependent on the excitation efficiency of the marker and the background fluorescence of unstained cells. For markers such as fluorescein, cells with only 10,000-20,000 dye molecules can be distinguished and currently the number of markers is limited by the excitation and detection systems available. The increasing availability of fluorochromes suitable for use with solid-state lasers heralds the next phase in this technology by allowing greater numbers of parameters to be gated so that the selection may be even more specific (Battye and Shortman, 1991).

Although the viability and functional capacity of cells may be affected by FACS, it is possible to use polymerase chain reaction and fluorescence in situ hybridisation to investigate fetal cells isolated by this method from maternal blood (vide infra).

Although FACS is the gold standard and reference point for all other cell separation procedures, it has limitations. Failure to detect a particular antigen does not necessarily imply absence of the antigen. For instance, minor glycosylation in the case of carbohydrate moieties might render the antigen 'hidden' and there is sometimes arbitrary distinction between negative and positive cells. If cells are too close together in suspension then rare positive cells may be missed and yields from FACS can be variable (Sieff et al, 1982).

Moreover, the capacity of FACS to handle large numbers of cells is limited. Basic machines can only sort at a rate of 2000-3000 cells/ second though more recent flow sorters are claimed to sort at 10,000 cells/ second. However, this is relatively slow if one considers that in 20ml of blood there are more than 10^{10} cells. Furthermore, usually about 0.1-1% of all the sorted cells are collected and considerable numbers of cells may need to be collected. Hence, the ability of FACS to detect rare cells is limited by the specificity of labelling and the time to sort for them. Furthermore, FACS machines are extremely expensive, requiring considerable expertise and technical back-up to be maintained (Manyonda et al, 1992).

If the isolation and cytogenetic analysis of fetal cells from maternal blood is to become a screening tool, applicable to all early pregnancies, the necessary enrichment techniques must be robust and relatively inexpensive and FACS probably does not fulfil these criteria. Although these limitations are recognised, it must be acknowledged that much of the recent groundwork and advances in the field of fetal cell retrieval remains dependent on FACS.

3.1.2 Advances in Molecular Biology

One of the major issues is to confirm that cells isolated from maternal blood are actually fetal and the majority of published data involves identifying male DNA (the Y-chromosome) to demonstrate this.

3.1.2.1 Quinacrine Mustard (QM) staining

The heterochromatin q12 region of the Y-chromosome can be stained with quinacrine mustard (QM) and is seen as a fluorescent spot when viewed appropriately. Unfortunately, QM autosome fluorescence is seen on chromosome 3 and chromosome 13 so there is a high false-positive rate. There is inter-male variability in the size of the Y-chromosome heterochromatin and, as with many techniques, the method is subject to the vagaries of inadequate sample preparation and staining conditions (Jonasson, 1986). Kirsch-Volders et al (1980) found that in 20% of women, 0.02% of cells were QM-positive. All males showed

positive cells though only 55.7 ± 4.0% of all male nuclei were positive. Clearly, QM -staining is not an ideal method for accurate sex determination.

3.1.2.2 Fluorescence in situ hybridisation (FISH)

This will be described in greater detail in Chapter 7 but suffice to say at this stage that FISH involves hybridising a probe which is complementary to nucleic acid sequences (whether DNA or RNA) in the cell. The probe is labelled with a reporter molecule that allows subsequent visualisation by immunological development in much the same way as immunocytochemistry. Using chromosome-specific probes, the number of signals within the nucleus reflects the number of copies of that chromosome in the nucleus.

3.1.2.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) involves amplification of specific DNA sequences so that, for instance, Y-chromosome-specific DNA from male fetal cells in maternal blood might be detected. PCR uses two oligonucleotide primers which are complementary to a specific sequence at the ends of a stretch of DNA. The DNA and primers are heat denatured and allowed to reanneal with primers hybridising to opposite strands of DNA, one on either side of the target sequence to be amplified. The primers allow DNA polymerase to start working only at these specific sites. The DNA polymerase adds nucleotide primers in a 5' to 3' direction so that extension progresses along the strands of the primers in opposite directions (figure 3.1). In theory each round of amplification doubles the amount of DNA so that amplification is exponential rather than simply linear. In practice PCR is about 85% efficient after 20 cycles. In order to detect the DNA sequence, the PCR products may be digested with restriction enzymes and the DNA fragments subjected to agarose gel electrophoresis with ethidium bromide added to the gel to intercalate with the DNA fragments. Smaller fragments move faster through the gel and their sizes can be compared to known standard DNA fragments by visualising the ethidium bromide (and hence the DNA fragments) under ultra-violet light.

PCR may be used on single cells (Li et al, 1988) but the corollary of such exquisite sensitivity is the increased likelihood of false positive results. Therefore, as with all molecular biological techniques, it is important to have both positive and negative controls and strict adherence to protocols.

It can be appreciated that non-specific binding occurring early in the PCR process will be amplified to a much greater extent than non-specific binding occurring later, giving a stronger false-positive signal. This may be limited by "nesting". Nested PCR involves amplifying a sequence of DNA using "external" primers. PCR is performed again using another set of "internal" primers within the original primers. This is advantageous as the initial pair of primers might be similar to other sequences in the genome which, too, will be amplified. Therefore if there are relatively few target cells e.g. fetal cells in maternal blood, the fetal sequence will be swamped by amplification of maternal sequences. In fact, most sequences between mother and fetus will be common and only certain regions of the Y-chromosome are unique. However, by amplifying the sequence within the original sequence, non-specific (maternal) sequences will not be amplified any further (figure 3.2).

Nested PCR provides greater amplification potential and higher specificity. Even so, if there is any contamination from extraneous sources, e.g. from a male technician, because of the exquisite sensitivity this will be amplified and false positive results may still be obtained.

It is unlikely that a pure population of fetal cells will ever be obtained from maternal blood. Therefore, maternal DNA will always be in excess. If we consider cells from a Down's syndrome fetus in maternal blood, the difference between the normal maternal constitution and the fetal constitution is simply one of dosage - 2:3 copies of chromosome 21. Using PCR, it would be impossible to diagnose Down's syndrome because the slightly elevated chromosome 21 dosage could not be demonstrated. However, if fetal cells can be identified positively and a pure cell population obtained, they may be subjected to quantitative PCR which might overcome this problem (chapter 10).

Figure 3.1
Schematic representation of Polymerase Chain Reaction

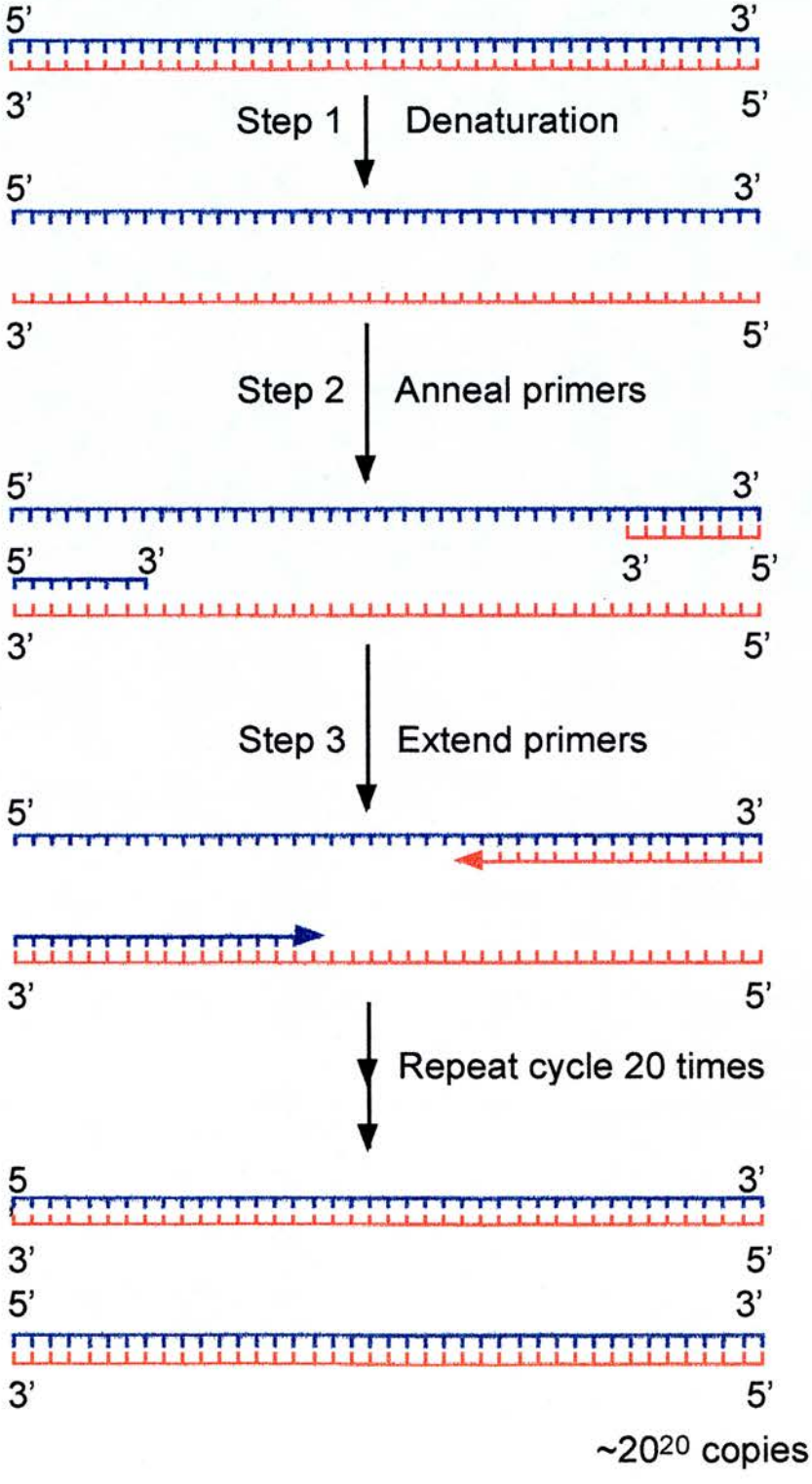
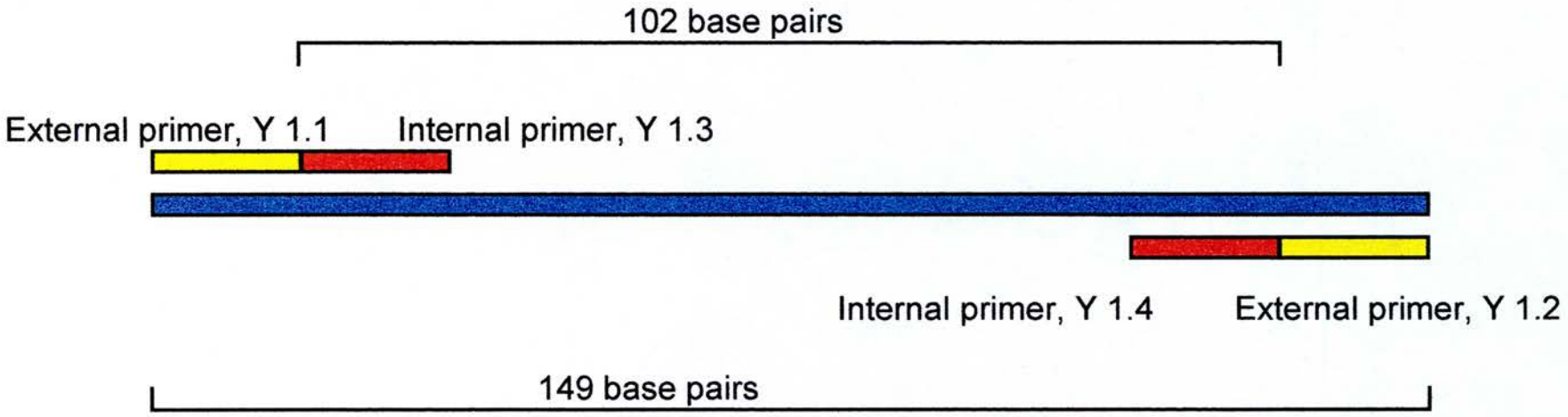


Figure 3.2 Nested PCR



(from Lo et al, 1989)

3.1.3 Statistical analyses

In the published data, most analyses have determined the probability of sexing a series of pregnancies based on the presence of Y-chromosomal DNA. Assuming that a fetus is as likely to be male as female, the chance of correctly assigning fetal sex is 0.5 in any one case and the chance of incorrectly assigning sex is also 0.5.

The following formula was used in all of the cited studies to determine the statistical significance of gender prediction where "N" is the total number of patients in the series, and "n" is the number of cases where fetal sex was wrongly assigned.

$$p = \frac{N!\,(0.5)N}{(N-0)!0!} + \frac{N!\,(0.5)N}{(N-1)!1!} + \frac{N!\,(0.5)N}{(N-2)!2!} + \ldots\ldots\ldots + \frac{N!\,(0.5)N}{(N-n)!n!}$$

The equations below have also been used to determine the sensitivity, specificity and other statistical parameters for prospective gender prediction. The 'Test result' was the sex assigned by the test so that, for instance, 'a' was the total number of cases in which the test result suggested a male and at birth the babies were male.

	Sex at delivery	
Test result	**Male**	**Female**
Male	a	b
Female	c	d

$$\text{Sensitivity} = \frac{\text{Total number of true positives in the test}}{\text{Total number of all positives in the population}} = \frac{a}{(a+c)}$$

$$\text{Specificity} = \frac{\text{Total number of true negatives in the test}}{\text{Total number of all negatives in the population}} = \frac{d}{(b+d)}$$

$$\text{Positive predictive Value} = \frac{\text{Number of true positives in the test}}{\text{Total number of positives seen in the test}} = \frac{a}{(a+b)}$$

$$\text{Negative predictive value} = \frac{\text{Total number of true negatives in the test}}{\text{Total number of negatives seen in the test}} = \frac{d}{(c+d)}$$

False positive rate =

$$\frac{\text{Number of false positives}}{\text{Total number of negatives in the population}} = \frac{b}{(b+d)} = 1 - \text{specificity}$$

False negative rate =

$$\frac{\text{Number of false negatives}}{\text{Total number of positives in the population}} = \frac{c}{(a+c)} = 1 - \text{sensitivity}$$

Finally, before considering the approaches used by different groups to enrich for fetal cells from maternal blood, it is important to consider the scale of the task.

3.1.4 The scale of the problem

Parks and Herzenberg (1982) cannulated placental blood vessels in Rhesus monkeys and transfused radiolabelled erythrocytes into the fetus. These could be topped up via the cannula. Once confident that continuing transplacental haemorrhage was not iatrogenic they estimated the daily transplacental haemorrhage of fetal blood to be 0-2µl. Eventually a steady state developed where cells were entering the maternal circulation at the same rate as they were removed. The most recent sensible estimates suggest there is perhaps 0.04-0.2µl of fetal blood in 20ml of maternal blood (Hamada et al, 1993). Table 3.1 shows that even if this higher figure is accurate, in 20ml of maternal blood at 15 weeks' gestation there might only be 420 fetal erythroblasts (0.2µl x 2.1 $x10^9$ erythroblasts/l) and 270 fetal lymphocytes. In 20ml of maternal blood there are 10^{10} cells, or one nucleated fetal cell for every 10^8 nucleated maternal cells. Therefore, looking for fetal cells in maternal blood is like looking for the proverbial needle in the haystack.

Table 3.1. Mean fetal erythrocyte and leukocyte values (Millar et al, 1985)

Weeks' gestation	15	16	17	18	19	20	21
Total erythrocytes ($x10^{12}$/l)	2.43	2.68	2.74	2.77	2.92	3.12	3.07
Mean cell volume (fl)	143	143	137	135	129	126	123
Erythroblasts ($x10^9$/l)	2.1	3.6	2.5	2.2	1.7	1.2	0.8
Total leukocytes ($x10^9$/l)	1.6	2.4	2.0	2.4	2.5	2.6	2.7
Neutrophils ($x10^6$/l)	113	198	127	161	134	129	192
Lymphocytes ($x10^9$/l)	1.36	2.17	1.70	2.12	2.16	2.33	2.33

Note the relative reduction in erythroblast concentration with increasing gestation compared to leukocytes.

Knowing the enormity of the task, the possibility of isolating fetal cells from maternal blood can be considered. Importantly, when reviewing the published studies it must be borne in mind that there is a tendency not to publish negative findings and to report only successful studies. Consequently, optimistic reports must be viewed with a degree of caution.

For simplicity the historical evidence will be considered according to cell type and in a chronological fashion. Although each is described in isolation, different groups have been working on enrichment of different cell types simultaneously. Throughout the narrative, when a certain number of "weeks' gestation" is quoted, it implies the number of weeks of amenorrhoea.

The large number of published studies by necessity compels omission of some and inclusion of others and studies on whole maternal blood will be discussed first.

3.2 Analysis of fetal cells from whole maternal blood

A number of groups have attempted to identify and analyse nucleated fetal cells from whole maternal blood with minimal enrichment (initial removal of only erythrocytes). Therefore, any type of nucleated fetal cell might be analysed.

Zilliacus et al (1975) searched for Y-chromatin-positive interphase cells based on QM staining. They found that 0.02 - 0.16% of all lymphocytes appeared to be positive which suggested that there were 10^3-10^4 maternal leukocytes for every nucleated fetal cell. They

correctly identified 7 of the 11 male fetuses with one false positive result ($p = 0.048$). They cultured the cells but could not identify any XY mitoses and could not find any non-nucleated fetal erythrocytes although they expected to find considerably more of these cells compared to nucleated cells. They concluded that if these cells were lymphocytes then they crossed the placenta by some form of active and selective transport rather than by haemorrhage. However, alternative explanations are that insufficient metaphase preparations were examined, particularly as fetal cells are now thought to be rarer than they assumed or, more likely, that the apparently high proportion of cells with a QM signal was due to false-positive autosomal fluorescence associated with QM staining.

Using direct blood smears and QM staining, Siebers et al (1975) reported on one hundred pregnant women none of whom had given birth to a male less than two years previously. In early pregnancy they found large nucleated cells which they assumed to be lymphoblasts and 77.8% of male-bearing pregnancies were Y-chromatin-positive by 12 weeks' gestation. They suggested that the proportion of fetal cells did not increase significantly with advancing gestation though closer scrutiny suggests that analyses were more reliable in the second and third trimesters. Twenty-four cases involved first trimester pregnancies and sex prediction was 75% accurate ($p < 0.02$). However, from the results of the second and third trimester cases, sex prediction was 96.7% accurate ($p < 0.0001$). A mean of 3.75% of lymphocyte-like cells were Y-chromatin positive and although the overall results were highly significant, the absolute numbers of apparent fetal cells was unlikely. Such a large figure would represent about 40ml of fetal blood in the maternal circulation and again, a more likely explanation is the false positive results associated with QM staining.

Because of such unlikely data, particularly as the only analytical technique available at that time was fraught with a high false positive rate, the interest in fetal cells in maternal blood waned. However, it was reawakened by the development of polymerase chain reaction (PCR).

In 1989 Lo et al analysed 19 pregnancies using nested PCR, amplifying a region of the DYZ1 locus of the distal long arm of the Y-chromosome. In none of the seven female-

bearing pregnancies was a Y-specific sequence identified but in all 12 cases involving male-bearing pregnancies a band was detected ($p < 0.0001$). They found that at least 15 cycles were required using the internal primers in late pregnancy whilst 20 cycles were required less than 11 weeks' gestation. However, beyond 20 cycles false-positive results were consistently obtained and in three non-pregnant female controls they had one false positive result. Although women performed all experimentation, they conceded that "nesting" was liable to detect even the tiniest amount of contaminating material and produce false-positive results. An alternative explanation for the false-positive results is that a 323 bp segment, which spanned the segment flanked by the external primers used is present in 10% of women (Nakagome et al, 1990).

Lo et al (1990) then reported the nested technique with primers which were purportedly more Y-specific. In 12 cases from 6-11 weeks' gestation, fetal gender prediction was 83.3% accurate ($p < 0.04$). In all 27 cases, up to term, gender prediction was 77.8% accurate ($p < 0.003$). They failed to generate Y-sequences in 4 cases involving a male fetus, possibly because there were very few or no circulating male fetal cells, and suggested that this may be overcome by multiple sampling of women. However, logistically this may not be practical and what is probably better is to determine the optimum time in early pregnancy when sampling is likely to be most rewarding. Furthermore, although their primers were purportedly more Y-specific, their false positive rate of 20% again demonstrated the extreme sensitivity of PCR to potentially extraneous DNA.

Haemoglobin Lepore-Boston is a haemoglobinopathy caused by a hybrid $\delta\beta$-gene. In 1990, Camaschella et al took blood from three mothers known to be carriers of β-thalassaemia, who had partners known to be carriers of the haemoglobin Lepore-Boston trait, and subjected it to gene sequence-specific PCR. Both cases of the condition were correctly identified at 8-10 weeks' gestation with results confirmed by conventional invasive methods. This was a major landmark as, for the first time, a genetic disease was diagnosed prenatally by non-invasive means.

However, the following year Nakagome et al (1991) reported their study of 18 patients using nested PCR. From control experiments they detected one male cell in 25,000 female cells. However, in none of the 8 cases involving a male fetus could they obtain a male-specific sequence, suggesting a much lower ratio of fetal to maternal cells. Suzumori et al (1992) investigated 100 women prenatally and 30 women immediately postpartum (15 males, 15 females). Using the original nested primers of Lo et al (1989) they correctly predicted the sex in 30 of 45 males whilst 10 of the 55 female-bearing pregnancies were also positive. Overall the gender prediction accuracy was 75% ($p < 0.0001$). In the postnatal studies, all the women who gave birth to males were PCR-positive, suggesting a greater volume of fetal blood in the maternal circulation following delivery. However, positive results were also obtained in 7 out of the 15 women who gave birth to females and the authors suggested that contamination from male workers and the previous birth of a male child might account for this. However, the proportion of false positive results that could be attributed to this latter factor (2 of the 10 antenatal patients and 3 of the 7 postnatal women) was small when compared to the total number of false positive results. The other possible explanation was the homology of the DYZ1 region with autosomal sequences which is only detectable with dual amplification (Nakagome et al, 1990). Whatever the reason, it emphasised the susceptibility of nesting to false positive results and better primers would be required if such an approach was to be applicable clinically.

In 1993 Hamada et al subjected the mononuclear cells from 50 primigravidae to FISH and nested PCR. Because they were mononuclear, any fetal cells were unlikely to be syncitiotrophoblasts or polymorphs but may have been cytotrophoblasts, lymphocytes or erythroblasts. By 15 weeks' gestation XY interphase cells were identified in all male pregnancies and correlated with PCR though FISH had greater specificity as it was less prone to false positive results. However, prior to 15 weeks' gestation, only two male (XY interphase) cells were found in the eight women carrying male fetuses and two positive cells were found in one of the 15 primigravidae carrying a female fetus.

In the same year Langlois and Wilson (1993) used nested PCR on whole maternal blood in 27 pregnancies at 10-11 weeks' gestation but gender prediction accuracy was poor (59%, $p > 0.05$). Liou et al (1993) investigated 31 pregnancies at 8-14 weeks' gestation again with nested PCR and they achieved 100% concordance in sex prediction ($p < 0.0001$). They first identified male DNA at 8 weeks' gestation and by 13 weeks' gestation they had demonstrated a Y-specific sequence in 95% of the male pregnancies.

Lo et al (1993) used PCR to amplify for the Rhesus D gene in Rhesus-negative women. They accurately predicted the fetal Rhesus status in 16 of 21 pregnancies ($p < 0.02$) and showed that fetal genotype implicated in fetal diseases could be demonstrated in maternal blood.

Thomas et al (1994), using nested PCR, accurately identified the fetal sex in all 5 in vitro fertilisation pregnancies before 6 weeks' gestation (less than 27 days' post-embryo transfer) which is probably the earliest demonstration of fetal DNA in maternal blood. This study continued to a total of 30 patients and, astonishingly, gender prediction from whole blood was 100% accurate ($p < 0.0001$). No male DNA could be detected eight weeks postnatally suggesting that regardless of the cell type analysed, there did not appear to be any persistence of fetal DNA which might confuse results in subsequent pregnancies (Thomas et al, 1995).

Smid et al (1997) employed nested PCR with the Y-specific sequence, DYS14, in 27 women taking samples from each trimester and their data suggested that the concentration of fetal cells in maternal blood was highest in the first and third trimesters. Although quite a small study, it was encouraging because the subsequent management of a complicated pregnancy is most easily planned from an early stage.

Bianchi et al (1997) took blood from 199 women carrying chromosomally normal fetuses and from 31 women with a male aneuploid fetus and subjected the blood to quantitative PCR with Y-chromosome-specific primers. Although there were false-positive DNA products detected in pregnancies bearing a female fetus there were quantitatively more products in male-bearing pregnancies ($p < 0.01$). Clearly this was neither a prospective nor blinded study

but significantly more fetal cells were detected in maternal blood when the fetus was aneuploid. This may have been because the invasive procedure used to initially diagnose the abnormality had increased the deportation of fetal cells in to the maternal circulation (Jansen et al, 1997) or that fetomaternal transfusion is increased because placentation is abnormal when the fetal karyotype is abnormal (see also chapter 7).

These studies suggest that fetal DNA sequences (usually male) may be found in maternal blood in early pregnancy which might be useful in X-linked recessive conditions or in cases where a gene is carried by the fetus but not the mother. However, the inability of PCR to detect fetal aneuploidies in cells from whole maternal blood is a significant limitation to its application. Furthermore, using PCR with the same primers, different groups have arrived at entirely different results, suggesting significant variation in fetomaternal transfusion and also highlighting the methodological problems associated with analysis of rare cells.

When the published studies are considered together, the large range of sensitivities (0-100%) without any preselection method underlines the need for an enrichment step. However, to achieve this, the enrichment technique must be reliable and probably enrich for a particular cell type to the exclusion of other potential nucleated fetal cells. The possibilities lie with fetal leukocytes, trophoblast cells or erythroblasts and each will be considered in turn.

3.3 Lymphocytes

Walknowska et al (1969) cultured maternal blood using the lymphocyte mitogen, phytohaemmaglutinin (PHA), and reported that 0-1.5% of the metaphase preparations showed 5 small acrocentric chromosomes. They presumed these corresponded to the two copies of chromosomes 21 and 22 and a Y-chromosome and of the 21 women who demonstrated this phenomenon, 19 gave birth to boys ($p < 0.0002$).

Because the cells resembled lymphocytes and responded to PHA, and normal placentae do not contain lymphoid cells, they suggested that they were fetal blood cells which found their way into maternal blood. If haemorrhage was the entry mechanism, the proportion of

putative fetal lymphocytes would be equivalent to more than 100ml of fetal blood. They did not believe that such a large volume could have resulted from a single episode of bleeding and suggested that small, recurrent bleeds were more likely. The alternative, or additional, explanation was that lymphocytes migrated through vascular endothelium from fetus to mother whilst other fetal blood cells could not breach this interface. They went on to suggest that the proportion of fetal lymphocytes was so high because they may be more sensitive to PHA, favouring their presence as mitotic cells over maternal lymphocytes. Furthermore, fetal lymphocytes might survive for longer in the maternal circulation so that a small but chronic transfer resulted in large numbers in maternal blood. Hence, the absolute volume of fetal blood in the maternal circulation might be small and yet the proportion of fetal lymphocytes could be quite high. Moreover, they suggested that lymphocyte transfer might actually be necessary for successful pregnancy outcome. As lymphocytes express HLA antigens, their escape into the maternal circulation may have some bearing on the mother accepting the fetus as "self".

The authors attributed the two false positive results to artefacts such as a broken chromosome but conceded that the persistence of XY cells from previous pregnancies due to fetal-maternal chimerism was a possibility. Their explanation of the three false negative results was that very few cells were cultured in these cases and so finding fetal metaphase spreads was less likely. However, closer analysis suggests that in these three cases a mean of 590 metaphase spreads were examined (range, 470 - 700) compared to 440 metaphase preparations overall (range, 192 - 900). If the "male" spreads were truly fetal, it is more likely that either the ratio was less than one in 900 or that there were no fetal cells in the original negative blood samples.

Schröder et al (1977), again using quinacrine staining on cultured lymphocytes in metaphase failed to predict fetal sex with any accuracy and concluded that fetal lymphocytes, if present, were unresponsive to the commonly used mitogens. However, they also suggested that the interphase cells which had been analysed by other groups might have been placental cells and not lymphocytes.

As long ago as 1962 Payne had established that HLA antigens were expressed on fetal lymphocytes from the twelfth gestational week. In 1979 Leonard Herzenberg and colleagues used fluorescence activated cell sorting (FACS) to enrich for HLA.A2-positive lymphocytes from HLA.A2-negative pregnant women, known to be carrying a male fetus (from prior amniocentesis). In 5 cases they found 2-7 cells which were quinacrine-positive and neonatal studies showed all five babies to be HLA.A2-positive. In the other cases no Y-chromatin cells were identified and all seven babies were HLA.A2-negative ($p < 0.0003$).

Kirsch-Volders et al (1980) cultured lymphocytes from 18 women from 6 weeks' gestation to term and using QM staining they found that 0.04-0.23% of peripheral blood mononuclear cells were positive, representing about 10ml of fetal blood. From metaphase preparations gender prediction was 89% accurate ($p < 0.004$). The first true positive Y-chromatin cell was at 10 weeks' gestation though, in fact, both false positive results were from cells at earlier gestations. They pointed out that this approach might only be practical in primigravidae because of lymphocyte longevity and the possibility of persistent fetomaternal chimerism (Ciaranfi et al, 1977). Importantly, they also emphasised that it was impossible to differentiate between a mother and her female fetus.

The group headed by Leonard Herzenberg then reported a further, much larger and complex study (Iverson et al, 1981). Of 108 women, 48 were HLA.A2-negative and known to be carrying a male fetus. Twenty-one of these had HLA.A2-positive partners. 20ml of maternal blood was flow sorted using an anti-HLA.A2 mAb and in all eight cases where the neonate was subsequently found to be HLA.A2-positive, between 1-7 Y-chromatin-positive cells were identified. The first positive cells were recovered at 15 weeks' gestation. In 1 case where the baby was HLA.A2-negative, they found two Y-chromatin-positive cells whilst in none of the other 12 cases (HLA.A2-negative male newborns) did they find any positive cells. In no instance where both parents were HLA.A2-negative did they find any positive cells. The remaining 19 HLA.A2-negative women gave birth to girls and in only one case were any Y-chromatin-positive cells found. Although this baby was HLA.A2-positive, quinacrine staining of her cells did not show any positive interphase signals. Of these

women, 13 had given birth to boys in the past. The authors concluded that fetal lymphocytes did not persist for any length of time in maternal blood and previous reports of this kind were probably due to autosomal fluorescence associated with quinacrine staining. These results were highly significant ($p < 0.0001$) and they estimated that there was 1 fetal lymphocyte to 1000-5000 maternal lymphocytes which was compatible with realistic minor, chronic transplacental haemorrhages. Although the sorted cells resembled lymphocytes they suggested that they could also be fetal haematopoietic progenitors as these also express HLA antigens.

Selypes and Lorencz (1988) then reported a novel enrichment technique. They subjected blood to conventional culture techniques and also to their "air culturing" technique. Using QM staining, sex prediction was 84.8% accurate ($p < 0.0001$). Based on the ratio of Y-chromatin-positive cells to maternal cells, they found "air culturing" to be superior ($p < 0.01$) and suggested that this technique preferentially cultured fetal cells. To date no-one has confirmed the improved results using "air" culturing, and an open mind must be kept about its applicability.

Tharapel et al (1993) enriched for fetal lymphocytes using FACS based on HLA differences between pregnant women and their partners. Using standard lymphocyte culture techniques they could not find any XY metaphase spreads in any male pregnancy and attempted to explain these negative findings in a number of ways. The numbers of fetal cells in maternal blood may vary depending on the cell type and gestational age so that at particular gestations it might be appropriate to enrich for particular cell types. Alternatively, fetal lymphocytes may be unresponsive to the standard mitogens. Using FACS they found that up to 2% of maternal cells which were negative for the mAbs used actually bound the antibody so that the relative enrichment of fetal lymphocytes was minimal. They only examined a mean of 135 metaphase preparations per sample (range, 0 - 613) and, given that fetal cells are very rare and enrichment was minimal, insufficient numbers of metaphase preparations were probably examined. Of course how fetal lymphocytes in maternal blood respond to the usual mitogens is unknown but we obtained umbilical cord blood from a

second trimester Down's syndrome fetus at termination of pregnancy and cultured the cells. We confirmed the 47 XX, +21 karyotype and, from our own first hand experience, it would seem that fetal lymphocytes in the second trimester are responsive to phytohaemmaglutinin (unpublished data). Tharapel et al's final point was that fetal cells in the maternal circulation may not be lymphocytes but erythroblasts. Fetal erythroblasts express HLA antigens in low density and if present might have been enriched by their technique. However, they probably do not respond to lymphocyte mitogens and they did not report interphase cytogenetic analysis.

It is apparent that the data regarding fetal "lymphocyte" interphase or metaphase cytogenetic analysis is conflicting. Quinacrine staining suggested implausibly large numbers of fetal lymphocytes in maternal blood but this may be due to its poor specificity. Moreover, the possibility that fetal lymphocytes persisted from previous pregnancies might confuse karyotyping. Morphologically fetal lymphocytes are indistinguishable from maternal lymphocytes and detailed HLA typing is probably impractical as a routine screening procedure for all pregnant women. Finally, lymphocyte surface antigens have never unequivocally identified the cell types actually analysed in these studies and some have suggested that they might be haematopoietic progenitors. For these reasons the isolation of fetal lymphocytes from maternal blood is probably not a practical solution to the problem.

3.4 Granulocytes

To date there has been only one report investigating fetal granulocytes in maternal blood (Wessman et al, 1992). Erythrocyte-depleted blood was subjected to absorption in situ hybridisation using a Y-chromosome probe and visualised with diaminobenzidine tetrachloride and overall, gender prediction accuracy was 90.9% ($p < 0.01$). In this study $0.13 \pm 0.08\%$ of polymorphs showed a Y signal and the authors proposed polymorphs as a fetal cell type suitable for enrichment because they have a short half-life so that persistence to a subsequent pregnancy is unlikely.

However, closer analysis is required. If we consider the data from Table 3.1, the estimated number of fetal granulocytes in the maternal circulation represented over 150ml of fetal blood. This is highly improbable. There is no evidence that polymorphs are actively transported across the placenta and the most likely explanation is the high false positive rate associated with absorption in situ hybridisation compared to fluorescence in situ hybridisation (Jonasson, 1986). This study was small and has yet to be corroborated by others. Furthermore, it would be impossible to differentiate fetal from adult granulocytes and, at present, enrichment of fetal granulocytes from maternal blood is not a realistic prospect.

3.5 Trophoblast cells

Trophoblast cells are in direct contact with maternal blood and, therefore, no breach in the fetomaternal interface is necessary for these cells to enter the maternal peripheral circulation.

In 1893 Schmorl described trophoblast cells in the lungs of women who had died of eclampsia, now a well-documented phenomenon (figure 3.3). However, trophoblast deportation may be a physiological phenomenon and possibly a prerequisite for successful pregnancy. As trophoblast cells do not express Class I and Class II MHC-antigens they may antigenically "present" the fetus to the mother so that she does not reject it as "foreign" (Adinolfi, 1992a). Furthermore, because they do not stimulate an aggressive immune response, they may not be removed rapidly from the maternal circulation.

Douglas et al (1959) analysed whole maternal blood with no attempt at enrichment or immunohistochemical identification. They found multinucleate cells in 61.5% of broad ligament veins at Caesarean section but only in 9.1% when sampling the inferior vena cava. They could not identify any trophoblast cells in 80 women following antecubital vein sampling. Their work was corroborated by Attwood and Park (1961) who suggested that trophoblast cells were removed rapidly by the pulmonary vasculature and did not circulate for very long.

Little was reported until Covone et al (1984) described a mAb, H315, which identified cytotrophoblast and syncitiotrophoblast cells. It did not, apparently, stain blood cells. Using FACS with H315 they isolated mononuclear and multinuclear cells in 50% and 80% of pregnancies respectively. There was no relation to gestational age and no attempt was made to sex the pregnancies prospectively. Unfortunately, 50% of controls also showed H315-positive multinucleated cells. Pool et al (1987) found that H315 reacted strongly with syncitiotrophoblast but variably with cytotrophoblast. Using FACS with this mAb they could not isolate positive cells in 25% of pregnant women and the population of sorted cells was not significantly different between pregnant and non-pregnant women. Furthermore, as the FACS-sorted cells were negative for other trophoblast markers, they concluded that H315 was unreliable. In a follow-up to their original study, Covone et al (1988) flow sorted H315-positive cells and using PCR with Y-specific primers, no Y-DNA fragment was observed in any pregnancy with a male fetus. Following lymphocyte incubation with cellular elements from chorionic villi they found that lymphocytes became H315-positive and suggested that circulating maternal leukocytes adsorbed the placental antigen on to their surface and were thus positively sorted.

In a slightly different approach, Adinolfi et al (1989) used immunomagnetic Dynabeads conjugated to H315 mAb in an attempt to enrich for trophoblast cells. Following isolation and PCR, no Y-DNA fragments were observed. They, too, suggested that H315 was unhelpful.

Mueller et al (1990) used Dynabeads with two mAbs to trophoblast surface protein antigens. The isolated cells were subjected to PCR with Y-specific primers and overall gender prediction was 92.3% accurate ($p < 0.002$). They suggested that the only false-positive case may have been due to a "male vanishing twin" with the abortus shedding large numbers of trophoblast cells into the maternal circulation. The group continue to screen for trophoblast-specific mAbs but, because of commercial considerations, have not made these available for testing by external laboratories.

Bruch et al (1991) used FACS with three anti-trophoblast mAbs (which did not react with erythroblasts) and identified Y-DNA by PCR in two of the three women carrying a male fetus.

In nine of twenty-three pregnant women, they failed to find any positive cells ($p = 0.186$) and the sorted cells did not look like trophoblasts by either light or electron microscopy. They suggested that perhaps trophoblast cells in maternal blood are altered morphologically and that maternal leukocytes adsorbed trophoblast-antigens on to their surface. Of course it may be that their mAbs were not trophoblast-specific and reacted with maternal blood cells as well. The same group, in a case report, addressed the problem of leukocyte contamination by a multiple stage enrichment process (Cacheux et al, 1992). Blood was taken from a woman known to be carrying a 47, XYY fetus and the erythrocytes were removed by density gradient centrifugation. The lymphocytes were removed immunomagnetically and the remaining cells flow sorted with two of their original mAbs. Using FISH with a Y-chromosome probe, 45 of the 1387 collected cells (3.2%) showed one Y signal and 14 (1%) showed two Y signals. In the original amniocentesis sample 66% of scored nuclei showed two Y signals. The false negative results might have been due to inefficient hybridisation (chapter 7.9.2) but an alternative explanation is that the two Y signals in cells were false positive signals as they did not report their results with control samples. In any case, in this situation PCR would have been unhelpful as it would not have permitted discrimination between one and two Y signals in the cells. From this example, the need for FISH to diagnose autosomal trisomies is apparent.

Thomas et al (1994, chapter 3.2) investigated 5 in vitro fertilisation pregnancies which had undergone pre-implantation sexing and, using nested PCR, they accurately identified both male-bearing pregnancies before 6 weeks' gestation. Fetal vessels are only present in the villus stroma from 8 weeks' so it was unlikely that fetal blood elements could escape into the maternal circulation as early as 5 weeks'. Because of this physiological precondition, they proposed that the most likely source of fetal DNA was trophoblast cells.

Durrant et al (1994) screened 19 oncofetal antibodies for trophoblast specificity but when coupled to immunomagnetic beads, even the most trophoblast-specific of these could not detect trophoblast cells in artificial admixtures. However, they subsequently reported their experience using immunomagnetics and positive sorting for both fetal erythroblasts and

trophoblast cells (Durrant et al, 1996). They subjected the potential trophoblast cells to PCR and found that gender prediction was poor ($p = 0.09$) whilst if potential fetal erythroblasts were analysed on their own, gender prediction was just significant ($p < 0.03$). However, if the two were combined, prediction was greatly improved ($p < 0.002$) because of a reduction in the large false negative rate associated with either cell types analysed on their own. They argued that cytogenetically analysing fetal erythroblasts could act as an internal control if confined placental mosaicism was suspected, although the problems associated with positive selection are discussed further in chapter 6.

Of all the potential cells for enrichment, trophoblast cells are the only ones which are fetal-specific and no breach is necessary for cells to enter the maternal circulation. Unfortunately, generation of specific mAbs is difficult as antigens may be adsorbed on to maternal leukocytes often resulting in a high false-positive rate. Furthermore, confined placental mosaicism could prevent definitive diagnoses being made from a maternal blood sample. All suspected aneuploid pregnancies would require invasive diagnostic testing (chapter 2.3) which would defeat the aim of non-invasive prenatal diagnosis. In summary, although isolating trophoblast cells has merits it also has considerable shortcomings and, therefore, many groups are now focusing on fetal erythroblasts.

3.6 Fetal erythroblasts

In 1990 Diana Bianchi and her colleagues enriched for fetal erythroblasts from maternal blood and there are a number of reasons why these might be good candidate cells for enrichment.

a) Relative proportions of erythroblasts at different gestational ages.

In the first and early second trimesters, as placentation establishes, there is rapid invasion of fetally-derived cells into maternal arterioles. Hence, it is plausible that around this time frequent breaches occur in the fetomaternal vascular interface, allowing more fetal blood cells into the maternal circulation. Erythroblasts are the most common type of

nucleated cell in early pregnancy but their proportion, relative to other nucleated cells, declines rapidly after 18 weeks' gestation as the main site of erythropoiesis switches to the spleen and bone marrow (figure 10.2, table 3.1). Therefore, the majority of nucleated fetal blood cells entering the maternal circulation from a minor transplacental haemorrhage in early pregnancy, should be erythroblasts.

b) Adult erythroblasts in the peripheral circulation.

The bone marrow is a dedicated organ, efficient at retaining its progenitor cells and in healthy pregnant woman, adult erythroblasts should not find their way into the peripheral circulation. However, pregnancy places additional demands on erythropoiesis and there is some evidence that maternal erythroblasts enter the peripheral circulation during pregnancy (Slunga-Tallberg et al, 1995). This emphasises that erythroblasts are not fetal-specific but, even so, if erythroblasts are recovered there remains a good chance that they are fetal.

There are a number of pathological conditions where erythroblasts escape into the peripheral circulation (Table 3.2). Of all these, severe iron deficiency anaemia, haemoglobinopathies and splenectomy are the most likely conditions to be encountered in a pregnant woman and thus confuse analyses. The others are generally life-threatening and the woman, in any case, is unlikely to conceive.

Table 3.2. Pathological conditions associated with peripheral erythroblasts (Harper, 1968)

- Post-splenectomy
- Severe anaemia: iron deficiency, megaloblastic, haemolytic
- Polycythaemia
- Haemoglobinopathies
- Di Guglielmo syndrome
- Leukaemias
- Neutrophilic leukaemoid reactions
- Leukocytoses with predominant neutrophilia (e.g. sepsis)
- Subacute or chronic haemorrhage
- Chronic myeloproliferative disorders
- Erythrocytosis

c) Transplacental haemorrhage.

Cohen et al (1964), using an anti-Rhesus antibody, showed that the frequency of fetal erythrocytes in maternal blood increased with advancing gestation and the likelihood of finding fetal erythrocytes approached 100% if enough samples were taken. They suggested that transplacental passage was an intermittent, recurrent event and a normal phenomenon of pregnancy. This was substantiated by the model of Parks and Herzenberg (1982, chapter 3.1.4) and if their model is pertinent to humans then it would suggest that fetal erythroblasts are escaping constantly into maternal blood. Fetomaternal haemorrhage probably increases as pregnancy advances but at later gestations a greater haemorrhage would be required for the same number of erythroblasts to find their way into the maternal circulation than at earlier gestations.

d) Peripheral mitoses.

Fetal erythroblasts are capable of mitosis (Tipton et al, 1990) and if they enter the maternal circulation they could be mitotically active and amenable to detailed cytogenetic analysis.

e) Removal of fetal erythrocytes from the maternal circulation.

Adults receiving incompatible blood group transfusions remove the foreign blood rapidly. However, it is possible that incompatible fetal blood does not initiate as great an immune response. At least 500µl of Rhesus-positive blood is needed to initiate an immune response as fetal erythrocytes have 75% fewer reactive A and B sites than adult erythrocytes (Voak and Williams, 1971) and erythroblasts express even fewer antigen sites than erythrocytes (Sieff et al, 1982). The Rhesus-D antigen is only expressed from the pronormoblast stage onwards and antigen expression reaches a maximum only when the erythrocyte is fully mature (Rearden and Chiu, 1981). Consequently, fetal erythroblast destruction may not be as rapid as in adult-to-adult incompatible transfusions and they may persist for their natural lifespan in maternal blood.

f) Erythroblast lifespan.

Little is known of the maturation time of early fetal multipotential stem cells but observations in adult bone marrow suggest a five day intramedullary maturation time (Erslev, 1990). If this applies to fetal erythropoiesis, they should not persist for any length of time in the maternal circulation. Therefore, if found consistently in maternal blood it implies that fetal erythroblasts are constantly removed or become enucleated and are replaced by chronic transplacental haemorrhage. This means that fetal erythroblast persistence to another pregnancy is improbable and unlikely to confuse cytogenetic analyses in the index pregnancy, offering a major advantage over fetal lymphocytes (chapter 3.3).

g) HLA incompatibility.

Erythroid precursors express HLA antigens but these are gradually lost during maturation (Sieff et al, 1982). Of course this could mean that, because of HLA incompatibility, fetal cells are removed rapidly from maternal blood. However, many groups have shown that fetal cells can be enriched by exploiting HLA differences (chapter 3.3) so that either there must be a degree of HLA-incompatible tolerance between mother and fetus and/ or a stable state of removal and replacement of fetal cells exists.

h) Morphological identification.

Erythroblasts have a characteristic morphology and are easily distinguishable from other cells. Fetal erythroblasts are large cells though their size decreases as pregnancy advances (table 3.1). We believe figure 3.4 demonstrates a fetal erythroblast which was enriched from the blood of a pregnant woman (chapter 6) as it is much larger than the surrounding adult erythrocytes.

Chromosomally abnormal fetuses, especially trisomies, have a significantly higher mean corpuscular volume than controls as they appear to have escaped normal erythropoietic control mechanisms or lag behind by a matter of weeks (Sipes et al, 1991). Although this phenomenon is unexplained, it represents an intrinsic red cell abnormality and this obvious morphological difference might allow even easier differentiation of potential fetal

erythroblasts on a microscope slide in karyotypically abnormal pregnancies, particularly if the slide was scanned by a robot (chapter 10.4).

i) Positive identification of fetal erythroblasts.

If fetal erythroblasts can be identified immunologically as well as morphologically, it would aid rapid identification of these cells on a slide and minimise false results with subsequent cytogenetic analyses. The composition of haemoglobin in fetal erythrocytes varies throughout gestation (figure 10.2) and if mAbs can be raised to embryonic haemoglobin chains it should be possible to positively identify fetal erythroblasts and, thereafter, subject them to cytogenetic analyses using fluorescence in situ hybridisation.

Enrichment of fetal erythroblasts from maternal blood

During cell proliferation, CD71 (transferrin receptor) expression increases 2-5 fold. CD71 is expressed on activated lymphocytes, a small proportion of other peripheral blood cells and erythroid progenitors up to and including the reticulocyte stage (Sieff et al, 1982) but is lost rapidly thereafter (Frazier et al, 1982).

Bianchi et al (1990) enriched for fetal erythroblasts by FACS based on CD71 expression and, from an original 20ml of maternal blood, using PCR they accurately identified 6 of 8 male-bearing pregnancies ($p < 0.003$). They estimated recovery of about 1ng of fetal DNA, equivalent to approximately 150 cells. At 17 weeks' gestation, 150 erythroblasts represents approximately 0.04µl of fetal blood, equivalent to 10µl in the entire maternal circulation, which is considerably less than other estimates (Parks and Herzenberg, 1982; Hamada et al, 1993). Enrichment procedures inevitably incur some cell loss and their calculations did not take this into account. Consequently, there might be even more fetal blood in the maternal circulation. Nevertheless, considerable enrichment is still necessary.

Glycophorin-A (GPA) is the erythroid-specific major membrane sialoglycoprotein and appears at the erythroblast stage, reaching a maximum at the normoblast stage (Kansas et al, 1990). There are no surface antigens which are erythroblast-specific and only glycophorin

and the Rhesus antigens are erythroid-specific (Yokochi et al, 1984). Rhesus cannot be used as a sorting parameter as it would only be appropriate for Rhesus-incompatible pregnancies. GPA, however, in combination with CD71 should identify erythroblasts and reticulocytes. The large size of fetal erythroblasts (table 3.1) also allows size to be used as a gating parameter with FACS. After enucleation, right-angle scatter of erythroid cells is increased, possibly because of the acquisition of the biconcave disc shape (Loken et al, 1987), and hence reticulocytes may be gated out of the sorted cells. Based on this rationale, Price et al (1991) from Tennessee flow sorted on cell size, cytoplasmic granularity, transferrin receptor and Glycophorin-A expression. They collected 0.4 ± 0.7% of cells from first trimester samples and 2.2 ± 3.6% of cells from the second trimester samples. Most were reticulocytes. Using nested PCR with Y-DNA primers on 18 samples they predicted fetal gender with 94.4% accuracy ($p < 0.0001$). Using FISH with a Y-chromosome-specific repeat sequence probe, about 10% of sorted cells showed a Y-domain in male-bearing pregnancies but their false positive rate was 25% and the false negative rate was 35.7%. Overall, gender prediction was only 68.2% accurate ($p > 0.05$). They also sorted cells one week after a patient underwent chorionic villus sampling and was found to be carrying a Down's syndrome fetus. Twenty-four of 62 cells analysed showed three domains using a chromosome 21-specific probe. Although many of these cells might have been introduced into the maternal circulation by the invasive procedure, if fetal erythroblasts only have a five day lifespan then those erythroblasts escaping at the time of CVS should have enucleated by the time enrichment was attempted.

The group also took blood from a woman prior to first trimester CVS, flow sorted the cells and performed FISH with probes for the Y-chromosome and chromosomes 18 and 21. From a total of 681 cells reported, 8.7% of nuclei showed a single Y domain, 8.5% (25 of 295) showed three chromosome 18 domains whilst only 3.0% (5 of 166) of cells showed three chromosome 21 domains. From these results they predicted the 47 XY, + 18 karyotype correctly. This report was a milestone. It demonstrated that fetal cells, probably erythroid, could be isolated from maternal blood in early pregnancy and diagnoses of fetal aneuploidy

could be made. The case report also illustrated that FISH, and not PCR, is necessary to diagnose fetal aneuploidy and that simultaneous X and a Y-chromosome probes are required to predict fetal sex accurately.

Bianchi et al (1991) amended their original protocol by removing leukocytes with FACS and then collected CD71-positive cells. The sorted cells were subjected to PCR and combining this data with that of their 1990 study, they suggested that Y-specific DNA fragments were easiest to obtain up to 16 weeks' gestation and declined thereafter. It is preferable to make a diagnosis of fetal abnormality early in pregnancy and, if these findings are correct, it would be advantageous.

The Tennessee group then reported a prospective diagnosis of Down's syndrome (Elias et al, 1992). In two cases, prior to first trimester CVS, they flow sorted maternal blood and used FISH with a chromosome 21 probe. Each observer scored one hundred nuclei and in one case 74% of the nuclei exhibited 3 signals. Using this probe in diploid cells, 7.5% (range = 0-12%) of nuclei exhibited 3 signals and they used this level as a cut-off for karyotype prediction. From their results they correctly predicted a 47, +21 karyotype. Unfortunately in the other reported case only 2.9% of sorted nuclei exhibited three signals which would have fallen below their threshold and, erroneously, the fetus would have been considered diploid. However, their data must be interpreted with caution. In the "diagnostic" sample they collected 37,000 nucleated cells and three independent observers each scored only 100 nuclei. It is highly unlikely that they examined the same cells and this would suggest that there were over 27,000 fetal erythroblasts in 20ml of maternal blood. The issue of fetal erythroblast persistence is unresolved but from current assumptions concerning their short half-life, this would represent an unlikely figure of 5ml of fetal blood in the maternal circulation.

Although, this report was encouraging, it illustrated a deficiency in FISH analysis. Cell fixation strips away the cytoplasm to improve probe access and it is then impossible to identify the sorted cells positively. Furthermore, the hybridisation efficiencies of individual probes, particularly with regard to the cell type, must be determined. Karyotype assignment

based on spot counts then becomes a statistical question related to the ratio of fetal to maternal cells. The number of sorted maternal cells is likely to remain reasonably constant from sample to sample but, as far as is known, there is considerable variation in the number of fetal cells between pregnancies. Therefore, in aneuploid pregnancies, if there are very few fetal cells they might not take the count of aneuploid cells above the threshold, as in the second reported case. It is clear that positive identification prior to FISH is imperative to allow cytogenetic analysis only of fetal cells.

Bianchi et al (1992) reported a case at 19 weeks' gestation involving a Down's syndrome fetus (47XY, +21) diagnosed by prior amniocentesis. They enriched for cells by FACS based only on CD71 expression and subjected the isolated cells to FISH using a probe for chromosome 21 and the Y-chromosome. Only three cells showed a single Y domain and three chromosome 21 domains simultaneously. One cell showed three chromosome 21 signals but no Y-probe signal. Because of the very small number of cells that suggested the true fetal karyotype it would have been impossible to arrive at any meaningful conclusion.

Gänshirt-Ahlert et al (1992) used positive selection based on CD71 expression with the Miltenyi magnetic activated cell sorting system (MACS). Their results are discussed in greater detail in chapter 6.4.4 but even in model systems they found that the relative enrichment of erythroblasts was poor. However, because of the expense and complexity of FACS, such alternative strategies are necessary if fetal cell retrieval from maternal blood is to become a routine early pregnancy screening procedure.

In 1993 Bianchi et al adopted a multiparameter approach to flow sorting to prospectively sex fetuses with PCR. Again they combined data from previous reports with newer data. Using CD71 expression alone, gender prediction was poor ($p = 0.28$). They used an additional marker, CD36, the thrombospondin receptor, which is apparently expressed from the colony forming unit-erythrocyte stage of fetal but not adult erythrocytes. In these cases gender prediction accuracy was 87.5% ($p < 0.05$). They enriched five maternal blood samples based on GPA expression alone and gender prediction was 100% accurate ($p < 0.05$). They sorted five samples on both CD36 and GPA expression so that, in theory, only

erythroid progenitors should be collected and again gender prediction was 100% accurate. In the three final women they used CD71 and GPA and although gender prediction was again 100%, these results could have arisen by chance. The authors found that 4.9-25.3% of sorted cells expressed both CD36 and GPA whilst only 0.4-1.7% of cells expressed both CD71 and GPA. They suggested that the dual antibody protocol identified different subpopulations of cells though in fact the CD71/GPA-positive population could have been a subset of the CD36/GPA population. If the 13 patients where GPA was used are considered (regardless of other mAbs) the chance of predicting fetal sex with such accuracy was less than 1 in 10,000. They concluded that, because the sorted fetal cells expressed GPA, it was convincing proof of their erythroid origin.

Following their original communication, Gänshirt-Ahlert et al (1993) reported an improved immunomagnetic enrichment method using a triple density gradient cushion (Bhat et al, 1993). The cells harvested at the upper interface were enriched further by MACS using an anti-CD71 mAb and the recovered cells stained with a simple haematological stain. Their data suggested that the number of fetal erythroblasts increased exponentially with advancing gestation and as many as 500,000 erythroblasts were recovered which is surprising as previous reports suggest far fewer cells. However, in seven cases they subjected the recovered cells to FISH using a Y-chromosome repeat sequence probe. In the male-bearing pregnancies a mean of 14% of nuclei showed a single Y signal and gender prediction was 100% accurate ($p < 0.008$). During pregnancy around 0.83% of maternal blood cells are CD71-positive (Adinolfi, 1992a). However, the data of Gänshirt-Ahlert et al suggest that around 0.1% of all nucleated cells in maternal blood should be fetal. This is extremely unlikely and their results must be considered with caution. The authors also subjected enriched cells from known or suspected fetal aneuploidies to FISH using appropriate chromosome probes. They found that the mean percentage distribution of cells with three signals in trisomic pregnancies did not overlap with the percentage distribution of cells with three signals in normal pregnancies. However, in the ten trisomy 21 cases, only one of the diagnoses was made prospectively i.e. prior to an invasive diagnostic procedure. Of the five

trisomy 18 cases, only one of the diagnoses was made prospectively and even this was at 33 weeks' gestation. Of all the common aneuploidies, trisomy 18 has the most characteristic ultrasound malformations (Nicolaides et al, 1992) and, given the late stage of diagnosis, fetal abnormality was possibly suspected because of other clinical abnormalities such as intrauterine growth restriction which may have prompted an ultrasound scan. Their data suggested that more fetal cells escaped into the maternal circulation in aneuploid pregnancies, a phenomenon which was also reported by Simpson and Elias (1993). In some way aneuploid placentation differs from normal pregnancies, with associated altered placental hormone production (chapter 2.8), and it may be that these placentae also allow fetal cells to cross into the maternal circulation in greater numbers as well. They followed patients postnatally to address the possibility of fetal erythroblast persistence in maternal blood and, using FISH in aneuploid pregnancies, the percentage of putative trisomic cells fell to control levels by 7-10 months postpartum. If the cells were fetal erythroblasts, and they do not persist beyond 10 months postpartum, they should rarely confuse analyses in subsequent pregnancies.

The same group reported that fetal erythroblasts could be identified from five weeks' gestation. They found that, up to 16 weeks' gestation, as many as 10,000 fetal erythroblasts in 91.3% of all pregnancies (Gänshirt-Ahlert et al, 1994). As so many putative erythroblasts were found, the validity of these results must be questioned. Phagocytic cells should be removed prior to MACS separation or they engulf the beads, the nuclei become pyknotic, the cells assume an erythroblast-like morphology (using simple Romanowski stains) and they are easily confused with true erythroblasts (Manyonda et al, 1992). The density gradient media used by Gänshirt-Ahlert et al preferentially collected neutrophils and as they did not use any other confirmatory test, such as immunocytochemistry or FISH, it might explain the high numbers of putative erythroblasts they recovered. Although encouraging, it emphasises that positive identification is imperative to minimise false results.

Zheng et al (1993) reported a study to prove simultaneously that the isolated cells were both erythroid and fetal. Erythrocytes were removed by density gradient centrifugation and

then the majority of leukocytes were removed with anti-CD45 and anti-CD32 mAbs using the immunomagnetic MACS system. In their model experiments (spiking adult blood with fetal erythroblasts), leukocyte removal was rather variable: relative to the number of leukocytes before and after enrichment, erythroblast enrichment ranged from a factor of 18- to 184-fold. However, from cytospin preparations of the enriched maternal blood samples, the remaining cells were identified on slide using a mAb to γ-globin (UCH γ, Allen et al, 1987) with Vector Red as the chromogen. The positions of the γ-globin-positive nucleated cells were recorded and the slides subjected to FISH using a repeat sequence Y-chromosome probe. The cells were relocated and, if negative for a Y-domain, FISH was performed again but using an X-chromosome centromeric repeat sequence probe. In five of the pregnancies (four males and one female) between 2 and 11 positive cells were identified and in all of these gender was correctly predicted ($p < 0.03$). In the other sample, no γ-globin-positive cells were identified. Immunocytochemistry prior to FISH reduced the hybridisation efficiency of the Y-chromosome probe from 98% (non-erythroblast male cells) to 64% (erythroblasts subjected to immunocytochemistry). They did not report the hybridisation efficiency of fetal erythroblasts which had not undergone prior immunocytochemistry but the effects of the dual procedures is discussed in greater detail in chapter 9.

Reading et al (1995) enriched for fetal erythroblasts using MACS by negative and then positive sorting. They subjected the recovered cells to immunocytochemistry with an anti-HbF mAb and thereafter FISH. In 5 of 40 cases there were no HbF-positive cells and in 23/40 FISH failed on the positive cells. Of the remaining 12 cases, gender prediction was 83.3% accurate ($p < 0.02$) but this must be taken in the context of the additional 28 cases where no result was obtainable. Slunga-Tallberg et al (1995) reported that erythroblasts could be enriched from 37 of 40 primigravidae in greater numbers than in non-pregnant women. The nucleated cells were all glycophorin A-positive and all women were known to be carrying a male fetus. They too found that hybridisation efficiency was reduced with erythroblasts (see also chapter 7) and, concerningly, none showed an XY karyotype. They

concluded that these cells must be maternal erythroblasts which enter the peripheral circulation during pregnancy.

Bianchi et al (1996) flow sorted maternal blood for erythroid precursors and subjected the collected cells to PCR for a Y-chromosome sequence. Both sensitivity and specificity were poor (68% and 69% respectively) and the false-positive rate was 31%. They suggested that the high false-positive rate was due to prolonged persistence of fetal DNA because of microchimerism between fetal and maternal cells. However, an alternative explanation would be contamination of the samples with DNA from male technicians but either way it demonstrated the major limitation of PCR in this context. Furthermore, it illustrated that absence of amplification products requires confirmation that fetal material is actually present rather than the test being falsely negative.

Sohda et al (1997) enriched for erythroblasts with FACS and subjected the recovered cells to Y-chromosome FISH. Of the thousands of cells recovered, almost 5% displayed a Y-chromosome signal. Although gender prediction was extremely accurate, given other estimates of the frequency of fetal erythroblasts in maternal blood, this large number is unlikely and again suggests that the cells should be identified positively in some way prior to cytogenetic analysis. Campagnoli et al (1997) positively sorted for erythroblasts using the immunomagnetic MACS system and correctly identified all seven male fetuses using Y-chromosome FISH.

Mavrou et al (1998), also using the MACS system, enriched for erythroblasts by depleting leukocytes and found between 1 and 15 γ-globin-positive cells in 35 of the 40 women. Unfortunately they managed to successfully perform FISH with sex chromosome probes in only five cases which suggests that very few cells can be recovered and, also, that the efficiency of FISH with fetal erythroblasts is poorer than with other cell types.

Bischoff et al (1998) reported their results on 40 women, from 10 to 27 weeks' gestation, using FACS with a combination of negative and positive enrichment for fetal erythroblasts and subsequent multi-colour FISH analysis. They only evaluated between 154 and 4212 cells from each woman and the gender prediction accuracy was only 72.5% because of the

high false negative rate (68.7%) in assigning the fetus as male. There were two cases of trisomy 21 and in both only one trisomic cell was identified in the 272 and 955 cells evaluated from each case. Given the small number of positive cells, it would be impossible to confidently assign the abnormal fetal karyotype. Furthermore, there was one case of trisomy 18 and they enriched cells at 27 weeks' gestation. Trisomy 18 is characterised by clinically detectable fetal growth retardation and pathognomonic ultrasound features and confirmatory invasive testing would be offered. The text only states that one of the 40 cases was performed because of abnormal ultrasound findings but, given the late gestation at testing, it is reasonable to assume that this was the one. Unfortunately they failed to diagnose the karyotype which is, of course, is concerning.

Al-Mufti et al (1999) reported positive immunomagnetic enrichment of erythroblasts on 230 pregnancies at 10-14 weeks' gestation, prior to invasive testing. Of these women, 36 (16%) were carrying a trisomy 21 fetus. The recovered cells were subjected to FISH with a probe for chromosome 21 and, in 22 cases, over 5% of nuclei displayed 3 signals. These data must be viewed cautiously as the 16% incidence of trisomy 21 is considerably higher than any other reported series for prenatal diagnosis and the high proportion of trisomic cells recovered from maternal blood is similarly much higher than other series even using FACS, the acknowledged gold standard for cell separation.

Most recently, Wang et al (2000) reported a direct comparison of FACS and MACS. This was simply a comparison as blood was taken immediately after termination of pregnancy when a larger number of fetal erythroblasts might be expected to leak into the maternal circulation. They subjected maternal blood to both FACS using an anti-γ-globin mAb and negative enrichment with MACS (depletion of CD45-positive cells) and staining the remaining cells with the anti-γ-globin mAb. The collected cells in both groups were subjected to X- and Y-chromosome FISH and they found that the yield of fetal cells was better in the FACS group. This confirmed that FACS remains the gold standard for enrichment but,

because of its technical disadvantages (*vide supra*), simpler, less expensive enrichment methods must continue to be investigated.

3.7 Summary

Although fetal cells can be found in maternal blood, they are extremely rare. Even the most recent reports suggest that very few cells can be recovered which emphasises the absolute necessity for positive identification of fetal cells prior to cytogenetic analysis. The apparent absence of fetal DNA does not necessarily mean that fetal DNA is not present or, vice versa, that cells which appear fetal morphologically, may actually be maternal.

PCR analysis of whole blood might allow the diagnosis of certain genetic disorders but is prone to false-positive results and it cannot be used to diagnose fetal aneuploidies. FISH analysis on whole maternal blood samples is impractical and some form of fetal cell enrichment is necessary.

The original analyses on putative fetal lymphocytes were dogged by the lack of specificity of both the enrichment and the cytogenetic techniques available at that time but, as these techniques improved, it appeared that they could be isolated reliably from maternal blood. Unfortunately, there is the possibility that fetal lymphocytes might persist from previous pregnancies which could confuse karyotyping. Furthermore, detailed HLA typing would be required to distinguish fetal from maternal lymphocytes which is impractical.

The only study analysing fetal neutrophils from maternal blood was flawed both theoretically and methodologically and most groups have dismissed the potential of fetal neutrophils as a candidate cell type.

Of all the potential cells, trophoblast cells are the only ones which are truly fetal-specific. Furthermore, no vascular breach is necessary for trophoblast cells to enter the maternal circulation. Unfortunately, generation of trophoblast-specific mAbs has proved extremely difficult and as trophoblast cells have limited mitotic capability it may restrict the efficiency of FISH. Finally, and most importantly, confined placental mosaicism could severely restrict the potential for definitive diagnoses to be made from a maternal blood sample alone.

Current opinion suggests that erythroblasts are the most promising fetal cell type for enrichment from maternal blood as transplacental haemorrhage appears to be a common, and possibly physiological, phenomenon and erythroblasts are likely to cross over in greatest

numbers in early pregnancy (Adinolfi, 1992b). They are morphologically and immunologically distinctive cells and they appear to be mitotically active. Fetal erythroblasts also express fewer antigens than their adult counterparts so they might not be removed rapidly from the maternal circulation despite blood group incompatibilities. Furthermore, the volumes in the maternal circulation might be insufficient to provoke an immune response. Unfortunately, hybridisation efficiency appears to be poorer than with other cell types and whether fetal erythroblasts persist for any length of time postnatally remains unresolved.

However, many of these statements are largely conjecture and the large number of small studies with varying results attests to the problems of enrichment and cytogenetic analysis of fetal cells from maternal blood.

3.8 Aims of this Thesis

If fetal cells isolated from maternal blood are to be used diagnostically, the test must be at least as good as the invasive tests it replaces. If used as part of a screening test, it must be as good as maternal serum and nuchal thickness screening.

Before this can happen, certain key questions must be answered. Can fetal cells be recovered in all pregnancies? What is the optimal time for blood sampling? How many cells can be recovered and how much maternal blood would be needed? How reliable is FISH on the recovered cells? Is it possible to prove that the recovered cells are fetal and thereafter to perform FISH on them? Are currently available cytogenetic techniques adequate to obviate invasive diagnostic techniques and could gene disorders be diagnosed? And finally, what factors may confound analyses? Because of the above factors, we have concentrated our efforts on fetal erythroblasts and by doing so, address some of these questions.

This study aimed to develop a simple, inexpensive and automatable technique to enrich for fetal erythroblasts from maternal peripheral blood and to cytogenetically analyse the enriched cells using fluorescence in situ hybridisation. Attempts were also made to identify the cells positively prior to FISH. The philosophy of automation was fundamental as only

then could it ever be applicable on a large scale. Robotics and automation was beyond the scope of this thesis but each of the steps had to be simple if this was to be achieved.

The methods were developed on models which were subsequently applied to maternal blood samples and the problems encountered are discussed in the following chapters. Although each of the chapters is described in isolation, it will become apparent that the different aspects of enrichment and cytogenetic analysis are inter-related, and alteration of one aspect had repercussions for subsequent steps in the procedure.

The techniques used throughout this project are described in a logical progression for the novice who might wish to repeat some of this work and all experiments conformed to, and were performed under, COSSH regulations.

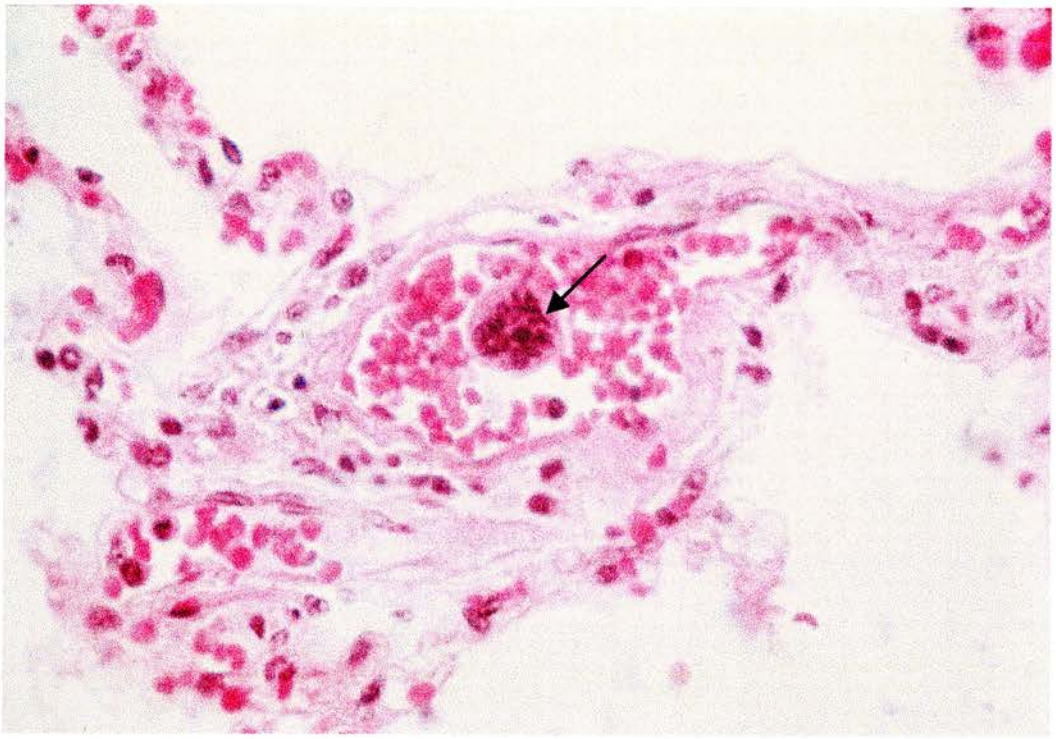


Figure 3.3 Trophoblast cell (arrowed) in a maternal pulmonary vessel at post mortem

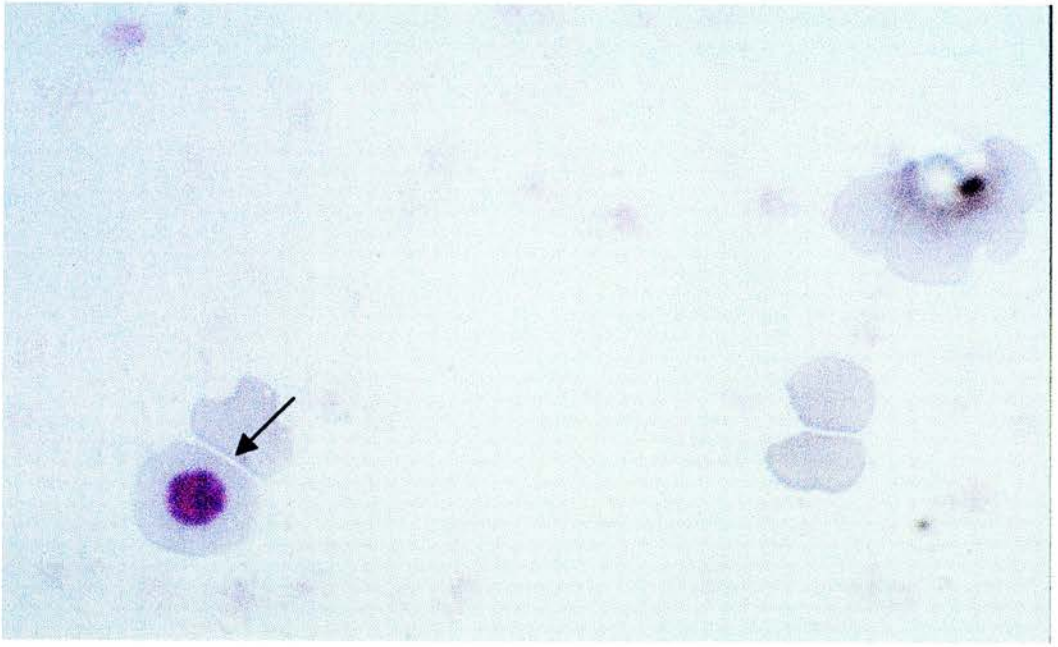


Figure 3.4 A fetal erythroblast (arrowed) enriched from the blood of a pregnant woman
Note the large size of the erythroblast relative to adult erythrocytes

Chapter 4. General Laboratory Techniques

4.1 Morphological Staining Techniques

4.1.1 May-Grünwald-Giemsa

Slide preparations (either whole blood smears or cytospin specimens) were fixed in 100% methanol for five minutes, immersed in neat May-Grünwald stain for 10 minutes and then immediately in 12% Giemsa (in pH 6.8 distilled water) for five minutes. The slides were rinsed in distilled water (pH 6.8) and air dried prior to mounting.

4.1.2 Reticulocyte stain

New methylene blue (1g) in 100ml of citrate-saline solution. Equal volumes of stain and whole blood were mixed in an Eppendorf tube and incubated at 37°C for 15 minutes. The blood film was examined at x100 magnification.

4.2 Cell counts

4.2.1 Nucleated cell count.

Diluting fluid: 2% acetic acid in distilled water (to lyse erythrocytes) with 3 drops of methyl violet to stain the nuclei. 50µl of whole blood was aspirated and the external surface of the pipettor tip carefully wiped clean. The blood was ejected into 950µl of diluting fluid and fluid was repeatedly drawn in and out of the pipettor tip to wash all the cells into the diluting fluid. This was then thoroughly mixed for five minutes. A drop of suspension was drawn under the coverslip of an improved Neubauer Counting Chamber by capillary action. The number of nuclei were counted in the four large squares at the corners and the central large square and this figure was multiplied by 40 to calculate the nucleated cell count x 10^3/ml.

4.2.2 Erythrocyte count

Diluting fluid: Sorenson's buffered saline with 1% formalin (to fix the cells). 5µl of whole blood was added to 995µl of diluting fluid in the same manner as for a nucleated count and mixed thoroughly. A drop was drawn under a Neubauer coverslip and the number of erythrocytes were counted in the four small corner squares and the central small square of

the central large square of the counting chamber. This figure was expressed as the total number of erythrocytes x 10^7/ml.

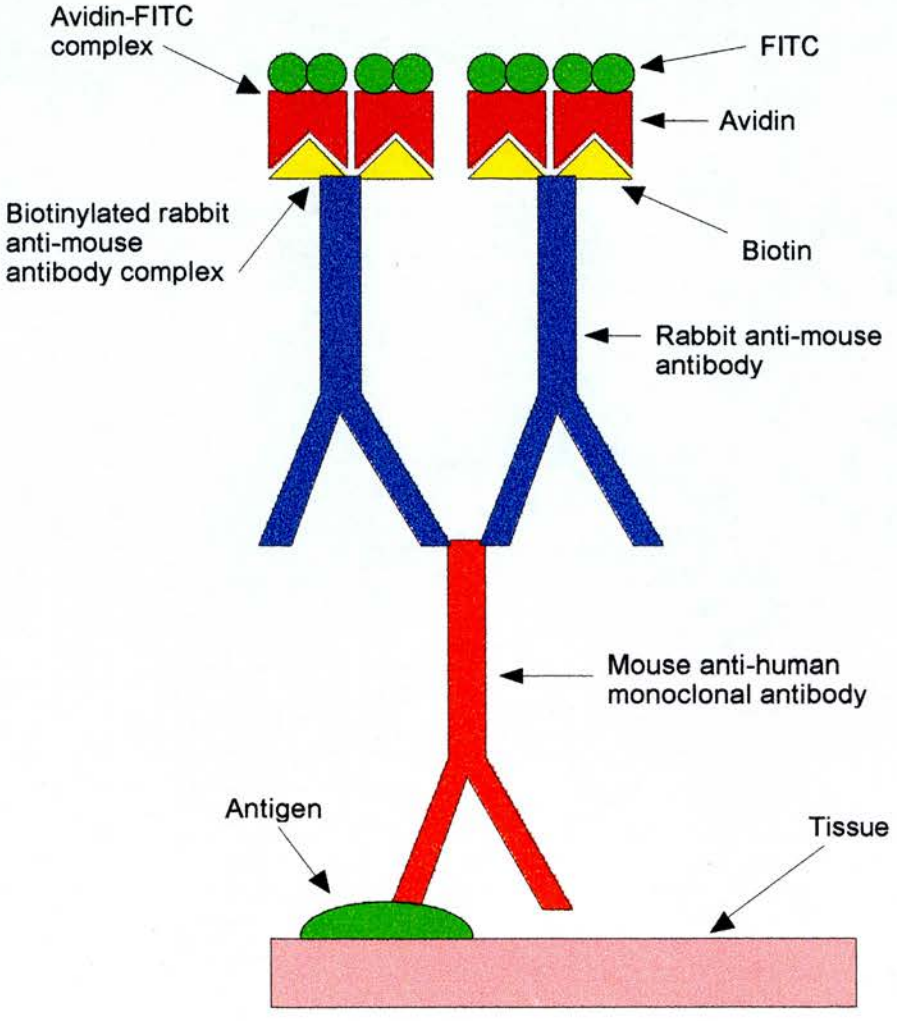
4.3 Immunocytochemistry

4.3.1 Principles of immunocytochemistry

Many of the methodologies described in subsequent chapters are dependent on immunological techniques and exploitation of monoclonal antibodies. Hence, an overview of the principles is given at this stage.

All tissues express certain antigens to which specific "monoclonal" antibodies (mAbs) may be raised. The primary mAb, often mouse anti-human, when incubated under appropriate conditions, will bind firmly to it's target antigen. The binding site can be visualised using a secondary antibody (perhaps a rabbit anti-mouse antibody with a marker, or chromogen, attached) directed against the original mAb. The chromogen may be a fluorescent marker or one visualised by enzyme precipitation to leave a known coloured stain. Because the secondary antibody has multiple binding sites many secondary antibodies bind to the primary mAb. The more secondary antibodies clustered around the original tissue antigen site, the greater the signal produced. Layers can be built up like an inverted pyramid depending on the intensity of signal required (figure 4.1). An indefinite number of layers can be developed but as secondary antibodies are often polyclonal they may react with different antigens, non-specifically binding to other tissues and increase "background" staining. At best this will confuse the picture but at worst can be misinterpreted as target antigen. Hence, there is a balance between signal intensity versus background staining, a dilemma encountered not only by the immunocytochemist but also by those performing in situ hybridisation.

Figure 4.1
The Principles of Immunocytochemistry



Specific reagents

In order to minimise non-specific binding of the secondary antibody to other tissue antigens, the species of blocking serum should be of the same species as the secondary antibody (e.g. normal rabbit serum with rabbit anti-mouse secondary antibody). Phosphate buffered saline with 1% bovine serum albumin should be used if there are multiple layers involving different species.

Assessment of signal intensity is subjective but generally follows a plateau distribution i.e. at very weak and very high primary mAb concentrations, signal intensity is poor whilst the greatest but equivalent signal intensities are seen over a broad band of mAb dilutions. If the primary mAb is too concentrated steric hindrance may result as too many mAbs compete for the same antigen. As a result of this crowding, the secondary antibodies cannot bind adequately and the subsequent signal intensity is reduced. Conversely, if the mAb is too dilute, too little binding also results in a weak signal. Hence, for economy, the weakest primary mAb concentration should be used without compromising the final signal.

The monoclonal antibodies used will be discussed in detail in the appropriate chapters.

4.3.2 Immunocytochemistry Method

All immunocytochemistry experiments were performed with a positive control (to ensure that the process had worked) and a negative control (no primary mAb) to ensure that secondary antibody binding was specific.

For best results, slides were fixed in methanol: acetone 50:50 for five minutes and air dried. Slides were washed in tris buffered saline (TBS) for 15 minutes and incubated with normal rabbit serum (NRS) for 10 minutes. The primary mAb was added for 30 minutes at room temperature and washed off with TBS for 10 minutes. The slides were then incubated with the secondary antibody for 30 minutes and washed again in TBS for 10 minutes. If the secondary antibody was directly-labelled with a fluorochrome the slides were mounted in

Vectashield/DAPI and viewed using a Zeiss Axioplan microscope. Images were captured on an Apple Macintosh Quadra 950 using IPLab Spectrum 2.4 software.

If the secondary biotinylated antibody required enzyme development the slides were incubated with streptavidin alkaline phosphatase for 30 minutes, washed in TBS for 10 minutes and then developed with the DAKO New Fuchsin kit for 20 minutes as per the DAKO protocol. The slides were rinsed for 5 minutes in tap water, air dried and counterstained with Giemsa before mounting in glycergel (DAKO).

The above method, with necessary adaptations, was used repeatedly throughout the following chapters and will be discussed in detail where appropriate.

Chapter 5. Density gradient removal of mature erythrocytes

The ratio of fetal erythroblasts to all maternal cells is estimated at 1 in 10^8-10^{10} (Chueh and Golbus, 1991; Simpson and Elias, 1993). The majority of blood cells are non-nucleated erythrocytes and hence enormous enrichment is necessary to reduce this to a more manageable figure.

Strategically it was logical to remove the major contaminating cell type (mature erythrocytes) as the first enrichment step and this was the object of density gradient centrifugation.

5.1 Principles of Separation

The relative density of different blood cell types increases from platelets, to monocytes, lymphocytes, basophils, neutrophils and eosinophils whilst non-nucleated erythrocytes are the most dense. Therefore, when centrifuged, erythrocytes gravitate most readily and a "buffy coat" of leukocytes and platelets collect at the top of the cell column.

In a continuous density gradient column, the density of the medium is greater at the bottom than at the top. Cells form layers where their densities are less than the underlying medium and this level is known as the isopyknic point. A discontinuous gradient has a constant density throughout the fluid column.

5.2 Development of separation media

The reaction between two compounds can be increased if it occurs at an interface and centrifugation promotes further the collisions necessary for such reactions to occur.

Bøyum (1964) created an iodine-based medium of known specific gravity, "Natrii" (N-methyl-3,5-diacetoamido-2,4,6-trijodbenzoas) and added methylcellulose to encourage red cell aggregation through interaction with carbohydrate moieties on the cell membrane. The resultant rouleaux were more dense and passed through the medium leaving unaggregated nucleated cells above the medium.

The degree of erythrocyte removal can be manipulated by altering the relative density, the methylcellulose concentration, the pH or the osmolality of the medium. Unfortunately, the trade-off is in target cell recovery which must be maximised for rare cells such as fetal cells in maternal blood. Moreover, the densities of a particular cell type follow a Gaussian distribution and there is overlap in densities between the most dense of one cell type and the least dense of another. Therefore, there will always be some contamination by unwanted cells.

Histopaque (Sigma) is a mixture of an iodinated compound, sodium diatrizoate, and the polysaccharide, ficoll 400. The proportion of diatrizoate can be varied to selectively enrich for particular cell types. Ficoll improves erythrocyte removal by rouleaux formation but because of this erythroblasts may also be removed.

Nycodenz is a non-ionic tri-iodinated derivative of benzoic acid with three aliphatic hydrophilic side chains. It lacks any red cell aggregating agent so that erythroblasts should not be lost in the red cell pellet if they are less dense than the medium. The relative density of Nycodenz can be specified depending on the proportions of Nycoprep and Nycodenz buffer.

A number of different density gradient media and different types of centrifugation tube were investigated to determine which removed most non-nucleated erythrocytes whilst maximising fetal erythroblast recovery.

5.3 Methods

The initial model involved umbilical cord blood taken at delivery, collected in EDTA tubes (Local Ethics Committee approval having been obtained) and three different density gradient media were investigated.

Erythrocyte and leukocyte counts and a differential cell count were determined with the differential count always based on the first 1000 nucleated cells in consecutive high power microscope fields.

5.3.1 Density gradient media on model systems

5ml of whole blood was carefully layered on to 3ml of density gradient medium and centrifuged at 500 x g for thirty minutes. The cells were harvested at the plasma/ gradient interface, washed in 2% (w/v) bovine serum albumin in phosphate buffered saline [2% (w/v) BSA in PBS] for two minutes and the supernatant centrifuged at 250 x g for fifteen minutes. The washing process was repeated twice more and the cells resuspended in 2% (w/v) BSA in PBS up to the original volume of 5ml.

Erythrocyte and leukocyte counts and a differential count were determined from the harvested cells, which allowed the proportion of recovered nucleated cells and fetal erythroblasts to be calculated.

The efficiency of separation using whole or washed blood was also compared. Whole blood was divided into equal aliquots and one aliquot was diluted in 2% (w/v) BSA in PBS, washed for five minutes and then the cells pelleted at 350 x g for fifteen minutes. The washing process was repeated once more and the pellet resuspended to its original volume in 2% (w/v) BSA in PBS. This sample and the original whole blood aliquot were then layered on to equal volumes of the density gradient medium and centrifuged as previously described.

Model systems of cord blood mixed with varying proportions of adult blood from healthy volunteers were investigated and again the efficiency of centrifugation was determined.

Samples were also obtained from the products of conception at first trimester suction termination of pregnancy (with Ethics Committee approval and informed patient consent). The whole sample was diluted in 2% (w/v) BSA in PBS and filtered through nylon stockings to remove solid matter leaving a fluid which was relatively rich in erythroblasts. Inevitably it also contained maternal blood. Cell and differential counts were determined. The samples were then mixed with varying proportions of adult blood from healthy volunteers as it was hoped that this model would simulate whole maternal blood more realistically. The minimum dilution investigated was 5µl of termination blood (fetal and maternal) diluted in 10ml of healthy, non-pregnant female volunteer blood.

In the later stages of the project, another gradient medium, Nycodenz, relative density of 1.085, was also investigated on fetal blood from these samples.

5.3.2 Centrifugation tubes

Using Histopaque 1119, four different centrifugation tubes were compared directly: conical Sterilin centrifugation tubes (catalogue number, AS144), Falcon polypropylene tubes (catalogue number, 2096) and translucent polystyrene tubes (catalogue number, 2099) which were claimed by the manufacturers to be smoother, facilitating erythrocyte removal. A major disadvantage of density gradients is the time and care required to layer whole blood without disturbing the interface. Greiner polypropylene LeucoSep tubes (catalogue number, 163290) have a porous filter fixed transversely towards the bottom of the test tube which allows whole blood to be poured on to the filter without disturbing the interface.

5.3.3 Maternal blood

15ml of venous blood was drawn from the antecubital fossa of pregnant woman at 7-15 weeks' gestation and collected in EDTA tubes (with Ethics Committee approval and informed patient consent). Whole blood was subjected to density gradient centrifugation using Histopaque 1119 as above and resuspended in 2% (w/v) BSA in PBS. The cell suspension was thereafter subjected to erythroblast enrichment using immunomagnetic Dynabeads (Chapter 6).

5.4 Results

Tables 5.1 - 5.5 present the results of the different variables investigated. The results are presented as mean values (± 1 standard deviation), where "n" is the number of samples studied and the results are tabulated for easier comparison of data. Student's t-test was used for statistical analysis and the 95% confidence intervals for the data were determined using SPSS (version 8.0) statistical programme.

Table 5.1 details the differential nucleated cell count from term cord blood, the results being similar to those published previously (Playfair et al, 1963).

Table 5.1 Cell and differential counts in term cord blood

Cell type	Cell count
Erythrocytes	4.43 (± 0.38) x 10^9/ml
Nucleated cell count	9.4 (± 1.0) x 10^6/ml
Polymorphs	59.0 ± 7.5%
Lymphocytes	35.3 ± 6.5%
Monocytes	0.2 ± 0.2%
Erythroblasts	5.6 ± 4.5%

Cells stained with May-Grünwald-Giemsa stain (n = 105).

Table 5.2 details the efficiency of erythrocyte removal and recovery of leukocytes and fetal erythroblasts. Both ficoll-hypaque and Histopaque 1077 resulted in better erythrocyte removal than Histopaque 1119 ($p < 0.001$) but Histopaque 1119 recovered significantly more erythroblasts ($p < 0.001$). This latter result is probably more important as, given the small number of fetal erythroblasts expected in maternal blood, maximum erythroblast recovery at each stage of the enrichment process is necessary.

Table 5.2. Nucleated cell recovery with different density gradient media using cord blood

Density gradient medium	Erythrocyte removal (%)	Leukocyte recovery (%)	Erythroblast recovery (%)
Ficoll-Hypaque (n = 57)	99.94 ± 0.09* (99.92 - 99.96)	20.2 ± 6.5** (18.5 - 21.9)	34.2 ± 23.9† (28.0 - 40.4)
H1077 (n = 57)	99.94 ± 0.09* (99.92 - 99.96)	33.2 ± 11.9** (30.1 - 36.3)	15.6 ± 7.5† (13.6 - 17.6)
H1119 (n = 145)	99.41 ± 0.79 (99.28 - 99.54)	64.2 ± 29.3 (59.4 - 69.0)	53.0 ± 28.6 (48.3 - 57.7)

Blood centrifuged at room temperature at 500 x g for thirty minutes.
*$p < 0.001$, erythrocyte removal comparing density gradient medium with Histopaque 1119.
**$p < 0.001$, leukocyte recovery comparing density gradient medium with Histopaque 1119.
†$p < 0.001$, erythroblast recovery comparing density gradient medium with Histopaque 1119.
95% confidence intervals in parentheses.

Washing blood prior to density gradient centrifugation resulted in poorer recovery of both leukocytes and erythroblasts (table 5.3) although this did not reach significance.

Table 5.3 Comparison of the effect of washing blood prior to density gradient centrifugation on nucleated cell recovery in term cord blood

Medium	Percentage recovery of nucleated cells (%)	Percentage recovery of erythroblasts (%)
H1077 (washed)	21.3 ± 6.9 (16.5 - 26.1)	11.4 ± 5.0 (7.9 - 14.9)
H1077 (unwashed)	58.4 ± 33.2 (35.4 - 81.4)	24.3 ± 16.8 (12.7 - 35.9)
H1119 (washed)	43.2 ± 28.6 (23.4 - 63.0)	35.8 ± 34.2 (12.1 - 59.5)
H1119 (unwashed)	79.7 ± 16.2 (68.5 - 90.9)	65.5 ± 26.8 (46.9 - 84.1)

Blood washed prior to centrifugation in 2% (w/v) BSA in PBS and then centrifuged at room temperature at 500 x g for thirty minutes (n = 8, each group).
95% confidence intervals in parentheses.

In direct comparisons of Histopaque 1119 with Nycodenz 1.085, the former appeared to be more efficient at removing erythrocytes although this did not reach statistical significance ($p > 0.05$, table 5.4). However, Nycodenz 1.085, because it lacks a red cell aggregating agent resulted in better erythroblast recovery ($p < 0.001$).

Table 5.4 Direct comparison of Histopaque 1119 and Nycodenz 1.085 using artificial admixtures of fetal and adult blood

Density gradient medium	Erythrocyte removal (%)	Leukocyte recovery (%)	Erythroblast recovery (%)
Histopaque 1119	99.95 ± 0.07 (99.91 - 99.99)	68.6 ± 23.7 (53.9 - 83.3)	16.6 ± 9.5 (10.7 - 22.5)
Nycodenz 1.085	99.87 ± 0.28 (99.70 - 100)	46.8 ± 24.5 (31.6 - 62.0)	38.4 ± 10.9 (31.6 - 45.2)
p	NS	NS	< 0.001

Centrifugation at room temperature at 500 x g for thirty minutes (n = 10 each group)
NS = not significant. Note the superior erythroblast recovery with Nycodenz (95% confidence intervals in parentheses).

Direct comparisons using different centrifugation tubes showed that there was little difference in erythrocyte removal or leukocyte recovery ($p > 0.05$) except that Greiner tubes recovered fewer leukocytes compared to the Sterilin tubes ($p < 0.001$, table 5.5).

Table 5.5 Comparison of different centrifugation tubes using H1119

Centrifuge tube	Erythrocytes removed (%)	Leukocytes recovered (%)
Sterilin	99.98 ± 0.01 (99.97 - 99.99)	46.6 ± 5.7* (46.5 - 46.7)
Greiner	99.97 ± 0.03 (99.95 - 99.99)	30.8 ± 1.7* (29.8 - 31.8)
Falcon (2099)	99.99 ± 0.01 (99.97 - 99.99)	36.1 ± 22.7 (23.3 - 48.9)
Falcon (2096)	99.98 ± 0.01 (99.97 - 99.99)	42.5 ± 21.5 (30.3 - 54.7)

Centrifugation at room temperature at 500 x g for thirty minutes (n = 12 each subgroup)
95% confidence intervals in parentheses.
*$p < 0.001$, difference between Sterilin and Greiner tubes on leukocyte recovery

5.5 Discussion

The experiments in model systems showed that ficoll-containing density gradient media resulted in good erythrocyte removal and the more dense the medium, the better the recovery of erythroblasts. Non-aggregating media resulted in poorer erythrocyte removal (though this was not significant) but better erythroblast recovery.

Washing resulted in increased loss of erythroblasts presumably because cells adhered to the test tube walls and/or clumped. Inevitably the more washing steps, the greater the

losses. Because fetal erythroblasts are extremely rare in maternal blood, and every effort must be made to maximise their recovery, washing was abandoned.

There were only marginal differences in erythrocyte removal using the different types of tubes. The Greiner tubes were easiest to use, but least efficient at erythrocyte removal (as erythrocytes tended to stick at the porous filter and were collected with the nucleated cells). They also recovered fewer leukocytes. The polystyrene Falcon tubes (2099) were best at removing erythrocytes but nucleated cell loss was greater. Erythrocyte removal and nucleated cell recoveries were similar for the Sterilin and polypropylene Falcon tubes (2096) but leukocyte recovery (and, presumably fetal erythroblast recovery) was more predictable using the Sterilin tubes.

Bhat et al (1990) enriched for fetal lymphocytes from homogenised fetal livers using a discontinuous dual density gradient system of Histopaque 1077 (H1077) on Histopaque 1119 (H1119). They found that lymphocytes preferentially collected at the plasma/H1077 layer whilst erythroblasts collected at the H1077/H1119 interface. Although the upper interface was relatively free of erythroblasts (less than 0.5% of the nucleated cells) overall 17.4% of all erythroblasts collected at this interface. They subsequently used a discontinuous triple gradient of H1077, H1107 and H1119 to enrich specifically for erythroblasts in model systems (Bhat et al, 1993). Erythroblasts express the transferrin receptor, CD71, and they examined the cells according to CD71 and leukocyte antigen (CD45 and CD13) expression. At the H1077/H1107 interface, 19.3% of nucleated cells were CD71-positive and leukocyte antigen-negative, suggesting that they were of erythroid origin. 1.1% of the nucleated cells at the plasma/ H1077 interface were erythroid, whilst at the H1107/H1119 interface, 1.5% of the nucleated cells were erythroid. They suggested that erythroblasts preferentially collected at the H1077/H1107 interface and, if enriching from maternal blood, harvesting this layer might be most rewarding. Unfortunately the authors did not report the absolute number of cells recovered at each interface. If, for instance, ten times as many cells were collected at the lower interface compared to the one above, the total number of fetal erythroblasts would be similar in each layer. In considering rare cells, such data is necessary as it could have

significant bearing on the density gradient used. Hahn et al (1999) compared a triple gradient with a single gradient and concluded that the latter was simpler and resulted in better recovery of fetal erythroblasts. Huber et al (1996), using an analytical proprietary liquid chromatography gradient, suggested that up to 80% of fetal erythroblasts were lost in the red cell pellet if relatively low density ficoll-hypaque was used, including gradients used currently by many groups. This is much greater than our experience with H1119 although our large standard deviation is testimony to the very large proportion of erythroblasts which can be lost. However, it confirms our belief that a single higher density gradient is simpler than dual or triple cushions and would maximise erythroblast recovery (although at the expense of slightly greater erythrocyte contamination).

The inclusion of an aggregating agent in H1119 resulted in better, and more predictable, erythrocyte removal than Nycodenz 1.085 which lacked an aggregating agent (table 5.5) although this did not reach statistical significance. However, Nycodenz resulted in statistically better erythroblast recovery. Unfortunately there were also almost three times as many contaminating erythrocytes after centrifugation which more than offset the improvement in erythroblast recovery as this many erythrocytes would occupy considerable space on microscope slides. Sekizawa et al (1999) compared two different densities of percoll (relative density 1.083 and 1.090) which does not contain an aggregating agent and found they could recover significantly more erythroblasts by using the denser medium. However, they also found that the proportion of contaminating cells was also increased. It would be ideal to use a medium such as Nycodenz to recover as many erythroblasts as possible but to do so would depend on removing the remaining contaminating erythrocytes at a later stage (chapter 10.1).

5.6 Summary

Histopaque 1119 was the best compromise for erythrocyte removal and erythroblast recovery. We concluded that blood should not be washed prior to density gradient centrifugation and centrifugation should be performed using conical based Sterilin tubes.

As expected, density gradient centrifugation was insufficient and the enrichment process was continued with immunomagnetic Dynabeads (chapter 6).

Chapter 6. Immunomagnetic enrichment of Erythroblasts

6.1 Principles of Immunomagnetic separation

Explanation of the principles of immunomagnetic separation is given here to allow better understanding of the methodologies that are described immediately after. Basically, immunomagnetics utilises magnetic particles onto which are attached specific antibodies. The antibodies are directed against cell surface antigens and when placed adjacent to a magnet the magnetic beads are drawn to the magnet, dragging with them any attached cells.

6.1.1 Dynabead constitution

Dynabeads are 4.5μm diameter particles consisting of a styrene divinylbenzene core and magnetite. The core is coated with a hydrophilic polymer containing epoxy and hydroxyl groups which binds the secondary antibody covalently. The secondary antibody can then bind the F_c portion of a primary monoclonal antibody (mAb). The magnetite in the bead is attracted to the magnet (Lea et al, 1985). Because of their size, Dynabeads are visible under the light microscope and also autofluoresce.

Dynal now produce beads which have IgG subclass-specific, species-specific secondary antibodies so that the subclass of the primary immunoglobulin determines the variety of bead to be used. This improved the efficiency enormously (table 6.3).

6.1.2 Methods of binding the secondary to the primary antibody

There are essentially two approaches - the indirect and direct methods.

The indirect method involves incubating the cells with the primary mAb, washing the cells to remove excess mAb and then incubating the cells with the Dynabeads. The beads attach to those cells that have bound the primary mAb and the cells are subsequently removed from suspension. The indirect approach has certain disadvantages. In tissues with high antigen expression, primary mAb crowding may reduce secondary antibody binding because of steric hindrance (chapter 4.3). Alternatively, the primary mAb may bind in a configuration

that is inaccessible to the secondary antibody. In these situations the direct method improves separation (Gee et al, 1991).

The direct approach involves first attaching the primary mAb to the secondary antibody and then incubating the beads with the cells. Once the beads have bound to the cells, the test tube is placed adjacent to a magnet, housed in the proprietary "magnetic particle separator". The beads are drawn to the magnet, taking attached cells with them (figure 6.1).

Whenever a new primary mAb was obtained, using the direct method the optimum concentration needed to be determined to maximise bead efficiency and the following strategy was used.

After removal of erythrocytes (chapter 5), differential and quantitative leukocyte counts were ascertained and immunocytochemistry performed using the primary mAb. This allowed the proportion and the absolute numbers of cells binding the specific mAb to be calculated. Varying concentrations of the new antibody had been previously made up with beads and the individual aliquots of beads incubated with equivalent samples of the leukocyte preparation. Following removal of the beads, a cell count and differential of the supernatant was made and immunocytochemistry using the same primary mAb again performed. Those preparations, post-immunocapture, which showed the fewest cells expressing the antigen suggested the optimum mAb concentration for maximal bead efficiency. The method was largely empirical and there was a relatively broad plateau for the optimal mAb concentration before efficiency was reduced significantly. In this way the lowest concentration of mAb could be used without compromising bead efficiency.

6.1.3 Optimising the number of beads per cell

If too few beads were used, cell removal was reduced. Using too many beads simply increased costs without improving cell removal and the mass movement effect of large numbers of beads increased the non-specific loss of erythroblasts (chapter 6.2.7). The direct approach was more economical with respect to both mAb and beads than the indirect method and, after initial comparisons between the two, all separations were performed using the direct approach.

6.1.4 Methods of cell enrichment

Two methods were tested for fetal erythroblast enrichment: positive and negative selection. Positive selection implies that the antibody used isolates the cell of interest. We used a monoclonal antibody to the transferrin receptor (CD71). Negative enrichment implies removing unwanted cells (leukocytes), leaving the desired cells (fetal erythroblasts) and contaminants in suspension (figure 6.2).

6.2 Method for Dynabead Preparation

1ml of beads was resuspended in 10ml of 2% (w/v) BSA in PBS/0.02% azide (2% BSA/PBS/azide) and placed in the magnetic particle separator for two minutes. The supernatant was pipetted off and the beads resuspended in 10ml of 2% BSA/PBS/azide, mixed for two minutes in an SB1 end-over-end blood tube rotator (Stuart Scientific Co. Ltd.) and then placed in the magnetic particle separator again. The washing process was repeated for a total of five washes to remove any unbound secondary antibody.

For a 1 in 10 dilution of primary mAb, 1ml of the mAb was added to 4×10^8 of the appropriate IgG subclass-specific Dynabeads and made up to 10ml with 2% BSA/PBS/azide. The beads and mAb were mixed at 4°C for at least 30 minutes, though usually overnight, and could be left in this state for several weeks. Using the direct technique, table 6.1 shows the optimum dilutions of the primary mAb.

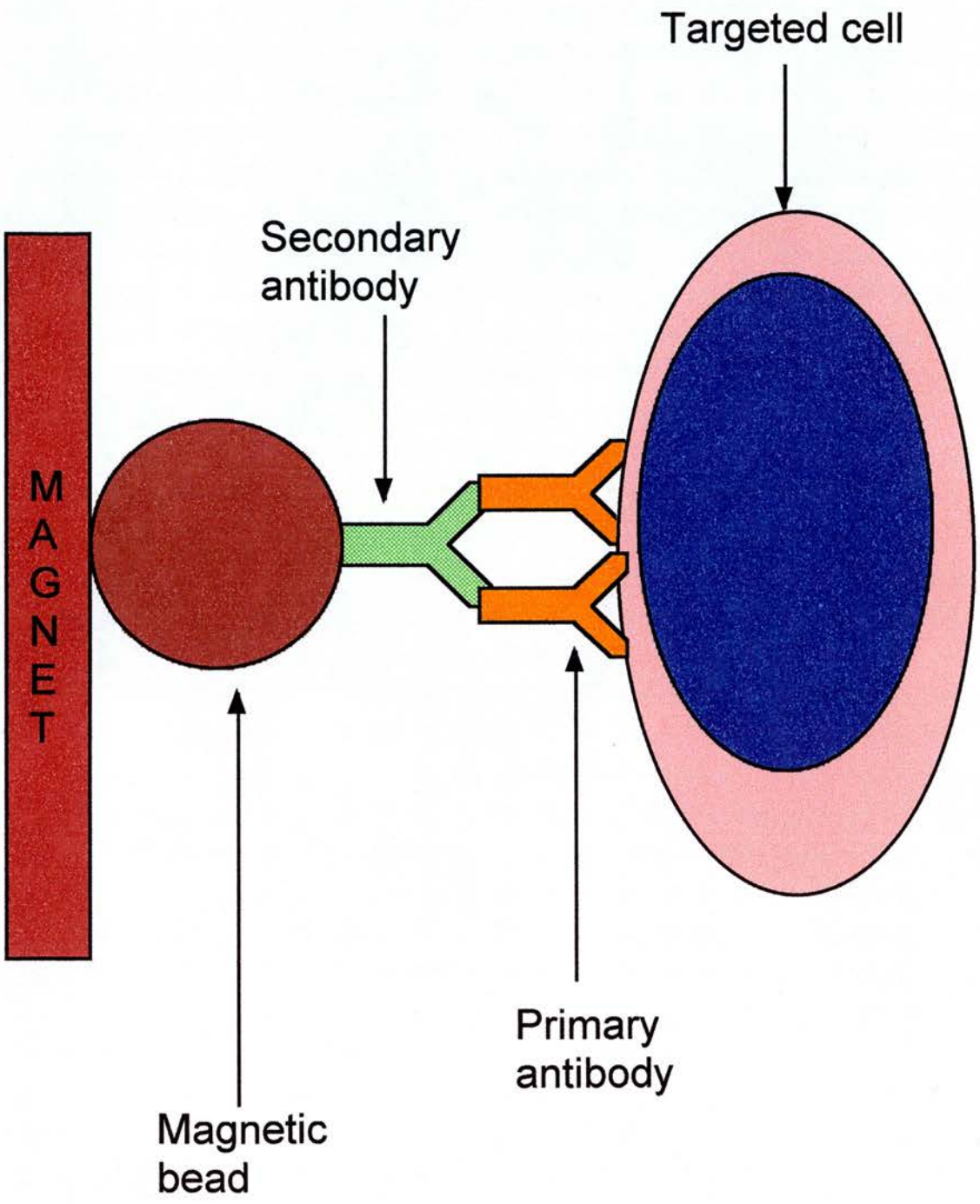
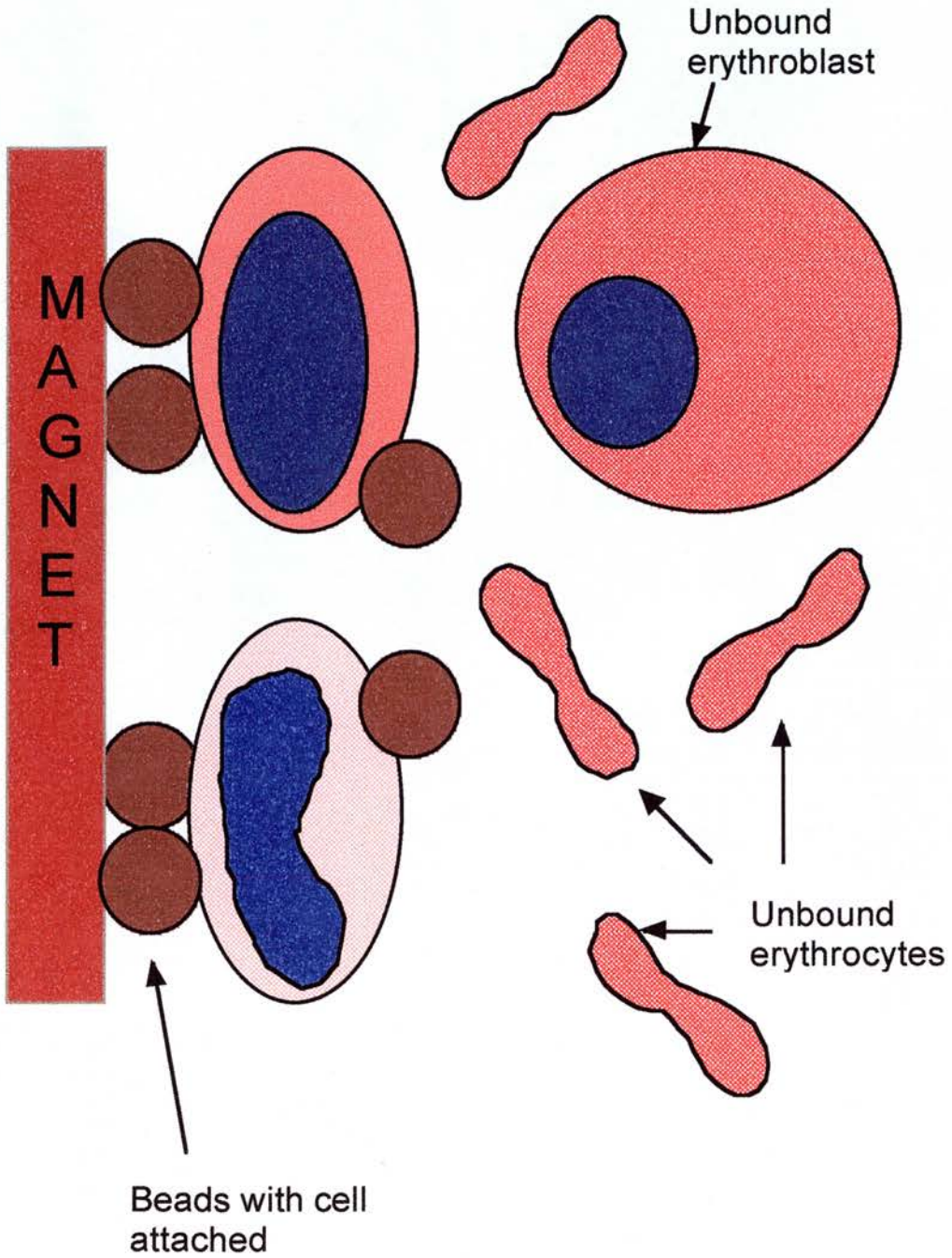


Figure 6.1.
The Principle of Immunomagnetics

The bead draws the attached cells to the magnet

Figure 6.2. The Principles of Negative Selection



The cells which are attached to the beads are retained on the magnet and discarded, leaving the target cells (erythroblasts) in suspension.

Table 6.1. Optimum primary monoclonal antibody dilutions and number of Dynabeads per cell

Antigen	Monoclonal antibody	Monoclonal antibody dilution	Optimum number of beads/ cell
CD71	BER-T9	1 in 50	5
CD18	HIG.125	1 in 10	4
CD2	RFT-11	1 in 10	3
CD3	UCHT1	1 in 10	3
CD45	EBZ17	1 in 10	4
HLA.DR	DA6.231	1 in 30	3

See Appendix II for mAb source.
Dynabeads and primary mAb incubated at 4°C in 2% BSA/PBS/azide.

6.2.1 Preparation of Dynabeads for cell enrichment

Following density gradient centrifugation, the total number of leukocytes were determined and an appropriate quantity of beads was washed five times in phosphate buffered saline (PBS). Suboptimal washing did not remove all the unbound primary mAb resulting in competition between bound and unbound mAb for antigen sites, so decreasing bead-cell binding.

6.2.2 Estimation of Dynabead efficiency

The major drawback of positive selection was the difficulty in detaching beads from the cells (vide infra) and, therefore, the inability to examine the selected cells morphologically. The only way to determine which cells had been selected was to analyse the cells left in suspension. Although less than ideal, by inference the cells which had been removed could be estimated. Using negative enrichment this was not a problem as the cells left in suspension included fetal erythroblasts.

Every deposition method incurs cell loss. This was not expected to be cell-specific but across all cell types. Simply by making a direct estimation of the number of nuclei that were deposited on to slides resulted in an overestimation of the efficiency of beads. Calculation of Dynabead efficiency, using either positive or negative selection, was therefore likely to be no

more than an estimate. However, a strategy to make this as accurate as possible was developed.

Cytocentrifuge preparations were made following Histopaque and immunomagnetic enrichment and then stained. The ratio of nucleated cells to erythrocytes following these two stages was calculated and from these ratios the percentage of fetal erythroblasts recovered or selected (in the case of positive selection) and leukocytes removed was determined. Because ratios and not absolute cell numbers were calculated, the results were likely to be more accurate. This method to calculate bead efficiency has been reported previously by Gänshirt-Ahlert et al (1993).

6.2.3 Optimising conditions for cell and bead incubation

(i) Incubation temperature

Samples of cells were taken from the same original cell suspension and mixed simultaneously in exactly the same way but at 4°C and 22°C. Using negative selection, the results of fetal erythroblast recoveries and leukocyte removal were determined.

(ii) Cell-bead mixing methods

The cell suspension was made up with 2% (w/v) BSA in PBS and using an equal amount of beads conjugated to anti-CD18, anti-CD2 and anti-CD3 mAbs, different methods of cell/ bead mixing were investigated.

(a) Peristalsis technique. The tip of a 1mm bore soft plastic tube was placed at the bottom of the test tube containing the cell suspension at 22°C and an aliquot of beads was added. A Minipuls 2 peristaltic pump (Gilson) generated small air bubbles that rose through the suspension at 1 bubble/ second, encouraging the beads and cells to mix. After 20 minutes the test tube was placed in a magnetic particle separator. The supernatant was decanted into a fresh test tube, another aliquot of beads added and the process repeated for four incubations.

(b) Dropping technique. Dynabeads, being denser than cells, gravitate faster. 75µl aliquots of beads were dropped every two minutes into a static upright test tube containing the cell suspension and allowed to descend under unit gravity until all the beads were exhausted.

(c) Rotor mixing. The cell suspension filled the test tube to approximately 70%. The cells were incubated with four sequential aliquots of beads, each for 20 minutes, at 22°C using an end-over-end blood tube rotator, SB1 (Stuart Scientific Co. Ltd.) angled at 30° to the horizontal, rotating at 26 revolutions per minute.

(d) Spiramix technique. The Denley Spiramix 5 was easiest to use as test tubes could be added and removed without having to stop the motor. The test tubes were approximately 70% full and the cells were incubated with four sequential aliquots of beads, each for 20 minutes, with the barrels rotating at 48 rpm.

Cell counts and differentials were calculated for each of the different mixing methods to determine the most efficient method of cell removal.

(iii) Length of incubations and the number of aliquots of beads

Two approaches to the length of time cells were mixed with beads were investigated. Firstly all the cells were incubated with all the beads for a prolonged but defined period, usually 90 minutes. Alternatively the same absolute number of beads were divided into three, four or five equal aliquots and all the cells incubated with successive aliquots for a shorter period (e.g. 20 minutes each) for approximately the same length of time overall.

6.2.4 Positive selection of fetal erythroblasts

An absolute quantitative leukocyte count, differential count and reticulocyte count was made of the cells after removal of erythrocytes by density gradient centrifugation.

A ratio of 5 beads, coated with anti-CD71 mAb (BER-T9), to one nucleated cell was optimal in model experiments. The beads were divided into 3 equal aliquots and these were incubated sequentially with the leukocyte suspension for 20 minutes at 22°C on the end-

over-end rotator. After magnetic bead removal the remaining supernatant was carefully pipetted into another test tube, the next aliquot of beads was added and the incubation repeated.

Following the three incubations, a reticulocyte and quantitative leukocyte count and differential cell count were calculated on the bead-free supernatant. A cytospin preparation was also subjected to immunocytochemistry using the original BER-T9 mAb to assess the efficiency of beads at removing CD71-positive cells.

Two effects were soon apparent. Firstly, a sizeable proportion of leukocytes, particularly polymorphs, appeared to be selected. Secondly, the morphology of the cells that were positively selected could not be determined as overlying beads obscured them (figure 6.3). In maternal blood samples, if this population included erythroblasts, cytogenetic analysis using fluorescence in situ hybridisation would be impossible.

To determine why so many leukocytes were removed, cell suspensions were incubated with raw beads and those cells remaining in suspension were analysed.

An attempt was made to detach the beads from the cells with Dynal proprietary "Detach-a-beads" using the manufacturer's protocol or by simply "flooding" the positively selected cell population with a 1 in 50 dilution of anti-CD71 mAb. This was performed by incubating BER-T9 with the beads for twenty minutes, placing the test tube in the magnetic particle separator and then making cytospin preparations to see which cells detached from the beads.

6.2.5 Miltenyi Magnetic activated cell sorting

In 1992 Gänshirt-Ahlert et al reported using the Miltenyi Magnetic Activated Cell Sorter (MACS) to enrich for fetal erythroblasts from cord blood. In an attempt to repeat their work, we obtained the MACS system from Becton-Dickinson.

Using the indirect method and BER-T9 (using the 10ml syringe columns and the protocol detailed by Miltenyi) three passes of the column were made. The bead-free supernatant was collected and cell counts and cytospin preparations made for morphological and immunocytochemical staining. The syringe column was removed from the magnetic bracket

and the steel wool flushed three times. The cells in the eluent were collected and analysed in the same way. From this, the removal and recoveries of fetal erythroblasts by MACS were calculated.

6.2.6 Negative enrichment for Erythroblasts

Following density gradient separation the total number of nucleated cells was determined and the absolute numbers of beads necessary were prepared (table 6.1).

The aliquot of anti-CD18-beads was divided into four equal aliquots and the first aliquot was incubated with the cell suspension for 20 minutes at 22°C on an end-over-end blood tube rotator. Following magnetic particle separation the supernatant was carefully pipetted off and placed in another test tube whilst the beads were discarded.

The anti-CD2- and anti-CD3-beads were divided into three aliquots and the individual aliquots and the anti-CD18-beads were added to the cell suspension for three more sequential incubations, each for 20 minutes. The supernatant became progressively clearer as more leukocytes were removed.

After the anti-CD18/CD2/CD3 mAb cocktail, approximately 80% of the remaining mononuclear cells were CD19-positive, indicative of B-cells. B-lymphocytes and activated T-lymphocytes express HLA.DR antigens and, therefore, an in-house mAb DA6.231 was added to target the B-cells (Guy et al, 1982).

After the final incubation the supernatant was placed in a sterile test tube. 500µl were removed, a cytospin preparation was appropriately stained and a differential count determined.

6.2.7 Investigation of erythroblast loss associated with negative enrichment

From the model systems it was apparent that fetal erythroblasts were lost at the bead stage. This appeared to be dependent on the length of incubation and the mAbs used and resulted in the different protocol permutations.

Cells were inevitably left on the walls of the test tube when the supernatant was removed but another possible mechanism of erythroblast loss was at the magnet stage. When the test

tube was placed in the magnetic particle separator, it was plausible that erythroblasts were literally "caught in the back and pushed" to the test tube wall because of the mass movement effect of the beads.

This was investigated by washing the beads three times in 2% (w/v) BSA in PBS and then replacing the test tube against the magnet. It was hoped that cells that were not immunologically bound would separate from their beads during the resuspension and thereafter remain in suspension rather than being pushed to the side wall again. The supernatant (of unbound cells) was pipetted off into a clean test tube and centrifuged to pellet the cells. A differential count and nucleated cell/ erythrocyte ratio were determined to calculate the number of erythroblasts in this supernatant.

6.2.8 Combining negative and positive enrichment

Neither positive nor negative enrichment gave completely satisfactory results and the two methods were combined in an attempt to reduce the final absolute number of cells. Firstly, leukocytes were removed and then CD71-positive cells were selected with BER-T9-conjugated beads using the protocols as above. The bead-cell conjugates were fixed in suspension in fresh methanol: acetic acid (3:1) to lyse the cell membranes so that the bare nuclei separated from the beads which were subsequently drawn to the magnet. The suspension was drawn off and centrifuged to pellet the nuclei. This was resuspended in a small volume of methanol: acetic acid for later in situ hybridisation.

6.3 Results of Immunomagnetic enrichment techniques

6.3.1 Positive selection

Table 6.2 shows that 70-83% of cord erythroblasts were CD71-positive and demonstrates the efficiency of positive selection of the positive cells. These data show that enrichment was only to one order of magnitude. Furthermore, raw beads (without a primary mAb attached) appeared to remove both polymorphs and lymphocytes possibly by phagocytosis or by

binding of the secondary antibody to cell surface antigens. Furthermore, the attached beads (figure 6.3) obscured the morphology of the positively selected cells.

Table 6.2. Positive selection with anti-CD71 mAb (BER-T9) (n=25)

CD71-positive cord erythroblasts (%)	70-83
Removal of CD71-+ve erythroblasts (%)	93.2 ± 10.7 (89.0 - 97.4)
Removal of leukocytes (%)	62.5 ± 24.4 (52.9 - 72.1)
Removal of polymorphs with raw beads (%)	74.9 ± 11.0 (70.6 - 79.2)
Removal of lymphocytes with raw beads (%)	34.4 ± 9.5 (30.7 - 38.1)

Dynabeads incubated with erythrocyte-depleted nucleated cells at room temperature.
95% confidence intervals in parentheses

Using the proprietary Detach-a-beads only 3.7% of all the CD71-bound cells could be detached and the final recovery, from all starting fetal erythroblasts, was only 0.8%. By flooding the beads with BER-T9 mAb, 7.5% of all the bound cells could be detached and the final recovery from all starting fetal erythroblasts was 15.3%.

6.3.2 Results using the Miltenyi Magnetic Activated Cell Sorter

We found that 56.7 ± 17.9% of fetal erythroblasts (n = 6) escaped immunocapture. However, of these, approximately 40% appeared to be CD71-negative. Furthermore, over 70% of leukocytes (particularly polymorphs) were not in the negative fraction and many of these came through with the positive eluent. More importantly, only 24.0 ± 5.7% of the selected fetal erythroblasts could be recovered from the steel wool column.

6.3.3 Negative selection

The ratio of beads to cells varied and generally, the more beads used, the greater the leukocyte removal. However, this increased the costs and, importantly, non-specific erythroblast loss.

Data is presented as the percentage removal of leukocytes from the total number of leukocytes after density gradient centrifugation and, similarly, the recoveries of erythroblasts

is presented as a percentage of fetal erythroblasts recovered after density gradient centrifugation.

HIG.125 is an IgG_{2a} mAb. Table 6.3 compared the old Dynabeads (where the secondary antibody was a polyclonal antibody) and the newer Dynabeads, where the secondary antibody is immunoglobulin subclass-specific. It demonstrates that leukocyte removal was more efficient with the newer Dynabeads ($p < 0.001$) but there was no difference in erythroblast recovery.

Table 6.3. Direct comparison of different Dynabeads using anti-CD18 mAb (HIG.125) on efficiency of negative enrichment

Secondary antibody	n	Leukocyte removal (%)	Erythroblast recovery (%)
IgG non-specific (polyclonal)	11	68.7 ± 11.5 (61.9 - 75.5)	86.6 ± 23.2 (72.9 - 100)
IgG_{2a} subclass-specific	11	97.3 ± 1.4 (96.5 - 98.1)	90.9 ± 12.9 (83.3 - 98.5)
p		< 0.001	NS

Dynabeads with polyclonal or sub-class-specific secondary antibody bound to HIG.125 incubated with cells at room temperature.
NS = not significant. 95% confidence intervals in parentheses.

Using different T-cell mAbs with the subclass-specific beads the possibility of additional mAbs was addressed. Table 6.4 shows that RFT-11 mAb was more efficient at removing T-cells than the mAb, UCHT1 ($p < 0.001$) but neither removed erythroblasts.

Table 6.4. Direct comparison of individual anti-T-cell mAbs on efficiency of T-cell removal

Primary antibody	n	T-cell removal (%)	Erythroblast recovery (%)
RFT-11 (anti-CD2)	5	84.9 ± 2.3 (82.9 - 86.2)	98.7 ± 2.1 (96.9 - 100)
UCHT1 (anti-CD3)	5	65.4 ± 6.0 (60.1 - 70.7)	99.4 ± 1.3 (98.3 - 100)
p		<0.001	NS

Incubations at room temperature. 95% confidence intervals in parentheses.

A single aliquot of EBZ17-coated Dynabeads (anti-CD45) was compared with sequential incubations of equal aliquots of beads, using the same total number of beads (Table 6.5).

Although additional aliquots improved leukocyte removal, this did not reach significance. However, using 5 aliquots of anti-CD45-coated beads significantly reduced the recovery of erythroblasts compared to one or three aliquots ($p < 0.001$) and fetal erythroblast recovery was substantially poorer when compared to the anti-CD18 mAb ($p < 0.001$).

Table 6.5 Comparing incubations using single and multiple aliquots of beads coated with anti-CD45-mAb (EBZ17)

No. of aliquots	n	Leukocyte removal (%)	Erythroblast recovery (%)
1 aliquot (90 minute incubation)	60	97.6 ± 3.6 (96.7 - 98.5)	25.9 ± 21.5 (20.5 - 31.3)
3 aliquots (20 minute incubations)	19	98.3 ± 2.2 (97.3 - 99.3)	23.8 ± 12.9 (18.0 - 29.6)
5 aliquots (20 minute incubations)	10	98.9 ± 0.6 (98.5 - 99.3)	6.7 ± 3.0* (4.8 - 8.6)

Incubations at room temperature. Note the fall in erythroblast recovery with increasing leukocyte removal. 95% confidence intervals in parentheses.
*$p < 0.001$, erythroblast recovery compared to 1 or 3 aliquot incubations.

Table 6.6 shows that the addition of an anti-HLA.DR mAb significantly increased leukocyte removal ($p < 0.001$) but that erythroblast recovery was dramatically reduced ($p < 0.001$). This latter result was more important because such losses would be intolerable given the small number of fetal erythroblasts expected in maternal blood.

Table 6.6. Efficiency of negative selection with different mAb cocktails

mAb cocktail	Anti-CD18/CD2/CD3	Anti-CD18/CD2/CD3/HLA.DR	p
n	35	44	
Leukocyte removal (%)	99.1 ± 0.4 (99.0 - 99.2)	99.8 ± 0.2 (99.7 - 99.9)	< 0.001
Erythroblast recovery (%)	82.0 ± 20.5 (75.2 - 88.8)	21.8 ± 11.0 (18.6 - 25.0)	< 0.001

Incubations at room temperature. 95% confidence intervals in parentheses.
Note the fall in erythroblast recovery with increasing leukocyte removal.

Using the two stage process of Histopaque 1119 and negative enrichment with Dynabeads, it was possible to recover fetal erythroblasts from a starting ratio of 1 fetal erythroblast: 10^8 maternal cells (1 in 10^6 leukocytes), approaching the ratios which are estimated in maternal blood. Overall, the final erythroblast recovery from whole blood was approximately 32%.

6.3.4 Estimation of erythroblast loss with negative enrichment

The results are presented from assays using only anti-CD18- and anti-CD3-mAbs. At each stage when the test tube was placed in the magnetic particle separator and the beads were drawn to the magnet, approximately 2-3% of the original number of erythroblasts could be recovered by washing the beads and dislodging any immunologically unbound cells. Overall, therefore, only around 6-10% of fetal erythroblasts might be recovered by washing the beads.

6.3.5 Comparison of incubating methods

Table 6.7 illustrates the relative efficiencies of the different incubation methods at 22°C using beads coated with anti-CD18, anti-CD2 and anti-CD3 mAbs.

Clearly the two formal mixing techniques (end-over-end mixer and Spiramix) were superior to either the peristalsis or dropping techniques ($p < 0.001$). However, compared to the Stuart end-over-end rotor, the Denley Spiramix was easier to use and leukocyte removal was also superior ($p < 0.001$).

Table 6.7 Comparison of incubation methods

Incubation method	n	Leukocyte removal (%)
Peristalsis technique	8	43.2 ± 15.6 (32.4- 54.0)
Dropping technique	8	16.3 ± 6.4 (11.9- 20.7)
Stuart SB1 end-over-end rotor	12	99.2 ± 0.2 (99.1- 99.3)*
Denley Spiramix 5	12	99.6 ± 0.1 (99.5- 99.7)*

95% confidence intervals in parentheses.
*$p < 0.001$, comparison of end-over-end rotor with Denley Spiramix using Dynabeads bound to anti-CD18, CD2 and CD3, incubated at room temperature.

6.3.6 Results of combining negative and positive enrichment

In model experiments, following leukocyte removal, an average of 43.7% of fetal erythroblasts were removed from the supernatant by the anti-CD71-conjugated beads. Therefore, using this three-stage approach, the final erythroblast recovery from whole blood was approximately 15%.

6.4 Discussion

6.4.1 Optimising the incubation method

(i) Bead-cell binding

The direct approach (incubating cells with Dynabeads with the primary mAb already attached to the beads) was superior to the indirect approach (data not shown). Beads could be prepared several weeks in advance and experimentation was more rapid on the day. It had the additional advantage of subjecting the cells to fewer washes and centrifugations, resulting in reduced cell loss. Finally, the direct method was more economical for both primary mAb and beads.

(ii) Incubation temperature

The laboratory was not equipped with a 37°C room but incubation at 22°C was more efficient than at 4°C. Antibodies work most efficiently at 37°C but at this temperature phagocytes may also be activated. At 4°C mAb-epitope binding is less efficient but bonds are stronger and hence if beads attach to a cell, there is a greater chance of dragging the

cell to the magnet. The greater efficiency at 22°C was probably a reflection of the mAb binding at a temperature nearer to its optimal working temperature.

(iii) Cell-bead mixing methods

It is desirable to encourage as many antibody-antigen collisions as possible to maximise the possibility for antibody binding and clearly the formal mixing methods were superior. It was hoped that the gentler mixing techniques would provide a longer time for antibody-antigen interaction and, as there was no sudden mass movement effect as beads were drawn to the magnet, non-specific fetal erythroblast loss would be minimised. However, mixing the supernatant on rotating barrels (Denley Spiramix 5) gave the best and most reproducible results. Because the mixer did not need to be stopped to remove the test tubes, it was also the easiest to use and was adopted as the incubation method of choice.

(iv) Length of incubations and the number of aliquots of beads

Theoretically only one bead is required to remove a cell but three beads is optimal (Pilling et al, 1989). For a finite number of beads, short incubations of multiple aliquots was superior to longer but fewer incubations (Table 6.5). Particular mAbs may have a greater affinity for certain cells and as more beads bound to that cell there were fewer beads available to react with the remaining cells. These higher affinity mAbs removed many cells with the initial aliquots and in subsequent aliquots there were relatively more beads to react with the remaining cells.

The length of each incubation period was crucial. The longer the incubation period, the greater the number of beads bound per cell. However, this also increased non-specific binding and phagocytosis of beads. This was especially important when the anti-HLA.DR mAb was used (Chapter 6.4.6).

6.4.2 Positive selection (CD71)

The rationale for using CD71 to enrich for fetal erythroblasts has been detailed in chapter 3.6. For live cell separation only cell surface markers can be used (unlike FACS analysis) and, therefore, it would seem that immunomagnetic separation is an ideal modality for fetal cell enrichment.

Removal of 93.6 ± 12.0% of CD71-positive cord fetal erythroblasts (almost 70% of all fetal erythroblasts) represented efficient selection. Frazier et al (1982) calculated that the available transferrin binding sites were less than predicted by radio-immunoassay because some of the receptor molecules were either embedded in the membrane or were intracellular and hence unavailable for transferrin binding. This might explain why a proportion of fetal erythroblasts was not selected with Dynabeads. However, it is also true that beads can never be 100% efficient at selecting cells.

However, the selection of over 60% of leukocytes was very disappointing and overall the relative enrichment of fetal erythroblasts, even in cord samples, was poor. Activated lymphocytes and other dividing cells are CD71-positive and removal of these cells might be expected. However, even during pregnancy approximately only 0.83% of leukocytes are CD71-positive (Adinolfi, 1992a). Therefore, immunological selection should not account for the large proportion of leukocytes removed. Raw beads (with no primary mAb) removed a substantial proportion of leukocytes probably by non-specific binding, engulfment of beads by phagocytic cells and activation of cells because of harsh collisions. In the leukocyte-rich layer following Histopaque 1119 centrifugation, polymorphs represented the greatest proportion of leukocytes. Many of these were removed by the anti-CD71-conjugated Dynabeads and accounted for the apparent selection of CD71-negative leukocytes.

6.4.3 Detachment of positively selected cells

Unfortunately our attempts at detaching selected cells from the beads were disappointing. The proprietary Dynal Detach-a-beads use an anti-F_{ab} goat anti-mouse antibody that binds to a site between the hinge and the actual antibody-binding site of the primary mAb. Rasmussen et al (1991) found that Detach-a-beads left no primary mAb (B-cell, anti-CD19 mAb) on the cell surface. However, other groups have reported difficulties with Detach-a-bead efficacy (Pilling et al, 1989; Manyonda et al, 1992) and Dynal admit that they are not very effective with CD71-positive cells (Roy Edward, Dynal, personal communication).

Attempts to detach selected cells by competitive inhibition using BER-T9 (antiCD71) were more successful but it was wasteful of antibody and only 15.3% of fetal erythroblasts were finally recovered.

Because of the poor relative enrichment (because of non-specific leukocyte binding), the inability to identify the selected cells after immunocapture and the inability to reliably and efficiently detach the Dynabeads from the cell surface, positive selection was abandoned.

6.4.4 Magnetic Activated Cell Sorting (MACS)

MACS beads do not interfere with proliferation assays, can be used with flow cytometry and the extremely powerful magnet means that few beads per cell are required to remove cells. Most importantly, because of their size, the beads do not obscure cell morphology so the selected cells can be investigated subsequently by immunocytochemistry or FISH.

Using MACS with anti-CD71 mAb over half the fetal erythroblasts escaped immunocapture, though two-fifths of these were CD71-negative. Moreover, of the erythroblasts that were selected, three-quarters could not be recovered from the steel wool and when dealing with rare cells, this degree of loss was intolerable. The areas of loss were thought to be from the multiple washing/ centrifugation steps and fetal erythroblasts remaining trapped in the steel wool. As with Dynabeads, a large proportion of leukocytes was also removed so that the relative enrichment was poor.

Although MACS and Dynabeads were not compared directly, the time required for processing was considerably longer with MACS (although with increased familiarity this may not be so pronounced). Moreover, fetal erythroblast removal was superior with Dynabeads. However, it must be remembered that the results for Dynabeads were calculated from inference of what was left in the supernatant (whereas the results using MACS were direct analyses) and subsequent cytogenetic analysis of the selected cells was impossible with Dynabeads.

Manyonda et al (1992) compared MACS with Dynabeads for positive and negative selection of CD45RO-positive cells. Dynabeads were more efficient and there was significantly greater cell loss with MACS because of the increased number of separation steps. Cell losses and recoveries varied more with MACS and it was much slower because of the greater number of washing steps required by the protocol. Finally, in a cost-benefit analysis, they found MACS to be much more expensive.

Gänshirt-Ahlert et al (1992) reported that using MACS on cord blood they could only enrich for fetal erythroblasts by a factor of 1.4 to 10.8, similar to our results and insufficient for the needs in maternal blood. Although some groups continue to investigate positive selection of erythroblasts using MACS, we did not pursue this avenue further.

6.4.5 Negative enrichment for fetal erythroblasts

Negative enrichment has been used successfully for different applications. Bertoncello et al (1991) used a cocktail of mAbs simultaneously to remove bone marrow cells and enrich for primitive haematopoietic cells and because of the problems of positive selection, negative enrichment was investigated.

Pilling et al (1989) found that the addition of mAbs directed against different epitopes of the same antigen did not improve the efficiency, but when two mAbs directed against different antigens on the same cell were used, depletion efficiency was better. Because of the mechanical and spatial factors of antigen distribution, two beads binding to different antigens can act synergistically to facilitate removal. They also found that negative

enrichment gave a higher yield of cells than positive selection, an important consideration with rare (fetal) cells. These principles allowed us to develop a cocktail of mAbs systematically.

6.4.6 Choosing the best monoclonal antibody cocktail

The anti-CD18 mAb, HIG.125, removed 97.3 ± 1.4% of leukocytes. It is an IgG_{2a} mAb directed against the common β-chain (CD18) of the α/β heterodimer of the leukocyte adhesion molecule (LAM-1, CD11a/CD18). As such it has pan-leukocyte activity (Uciechowski and Schmidt, 1989). It was developed in-house by Dr. Veronica van Heyningen and colleagues and obtained as a culture supernatant.

The anti-CD2 mAb, RFT-11, removed 84.9 ± 2.3% of T-cells. CD2 is a transmembrane single chain 50 kDa glycoprotein expressed on resting and activated T-lymphocytes. It is involved in cell adhesion and the alternative pathway for T-cell activation (Meuer, 1989). RFT-11 is an IgG_1 subclass anti-CD2 mAb, and was obtained free of charge from the Scottish Antibody Production Unit (SAPU) as a culture supernatant.

The anti-CD3 mAb, UCHT1, removed 65.4 ± 6.0% of T-cells. CD3 is a receptor complex of at least five chains with molecular weights ranging from 16 to 28 kDa. It is involved in T-cell activation and signal transduction and has pan-T-lymphocyte expression (Kurrle, 1989). The mAb, UCHT1, an IgG_1 subclass immunoglobulin, was also obtained free of charge from SAPU as a culture supernatant.

The anti-CD45 mAb, EBZ17, removed 98.3 ± 2.2% of all leukocytes. CD45 is a single chain glycoprotein present on all leukocytes and is involved in signal transduction by modifying signals from other surface molecules. There are four isoforms with molecular weights of approximately 185 kDa, 200 kDa, 215 kDa and 230 kDa. T-cells express all four chains whilst B-cells express mainly the 230 kDa chain (Thomas, 1989). The mAb, EBZ17, also obtained from SAPU as a culture supernatant, blocks all four glycoprotein chains (Smith et al, 1985).

It was puzzling that although EBZ17 and HIG.125 removed similar proportions of leukocytes, erythroblast recovery was substantially less with EBZ17 (table 6.6) and frequently erythroblasts were found with a bead attached (Figure 6.4).

The leukocyte adhesion molecule (CD11a/CD18) is expressed on all leukocytes but not on erythroid lineage cells (Kansas et al, 1990). Therefore HIG.125 should not react with fetal erythroblasts. The mAb, HLe-1 (Beverley et al, 1980), recognises the 200 kDa chain of CD45 and also reacts with epitopes on red cell precursors (Loken et al, 1987). Hence, CD45 is expressed on erythroid cells at a low level and even after CD71 expression has peaked (Kansas et al, 1990). Presumably, as more leukocytes were removed by the mAb cocktail, there were fewer cells which were strongly CD45-positive and the EBZ17-beads attached to any cell with low CD45 expression. It would certainly account for the difference in recoveries between the two pan-leukocytes mAbs and because of this anti-CD45 mAbs were abandoned.

This reduction in fetal erythroblast recovery was not seen with multiple incubations using the anti-CD18, anti-CD2 and anti-CD3 mAbs and the principle of multiple aliquots of beads was maintained.

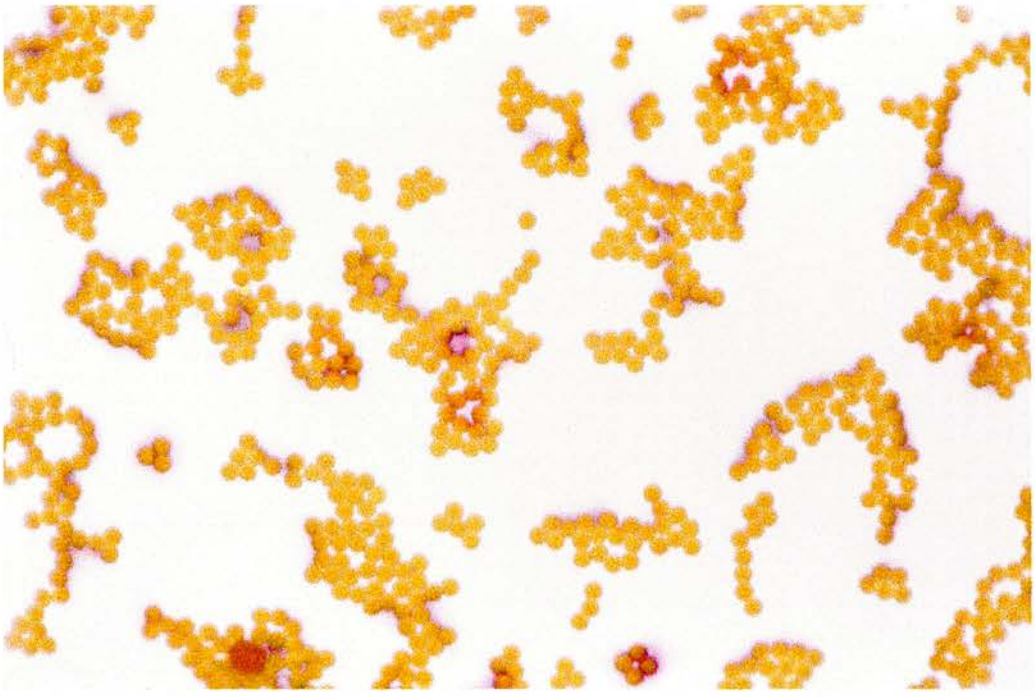


Figure 6.3 Positive selection of fetal erythroblasts using Dynabeads
Cytospin preparation. Note that the morphology of the selected cells (stained nuclei) is obscured by the immunomagnetic Dynabeads beads (orange)

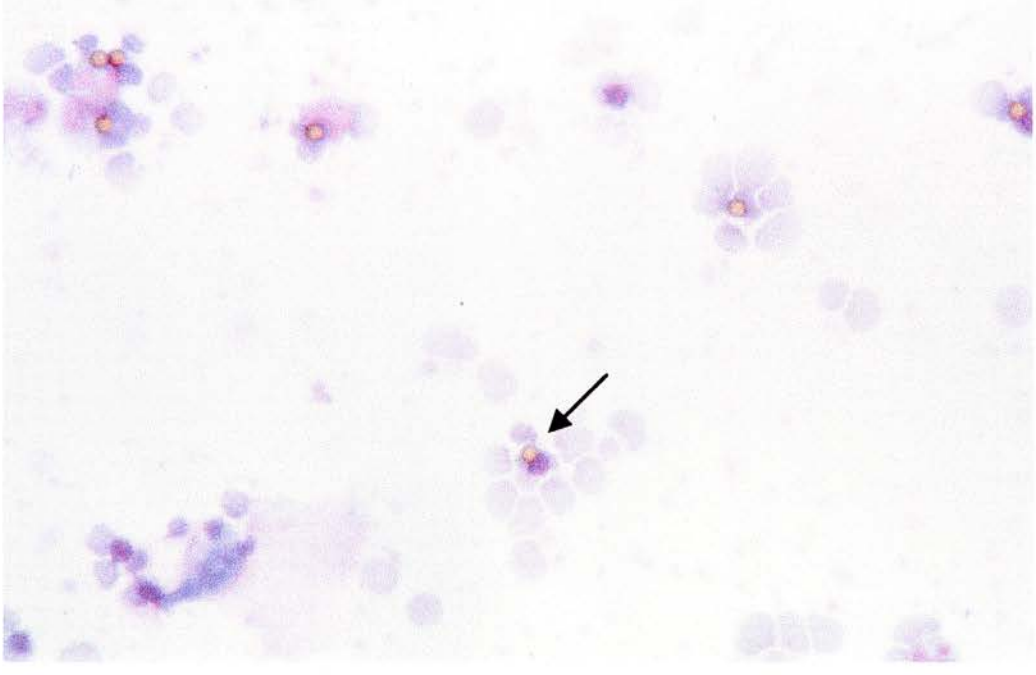


Figure 6.4 Fetal erythroblast with anti-CD45-conjugated bead (EBZ17) attached
Cytospin preparation. Note that the erythroblast (arrowed) has an immunomagnetic bead attached to it. Hence, it could be drawn to the magnet and removed from suspension.

The addition of the anti-HLA.DR mAb, DA6.231, improved the leukocyte removal but substantially reduced erythroblast recovery (table 6.6). DA6.231 is an IgG_1 mAb and was made in-house (Guy et al, 1982). It is directed against the β-subunit of HLA class-II molecules and was added specifically to target B-cells. However, erythroid precursors also express HLA.A, B, C and DR antigens and, using FACS, HLA.DR was demonstrated on 28.0% (range, 9.3-34.0%) of erythroblasts (Sieff et al, 1982). The authors pointed out that FACS will not detect very low antigen expression and an even greater proportion of erythroblasts might express undetectable HLA.DR. As more leukocytes were removed, there were fewer leukocytes to react with DA6.231-conjugated beads and these could react with any other HLA.DR-expressing cells, including erythroblasts. This would certainly explain the decline in recovery with increasing leukocyte removal when DA6.231 was included in the mAb cocktail. Although leukocyte removal was improved, because of these results, the use of DA6.231 was discontinued. However, it should be possible to use a different anti-B-cell mAb that does not react with fetal erythroblasts to improve enrichment if this is necessary.

6.4.7 Estimation of erythroblast loss during negative enrichment

The detrimental mass movement effect of beads "pushing" erythroblasts to the magnet was likely to be greatest in a small volume containing a high concentration of beads. Manyonda et al (1992) found that cell loss in this way amounted to a fairly consistent 12%. Unfortunately, these conditions maximised the number of collisions between beads and target cells. Diluting the cell/ bead supernatant prior to magnetic separation only recovered about 6-10% of fetal erythroblasts and other cells came away as well, reducing the relative enrichment. Considering what was required, it was not felt that the return from this fraction warranted the additional steps. However, if insufficient numbers of fetal erythroblasts are recovered from maternal blood after the complete enrichment process, retrieval of these trapped erythroblasts may be necessary.

6.4.8 Enrichment of other fetal cells

Negative selection should enrich for trophoblast cells as they do not express antigens to which the mAbs used were directed (figure 6.5). To date unequivocal syncitiotrophoblast cells have not been identified. Multinucleate cells have been encountered but, following FISH, the individual nuclei have shown two X chromosome signals and no Y signal. In one of these cases XY interphase cells were also found and the woman subsequently delivered a male. It is most likely that these were megakaryocytes. Of course it is possible that mononuclear cytotrophoblast cells have been analysed by FISH but they have not been identified positively. However, because of potential confined placental mosaicism (chapter 2.3), it is arguable whether enrichment of trophoblast cells is, in any case, desirable.

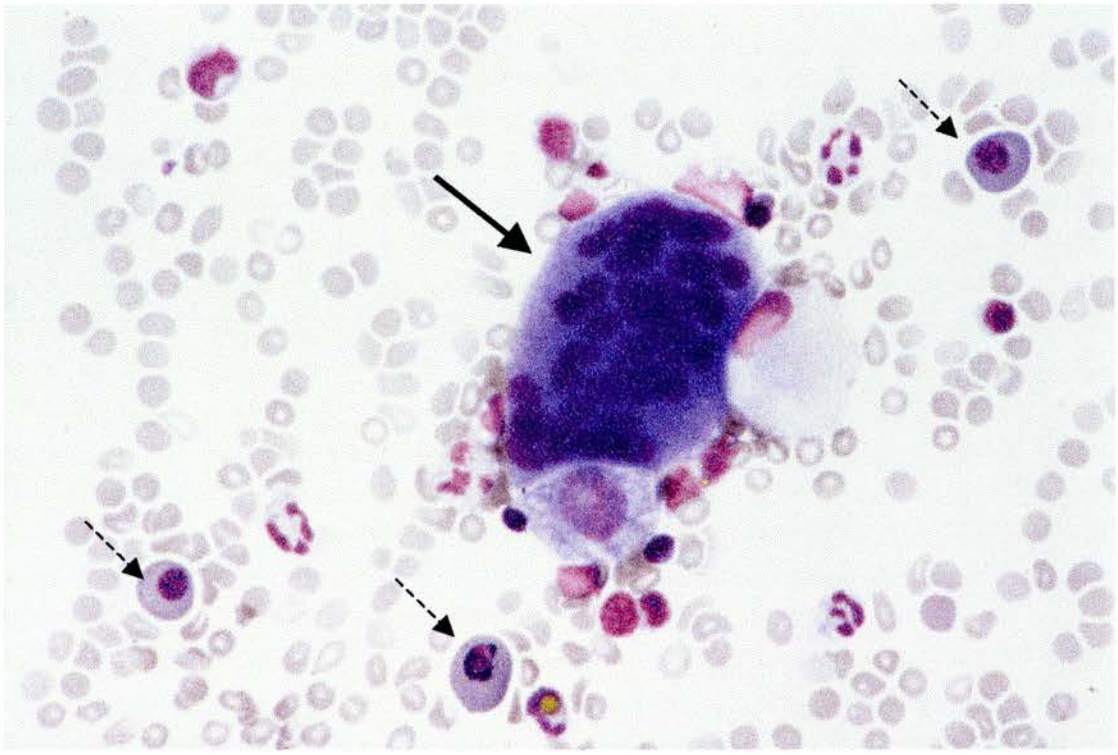


Figure 6.5 Multinucleated syncitiotrophoblast enriched from an artificial admixture of termination and adult blood.
The syncitiotrophoblast cell (solid arrow) is surrounded by several erythroblasts (dashed arrows).

6.4.9 Combining negative and positive enrichment

Using this approach, just over 40% of fetal erythroblasts were removed from the supernatant and in model experiments, 61.6% of the cells showed a Y-chromosome signal (in the case of a male fetus). However, given that almost 60% of fetal erythroblasts remained in the unselected fraction the absolute recovery of fetal erythroblasts was poor. Following fixation and lysis in methanol: acetic acid it was impossible to identify the fetal erythroblasts positively and we were unable to determine exactly how many cells were actually dislodged from their beads. Therefore, although the ratio of male fetal cells to maternal cells was large, the absolute numbers of recovered fetal erythroblasts was small and this three-stage method of enrichment was unsuitable for the required task.

6.5 Summary

Using the described negative enrichment technique in model systems, it was possible to recover fetal erythroblasts from a starting ratio of 1 in 10^8. Unfortunately, it is not known what fetal erythroblasts in maternal blood look like, how they behave or how frequently they occur. Therefore, enrichment from maternal blood can only be approached asymptotically by the models we have designed.

Figures 6.6, 6.7 and 6.8 demonstrate the enrichment from whole blood, through density gradient centrifugation to removal of leukocytes. Note the reduction in the relative number of erythrocytes (following density gradient centrifugation) and then their relative increase once the majority of leukocytes have been removed by Dynabeads. Figure 6.9 suggests hypothetical results based on current knowledge and our results which, overall, represents enrichment by three orders of magnitude.

Figure 6.6 Whole blood
Note the large ratio of erythrocytes to nucleated cells

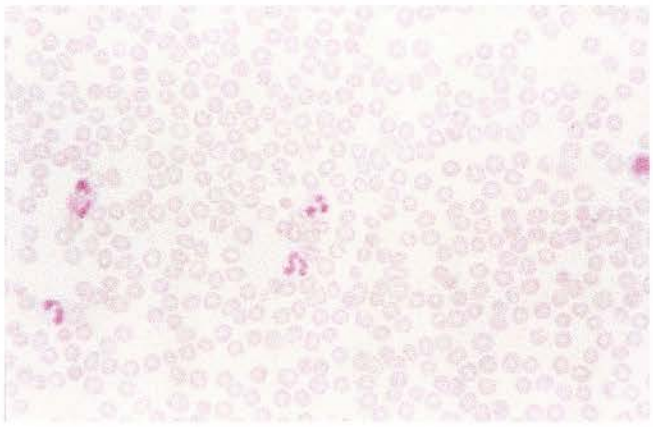


Figure 6.7 Blood following density gradient centrifugation with Histopaque 1119
Note that the majority of erythrocytes have been removed

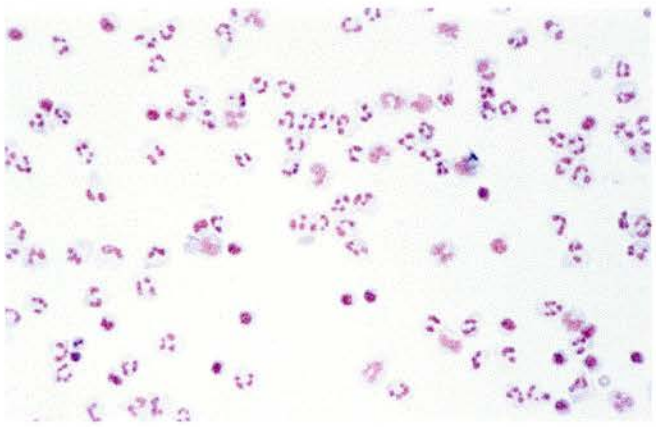


Figure 6.8 Cells recovered after complete negative enrichment
Enriched fetal erythroblast is arrowed. Note that there are now very few nucleated cells and the majority of cells are now erythrocytes

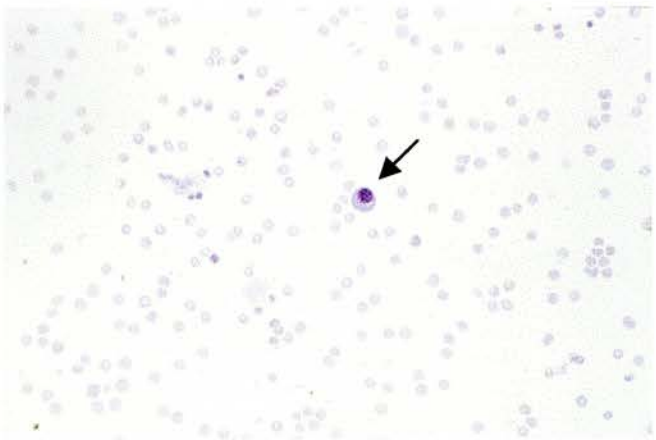


Figure 6.9 Hypothetical recovery and enrichment

	Whole blood	After H1119 centrifugation	After negative enrichment with Dynabeads
Total number of erythrocytes	1×10^{11}	5×10^{7}	5×10^{7}
Total number of leukocytes	1×10^{8}	5×10^{7}	5×10^{5}
Total number of fetal erythroblasts	100	40	32
Ratio of Erythroblasts: Leukocytes	1×10^{6}	1.25×10^{6}	1.6×10^{4}
Ratio of Erythroblasts: All cells	1×10^{9}	2.5×10^{7}	1.6×10^{6}

It would be ideal to deposit the remaining cells on to as few slides as possible, prior to FISH, whilst minimising the cell loss during deposition. This could allow positive identification of fetal erythroblasts but it presents us with a predicament. Optimal probe access requires removal of cytoplasmic and perinuclear proteins but this would prevent using cytoplasmic markers for positive identification.

Each of these issues will be addressed separately. Cytogenetic analysis of fetal cells was the primary goal and will be dealt with first. However, it must always be borne in mind that the key areas of enrichment, deposition, positive identification and cytogenetic analysis were complexly inter-related.

Chapter 7. Cytogenetic analysis of fetal erythroblasts

7.1 Introduction

Even with the best enrichment, maternal cells far outnumber any putative fetal cells. If the primary goal is aneuploidy detection then polymerase chain reaction is inappropriate and in situ hybridisation is necessary to determine the copy number of specific chromosomes in a nucleus (chapter 3).

Fluorescence in situ hybridisation (FISH) involves hybridising a probe which is complementary to a nucleic acid sequence (whether DNA or RNA) in the cell. The probe is labelled with a reporter molecule which allows subsequent visualisation by immunological development in much the same way as immunocytochemistry. Using chromosome-specific probes, the number of signals within the nucleus reflects the number of copies of that chromosome in the nucleus.

The proceeding sections will outline the principles and methods of fluorescence in situ hybridisation so that its application to enriched maternal blood samples can be better understood. The reagents are listed in Appendix II, with abbreviations in Appendix I.

7.2 Overview of in situ hybridisation

Non-radioactive in situ hybridisation, especially fluorescence in situ hybridisation (FISH), has certain advantages over radioactive in situ hybridisation. FISH is faster, gives better spatial resolution of individual hybridisation signals and signals are more stable. The entire genome, entire chromosomes, chromosomal subregions or single-copy sequences can be targeted specifically depending on the complexity of the probes used and as the technology has developed, the sensitivity of FISH is now approaching that of isotopically labelled probes (2-5 kbp). Furthermore, a variety of probe-labelling systems allow simultaneous detection of different probes in the same nucleus using different fluorochromes. FISH can be performed on metaphase or interphase cells, including cells fixed in suspension so that the three

dimensional structure of the cell can be preserved. These probe-labelling and detection systems are commercially available and the quality of microscopes and cooled charged coupled device (CCD) cameras has improved steadily resulting in excellent images (Trask, 1991).

Plasmids are circular self-replicating molecules. The DNA probe is inserted and the plasmid can then infect a bacterium such as E. coli which, as it multiplies, replicates the total number of plasmids and hence the total number of probes. The probe can then be purified from its vector. Cosmids are very large plasmids. Phage-λ has a Cos (cohesive) site at each end with an area of DNA in the middle which can be removed using specific enzymes and the probe-DNA inserted. Once replicated, another enzyme can be used to recognise and break the Cos sites, releasing the probe.

To improve the specificity of the final signal, hybridisation of the labelled probe to repetitive DNA sequences in the genome can be suppressed by prehybridising with unlabelled genomic DNA, for example with Cot1 DNA. This is crucial for localisation of unique sequences contained within large insert probes such as cosmids.

7.3 Preparation of Probes

7.3.1 Plasmid DNA purification and preparation

7.3.1.1 Bacterial culture

E. coli glycerol stock (with incorporated plasmid) was plated on L-agar containing Ampicillin (50µg/ml) and incubated overnight at 37°C. Individual colonies of E. coli were placed in separate sterile culture tubes containing 5ml LB-broth (with 1:100 phosphate and ampicillin 1:1000 dilution) as per Sambrook et al (1989) and incubated at 37°C overnight in a culture shaker.

7.3.1.2 TELT method for DNA purification

1ml of the culture suspension was centrifuged in an Eppendorf centrifuge and the supernatant discarded. The bacteria were resuspended in 200µl of TELT buffer. Lithium chloride in the buffer removed RNA and protein aggregates from lysates. Fresh lysozyme (10mg/ml) was made up in TELT buffer and 10µl added to the bacterial suspension to destroy the bacterial cell membranes. The Eppendorf tubes were heated to 100°C for 1 minute on a hot-block and then plunged deep into ice and allowed to cool for 5 minutes. Following high speed centrifugation, the supernatant was decanted into fresh Eppendorf tubes and an equal volume of propan-2-ol (i.e. approximately 200µl) was added to precipitate the DNA. This was ice cooled for 20 minutes, centrifuged and the supernatant decanted off without disturbing the pellet at the bottom of the tube. The remaining propan-2-ol was evaporated in a spin vacuum, resuspended in 50µl of Tris-EDTA (pH 8.0) and dissolved in a 37°C waterbath for 30 minutes. The DNA was digested with the appropriate restriction endonuclease and gel electrophoresis performed to confirm the presence of the DNA insert (probe).

For the X-chromosome probe, pSV2neoX5 the following reagents were used:

BAM H1 restriction endonuclease	0.5µl
Buffer B (x10 stock)	2.0µl
Probe DNA	10.0µl
Spermidine (100mM)	1.0µl
Distilled water	6.5µl
Total	20.0µl

We made up 20µl of digest. Spermidine stabilises DNA enzyme complexes. The DNA was digested at 37°C in a waterbath for 60 minutes. From in-house experience, HaeIII restriction endonuclease worked best with Buffer M for the Y-chromosome probe, pHY2.1.

Standard gels were 0.7% solution of agarose in Tris-EDTA with ethidium bromide. The gel loading buffer contained bromophenol blue (0.25%), xylene cyanol FF (0.25%) and ficoll (15%) in distilled water (Sambrook et al, 1989). Ficoll increased the density to encourage

sinking and EDTA stopped the enzyme reaction. The dye allowed direct visualisation of the migration front.

10µl of a known λ-marker, e.g. HindIII, and the DNA digests were added to the wells and subjected to 50mV for 60 minutes. Internal controls of uncut DNA, i.e. no restriction enzyme, were run simultaneously to estimate the size of the entire vector and confirm that the insert had been cut from the vector. The bands corresponding to DNA fragments were visualised under UV light and the size determined by reference to the λ-standards used. Large amounts of DNA probe could then be purified.

7.3.1.3 Maxiprep. Preparation of large quantities of probe.

DNA purification was performed using the Promega Wizard Maxiprep Purification System. There were minor modifications based on in-house experience and hence the method is given in full.

The culture tube with the colony shown to be containing the vector was cultured overnight in 500ml LB-broth in a shaker at 37°C. The broth (with no added magnesium) contained a 1:10 dilution of phosphate supplement and ampicillin (50µg/ml) 1:1000 dilution. The following morning the broth was poured into sterile Sorvall High Speed Centrifuge Pots and accurately balanced on weighing scales. The pots were centrifuged at 14,000 x g at 20°C for 15 minutes, the broth decanted off and the pellet of bacteria resuspended in 15ml of Cell Resuspension Solution until no clumps were visible. 15ml of Cell Lysis Solution was added and the cells mixed by inversion until the solution became very viscous and then cleared. This was put on ice for 20 minutes and then transferred to Falcon tubes where 15ml of the Neutralisation Solution was added and mixed. The solution became very viscous again. This was put on ice for a further 20 minutes and then centrifuged at 14,000 x g at 4°C for 15 minutes.

The cleared supernatant was carefully decanted to a clean Falcon tube, avoiding the white precipitate, and 0.6 x volume of propan-2-ol was added and mixed. This was left to

stand at room temperature for one hour and then centrifuged at 14,000 x g at 4°C for 15 minutes. The supernatant was decanted off and discarded and the DNA pellet resuspended in 75% ethanol. This was centrifuged at 14,000 x g at 4°C for ten minutes. The ethanol was decanted off and the Falcon tube turned upside down and placed on filter paper to encourage the remainder of the ethanol to drain off to allow the DNA to dry completely.

The Wizard Maxiprep DNA purification resin was mixed thoroughly and 10ml added to the DNA. It was mixed by swirling until it got quite viscous. The Maxicolumn tip was inserted into the vacuum manifold and the resin/DNA mix poured in. The vacuum was applied to pull the resin through the Maxicolumn. To transfer all the DNA/resin mix to the Maxicolumn, 13ml of the column wash solution was added to the Falcon tube which contained the DNA/resin mix, swirled and immediately poured into the Maxicolumn. The vacuum was applied again, a further 12ml of the column wash solution was added and the vacuum applied again. 5ml of 80% ethanol was added to the Maxicolumn and the vacuum applied to rinse the resin which was dried by continuing to draw the vacuum for a further 10 minutes.

The Maxicolumn was placed in its original sterile Falcon tube and 1.5ml of preheated Tris-EDTA (65-70°C) was added for one minute. This was centrifuged at 1300 x g for 5 minutes, the Maxicolumn was removed and the purified DNA eluate was left in the bottom of the Falcon tube.

7.3.1.4 Spectrophotometry

A 1:100 dilution of DNA/Tris-EDTA (pH 7.4) was made up in an Eppendorf tube and the spectrophotometer set to 260nm (the wavelength necessary for DNA analysis). A quartz cuvette for DNA analysis was always used. The control Tris-EDTA (with no DNA) was put in the cuvette, allowing the spectrophotometer to be zeroed. The diluted DNA was substituted and the optical density (OD) determined. 50µg/ml of double-stranded DNA has an absorbence of 1 at 260nm and hence an OD of 1 corresponded to approximately 50µg/ml of double-stranded DNA. Bearing in mind that it is usual to only label about 500ng of DNA at a

time, a large amount of very pure DNA was generated using this method. The DNA was diluted in Tris-EDTA and stored at -20°C in individual aliquots (to avoid repeated freeze/ thawing of DNA) for future labelling.

To ensure that the probe had been purified a midi-gel was run as described previously.

7.3.2 Principles of Nick Translation

7.3.2.1 Probe labelling by Nick Translation

Deoxyribonucleic acid, DNA, is made up of four nucleotides in varying sequences: adenine, guanine, cytosine and thymidine with a polysugar-phosphate backbone. Nick translation was described by Langer-Safer et al (1992) whereby occasional thymidine bases are replaced with deoxyuridine triphosphate (dUTP) bases on to which a reporter molecule is attached. The reporter permits visualisation of the binding site and is often biotin or digoxigenin, but occasionally direct labelling using a fluorochrome such as fluorescein isothiocyanate (FITC) may be used.

DNase-1 "nicks" the DNA reasonably randomly and the number of "nicks" is proportional to the amount of DNase added. If too much DNase-1 is used, then the DNA fragments will be too small and the optimal amount should give 500bp fragments. When the DNA-polymerase (Pol-1) sees a nick it repairs it with a similar base, chews the next base away and replaces it with the appropriate base. It stops chewing/ repairing when it gets to the next nick. Thymidine bases may be replaced by dUTP. Biotin is a small molecule so every thymidine base is replaced by a biotin-dUTP base. Digoxigenin is large, so only 2 in 5 thymidine bases are replaced by digoxigenin-dUTP and the other 3 thymidine bases are replaced by dTTP. For direct-labelled probes, FITC is a large molecule so only 2 in 5 thymidine bases is replaced by FITC-dUTP.

Normally 500ng of probe was labelled. Probes were clean as the method is very sensitive to contaminants and all reagents were kept on ice and labelling performed in an unhurried but efficient manner.

10 x Nick Translation Salts (2.0µl), dATP (2.5µl), dCTP (2.5µl), dGTP (2.5µl), biotin-16-dUTP (2.5µl), 1.0µl of 1:1000 diluted DNase, 500ng of probe and a volume of distilled water to make the total volume up to 19µl were mixed in an Eppendorf tube. 1µl of Pol-1 was added and the Eppendorf put in a 15°C waterbath for 90-120 minutes. The reaction was stopped by adding 2.0µl of 0.2M EDTA (to mop up the magnesium) and 1.0µl of SDS (a detergent which denatured the enzymes). After the labelling reaction had been stopped, the probes were separated from the unincorporated nucleotides by Pharmacia Nick Spin Columns.

7.3.2.2 Nick Spin Columns

Nick Spin Columns contain G50 sephadex beads that have tiny pores. These pores permit the penetration of unincorporated nucleotides, thus retarding their elution from the column. The fluid was allowed to drain out of the column and 3ml of Tris-EDTA added and allowed to drain through. The excess was discarded and the column centrifuged at 500 x g for 4 minutes.

Because of the theoretical risk of diluting out the EDTA/SDS and reactivating the pol-1, only at this stage was the probe made up to 75-100µl with Tris-EDTA. It was immediately layered on top of the Sephadex beads and the column centrifuged at 500 x g for 4 minutes. The probe was collected in a labelled Eppendorf tube at the bottom of the test tube.

7.3.2.3 Estimation of Probe concentration

Nitrocellulose filters were prepared according to standard methods by washing in distilled water for two minutes and then in 20xSSC for twenty minutes and air-dried. 1: 10^3 and 1: 10^4 dilutions of the probe were prepared in Tris-EDTA (pH7.5) and dotted on to the filter papers to give concentrations of 20pg/µl, 10pg/µl, 2pg/µl and 1pg/µl.

The λ-standard was diluted to the same concentrations and dotted on to the filter paper in their corresponding positions. The filter paper was placed in a Biorad GS Gene ultraviolet (UV) cross-linker and subjected to 250mJ UV radiation. Biotin or digoxigenin probes were

detected with streptavidin alkaline phosphatase and anti-digoxigenin alkaline phosphatase respectively using BCIP/NBT Vector kit protocols (Vector). The filter paper was sealed and incubated in the dark at room temperature overnight and the probe concentration subjectively determined by comparing the colour intensity of the probe with that of the λ-standards.

7.3.2.4 Principles of probe hybridisation and visualisation

Cot-1 DNA is a highly repetitive, widely occurring genomic DNA sequence. Especially with contiguous cosmid probes, it is frequently necessary to pre-incubate the probe with cot-1 to bind complementary areas of DNA on the probe. This prevents the repeat-containing areas of the probe from binding to genomic DNA which has the same sequence, thus improving probe specificity (reducing background staining), and is termed preannealing.

Denaturation allowed the double-stranded genomic and probe DNA to separate. The length of incubation and any refining steps (the stringency) then depended on the assay.

After washing to remove any unbound probe the hybridisation bodies were visualised using fluorescent affinity agents. Texas Red and FITC were the only fluorochromes used.

From the above, it can be seen that FISH employs the principles of indirect immunofluorescence (chapter 4) and a schematic representation is given in figure 7.1.

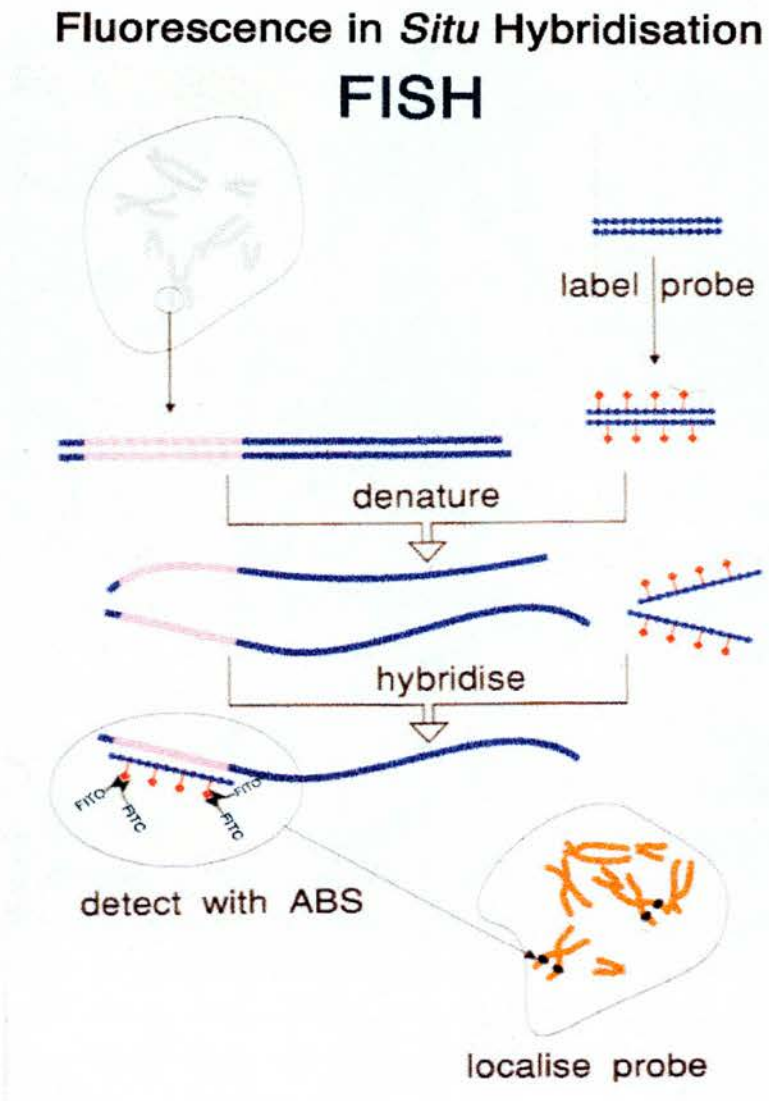


Figure 7.1 The principles of fluorescence in situ hybridisation

1. Double-stranded nuclear DNA and probe DNA (labelled with a reporter molecule e.g. biotin) are heat-denatured.
2. Single-stranded probe and nuclear DNA are hybridised overnight under stringent conditions.
3. Unbound probe is washed off.
4. The probe-nuclear DNA binding site is visualised by incubating with an anti-reporter molecule (e.g. avidin) which has a fluorochrome attached (e.g. FITC).

7.4 Fluorescence in situ hybridisation using chromosome-specific repeat sequence probes.

These are repetitive DNA sequences that hybridise to specific areas on specific chromosomes and are ideal for interphase analysis as the signals are large, intense and well-localised. However, there may be similar sequences in other chromosomes increasing the potential for false-positive results.

Both repeat sequence probes used in this study were made in-house, allowing limitless supplies at minimal cost and, importantly, our scientists were already familiar with their use. They were always tested with uncultured diploid cells as controls. Stimulated, cultured lymphocytes are frequently arrested in mitosis which may result in multiple nuclear signals and misrepresent the ploidy of the cell. Hybridisation efficiency is never 100% so that four copies of a chromosome may only be represented by three FISH signals and will falsely suggest a trisomic cell. Our experiments always involved uncultured cells as controls to minimise this risk, particularly as the cells from maternal samples were also uncultured.

As the nucleus is a three dimensional structure, FISH signals may be at different planes. Therefore, although a cell is trisomic, the third signal might be on a different plane and not seen and the cell wrongly considered diploid. This will, of course, increase the false-negative rate.

The Y-chromosome probe used was pHY2.1 (Cooke et al, 1982) and the X-chromosome probe used was pSV2neoX-5 (Waye and Willard, 1985).

7.4.1 Y-chromosome probe, pHY2.1

The long arm of the human Y-chromosome carries 2000 copies of a tandemly repeated 2.47kb sequence constituting about 20% of Y-chromosome DNA. This heterochromatin, which fluoresces brightly with quinacrine, is not involved directly with sex determination as it is occasionally found in women. Within the 2.47kb sequence is a 2.1kb sequence flanked by HaeIII sites. pHY2.1 (Cooke et al, 1982) was labelled with digoxigenin and visualised with

anti-digoxigenin-Texas Red. There are about 100 copies of a very similar 2.0kb sequence on autosomes (notably: 2q, 3p, 5p, 6p, 7q, 10p, 11p, 13p, 14p and 14q, 15p, 18p, 21q, 22q and also Xp and Xq) which can give much smaller, "background" signals.

7.4.2 X-chromosome probe, pSV2neoX-5

When DNA is run out on a caesium chloride gradient there is a large band and then smaller satellite bands, one of which is known as the "α" band. α-satellite DNA is repeat sequences in the pericentric regions of all chromosomes.

The pericentric region of the human X-chromosome is characterised by a tandemly repeated family of 2kb DNA fragments of which there are approximately 5000 copies. These fragments are each made up of twelve α-satellite monomers, each approximately 171bp in length. These monomers are 65-85% identical in sequence to each other which permits a consensus monomer sequence. pSV2neoX-5 is a 242bp fragment (i.e. spans one-and-half monomers) within the 2kb fragment. It is exclusive to the human X-chromosome so that under conditions of high stringency there should be little or no cross-hybridisation (Waye and Willard, 1985). Direct labelling of pSV2neoX-5 with FITC gave large, reliable signals with very little background fluorescence.

7.4.3 Method for fluorescence in situ hybridisation using chromosome-specific repeat sequence probes

Formamide is irritant and potentially teratogenic and gloves were always worn.

200ml of 2xSSC with 2ml of thawed RNase was warmed to 37°C and 200ml of 2xSSC was kept at room temperature. The slides were incubated in the warmed 2xSSC/RNase for one hour. Slides were immersed in the remaining 2xSSC and then sequentially dehydrated through 70%, 90% and 100% alcohol for two minutes each. They were then dried in a vacuum for 5 minutes. If slides were more than ten days old, they were subjected to proteinase K (PK) treatment. If the slides were more than three weeks old FISH was

unreliable. 400ml of PK buffer was divided into two 200ml aliquots and one aliquot heated to 37°C. Proteinase K digested proteins from around and within the nucleus, effectively opening up the nucleus and improving probe access. If cells were left in PK for too long then non-specific DNA binding, and hence background signals, were increased. 10µl of stock PK was added to the buffer at 37°C, slides immersed for two minutes and then immediately immersed in the PK-free buffer. They were sequentially dehydrated through 70%, 90% and 100% alcohol each for two minutes followed by vacuum drying. The amount of probe necessary for each coverslip was determined from the estimate of the probe concentration (chapter 7.3). For repeat sequence probes approximately 20ng of probe was applied per 22mm x 22mm coverslip and 60ng for a 22 x 50mm coverslip. The neat probes were put in an Eppendorf centrifuge tube. 0.5mg of salmon sperm DNA per 10µl of the final calculated volume of the hybridisation mixture and double the volume of absolute alcohol was added. The alcohol was evaporated in a vacuum centrifuge for one hour.

100µl of hybridisation mixture was made up of Formamide (55µl); 20xSSC (5µl); Tween 20 (1µl); distilled water (19µl) and dextran sulphate (20µl). This was added to the dried down probe and left to dissolve for one hour at room temperature. Coverslips were kept in absolute alcohol with 1% concentrated hydrochloric acid to remove grease and destroy bacteria. 10µl of the hybridisation mixture was placed on a clean 22 x 22mm coverslip or 30µl on to a 40 or 50 x 22mm coverslip. Slides were mounted in the hybridisation mixture, bubbles expelled and the coverslip sealed around the edges with "Tip Top" rubber solution. The slides were laid in an enamel tray, covered with tin foil and placed in a 70°C waterbath for 5 minutes to denature the chromosomes and the probes. The tray was then transferred to a 37°C waterbath and the chromosomes and probes left to hybridise overnight.

If α-satellite probes were used, overnight hybridisation was performed at 45°C to increase the stringency (reduce background staining). Other options to increase stringency were (a) increasing the concentration of formamide in the hybridisation mixture, (b) decreasing the salt (SSC) concentration in the hybridisation mixture, (c) washing the slides the following

morning at a higher temperature and lower salt concentration (e.g. 0.1xSSC at 60°C) and (d) decreasing the concentration of the probe to reduce non-specific binding.

7.5 Fluorescence in situ hybridisation using contiguous cosmid probes.

7.5.1 Chromosome 21 contiguous cosmid probe, cCMP21.a

Single-copy sequence probes allow identification of very specific areas of chromosomes. The smaller the probe, the more chromosome-specific it becomes but there is less reporter molecule available so that the signal is not as large. However, the signal size and reliability can be increased by using contiguous cosmids. Here two cosmids, which are very close together, or indeed whose ends overlap, are used simultaneously. Even if one of the cosmids fails to hybridise, hopefully the other will hybridise to give a reliable signal.

Analysis of chromosome 21 in interphase is more difficult than for other chromosomes because there is no reliable chromosome 21-specific repeat probe. A repeat sequence probe is available but it hybridises to both chromosomes 21 and 13 which limits its use (chapter 7.10.2).

The 21q22 region (the 22 region of the long arm of chromosome 21) is the region which confers "Down's syndrome" on an individual. In this study the chromosome 21-specific probe, cCMP21.a, the kind gift of Dr. Nigel Carter, was used (Zheng et al, 1992). cCMP21.a was produced from two cosmids, cCMP21.2 and cCMP21.6 which overlap to give a 55kb probe, highly specific for the 21q22 subregion. Because of its specificity, both signals could be resolved in common Robertsonian translocations involving duplication of chromosome 21q.

7.5.2 Method for fluorescence in situ hybridisation using chromosome-specific contiguous cosmid probes

The methodology for using cosmid probes required certain modifications compared to repeat sequence probes.

Slides were kept in a metal rack throughout the procedure to avoid altering the temperature of the water baths dramatically and because glass racks may crack with sudden temperature changes. The probe concentration was around 50ng per small coverslip. Sequences occurring within the cosmid probe and occurring elsewhere in the genome were competed out with excessive Cot-1 human DNA (1µg/µl/22mm x 22mm coverslip). Salmon sperm DNA was added to achieve a final concentration of 0.5mg/ 10µl of hybridisation mixture. Twice the total volume of absolute alcohol was added and the probes vacuum dried.

FISH using cosmid probes was performed at lower stringency and hence a hybridisation mixture containing only 50% formamide was used. This was added to the dried down probe and left to stand for one hour. 200ml of 70% formamide: 2xSSC was heated to 70°C. 70% alcohol was made up and put on ice. Cellular DNA was denatured in a 70°C oven for 5 minutes, immediately put in the 70% formamide: 2xSSC at 70°C for 3 minutes and then immediately transferred to the ice-cold 70% alcohol. The timing was crucial as this stopped the denaturation reaction. After the vacuum-dried probe had dissolved it was put in the 70°C waterbath (in a polystyrene floater) for 5 minutes. This allowed the double-stranded DNA of both the probe and the cot-1 human DNA to become single-stranded. It was then transferred to the 37°C waterbath for 15 minutes, allowing the single-stranded DNA to reanneal.

The probe was put on to a clean 22 x 22mm coverslip and mounted on the slide. Because the cellular DNA and the probe had already been denatured, no further denaturation step was required. The coverslip was sealed with "Tip Top" rubber solution, the slides laid in an enamel tray and covered with tin foil. The tray was put in the 37°C waterbath for overnight hybridisation. Cosmids were never hybridised at any higher temperature.

7.6 Development of hybridisation signals

The following morning, regardless of the type of probe used, the protocol was the same.

From this stage onwards, care was taken to prevent the slides drying to minimise background staining. The type of probe-labelling and the desired colour to visualise the hybridisation bodies determined which antibodies were used. For biotin-labelled probes an avidin molecule, with an appropriate fluorochrome attached, was used. The second layer was a biotinylated anti-avidin goat monoclonal antibody and the third layer avidin-fluorochrome again. For digoxigenin-labelled probes, two layers (a mouse-anti-digoxigenin mAb and anti-mouse-FITC) permitted adequate visualisation with minimal background staining. The antibodies were made up with blocking buffer (see appendix II) and centrifuged at 4°C. 1ml of blocking buffer was also centrifuged to remove large conjugates.

The rubber seal was peeled off and the slides put in a rack. During the subsequent washing, the coverslips fell off the slides. The slides were washed four times, each for three minutes, at 45°C in 50% formamide/ 2xSSC, the washes repeated at 45°C in 2xSSC and finally at 60°C in 0.1xSSC. After the final wash the slides were put in 4xSSC/ Tween 20 at room temperature and 2 litres of 4xSSC with 0.1% Tween 20 was warmed to 37°C. 10µl of blocking buffer (to reduce non-specific background staining) was put on a fresh coverslip (40µl for large coverslips) and mounted on the slide for five minutes. The coverslip was flicked off and the first layer of antibodies applied to the same coverslip and remounted on to the slide. The slides were laid in a sandwich box, containing benchcote moistened with 4xSSC/0.1% Tween 20, and put in a 37°C incubator for 30 minutes.

Following incubation the slides were washed three times in 4xSSC/ 0.1% Tween 20 at 37°C, each for 2 minutes, and the second layer of antibodies applied on clean coverslips. The incubation and washing was repeated for the desired number of layers. The excess fluid was drained off, 10µl of Vectashield/ DAPI/ propidium iodide was applied to a coverslip, the bubbles were expelled and the coverslips sealed with rubber solution.

7.7 Maternal blood samples

Using the enrichment process described in chapters 5 and 6 a prospective study of 15 women in early pregnancy was performed and prospective fetal sexing attempted.

Following enrichment, apart from some cytospin preparations, the remaining cells were fixed in suspension in fresh methanol: acetic acid (3:1) and deposited on to clean microscope slides. The cells were subjected to FISH, initially using only a Y-chromosome probe and subsequently using X and Y-chromosome probes simultaneously. The Y-chromosome, pHY2.1, was labelled with digoxigenin and visualised with anti-digoxigenin-Texas Red and the X-chromosome probe, pSV2neoX-5, was directly labelled with FITC.

In two cases (patients 10 and 12) the women agreed to have blood taken from them every two weeks from first presentation until twenty four weeks' gestation to see if there was any trend in recovery of potential fetal cells as pregnancy advanced.

Blood was also taken from the antecubital fossa of a 39 year old woman prior to vaginal prostaglandins and suction termination of pregnancy, seven days after CVS. CVS (for advanced maternal age) performed at 11 weeks' gestation had demonstrated a 47,XX+21 fetal karyotype. The blood was enriched as previously described and the cells fixed in methanol: acetic acid (3:1). The slides were subjected to FISH using the biotinylated chromosome 21 contiguous cosmid probe, cCMP21.a, and the signal developed with avidin-FITC.

7.8 Imaging

Slides were scanned manually using a Zeiss Axioplan fluorescence microscope with a triple band-pass filter and images were captured on an Apple Macintosh Quadra 950 using IPLab Spectrum 2.4 software.

Nuclei were inspected for a Y-hybridisation body and, when the two sex chromosome probes were used simultaneously, for X and a Y-hybridisation bodies. If signals were considered reliable then scanning was stopped for that particular maternal sample and the

fetus was assigned as male. All the slides for any particular maternal sample were scanned in entirety until such times as X and Y-hybridisation bodies were observed; if none was found, then the fetus was assigned as female.

Using the contiguous cosmid probe, for the cell to be considered as showing two copies of chromosome 21, the distance between the two signals had to be more than the diameter of either of the two signals. Similarly, if three potential signals were identified then the distance between two adjacent signals had to be more than the diameter of either of the two adjacent signals.

The nucleus is a 3-dimensional structure with probe signals at different planes. Therefore, the microscope objective had to be adjusted to examine the entire depth of each nucleus and, despite enrichment, it took several days to scan a single maternal sample.

7.9 Results

7.9.1 Hybridisation efficiencies of the chromosome probes

The following data for chromosome probe hybridisation efficiencies was accumulated on uncultured adult diploid leukocytes fixed in suspension in fresh methanol: acetic acid (3:1). Table 7.1 shows that the repeat sequence probes, pSV2neoX-5 and pHY2.1, were highly efficient.

Table 7.1 FISH analysis using single chromosome probes on adult leukocytes

Cell Karyotype	Female	Female	Male
Chromosome Probe	X (pSV2neoX-5)	Y (pHY2.1)	Y (pHY2.1)
No. of samples	5	5	5
No. of cells counted	10,000	50,000	10,000
2 signals	9520 (95.2 ± 2.7%)	0 (0%)	0 (0%)
1 signal	390 (3.9 ± 2.0%)	2 (0.01%)	9630 (96.3 ± 3.6%)
0 signals	90 (0.9% ± 0.4%)	49,998 (99.99 ± 0.01%)	370 (3.7 ± 3.6%)

FISH performed using protocol detailed in Chapter 7.4 and 7.6.
95% confidence intervals in parentheses.

Table 7.2 shows that the repeat sequence probes, pSV2neoX-5 and pHY2.1, for chromosome X and Y respectively could be used reliably to assign gender in uncultured leukocytes. Even so, there were false-positive results in female control samples and, likewise, there were 'female' false-positive results in male control samples. This must be borne in mind when attempting to assign gender in enriched maternal blood samples.

Table 7.2 FISH analysis using X and Y-chromosome probes simultaneously on adult leukocytes

Cell karyotype	Female	Male
No. of samples	7	3
Total no. of cells counted	35,000	15,000
Chromosome Signal: XY	11 (0.03%)	13620 (90.8%)
Chromosome Signal: XXY	7 (0.02%)	180 (1.2%)
Chromosome Signal: XX	32819 (93.8%)	9 (0.06%)
Chromosome Signal: XO	2054 (5.87%)	90 (0.6%)
Chromosome Signal: OO	105 (0.3%)	675 (4.5%)
Chromosome Signal: OY	4 (0.01%)	426 (2.84%)

FISH performed using protocol detailed in Chapter 7.4 and 7.6.
Note the high hybridisation efficiency with adult leukocytes

Table 7.3 shows the hybridisation efficiency of fetal erythroblasts. These data demonstrate that the hybridisation efficiency of erythroblasts is poorer than for adult leukocytes when compared with the results in table 7.1 ($p < 0.001$, female cells using X-chromosome probe alone; $p < 0.001$, male cells using Y-chromosome probe alone).

Table 7.3 FISH analysis using X or Y-chromosome probes on fetal erythroblasts

Erythroblast karyotype	Female	Male
No. of samples	9	3
Total no. of cells counted	18,000	6,000
Chromosome signal: XX	12,492 (69.4 ± 7.5%)	-
Chromosome signal: XO	3816 (21.2 ± 8.3%)	-
Chromosome signal: OO	1692 (9.4 ± 4.8%)	2304 (38.4 ± 2.4%)
Chromosome signal: OY	-	3696 (61.6 ± 8.2%)
Chromosome signal: YY	-	0

FISH performed using protocol detailed in Chapter 7.4 and 7.6.
Note the poorer hybridisation compared to leukocytes in Table 7.2

Table 7.4 shows that the hybridisation efficiency of the contiguous cosmid probe, cCMP21.a, with uncultured adult leukocytes was not as efficient as the repeat sequence probes. Only 66.2 ± 6.6% of cells showed two chromosome 21 signals simultaneously although very few appeared to be trisomic.

Table 7.4. FISH signals using the chromosome 21 probe, cCMP21.a, on uncultured adult leukocytes

0 signals	6880 cells (17.2 ± 5.1%)
1 signal	6602 cells (16.5 ± 5.2%)
2 signals	26,478 cells (66.2 ± 6.6%)
3 signals	12 cells (0.03 ± 0.02%)
4 signals	28 cells (0.07± 0.02%)

FISH performed using protocol detailed in Chapter 7.5 and 7.6.

7.9.2. Results of prospective in utero fetal sexing

In pregnancies involving a male fetus between 1 and 70 cells showed simultaneous X and Y signals (figure 7.2) and table 7.5 shows the fetal sex assigned by FISH and the sex at delivery (Gaudoin, 1995). Cases 1-7 used only the Y-chromosome probe whilst cases 8-15 used X and Y-chromosome probes simultaneously. Gender prediction was 86.7% accurate ($p < 0.004$, 100% sensitivity, false positive rate 25%). However, the two false-positive results emphasise the need for an additional test to confirm that the analysed cells were truly fetal in origin.

The results of enrichment and FISH analysis in the two cases that were sampled serially are summarised in table 7.6. It is tempting to suggest that the number of fetal erythroblasts increase in maternal blood up to 14 weeks' gestation and decline thereafter (case 10) but, unfortunately, because the pregnancy in the other case involved a female fetus, little can be concluded from this data.

Table 7.5. Results of prospective in utero fetal sexing

Case No.	Gestation at sampling	Chromosome probes used	No. of male cells counted	Sex assigned prospectively	Sex at delivery	True or false
1	8	pHY2.1	0	Female	Female	TN
2	12	pHY2.1	1	Male	Female	FP
3	12	pHY2.1	3	Male	Male	TP
4	14	pHY2.1	3	Male	Female	FP
5	12	pHY2.1	1	Male	Male	TP
6	10	pHY2.1	0	Female	Female	TN
7	10	pHY2.1	0	Female	Female	TN
8	15	pHY2.1, pSV2neoX-5	3	Male	Male	TP
9	11	pHY2.1, pSV2neoX-5	0	Female	Female	TN
10	14	pHY2.1, pSV2neoX-5	70	Male	Male	TP
11	16	pHY2.1, pSV2neoX-5	4	Male	Male	TP
12	16	pHY2.1, pSV2neoX-5	0	Female	Female	TN
13	16	pHY2.1, pSV2neoX-5	2	Male	Male	TP
14	11	pHY2.1, pSV2neoX-5	1	Male	Male	TP
15	13	pHY2.1, pSV2neoX-5	0	Female	Female	TN

FISH performed using protocol detailed in Chapter 7.4 and 7.6.
TP = true positive result, TN = true negative result, FP = false positive result, FN = false negative result

Table 7.6. Results of serial maternal blood tests

Gestation in weeks at sampling	8	10	12	14	16	18	20	22	24
No. of XY interphase nuclei Case 10	0	2	5	70	38	2	2	0	0
No. of XY interphase nuclei Case 12	0	0	0	0	0	0	0	0	0

FISH performed using protocol detailed in Chapter 7.4 and 7.6.

7.9.3 Results from first trimester Trisomy 21 case

In the case of the woman carrying a Down's syndrome fetus over 200 trisomy 21 cells were counted from less than one slide alone. Analysing 2000 consecutive nuclei, 16 nuclei (0.8%) showed three signals (Gaudoin, 1995; figure 7.3). When comparing this maternal sample with the control, using the χ^2 test, the difference in proportions is highly significant ($p < 0.001$).

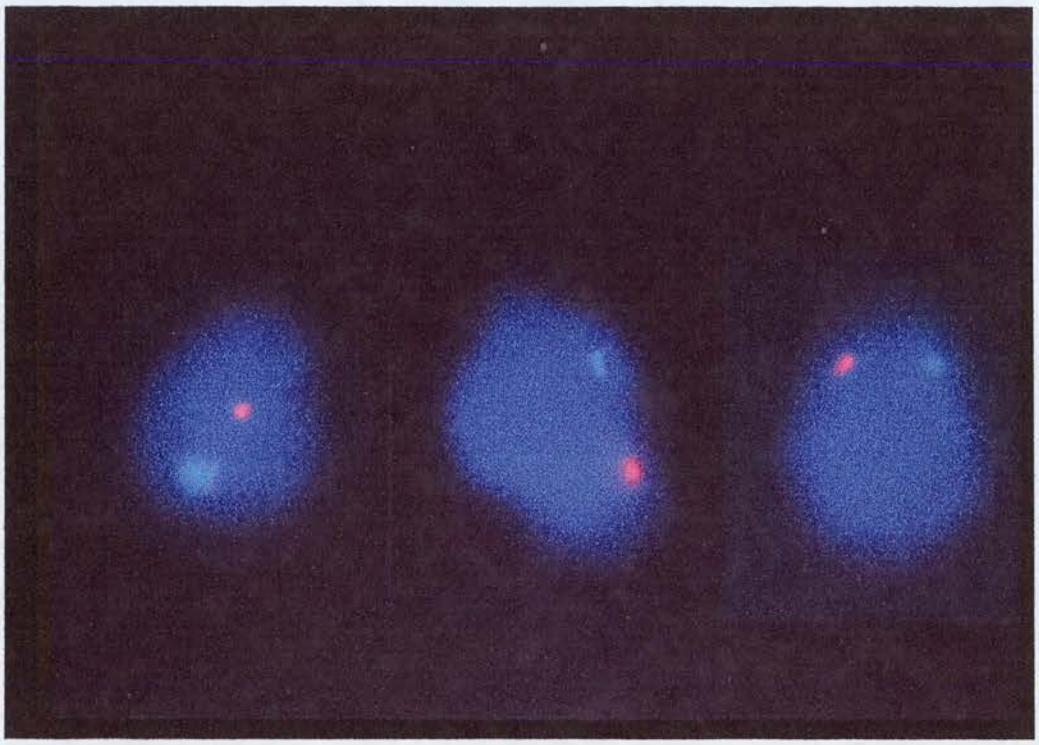


Figure 7.2 XY interphase cells enriched from the blood of pregnant women
X-chromosome probe labelled with FITC, Y-chromosome probe labelled with Texas red.

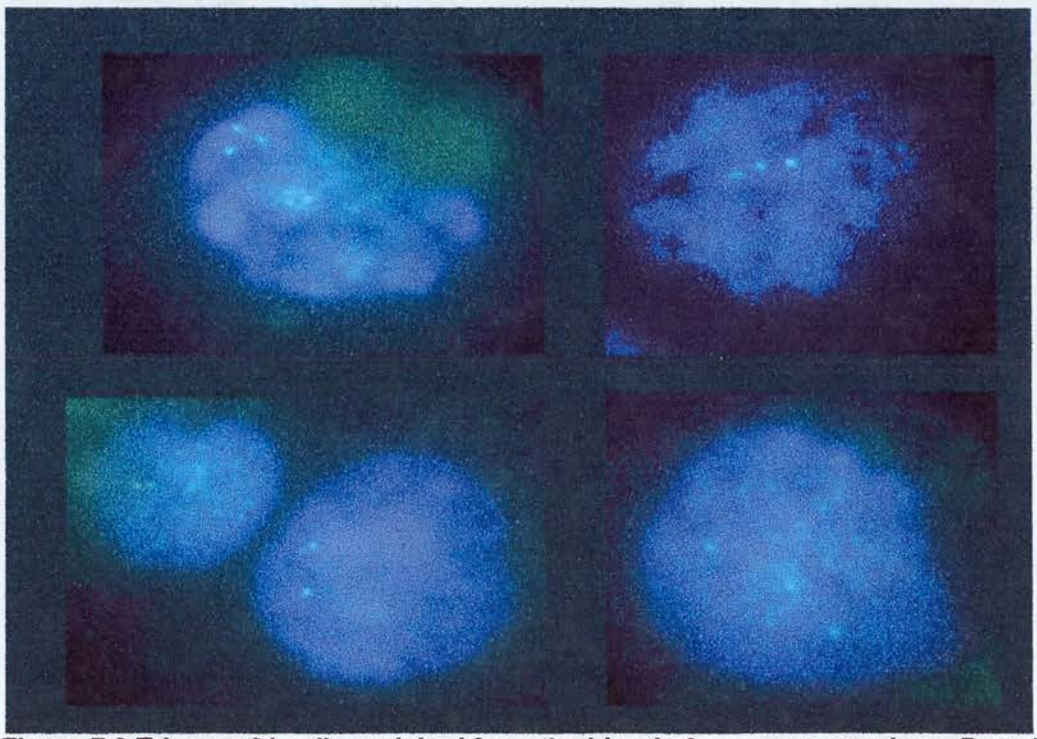


Figure 7.3 Trisomy 21 cells enriched from the blood of a woman carrying a Down's syndrome fetus
Chromosome 21 probe labelled with FITC. Note that the three-dimensional structure of the nucleus means that not all the probe signals are clearly visible in a single plane.

7.10 Discussion

7.10.1 FISH used to analyse putative fetal cells in maternal blood

Despite enrichment, it is unlikely that a pure sample of fetal cells from a maternal blood sample can be obtained and FISH is necessary to diagnose fetal aneuploidy in such circumstances.

As our attempts at prospective fetal sexing were significant (correctly assigning fetal sex in 13 of 15 cases), it suggests that fetal cells can be enriched from maternal blood in early pregnancy, albeit in small numbers. In the first seven maternal cases, FISH was performed with only the Y-chromosome probe and there were two false-positive results. It is very unlikely that these cells were lymphocytes from previous pregnancies (chapter 3.3) as both women were purportedly primigravidae and, furthermore, enrichment specifically depleted lymphocytes. It is more likely that the false signals were either the result of non-specific fluorescent debris coinciding with a nucleus or the probe binding to an autosome. However, it emphasises the necessity of confirming that the cells were indeed fetal. Using FISH alone, the simplest method was to use X and Y-chromosome probes simultaneously and ever since this was performed, no false positive results were incurred, despite the small numbers of cells recovered.

Two women were followed throughout the first and early second trimesters to try and establish any temporal relationship in the frequency of fetal cells in maternal blood. Although fetal sex was correctly assigned in both cases, it was unfortunate that case 12 delivered a girl. From case 10, it is tempting to suggest that the number of (male) fetal cells rises up to the early second trimester and then declines (table 7.6). This has been reported previously and may have a physiological basis (chapter 3.6), but as this was only one case, and the slides were searched in their entirety (unlike the other cases), any temporal relationship is unproven and this issue remains unresolved.

Differentiating male from female cells using multicolour FISH is relatively straightforward but it is a different matter if the cells (both fetal and maternal) have the same chromosomes

but different copy numbers of a particular chromosome. If the aggregate probability of detecting a single target chromosome is 0.9 (90%), then the chances of correctly detecting 3 chromosomes in a nucleus is 72.9% (0.9 x 0.9 x 0.9 = 0.729). Furthermore, even disomic cells may occasionally appear trisomic using FISH (table 7.4). Therefore, analyses of cells becomes a statistical exercise when counting the number of hybridisation signals within individual nuclei and assignment of ploidy by FISH is based on a statistical pattern. Hence it is necessary to define specific criteria for such assignment.

Compared with the control cells, there was a highly significant difference in the proportion of cells showing three chromosome 21 signals, enriched from the blood of the woman about to undergo termination of pregnancy for a Down's syndrome fetus. The confirmation of fetal aneuploidy is an important verification of our negative enrichment technique and demonstrates the need for in situ hybridisation. However, we cannot disregard the possibility of an iatrogenic increase in the number of fetal cells in this case as substantially more apparent fetal cells were found than in cases which had not undergone prenatal testing. Little is known of the persistence of fetal erythroblasts in the maternal circulation but there is some evidence that proportionately more cells enter the maternal circulation in aneuploid pregnancies, possibly because of abnormal placentation (chapters 3.6 and 10.3), and our findings are in keeping with other workers.

Gänshirt-Ahlert et al (1994) claimed that up to and including 16 weeks' gestation, they could detect as many as 10,000 fetal erythroblasts in 91.3% of all pregnancies. However, in this study a maximum of 70 male cells were identified at 14 weeks' gestation whilst in most cases very few XY interphase cells were identified. There may be a number of explanations for this difference.

Firstly, the obvious explanation is that positive selection using the MACS system is considerably better than our negative enrichment approach. However, even using multiparameter FACS, very few putative fetal cells have been recovered (chapter 3.6) and we, like others, have found MACS inferior to Dynabeads (chapter 6.4.4). An alternative explanation is that the analyses of Gänshirt-Ahlert et al were based solely on cell

morphology using Romanowski stains with no other corroborating evidence such as prospective fetal sexing or immunocytochemistry to confirm the identity of the cells. It is plausible that the cells were polymorphs which had engulfed MACS beads and the nucleus had then become pyknotic. From our own experience these cells can appear very similar to normoblasts but the cytoplasm has a glassy appearance.

Another reason why there might have been so few apparent fetal cells is the decreased hybridisation efficiency of erythroblasts (table 7.3). As cells degenerate, loss of cellular target DNA results in poorer hybridisation efficiency (Ward et al, 1993). Certainly normoblasts (whether fetal or adult) have pyknotic nuclei which are about to be extruded and probe access to erythroblast DNA is poorer than for peripheral blood leukocytes. Therefore, although there might be many more fetal erythroblasts in the final complement of cells, efficient hybridisation is not occurring.

Despite the Dynabead enrichment process there were still hundreds of thousands of nuclei. Examining every single 3-dimensional nucleus was extremely arduous and tiring, taking several days to scan an enriched sample in fluorescence. We also had to be pragmatic as the microscopes were required by many others. If we were sufficiently confident about the quality of XY signals, scanning was stopped and fetal sex was predicted on the basis of very few XY cells. Therefore there may have been more fetal cells present but the ability to search for them manually was the limiting factor. This emphasises the need for an automated slide search capability if the concept of analysing fetal cells from maternal blood is to become reality (Piper et al, 1994; Tanke et al, 1996).

Of course, no data has determined just how many fetal erythroblasts are consistently present in the maternal circulation and there may yet only be a handful in 20ml of blood.

7.10.2 The potential of FISH in Prenatal Diagnosis

There are advantages to rapid karyotyping in antenatal diagnosis. Speedy results may have a positive psychological effect and termination of pregnancy performed earlier in pregnancy is physically and psychologically less traumatic. Furthermore, if there are time constraints between the test and when management decisions must be made, rapid results can be advantageous. FISH is less labour-intensive than traditional cytogenetics and is applicable to tissues with a low mitotic index.

a) FISH and Amniocentesis

In 117 of a series of 556 uncultured amniocenteses, Klinger et al (1992) used five contiguous cosmid probes to chromosomes 21, 13, 18, X and Y simultaneously and in less than 2% was there any dubiety in fetal karyotype normality using their probe sets. However, hybridisation efficiency was lower in trisomic samples principally, the authors claimed, because pregnancy was more advanced at the time of sampling and these showed poorer hybridisation. Although promising, the authors did not say why this was the case. Possibly a fetal anomaly was detected on ultrasound scan, prompting the amniocentesis. This is a reasonable supposition as, in almost 80% of cases, not all the probes were used. Their choice of probes for a particular sample may have been guided by the suspected anomaly and if so, it cannot be described as a truly blind study.

Seres-Santamaria et al (1993) compared standard amniocyte metaphase analysis with interphase FISH, using an α-satellite probe which hybridised to both chromosomes 13 and 21 and only detected 12 of 16 Down's syndrome cases. However, this might have been anticipated as there should be five hybridisation bodies (three chromosome 21 domains plus two chromosome 13 domains) if hybridisation is 100% efficient. If the hybridisation efficiency is only slightly reduced, a nucleus will show perhaps four domains and erroneously appear diploid. Hence, a balance must be struck between obtaining a large, easily identified signal or a smaller, but more specific, signal and demonstrates the importance of choosing probes appropriately.

More recently Eiben et al (1998 and 1999) reported their experience with FISH in over 3000 amniocenteses. There were no false-positive results and all cases of trisomy 13, 18 and 21 were detected apart from one case of trisomy 21 which was missed because of hybridisation failure. This suggests that if a result is positive, further counselling may take place immediately. However, when considering that all other trisomies were detected with standard karyotyping, it confirms that cytogeneticists still cannot rely solely on the FISH results and must wait until the final culture to ensure they have not missed any trisomies or translocations.

b) FISH and chorionic villus sampling

The tissue obtained at chorionic villus sampling is metabolically active and hence FISH results should be reliable and available very soon after the procedure. This should permit CVS to be performed late in the first trimester (minimising the risks of limb reduction defects, chapter 2.3) but sufficiently early to allow suction termination of pregnancy if requested.

Evans et al (1992) correctly identified the only case of trisomy 21 in a small study and found that the hybridisation efficiency for uncultured chorionic villi was higher than for uncultured amniocytes. Bryndorf et al (1996) reported a series of over 2700 CVS cases in which they confidently assigned fetal karyotype. However, they were explicit in highlighting the increased incidence of mosaicism in placental samples, which necessitated a further invasive test, a problem that has dogged CVS from the start (chapter 2.3).

7.11 Summary

Our results suggest that it is possible to sex fetuses using FISH in early pregnancy from a peripheral maternal blood sample and, possibly, more cells might be isolated before 16 weeks' gestation. Furthermore, it is possible that more fetal cells may be isolated from aneuploid pregnancies. All these findings suggest that non-invasive prenatal diagnosis of fetal aneuploidy in early pregnancy is feasible.

However, this is a rather bold statement as so few cells were recovered in the karyotypically normal cases, which will be the vast majority of pregnancies. Undoubtedly more fetal cells must be recovered from maternal blood to improve diagnostic confidence.

FISH is now an established investigative modality but even with a pure sample of fetal amniocytes there are false-negative results and, earlier in pregnancy, there are false-positive results with CVS. Clearly prospective fetal sexing, to prove that such cells are fetal, is a step in the right direction. However, half of all pregnancies involve a female fetus. Positive identification of isolated cells would permit greater confidence in karyotype assignment and until then they can only be described as "putative" fetal cells. The ideal way would be simultaneous karyotyping and positive cell identification and the approaches to this will be discussed in chapter 9.

Chapter 8. Cell Deposition

At the end of enrichment the vast majority of cells remaining were non-nucleated erythrocytes and maternal leukocytes (figure 6.8). Consequently, there were several aspects to the deposition stage to be addressed.

Firstly, as many cells as possible must be deposited onto slides without incurring great losses of rare fetal cells. Secondly, the total number of cells from each sample should be deposited on three or four slides. If there are fewer slides and FISH failed, or a slide was damaged, then the sample may be useless for diagnostic purposes. If more slides are used then handling becomes a burden. And thirdly, cells should be deposited as a monolayer. Mature erythrocytes do not show chromosome signals but if one covers a nucleated cell it could interfere with hybridisation. Cells adequately separated might also allow FISH to be combined with a method that has previously identified the fetal erythroblasts positively (chapter 9). This would mean that only the positive cells need to be examined whilst FISH signals in other nuclei could be disregarded.

Using model systems of fetal blood mixtures enriched in the way already described, different deposition methods were investigated such that an identification system might be combined with FISH.

8.1 Deposition methods

8.1.1 Cytocentrifuge preparations

The enriched cells were washed, centrifuged and resuspended in 5ml of 2% (w/v) BSA in PBS. 500µl aliquots were loaded into cytocentrifuge buckets and centrifuged at 100 x g for 5 minutes onto clean microscope slides using a Shandon II cytocentrifuge (Shandon). A non-overlapping monolayer could be produced on 10 slides. The slides were fixed for subsequent May-Grünwald-Giemsa staining, immunocytochemistry and/ or FISH.

8.1.2 Settling technique

A wax ellipse, approximately 5cm^2 (5 x 10^8 µm^2) was drawn on the slide using a DAKO wax pen (catalogue number, S2002) and allowed to dry. 2ml of the cell suspension were then carefully loaded onto the slide within the ellipse. The cells were allowed to settle by unit gravity in a 37°C incubator which encouraged evaporation of the suspension medium.

8.1.3 Methanol: acetic acid fixation

After the final centrifugation following enrichment (chapter 6.3.3), the 2% (w/v) BSA in PBS was pipetted off to just above the tiny red cell pellet and the test tube was vortexed to resuspend the nuclei. Fresh Carnoy's methanol: acetic acid (3:1) was dropped, one drop at a time, onto the cells using a Pasteur pipette whilst vortexing continued. Once the test tube contained about 7ml of fixative it was left to stand at 4°C for fifteen minutes, centrifuged at 250 x g for 10 minutes and the fixative pipetted off to just above the cell pellet, which removed the lysed red cell membranes. The pellet was vortexed again and three drops of fixative was added. Using a fine-tipped Pasteur pipette a drop of the cell suspension was dropped onto an ethanol-cleaned microscope slide from a few centimetres above the slide and the fixative allowed to evaporate rapidly. The slide was inspected using a phase contrast microscope to ensure that there were sufficient nuclei on the slide. This was repeated until all the cell suspension was exhausted which usually required two or three slides. Slides were then subjected to immunocytochemistry and/ or FISH.

Fixation, as above, was then investigated using varying ratios of methanol: acetic acid from 5:1 to 100:1 to see if some cytoplasm could be retained to allow subsequent positive cell identification.

8.1.4 Smear technique

In the latter stages of the project, a technique initially developed by Mrs. Patricia Eason and subsequently modified and optimised by Mrs. Heather Finlayson and Dr. Alison Hill, appeared to be very promising.

After enrichment, the remaining cells were resuspended in a small volume of fetal calf serum with 1% dimethyl sulphoxide (DMSO). A smear was made in exactly the same way as for whole blood i.e. depositing a drop of the cells on a microscope slide and drawing the edge of another slide over the drop, along the length of the original slide. This allowed all the remaining cells to be deposited onto six to eight slides with the majority of nucleated cells concentrating at the feathered tail of the smear.

8.2 Results

Cytospin preparations gave excellent morphology and cells could be identified easily. Fetal erythroblasts were also readily identified by γ-globin immunocytochemistry (chapter 9). However, there were frequent instances where no, or very few, cells could be found on the slide and a pink halo was evident in the filter paper around the hole. These phenomena imply that a significant proportion of cells irretrievably leach into the filter paper. The cytospin is a 6mm diameter circle (radius, 3mm or 3000μm) so the deposition area is $2.83 \times 10^7 \mu m^2$ (πr^2). An erythrocyte has a diameter of 8μm and area of $50 \mu m^2$. Therefore, if all the cells were perfectly tessellated, there would be 5.6×10^5 cells on the slide. Clearly this is not the case as there is always additional (wasted) space between cells. However, even if this were the case, in ten cytospin preparations there would be 5.6×10^6 cells. At the end of the two-stage enrichment process around 5×10^7 erythrocytes remained (figure 6.9). Therefore, to deposit all the remaining cells would require around 100 slides and it is evident from these simple calculations that cytocentrifuge slide preparations lost at least 90% of cells. This degree of loss is supported by simply counting the total number of nucleated cells on the slide and comparing this with the total number of nucleated cells known to be in the final, enriched suspension.

Assuming that this cell loss was non-selective, 90% of fetal erythroblasts would also be lost. This was intolerable and cytospin preparations of enriched maternal blood samples were abandoned.

The wax ring method provided an area of approximately 5×10^8 μm^2. If we consider that 5×10^7 erythrocytes occupy 25×10^8 μm^2, then all the remaining cells might be accommodated on five slides if all the cells were reasonably approximated. Using this approach cell loss should be minimal because the cells should have settled before any significant evaporation took place. Unfortunately, because of the surface tension effect of the ever-decreasing pool of fluid, the cells were pulled more centrally into the wax ellipse. The resulting cell morphology was poor because all the cells heaped on each other and investigations were pursued no further.

Fixation in suspension using 3:1 methanol: acetic acid fixed cells firmly onto slides and lysed the remaining non-nucleated erythrocytes so that all the nuclei could be deposited onto three slides. Having removed the cytoplasm and perinuclear proteins, probe access and hybridisation was best. The disadvantage was that cell morphology was lost and positive identification of cells was impossible. However, the results of the prospective study of fetal sex prediction were obtained from cells treated in this manner (table 7.5).

More diluted acetic acid did not lyse all the erythrocytes but it retained the cytoplasm of nucleated cells better. A 1:40 dilution resulted in reduced hybridisation efficiency but was the best compromise between retaining cell cytoplasm for immunocytochemistry and later FISH (chapter 9.3).

The reagents used with the smear technique were compatible with subsequent immunocytochemistry but the problems of reduced hybridisation efficiency remained because of the intact cytoplasm (chapter 9). This should not be an insurmountable problem and, importantly, it should be possible to automate this technique using a robot arm which would minimise inter- and intra-operator variation for monolayer cell preparation, a principle which is fundamental to the whole approach.

8.2 Discussion

The ideal deposition method should produce a monolayer of cells, covering a large area of the slide so that many cells can be deposited onto a few slides, but without incurring significant cell losses. The smear technique appeared to be most promising but it was apparent that the major obstacle to depositing the remaining cells onto three slides is the large number of remaining erythrocytes. This will be considered further in chapter 10.

Chapter 9. Positive identification of fetal erythroblasts

Although we have successfully enriched for fetal cells from maternal blood and prospectively sexed pregnancies, a number of issues are unresolved.

Fetal erythroblasts are end-stage nucleated cells, the nuclear material is tightly packed and FISH is not as efficient as for other cell types (chapter 7). Our data suggest that very few fetal cells were recovered. Alternatively, reasonable numbers of fetal erythroblasts might have been recovered but, because of poor probe access, they were of no predictive value. Furthermore, even among diploid cells, some cells show one or three FISH signals, incorrectly suggesting aneuploidy. Male fetal cells are easily differentiated from maternal cells by FISH, but 50% of pregnancies involve a female fetus. Finding no aneuploid cells, all bearing two X chromosomes may indicate a normal female fetus but it may also mean that no fetal cells were isolated in the first place. Finally, even after enrichment, the vast majority of nucleated cells are maternal. However, as optimum FISH signals are obtained when the cytoplasm has been removed, the actual cell type cannot be determined and every nucleus must be examined.

To overcome these problems it is imperative that fetal cells are identified positively. Using model systems of adult/ fetal blood mixtures, we attempted to identify fetal erythroblasts in three ways and, thereafter, subject them to FISH. All the cells were subjected to cytospin deposition to maintain optimal morphology but the limitations of this deposition method have been highlighted in chapter 8.

9.1 May-Grünwald-Giemsa

Standard May-Grünwald-Giemsa staining (chapter 4) was combined with FISH, using the Y-heterochromatin probe, pHY2.1 (table 9.1). In only one case involving maternal blood has a definite erythroblast been observed (figure 3.4). Unfortunately, this slide had already been mounted and when the coverslip was removed and the slide subjected to FISH, there was no hybridisation. Furthermore, it was not known if this was a maternal or a fetal erythroblast although its size suggested that it was probably fetal.

9.2 Kleihauer-Betke Acid Elution

Fetal haemoglobin is more resistant to acid elution or alkali denaturation than adult haemoglobin and is the basis of the Kleihauer-Betke-Braun reaction (1957).

Fetal blood samples were fixed on clean microscope slides for five minutes in 80% ethanol. After air-drying the slides were dipped in the haemoglobin eluent (8.3mM hydrochloric acid, 7.5g/l haematoxylin) for twenty seconds, washed for two minutes in tap water and then air dried. Slides were then stained with erythrosin B (1g/l) for three minutes, washed in tap water and air-dried. Those cells retaining haemoglobin, i.e. fetal red cells, were readily apparent against the ghosted adult erythrocytes and leukocytes (figure 9.1). Their positions on the slide were manually recorded using a New England Finder and FISH was performed using the protocol described in chapter 7 (Gaudoin et al, 1994; figure 9.2).

Using this identification method, despite exhaustive searching of enriched maternal cytospin preparations, no cells were identified which were unequivocally fetal erythroblasts. Of course cytospin preparations incur large losses (chapter 8) so any rare fetal cells which might have been present could have been lost at the deposition stage.

Figure 9.1 Fetal erythroblast following Kleihauer-Betke reaction
Note the erythroblast's large size (solid arrow) and the smaller, ghosted surrounding adult erythrocytes (two collections of ghosted erythrocytes shown with dashed arrows)

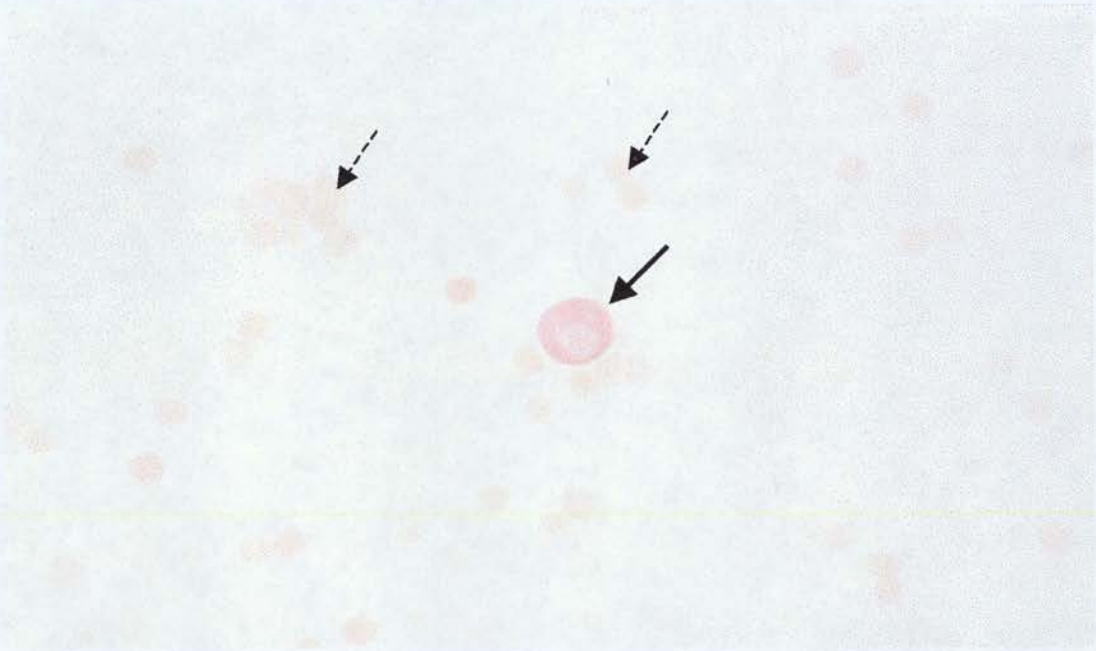
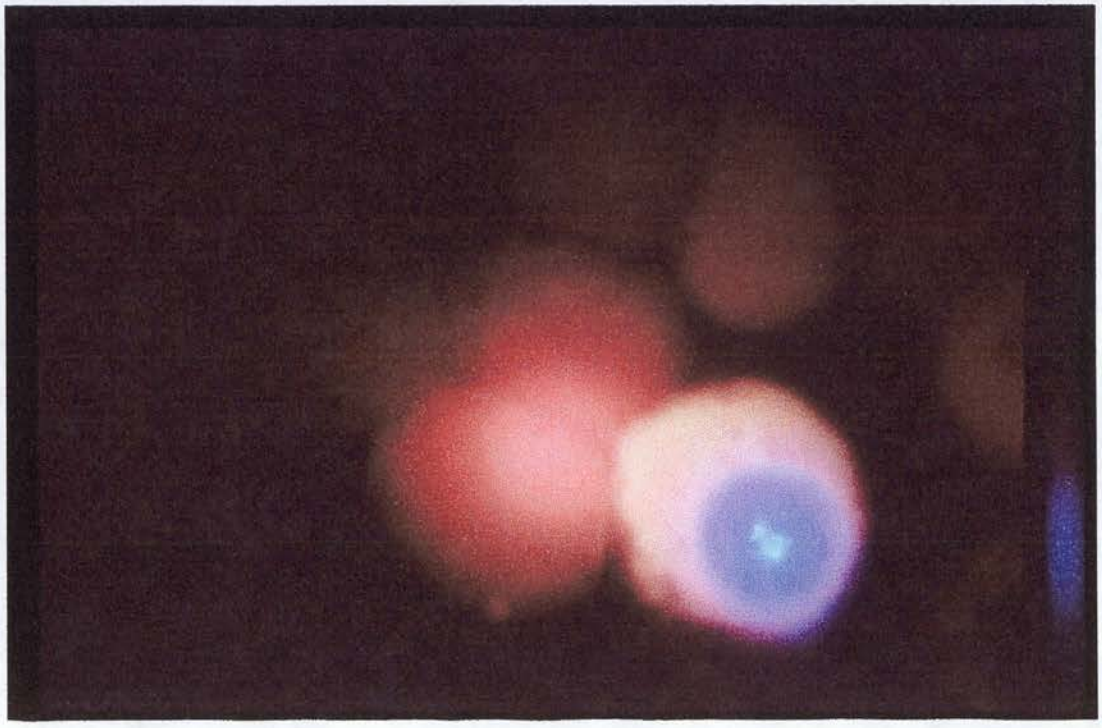


Figure 9.2 FISH performed on fetal erythroblasts after Kleihauer-Betke reaction
Y chromosome probe visualised with FITC. Note the higher intensity cytoplasmic staining of the Kleihauer-positive erythroblast compared to surrounding Kleihauer-negative cells.



9.3 γ-globin immunocytochemistry

The method for immunocytochemistry has been detailed in chapter 4.3. The monoclonal antibody (mAb), UCHγ, was an ascites preparation and the kind gift of Professor Peter Beverley (Allen et al, 1987). It was a mouse IgG_1 mAb against human γ-globin and was optimised to a 1 in 800 dilution for immunocytochemical work. Figure 9.3 (right hand photomicrograph) shows clear differentiation between adult and fetal red cells with UCHγ. For optimal immunocytochemistry, cells were fixed in methanol: acetone (1:1). Unfortunately, after γ-globin detection, when FISH was performed there was extensive background fluorescence rendering FISH signals uninterpretable.

FISH worked best after cells were fixed in methanol: acetic acid (3:1). However, this lysed the cell cytoplasm, so combining immunocytochemistry with FISH was impossible. Therefore, varying concentrations of methanol: acetic acid, ranging from 5:1 to 100:1 were investigated to determine how this affected the degree of cell lysis and subsequent FISH analysis (chapter 8.1.3). It was possible to fix the cells for one minute in methanol: acetic acid, 40:1, and then drop them on to clean microscope slides. This did not lyse all the erythrocytes but immunocytochemistry could be performed thereafter. In model experiments, the position of positive cells was recorded and FISH was subsequently performed (figure 9.3, left hand photomicrograph). Background fluorescence was greater than with fixation in 3:1 but it is promising for maternal blood samples.

To date we have not seen cells which were unequivocally fetal erythroblasts identified by UCHγ. Figure 9.4 shows a central cell in a cluster which has a perinuclear halo following immunostaining and development with the New Fuchsin kit. It does not look like erythroblasts recovered from termination samples but it is not known exactly what such cells look like once they have spent some time in the maternal circulation. This cell had been fixed and mounted and was not subjected to FISH analysis.

We have found γ-globin-positive cells from an enriched maternal blood sample (case 14), but which were undoubtedly polymorphs. When subjected to FISH, these cells showed two

X-chromosome signals and the woman went on to deliver a male fetus (which was predicted by FISH when other cells were fixed in 3:1 methanol: acetic acid).

Table 9.1 shows that prior positive identification reduces the hybridisation efficiency of fetal erythroblasts using either a Y-chromosome probe or X and Y probes simultaneously. Methanol: acetic acid (3:1), because it lysed the cell membrane and stripped the perinuclear proteins, resulted in the best hybridisation efficiency but positive identification was impossible. Kleihauer staining resulted in poorer hybridisation efficiency, which was reduced further when two probes were used simultaneously. Methanol: acetic acid (40:1) allowed positive identification of fetal erythroblasts prior to FISH although, in model experiments, only 43% of erythroblasts from a male fetus showed simultaneous X and Y signals. Hybridisation efficiency was similar to Kleihauer identification but the latter had the disadvantage that it did not lyse any cells so many slides would be needed to deposit all the cells after the enrichment process.

Table 9.1 Hybridisation efficiency of fetal erythroblasts using FISH combined with prior cytoplasmic staining

Fixation and Stain	Hybridisation efficiency	
	Y-chromosome probe	X and Y-chromosome probes
Methanol: acetic acid (3:1)	61.6 ± 8.2%	-
May-Grünwald-Giemsa*	32.0 ± 7.8%	-
Kleihauer-Betke†	45.9 ± 6.4%	37.5 ± 9.2%
Methanol: acetic acid (40:1) + γ-globin	-	43.2 ± 30.0%

Cytoplasmic staining performed using protocols detailed in Chapters 4.1* and 9.2†.
FISH performed after cytoplasmic staining using protocol detailed in Chapter 7.4 and 7.6.
Note the superior hybridisation efficiency in the absence of cellular cytoplasm (fixation in methanol: acetic acid, 3:1) compared to the staining techniques which leave the cytoplasm intact.

Figure 9.3 Combining UCHγ immunocytochemistry with FISH
Right side: Immunocytochemistry using UCHγ mAb on artificial admixture of cord and adult blood. Top right cell is UCHγ-positive, other two cells (bottom left) are UCHγ-negative.
Left side: Same cells following FISH using an X-chromosome probe. Probe labelled with digoxigenin and visualised with anti-digoxigenin-Texas Red.

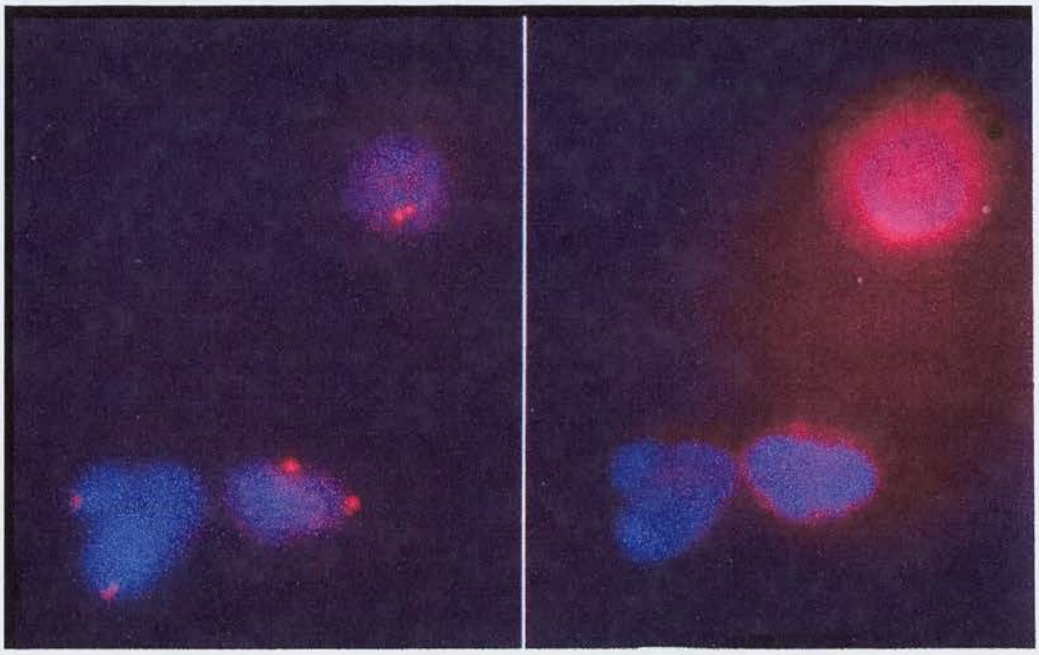
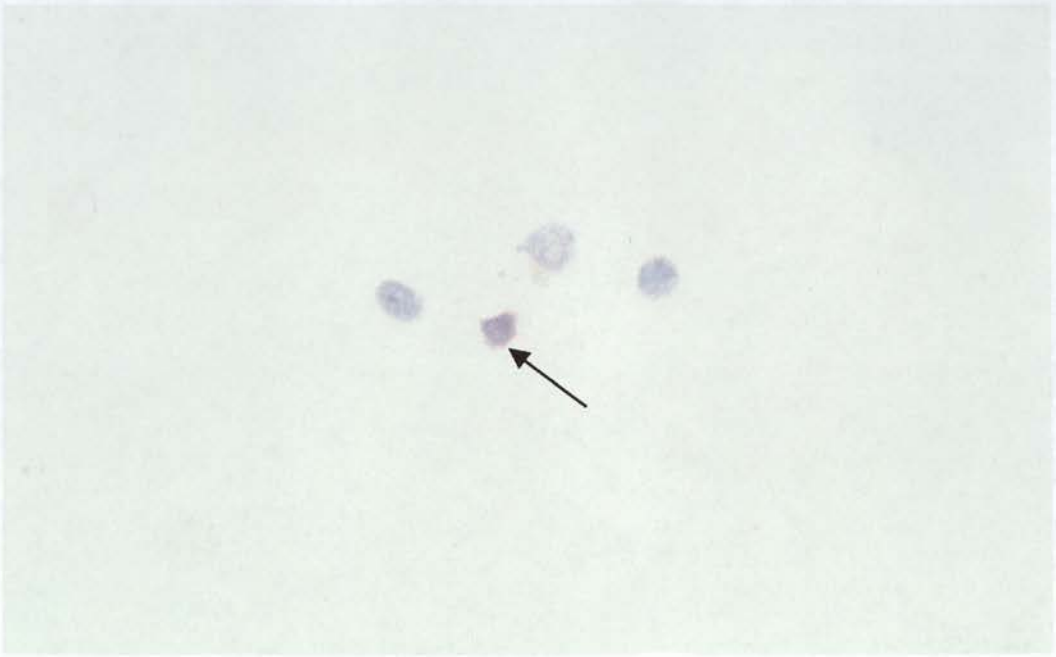


Figure 9.4 γ-globin-positive nucleated cell enriched from the blood of a pregnant woman
The central lower cell (arrowed) shows some unconvincing immunostaining (developed with the New Fuchsin kit and visualised with light microscopy) and it does not have the classic erythroblast appearance.



9.4 Discussion

Fetal erythroblasts do not hybridise as efficiently as leukocytes and attempting to positively identify them prior to FISH, by leaving the cytoplasm intact, resulted in even poorer hybridisation efficiency. Heavy metal salts interfere with FISH and these are present in both the acid elution solutions and in Romanowski stains which explains why hybridisation efficiency was compromised further using these modalities. Stripping the cytoplasm, even in a limited fashion with more dilute preparations of methanol: acetic acid, improved the hybridisation efficiency but positive identification was more difficult.

However, positive identification is a prerequisite because it would reduce the false-positive and false-negative results associated with FISH and would enable pregnancies involving female fetuses to be investigated. Hence, it would increase confidence in karyotype assignment. Importantly, positive identification would also allow an automated slide search capability to be developed. A scanning device could log the position of positive cells whilst surrounding cells (contaminating maternal cells) could be disregarded. This would obviate the arduous task of examining every nucleus for FISH signals, speed up search times and permit large numbers of pregnancies to be evaluated (Piper et al, 1994; Tanke et al, 1996). In turn, this would allow development of other methods of aneuploidy detection (chapter 10) and more rapid optimisation of fetal cell enrichment techniques, so maximising the recovery of fetal cells at the appropriate stages of pregnancy.

One of the major obstacles is that we still do not know what fetal erythroblasts look like, particularly if they have been in the maternal circulation for a short while. There are no fetal erythroblast-specific markers which limits the possibilities of positive selection (chapter 3.6) and hence, there is no specific marker to identify fetal erythroblasts by immunostaining prior to FISH analysis.

Fetal haemoglobin (HbF, $\alpha_2\gamma_2$) is the predominant haemoglobin produced up to 30 weeks' gestation. Adult haemoglobin (HbA, $\alpha_2\beta_2$) is produced from about 8 weeks' gestation onwards, reaching a steady state of approximately 10% of total haemoglobin production until

30-34 weeks' gestation (figure 10.2). Allen et al (1987) reported that 1-3% of adult erythrocytes were UCHγ-positive but did not report if it had been tested against leukocytes. Certainly our false-positive results are concerning as it may not be completely specific to the haemoglobin γ-chain.

Whether a Kleihauer-type reaction or monoclonal antibodies to γ-globin is used, a potential problem is that pregnancy is the only physiological condition in which fetal haemoglobin production is switched on again. Seventeen percent of women show a rise above normal levels in the first trimester, generally before the tenth week. These F-cells are maternal as the amino acid sequence of the γ-chain is of the adult variety, which is different from the fetal γ-chain sequence (Weatherall et al, 1974). HbF production can be reactivated in pathological conditions (table 3.2) but these are rare in pregnant women and are likely to be detected because of other clinical manifestations. A possible confounding variable is hereditary persistence of fetal haemoglobin production, sickle cell disease and thalassaemias. In these conditions there is also likely to be a substantial proportion of adult erythroblasts in the peripheral circulation. In some parts of the United Kingdom (particularly areas of London), and of course worldwide, this would become a sizeable problem. Therefore, identification of fetal erythroblasts based on γ-globin content may not be applicable on a global scale.

Holcomb et al (1990) predicted that the 0.04% "background rate" of F-cells in pure adult blood would render the Kleihauer-Betke test unreliable at cell counts below 0.1%. Furthermore, there is growing evidence that many nucleated erythrocytes in maternal blood are maternal (Slunga-Tallberg et al, 1995; de Graaf et al, 1999). This suggests that alternative markers are necessary to confirm the fetal origin of the erythroblasts. Monoclonal antibodies to the early embryonic haemoglobins (Hb-Gower: ε_4; Hb-Portland: $\zeta_2\gamma_2$; and Hb-Bart's: γ_4,) may be more fetal-specific than UCHγ and if these can be produced reliably they may avoid the problems associated with γ-globin (chapter 10).

An exciting development has been the positive identification of fetal erythroblasts on slide using mAbs to HbF or other embryonic haemoglobins, dissecting only the positive cells off

the slide and then subjecting them to PCR. Cheung et al (1996) identified between 7 and 22 cells from 16-18ml of maternal blood in a series of 10 cases and correctly (albeit retrospectively) predicted a normal fetal karyotype in two cases at risk of haemoglobinopathies. They stated a preference for ζ-globin as they felt it should be more fetal-specific. They would also have preferred to recover more cells as they often saw extraneous DNA bands because of the multiple amplification steps required. If there was the slightest contamination, this would also be amplified and reduce the predictive value of the results. Moreover, multiple cells would minimise the problems of DNA-degradation of one of the primer alleles which would minimise the false-negative rate, particularly as fetal erythroblasts are end-stage cells. Zheng et al (1993, chapter 3.6) and Cheung et al (1996) deposited the enriched cells using cytospins. This deposition method is unreliable (chapter 8) but if a better method can be developed then more cells might be recovered which would open up the whole field as any chromosome or gene disorder might be diagnosed in this way.

Positive identification is one of the prerequisites if fetal cell analysis from maternal blood is to become a reality. To date there have been very few publications of positively identified fetal erythroblasts in maternal blood which have subsequently undergone cytogenetic analysis. Unfortunately the data presented in this and preceding chapters emphasises again the complex inter-dependence between enrichment, deposition, identification and cytogenetic analysis.

Chapter 10. Future Developments

Although we have successfully sexed fetuses in early pregnancy it is apparent that there remain a number of problems. Firstly, at the end of enrichment there are too many erythrocytes. Secondly, the deposition process is far from optimised. Thirdly, the method of positive identification is not yet established and the hybridisation efficiency, already poor with fetal erythroblasts, is reduced further if performed after positive identification. Finally, it is imperative that the whole process is automated if such an approach is to be used on a large scale.

10.1 Differential lysis of adult erythrocytes

At the end of enrichment there were too many erythrocytes to be deposited on to three or four slides (chapter 8) and a simple preliminary step is necessary to remove these excess cells.

Boyer et al (1976) developed a technique to aid the antenatal diagnosis of fetal haemoglobinopathies in amniotic fluid samples contaminated with maternal blood by modifying the standard ammonium chloride red cell lysis buffer (the Ørskov-Jacobs-Stewart reaction) to exploit the biochemical differences between adult and fetal erythrocytes. Carbon dioxide carriage in blood is largely dependent on diffusion into erythrocytes and binding with water to form carbonic acid (H_2CO_3). This dissociates into hydrogen (H^+) and bicarbonate (HCO_3^-) ions. Red cell lysis is achieved by the addition of ammonium chloride. Bicarbonate exchanges with chloride which is matched by an inward diffusion of ammonia to form ammonium (NH_4^+) ions. The intracellular increase in ion pairs osmotically draws in water, the cell swells and eventually bursts. The rate-limiting step of haemolysis is carbonic anhydrase-dependent and can be reduced by the addition of carbonic anhydrase inhibitors such as acetazolamide (figure 10.1). Fetal erythrocytes have 100-fold less carbonic anhydrase I than adult red cells whilst acetazolamide penetration times are similar. F-cells (adult erythrocytes containing large amounts of fetal haemoglobin) do not preferentially survive haemolysis so they should not confuse the situation. Furthermore, the differences may be more pronounced

less than 20 weeks' gestation because of the marked difference in fetal red cell physiology in early pregnancy (Boyer et al, 1983). Clearly these observations would be advantageous with respect to fetal erythroblasts enriched from maternal blood for prenatal diagnostic purposes. Importantly, nucleated erythrocytes are more resistant to haemolysis than their non-nucleated counterparts which would facilitate further enrichment (Takeda and Nagai, 1981).

Differential lysis appears to be a simple but extremely promising avenue to improve the enrichment process and work was beginning on this, under the supervision of Dr. Alison Hill, at the end of my work in the Human Genetics Unit. Clearly the optimum concentration of acetazolamide, the effects these chemicals have on methods to identify fetal erythroblasts positively and the effects on subsequent FISH analysis will need to be determined.

10.2 Novel enrichment techniques: Charge flow separation

Wachtel et al (1996 and 1998) reported a novel technique, charge flow separation, which subjected cells to a cross flow electric field. The cells were channelled into different compartments, depending on their surface charge densities, and erythroblasts focussed like erythrocytes and some lymphocytes. FISH was performed on the several thousand cells collected from each maternal sample but gender prediction only tended towards significance ($p = 0.07$). Y-DNA PCR on the recovered cells achieved similar predictive results ($p = 0.09$). The data suggest that the large number of putative erythroblasts was unlikely, especially as it is considerably more than any other group have claimed. However, the authors suggested that because charge flow separation was inexpensive, rapid and reproducible and did not require monoclonal antibodies, it could provide a helpful modality, even if an additional enrichment step is necessary to improve specificity.

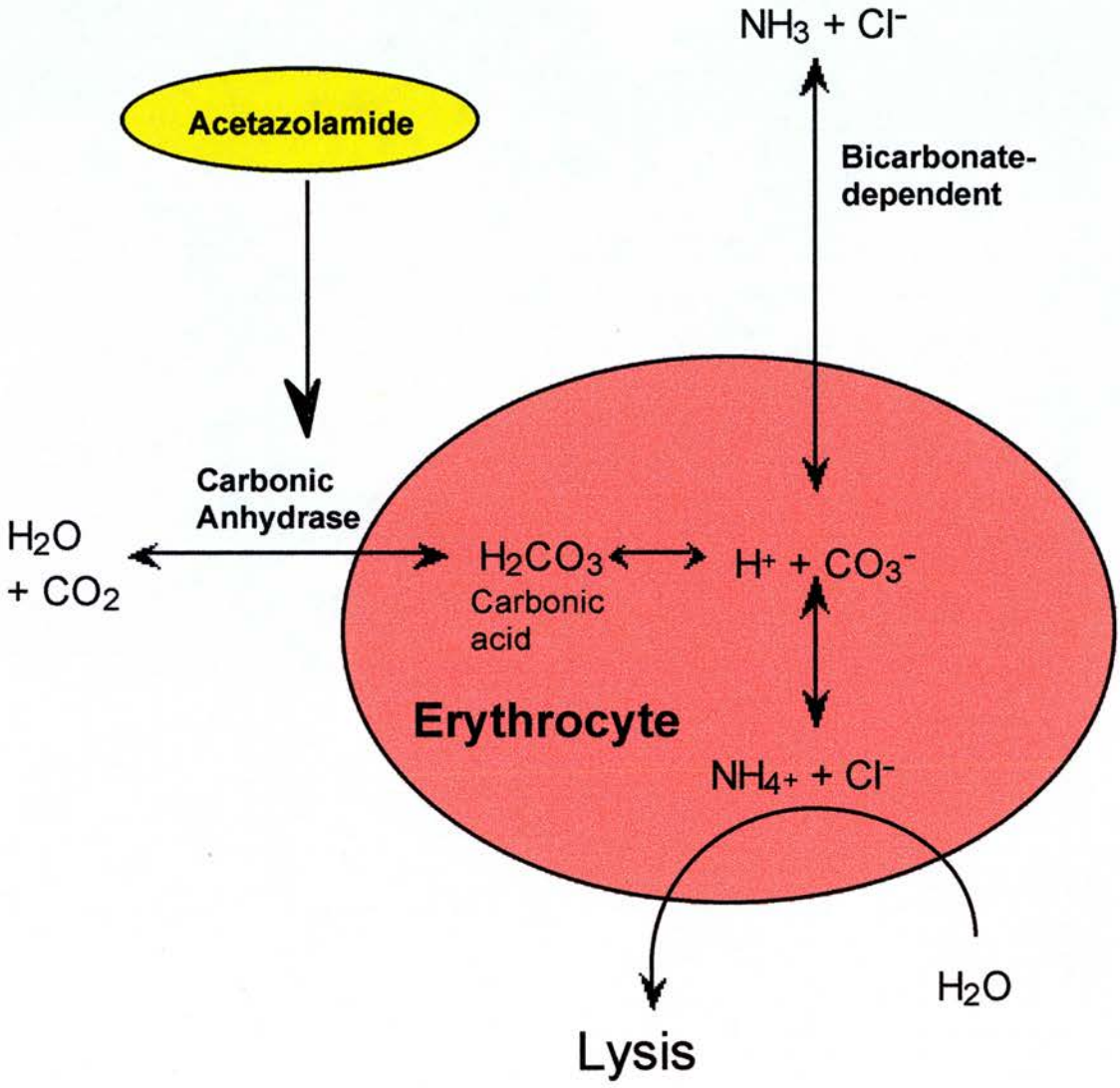


Figure 10.1 Differential Erythrocyte Lysis
(Ørskov-Jacobs-Stewart reaction)

Note that the rate-limiting step is carbonic anhydrase-dependent and is influenced by the addition of acetazolamide

10.3 Fetal cell culturing

Lo et al (1994) reported that fetal blood cell progenitors were more sensitive to erythropoietin than adult progenitors. They cultured whole blood from five pregnant women and subjected the proliferating cells to Y-chromosome FISH. Prior to culturing there were no Y-positive cells but after culturing there was 1 Y-positive cell to 400-9500 Y-negative cells. Hence, it appeared that culturing increased the relative and absolute number of fetal cells. Valerio et al (1996) enriched for erythropoietin-positive cells, cultured the recovered colony-forming unit-erythroid (CFU-E) and burst-forming unit-erythroid (BFU-E) cells and subjected these cells to PCR. Although fetal sex was already known in 5 of the 8 cases, gender prediction was 100% accurate ($p < 0.004$). They also reported that fetal erythroid progenitors were preferentially cultured. Some of the samples were subjected to interphase FISH analysis including two cases involving fetal trisomies. In these two cases there were more CFU-E and BFU-E cells with a higher proportion of trisomic cells. This supports the growing impression of greater fetal cell deportation into maternal blood in aneuploid pregnancies (chapter 7.9.3) and improves the prospects of definitive non-invasive prenatal diagnosis of aneuploidies. Little et al (1997) also cultured flow-sorted haematopoietic progenitor cells and similarly found a slight preferential expansion in detectable fetal cells. Perhaps of greater significance, however, was their observation that culturing improved the quality of the FISH preparations which, given the poor hybridisation of fetal erythroblasts (chapter 7), may be an important consideration.

Cell culturing has the disadvantage that it takes time and the speed of sample-processing is fundamental to the ethos of mass screening. Of course, if only a handful of cells can be recovered but successfully replicated, then it may be necessary and it may also result in good metaphase preparations which would allow structural chromosome abnormalities to be diagnosed as well.

However, even in enriched and cultured samples, maternal cells will probably outnumber fetal cells and positive identification prior to cytogenetic analysis remains necessary to minimise false results. It would be ideal to avoid cell culturing if possible but given the few

fetal cells recovered it may become necessary and further investigation of differential culturing is still required.

10.4 Positive identification

UCHγ monoclonal antibody did not appear to be sufficiently specific for fetal haemoglobin for our purposes (chapter 9.4). Figure 10.2 shows the temporal relationship of the synthesis of the various fetal haemoglobins during gestation (Grosveld et al, 1993). Fetal red cells contain embryonic haemoglobins until at least 18 weeks' gestation and there is no known reactivation of these haemoglobins during normal pregnancy. Therefore, if monoclonal antibodies can be raised to ζ- or ε-chains, they might be more specific than UCHγ (Cheung et al, 1996; Mesker et al, 1998).

In a slightly different approach, Hengstschlager and Bernaschek (1996) reported that fetal cells appeared to express considerably higher intracellular thymidine kinase activity than adult blood cells which could provide an additional marker to sort for, or positively identify, target cells and further investigation into both these areas is necessary.

10.5 Other molecular techniques

The explosion in molecular biological techniques in the past twenty years has allowed much of the work contained in this thesis to be performed. Ideally a diagnostic test should be applicable to one cell but several cells would be preferable to minimise inconclusive results and techniques continue to develop which might be applied to the handful of recovered fetal cells.

10.5.1 Primed in situ DNA synthesis

Primed in situ DNA synthesis (PRINS) combines the sensitivity of PCR with the visual appeal of FISH such that small, chromosome-specific DNA sequences can be amplified and visualised within a nucleus. Although in its infancy, in time it may allow disorders at the gene level to be diagnosed rapidly from enriched fetal cells rather than simply the common aneuploidies which are, relatively, gross genetic abnormalities (Gosden and Scopes, 1996; Orsetti et al, 1998).

10.5.2 Quantitative PCR

If fetal cells can be identified positively on a slide they could be micro-dissected off the slide and subjected to quantitative PCR which could overcome the problems of reduced hybridisation efficiency associated with erythroblasts (Cheung et al, 1996, chapter 9.4). Toth et al (1998) reported fluorescent PCR amplification of polymorphic small tandem repeat sequences on very few cells for chromosomes 21 and 18 and believe that it could be applied to fetal cells isolated from maternal blood, provided these can be positively identified on-slide prior to cytogenetic analysis.

Mosaic cells may give false-negative or ambiguous results and structural aberrations may go undetected but this is a promising avenue. Even more exciting is that it should be possible to diagnose genetic disorders which potentially takes the whole field to another developmental level.

10.6 Slide searching and Automation

Even after enrichment, there are too many cells to search the slide manually (chapter 7) and this must be taken in the context of a mass screening programme involving what is likely to be the majority of pregnant women. It may be that fetal cells are present but they have not been seen because of the inability to search the slides in their entirety.

For the reasons outlined in chapter 9, an automated approach is fundamental to the whole concept of fetal cells enriched from maternal blood. An automated slide-search capability should preferably incorporate a confocal microscope as this takes slices through a

3-dimensional structure (such as a nucleus). To achieve this would require considerable expertise but is not beyond the present capabilities of pattern recognition scientists (Piper et al, 1994). Figure 10.3 shows such a device, developed at the MRC Human Genetics Unit, Edinburgh. It is part of a robotic system which automatically prepares a monolayer of cells on microscope slides from a suspension of cervical cells, stains the slides and scans them. The robot records the position of potential dyskaryotic cells for later review by a cytologist.

Using high numerical aperture lenses to gather the maximum amount of light from fluorescent signals we have calculated that it would take about 16-20 hours to scan three slides for intranuclear FISH signals and log the position of possible karyotypically abnormal cells for later manual review. This is too slow for practical purposes but it would be accelerated dramatically if a machine could search simply for a positive marker and thereafter return to those particular cells to analyse the FISH signals (Tanke et al, 1996).

Scientists at the MRC-Human Genetics Unit, where all the work contained in this thesis was carried out, were beginning to work on this aspect of the project. Rather shortsightedly, in my view, the Pattern Recognition Department of the Unit was disbanded with the retirement of its section head, Dr. Denis Rutovitz. Denis Rutovitz was instrumental in setting up this project and until the absolute necessity for this stage is appreciated it is unlikely that this will ever be carried out.

10.7 Ethical considerations

Fetal cell isolation and cytogenetic analysis also raises ethical and moral issues that have yet to be debated. Prenatal screening is concerned with increasing choice and, in this case, it is the choice between having and not having a test. First trimester termination of pregnancy is considerably less distressing than a second trimester procedure but first trimester diagnosis may not always be necessary as many of these pregnancies would, in any case, go on to abort spontaneously. Therefore, offering women termination of an affected pregnancy may place them in a moral dilemma. Furthermore, in some areas of the world fetal sexing may be of primary importance and such a capability is open to abuse.

Particularly in the Western World, expectations are undoubtedly changing and "the perfect baby" is often anticipated. Non-invasive prenatal diagnosis will alter these expectations further and defensive medicine then becomes an issue. If a particular test is not offered and the baby has Down's syndrome it exposes the obstetrician to a possible lawsuit.

With current technologies fetal cell isolation and cytogenetic analysis could be applied only to a handful of common aneuploidies and will not detect non-genetic structural anomalies or gene defects. However, the recent, highly publicised completion of the Human Genome Project opens the field up even further as, in time, any genetic problem may be detected from a simple maternal blood test. Therefore, a credible body is needed to determine the conditions for which screening is worthwhile and doctors must be able to follow these standards without fear of litigation.

10.8 An International Programme

If fetal cell recovery is to become routine, large numbers of pregnancies require evaluation. Studying pregnancies by matching cell type with Y-chromosome FISH analysis would determine the proportion of pregnancies in which erythroblasts might be found. The method could then be extended to aneuploidy detection using previously known aneuploid pregnancies from, perhaps, chorionic villus sampling.

A number of groups worldwide (notably Diana Bianchi, Sherman Elias, Joe Leigh Simpson, Wolfgang Holzgreve and Dorothy Gänshirt-Ahlert) have now set up such a venture to try and address many of the issues highlighted in the previous chapters (Holzgreve et al, 1998). To date there have been no firm conclusions reported but international collaboration is essential if the field is to progress.

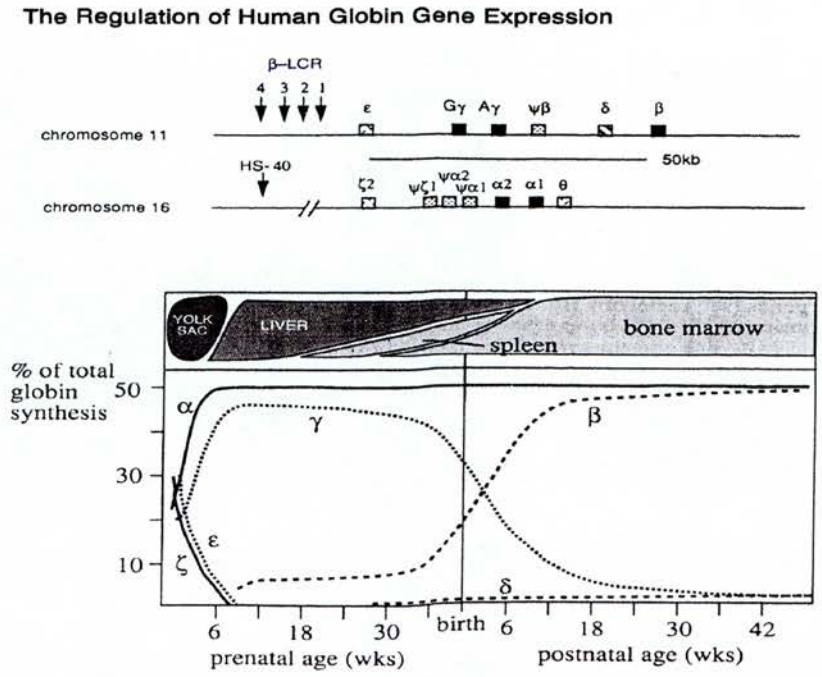


Figure 10. 2 Fetal haemoglobin production
From Grosveld et al (1993). Note the rapid fall in ε- and ζ-globin synthesis in the first trimester

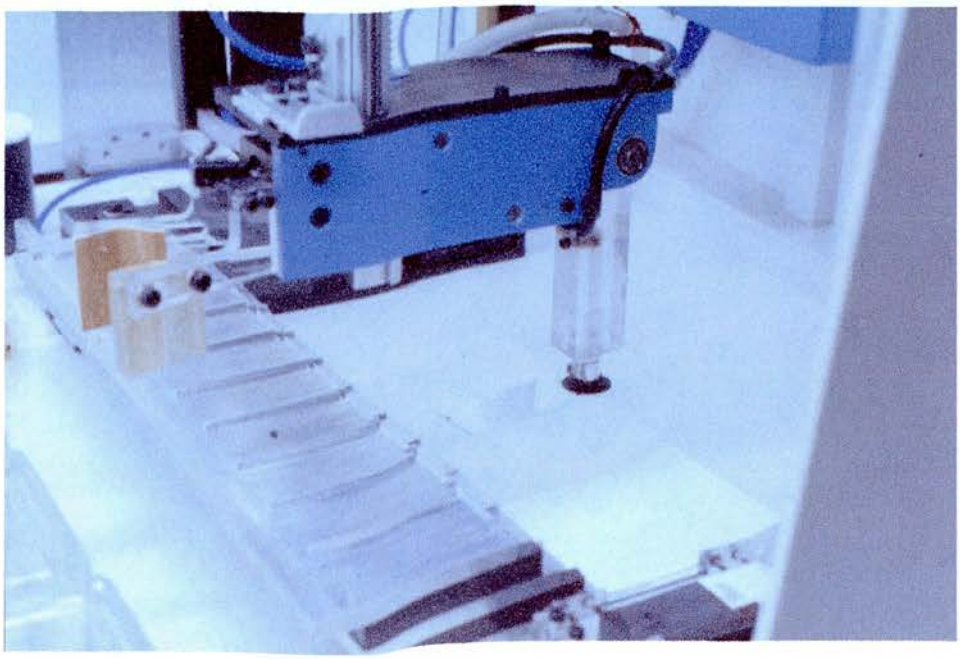


Figure 10.3 Automated slide preparation robot
The robot is picking up a prepared slide and transferring it to a microscope stage for automatic scanning

Chapter 11. Discussion

A significant proportion of pregnancies warrants some form of prenatal investigation and currently there are over two thousand conditions which can be diagnosed antenatally.

For a diagnostic procedure to be acceptable, it should be of no or low risk and preferably be offered early in pregnancy so that if a termination is requested it can be performed less traumatically. It should have a high specificity and accuracy, should not require a high rate of resampling, should be easy to administer and easy to understand. The cost: benefit ratio should be low and the technique should be applicable to many conditions with only a variation in the end point testing required for changing the object of the test.

Many of these criteria are fulfilled by current invasive diagnostic tests. The "risk" of the test to the pregnancy must be weighed against the risks of the fetus having a particular condition and a couple's perception of that condition. Ultrasound is the only non-invasive test but it cannot definitively diagnose most aneuploidies and, therefore, invasive testing can only be offered to certain women.

Down's syndrome is the most common clinically serious aneuploidy at term and rates increase with increasing maternal age. Age, however, can no longer be the sole screening criterion as maternal serum biochemical screening has improved detection rates. Nevertheless, an invasive procedure is still required and most often the suspicion is unconfirmed. These qualifications make isolating and cytogenetically analysing fetal cells from maternal blood a very attractive concept.

Of all potential fetal cell types, trophoblast cells and erythroblasts are the most promising because they are most readily distinguished from maternal cells. Unfortunately fetal cells probably occur in ratios of 1 in 10^6-10^8 maternal leukocytes and enormous enrichment is necessary to permit meaningful genetic analysis. Polymerase chain reaction cannot be used to diagnose fetal aneuploidies because, even in enriched samples, maternal cells, and therefore maternal chromosomes, will always outnumber the abnormal copy number of the fetal chromosomes. Hence, fluorescence in situ hybridisation is required.

There is always a tendency to publish only positive findings but even amongst the published data, different groups using similar enrichment techniques have obtained different results ranging from very optimistic to very discouraging. Furthermore, some studies suggest that fetal cells persist for a considerable time in the maternal circulation after delivery whilst others report they do not. This probably suggests that there is substantial inter-pregnancy variation in the incidence of fetal cells and/ or there is considerable variation in similar enrichment and analytical techniques in the hands of different groups. Clearly these techniques must become more reproducible and robust.

If the test is to be diagnostic, it must be at least as good as the invasive tests it replaces. As a screening test, it must be as good as maternal serum screening and ultrasound screening for nuchal thickness. Furthermore, any procedure must be amenable to automation if it is to be used on a large scale for screening and it must also be inexpensive.

Fluorescence activated cell sorting is too expensive and, at present, the most hopeful avenue is density gradient centrifugation followed by immunomagnetic enrichment and thereafter FISH.

Positive selection using immunomagnetic beads probably has limited potential as raising specific anti-trophoblast monoclonal antibodies is difficult whilst there are no specific cell surface markers for fetal erythroblasts. Using Dynabeads we were able to select 93.2 ± 10.7% of CD71-positive fetal erythroblasts but this lacked specificity as it also removed 62.5 ± 24.4% of leukocytes. Overall, this represented enrichment by only one order of magnitude.

Therefore, we based our investigations on negative enrichment i.e. removing unwanted cells. We specifically attempted to enrich for fetal erythroblasts but this approach might, simultaneously, also enrich for trophoblast cells. The techniques were developed on model systems and thereafter applied to maternal blood samples.

In model systems using Histopaque 1119, we removed 99.95 ± 0.07% of erythrocytes and recovered 53.1 ± 26.9% and 43.2 ± 34.4% of leukocytes and fetal erythroblasts respectively. Ficoll-containing media, such as Histopaque, resulted in excellent removal of erythrocytes but inevitably erythroblast recovery was less than leukocyte recovery because of additional

red cell aggregation. In a direct comparison of Histopaque 1119 with Nycodenz 1.085 (which does not contain any aggregating agent), the latter medium resulted in better erythroblast recovery and is a promising avenue for future research.

Once the majority of the erythrocytes were removed by Histopaque 1119, a simple cocktail of monoclonal antibodies conjugated to immunomagnetic Dynabeads, removed 99.1 ± 0.4% of leukocytes and recovered 82.0 ± 20.5% of erythroblasts i.e. an overall recovery of approximately 32% of all starting fetal erythroblasts. This represented enrichment by three orders of magnitude. Leukocyte removal could be increased further by targeting B-cells but the anti-HLA.DR monoclonal antibody we used also bound to receptors on the erythroblasts which resulted in unacceptably low fetal erythroblast recovery.

Once the two-stage enrichment was completed, cells were fixed in suspension in methanol: acetic acid to lyse the cytoplasm. This allowed all the remaining nuclei to be deposited on to three slides and improved nuclear access for the chromosome probes, so improving the quality of the FISH signals. Using a Y-chromosome probe singly, or later X and Y probes simultaneously, the cells were subjected to FISH and we successfully predicted fetal sex by the middle of the second trimester in 13 of 15 pregnancies ($p < 0.004$). However, this was achieved on the basis of very few cells in most samples. Furthermore, the two false-positive results demonstrate that other markers are necessary to confirm the fetal origin of the cells.

We also obtained blood from a woman who was carrying a Down's syndrome fetus, diagnosed by CVS, prior to first trimester termination of pregnancy. The blood was enriched using the two-stage method and the recovered cells subjected to FISH with a chromosome 21 contiguous cosmid probe. There were proportionately more trisomy 21 cells in this enriched blood sample than in the control sample so not only was it possible to confirm the abnormal fetal karyotype but it possibly suggests that more fetal cells escape into the maternal circulation in aneuploid pregnancies. These are encouraging findings and similar to results reported by others.

However, there is still much to be done. Amongst the issues to be resolved is the optimal cell type for enrichment, the ideal enrichment method, what level of enrichment can be achieved and indeed what level of enrichment is necessary. We did not identify as many cells as we had hoped. This may have been because there were so few fetal cells in the original sample, the technique incurred large losses of fetal cells or, because of the arduous nature of searching slides manually, we could not analyse sufficient numbers of nuclei.

Whether sufficient numbers of fetal cells can be recovered in all early pregnancies is not known. Ideally blood should be taken within the first half of pregnancy but the optimal time has not been determined. It might be necessary to sample women repeatedly to recover sufficient cells for cytogenetic analysis which may be unacceptable and, in addition, processing large numbers would present logistical problems.

Depositing all the cells onto three slides was ideal for slide handling purposes but was only possible because we lysed the cytoplasm of the remaining cells. These were mostly erythrocytes which occupy valuable space on the slide and ideally we require enrichment by another order of magnitude whilst maintaining cellular cytoplasm. Differential red cell lysis could be a way forward, particularly as the majority of erythroblasts were lost at the density gradient centrifugation stage, and if an alternative enrichment modality were employed, erythroblast recovery might be improved.

Maximising the number of cells which are deposited onto a slide, whilst maintaining cellular cytoplasm, should allow positive identification of fetal erythroblasts by fetal (embryonic) haemoglobin content and facilitate the development of an on-slide search capability. We have attempted to do this with a novel deposition technique similar to making a blood smear that concentrated the majority of the nucleated cells at the feathered end of the smear. Thereafter, immunocytochemistry could be performed using an anti-γ-globin mAb and the positive cells subjected to FISH.

The problems associated with in situ hybridisation must be overcome. Background fluorescence following positive identification was unacceptably high but all groups (whether commercial or governmental) must appreciate the necessity for better deposition techniques

and positive identification prior to FISH. This will establish the number of cells that can be recovered and analysed and will determine the level of sensitivity and specificity that would permit established tests to be obviated. However, this will require evaluation of hundreds of thousands of pregnancies.

The small number of cells recovered in the majority of studies raises questions about the accuracy of fetal cell analysis at the clinical level. If no, or very few, cells can be recovered following repeated maternal blood sampling by the end of the first trimester, CVS might still be an (undesirable) option. The question of confounding factors (particularly previous pregnancies, multiple pregnancy, reactivation of fetal haemoglobin production, incompatible blood groups and other antigen incompatibilities) must be addressed and, importantly, the ethical issues raised by this whole concept must also be debated fully.

We envisage that diagnoses of fetal abnormality made from maternal blood samples would initially require confirmation by established invasive prenatal tests but this can only be achieved once the above issues have been resolved.

Despite suggesting that a small number of fetal cells can be recovered and cytogenetically analysed from maternal blood in early pregnancy, the work encompassed in this thesis has been too limited to address these issues. Many groups around the world are working in this area, but after the enthusiasm in the early and mid-1990's it is disappointing that there have been relatively few new studies published in the last two or three years. What series have been published, have been small which suggests that much is still required despite claims in the media that a non-invasive test "is just around the corner". Unless thorough scientific investigation continues, unhampered by commercial interests, we risk jeopardising an extremely promising development in prenatal diagnosis. It must be remembered that it was over fifteen years between the first reports of chorionic villus sampling and its incorporation into common practice.

Fetal cells isolated from maternal blood have considerable potential. Whether the above problems can be overcome remains to be seen but in time it is hoped that definitive, non-invasive prenatal diagnosis would be available for those who need or wish it.

APPENDIX I. Abbreviations

Ab	Antibody
bp	base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CVS	Chorionic villus sampling
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein isothiocyanate
GPA	Glycophorin-A
HLA	Human leukocyte antigen
kbp	kilobase pair
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex
NRS	Normal rabbit serum
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemmaglutinin
PK	Proteinase K
QM	Quinacrine mustard
SDS	Sodium dodecylsulphate
SSC	Sodium saline citrate
TA-CVS	Transabdominal chorionic villus sampling
TAE	Tris-Sodium acetate-EDTA
TBS	Tris buffered saline
TC-CVS	Transcervical chorionic villus sampling
TELT	Tris-EDTA-Lithium Chloride-Triton X
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol, $C_4H_{11}NO_3$

APPENDIX II

Morphological stains

May-Grünwald stain	BDH, product number, 35025
Giemsa stain	BDH, product number, 35086
New methylene blue	Sigma, catalogue number, M 9140
Citrate-saline solution	1 volume sodium citrate (30g/l) + 4 volumes sodium chloride (9g/l)

Immunocytochemistry materials

Bovine serum albumin (BSA)	Product No. A-7030 (Sigma)
New Fuchsin Kit	DAKO, K698
Normal rabbit serum (NRS)	= 1 part normal rabbit serum + 4 parts TBS (Scottish Antibody Production Unit)
Streptavidin alkaline phosphatase	DAKO, D396 Diluted 1: 300 in TBS
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol, C4H11NO3
Tris Buffered Saline (TBS)	250ml distilled water + 6.05g Tris = Solution A 192ml distilled water + 3.4ml concentrated Hydrochloric acid (7N) = Solution B Add Solution A to Solution B. This is the working solution of TBS.

Secondary antibodies

FITC-sheep anti-mouse	IgG (SAPU)
TRITC-rabbit anti-mouse	IgG (Sigma)
RAMBo	Biotinylated rabbit anti-mouse IgG (DAKO, Denmark, Code E354) diluted 1:200 with NRS

Density gradient media

Histopaque 1077	Sigma, catalogue number, 1077-1 Sodium diatrizoate and ficoll 400
Histopaque 1077	Sigma, catalogue number, 1119-1 Sodium diatrizoate and ficoll 400
Nycodenz Buffer	Tris 5mmol/l, Potassium chloride 3mmol/l, Disodium calcium EDTA 0.3mmol/l, Sodium Chloride 7.5%, dissolved in 1 litre of distilled water and made up to pH 7.5 with concentrated HCl.
Nycoprep	Nycomed Pharma AS,Prod. No. 1002417 5 - (N-2, 3-dihydroxypropylacetamido)-2, 4, 6-tri-iodo-N, N'-bis (2, 3 dihydoxypropyl) isophthalamide Molecular weight 821

Immunomagnetics

IgG_1-specific Dynabeads	Product Number 11011 (Dynal, UK) 2ml, 4 x 10^8 beads/ml
IgG_{2a}-specific Dynabeads	Product Number 11013 (Dynal, UK) 2ml, 4 x 10^8 beads/ml
PBS/BSA/Azide	Phosphate Buffered Saline/ 2% BSA/ 0.02% Sodium azide

Monoclonal antibodies

DA6.231	IgG_1, anti-HLA.DR mAb (Guy et al, 1982) Tissue culture supernatant, 90µg/ml.
HIG.125	IgG_{2a}, anti-CD18 mAb (van Heyningen and Guy, unpublished). Tissue culture supernatant, 60µg/ml.
UCHT1	IgG_1, anti-CD3 mAb (Beverley and Callard, 1981) Tissue culture supernatant, 4µg/ml.
RFT11	IgG_1, anti-CD2 mAb (Verbi et al, 1982) Ascites, 0.1mg/ml.
UCHγ	IgG_1, anti-γ-globin mAb (Allen et al, 1987). Ascites, IgG concentration undetermined (P. Beverley, personal communication).
EBZ17	IgG_{2a}, anti-CD45 mAb (Smith et al, 1985) Tissue culture supernatant, IgG concentration 4µg/ml.
BER-T9	IgG_1, anti-CD71 (DAKO, catalogue number M734) Tissue culture supernatant, IgG concentration 175µg/ml.
Mouse IgG_1	Irrelevant mAb (Sigma)

Fluorescence in situ hybridisation materials

DNA purification

TELT Buffer	Tris 50.0mM, 6.05g EDTA 62.5mM (23.27g) Lithium Chloride 2.5M (106.00g) Triton X 0.4%, pH 7.5
TAE Buffer	Tris 40mM (pH8.2) Sodium acetate 20mM EDTA 1mM Ethidium bromide, 5µl/50ml of gel

Promega Wizard Maxiprep

Promega Maxipreps DNA Purification Systems
Catalogue number, A7100

Cell resuspension solution	Tris-HCl, pH7.5, 50mM EDTA 10mM RNase A, 100µg/ml

Cell lysis solution	NaOH, 0.2M Sodium dodecylsulphate (SDS), 1%
Neutralising solution	Potassium acetate, pH4.8, 1.32M
TE buffer	Tris-HCl, pH7.5, 10mM EDTA , 1mM
Column wash solution	NaCl, 200mM Tris-HCl, pH7.5, 20mM EDTA, 5mM Dilute 1:1 with 95% ethanol

DNA probe labelling

dNTP diluted bases	100mM, diluted 1:200 with distilled water (Pharmacia)
Digoxigenin-11-dUTP	1nmol/µl (Boehringer Mannheim GmbH)
Biotin-16-dUTP	1nmol/µl (Boehringer Mannheim GmbH)
Fluorescein-12-dUTP	1nmol/µl (Boehringer Mannheim GmbH)
DNAse	1mg/ml in 0.15M sodium chloride; 50% glycerol. Made up fresh as a 1:500 dilution with distilled water (Boehringer Mannheim GmbH)
Pol-1	10U/µl (Gibco BRL)
Nick Translation Salts	10 x concentration: 0.5M Tris, pH7.5; 0.1M MgSO4; 1mM dithiothreitol; 500µg/ml (BSA fraction V Sigma)
EDTA	Ethylenediaminetetraacetic acid, $C_{10}H_{16}N_2O_8$, mw 292.2, (Sigma)
SDS	Sodium dodecylsulphate, $C_2H_{25}O_4SNa$, mw 288.5, 5% solution (Sigma)
TE	Tris EDTA (10mM Tris, pH 7.5 and 1mM EDTA, pH8.0)

Estimation of Probe concentration

Biotin λ-standard	200pg/µl, Blugene Kit (Gibco-BRL)
BCIP/NBT Vector kit	SK 5400 (Vector)
Buffer 1	0.1M Tris, 0.15M NaCl, pH 7.5
Buffer 2	0.1M Tris, 0.15M NaCl, 3% BSA Fraction V, pH 7.5
Buffer 3	0.1M Tris, pH 9.5
Digoxigenin λ-standard	200pg/µl (Boehringer)
Streptavidin alkaline phosphatase	Gibco-BRL
Antidigoxigenin alkaline phosphatase	Boehringer

Fluorescence in situ hybridisation

Anti-mouse-FITC	1:100 dilution of stock (Vector) 10µl of avidin-FITC in 1ml of blocking buffer
Avidin-Texas red	1:500 dilution of stock (Vector) 2µl of avidin-Texas red in 1ml of blocking buffer
Avidin-FITC	1:500 dilution of stock (Vector):
Biotin anti-avidin	Goat monoclonal antibody, 1:100 dilution of stock (Vector)
Blocking Buffer	4xSSC/ 5% skimmed milk (Marvel): 2ml of 20xSSC + 8ml of distilled water + 0.5g of skimmed milk Centrifuged at 4°C for 15 minutes to remove conjugates
Cot-1 human DNA	1mg/ml, sonicated to 500 base pair lengths (Gibco-BRL)
DAPI	50µg/ml diluted to a final concentration of 500ng/ml (Sigma)
Dextran sulphate	For hybridisation mixtures: 50% in distilled water, Pharmacia
Formamide	For hybridisation mixtures: AnalaR grade, BDH, deionised with Amberlite monobed NB-1 resin, BDH
Mouse-anti-digoxigenin	1:500 dilution of stock (Vector)
PK buffer:	8ml stock 0.1M calcium chloride, 8ml of stock 1M Tris and 384 ml distilled water
Propidium Iodide	0.5µl: 1mg/ml (Sigma) diluted in 0.5ml Vectashield to a final concentration of 1ng/ml
Proteinase K (PK)	600µg/ml (Boehringer)
Salmon sperm DNA	10mg/ml, (Sigma)
RNase	10mg/ml, R9009 (Sigma)
SSC (Sodium saline citrate)	Sodium saline citrate, 20 times concentration: sodium chloride, 170.53g; trisodium citrate, 80.82g and made up to one litre with distilled water
Vectashield	Vector Laboratories Inc. 0.5ml of Vectashield + 5µl of 50µg/ml stock DAPI ± 0.5µl of 1mg/ml stock propidium iodide

APPENDIX III. Publications

each year over 400 000 Finnish citizens travel each year to Russia and about 200 000 Russians visit Finland. The patient described here and the fellow traveller with a positive throat culture both had a contact with a local inhabitant, during which exchange of saliva occurred. Although diphtheria is endemic in Russia, the risk for an average traveller is not very big. However, a booster of diphtheria vaccine is recommended, even for a short trip to Russia, if the traveller is not vaccinated or has not had a booster dose within 10 years.

The risk of spread of diphtheria in Finland, where the coverage of childhood vaccination is good, is improbable. However, in several countries of western Europe, surveys have shown that immunity to diphtheria is poor, especially among women over 40.[3] Male individuals are better protected because they are given an additional booster dose during military service. There have been small outbreaks of diphtheria in Sweden and Denmark recently.[4] With increasing travel to and from countries endemic for diphtheria, booster vaccinations of the adult population in western European countries may have to be reconsidered; and health care workers, who are at greatest risk of exposure to the saliva of diphtheria patients, should be adequately immunised.

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1 The Official Statistics of Finland XI:72,73. Helsinki: Public Health and Medical Care, 1974: 125.
2 WHO. Expanded program of immunization: outbreak of diphtheria, update. *Wkly Epidemiol Rec* 1993; **68:** 134–38.
3 Christensson B, Böttiger M. Serological immunity to diphtheria in Sweden in 1978 and 1984. *Scand J Infect Dis* 1986; **18:** 227–33.
4 Karzon DT, Edwards KM. Editorial: diphtheria outbreaks in immunized population. *N Engl J Med* 1988; **318:** 41–43.

Falloposcopic instillation of prostaglandin in tubal pregnancy

SIR—Falloposcopy, the transcervical endoscopic examination of the fallopian tube by a linear everting catheter (LEC), was reported in 1990.[1] It has been used diagnostically in infertile patients. Visualisation of the tubal lumen may assist in planning rational treatment of infertility, permitting surgery or tubal transfer of embryos or gametes if the mucosa is normal. A further possible application is the diagnosis of tubal pregnancy. The LEC may also be useful in the non-surgical treatment of unruptured tubal pregnancies. We tried to manage an unruptured tubal pregnancy with high initial beta-human chorionic gonadotropin (β-hCG) levels by prolonged prostaglandin (PG) $F_{2\alpha}$ application via LEC. Previously, non-surgical treatment of tubal pregnancy by local $PGF_{2\alpha}$ instillation had been limited to cases with initial β-hCG under 2500 mIU/mL serum; higher concentrations were correlated with unfavourable outcome.[2,3]

In a 25-year-old patient with a 3-year history of infertility, tubal pregnancy was diagnosed by vaginal ultrasound. β-hCG was 4506 mIU/mL. Under general anaesthesia and laparoscopic control, the LEC was introduced into the relevant tube. The tip of the catheter was positioned directly on the gestational sac. After visualising the lumen of the fallopian tube and the tubal gestation, 10 mg $PGF_{2\alpha}$ was injected. Laparoscopy was ended with the LEC left in place. 3 hours later, a second dose of 5 mg was injected with no adverse effects and the catheter was removed. The patient was discharged the next day. Serial β-hCG rapidly fell, and was undetectable after 4 weeks (figure). Vaginal ultrasound revealed that the tubal pregnancy had disappeared.

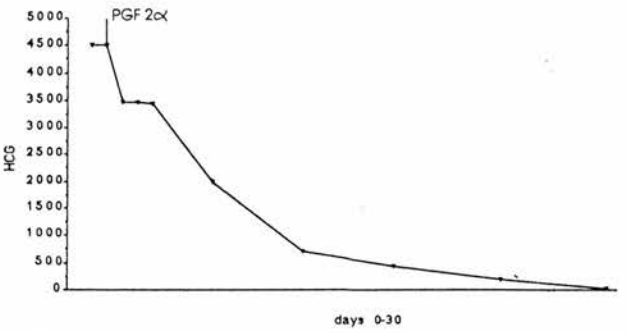

Figure: **β-hCG after falloposcopic prostaglandin**

Falloposcopy by means of LEC may lead to a new approach in the non-surgical management of unruptured tubal pregnancies. Repeated injections of $PGF_{2\alpha}$ via LEC may allow adequate doses, depending on initial β-hCG. However, $PGF_{2\alpha}$ is not necessarily the only drug to be used in this indication; methotrexate[4] or even hypertonic glucose solution[5] could also be used.

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1 Kerin J, Daykhovsky L, Grundfest W, Surrey E. Falloposcopy, a microendoscopic, transvaginal technique for diagnosis and treating endotubal disease incorporating guide wire cannulation and direct balloon tuboblasty. *J Reprod Med* 1990; **35:** 606–12.
2 Egarter Ch, Fitz R, Spona J, Vavra N, Husslein P. Treatment of tubal pregnancy with prostaglandins: correlation between hormone profile and success. *J Obstet Gynecol* 1991; **11:** 119–22.
3 Egarter Ch, Husslein P. Treatment of tubal pregnancy by prostaglandins. *Lancet* 1988; i: 1104–05.
4 Pansky M, Bukovsky I, Golan A, et al. Tubal patency after local methotrexate injection for tubal pregnancy. *Lancet* 1989; ii: 967–68.
5 Lang PF, Tamussino K, Hönigl W, Ralph G. Treatment of unruptured tubal pregnancy by laparoscopic instillation of hyperosmolar glucose solution. *Am J Obstet Gynecol* 1992; **166:** 1378–81.

Non-invasive antenatal diagnosis

SIR—Lo and colleagues (May 1, p 1147) attempt to determine the rhesus status of fetuses in utero by non-invasive means. They admit that this method has not reached the precision required for routine application (because a 20% false negative and almost 20% false positive rate is too high), but it shows great promise. Furthermore, it will contribute to a better understanding of normal fetomaternal physiology and to the quest for a non-invasive but definitive antenatal diagnostic procedure that would carry no risk to the fetus. Research into the isolation and genetic analysis of fetal nucleated cells in the maternal circulation is gaining momentum, and three types of cell—trophoblast,[1] lymphocytes,[2] and nucleated fetal erythrocytes (NFEs)[3]—are the main focus of attention. For several reasons the first and last of these are the most likely candidates and each has its proponents. Trophoblast is physiologically shed into the maternal circulation, whereas, in early pregnancy, NFEs are the most common type of nucleated fetal cell in the fetal circulation.[4] However, there is a growing opinion favouring NFEs.[5]

Because Lo et al did not use any relative enrichment techniques, it is not known if they analysed one particular type of cell or a combination of types. However, from their polymerase chain reaction results, it seems that in most cases there is 1 fetal cell for every 10^5–10^6 maternal cells. These workers clearly demonstrate the ability to detect signals in patients with substantial antibody concentrations, but do not

state whether in these cases there was a rising titre of anti-D; this, however, can be construed because they went on to do cordocentesis in the patients with high values. If the analysed cells were NFEs, then at 22 weeks' gestation (case 8) this should represent a transplacental haemorrhage of about 0·5 mL,[4] which would certainly be sufficient to stimulate a secondary immune response in an isoimmunised mother. If so, maternal antibodies would probably have mopped up the NFEs before their analysis, suggesting that the analysed cells are of a different type.

Little is known of fetomaternal cell transfer at much earlier stages of pregnancy, though there is growing evidence that NFEs occur in proportionately greater numbers in the maternal circulation at earlier gestations.[3] The question of which type of cell is most likely to give the desired result remains unanswered, but work such as theirs will help to elucidate this matter. In combination with methods of selective cell enrichment and prospects such as fluorescence in-situ hybridisation, it may ultimately lead to routine definitive diagnoses of fetal genetic abnormalities from simple maternal blood tests.

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1 Mueller UW, Hawes CS, Wright AE, et al. Isolation of fetal trophoblast cell from the peripheral blood of pregnant women. *Lancet* 1990; **336:** 197–200.
2 Herzenberg LA, Bianchi DW, Schröder J, Cann HM, Iverson GM. Fetal cells in the blood of pregnant women: detection and enrichment by fluoroscence-activated cell sorting. *Proc Natl Acad Sci USA* 1979; **76:** 1453–55.
3 Bianchi DW, Stewart JE, Garber MF, Lucotte G, Flint AF. Possible effect of gestational age on the detection of fetal nucleated erythrocytes in maternal blood. *Prenat Diag* 1991; **11:** 523–28.
4 Millar DS, Davis LR, Rodeck CH, Nicolaides KH, Mibashan RS. Normal blood values in the early mid-trimester fetus. *Prenat Diag* 1985; **5:** 367–73.
5 Adinolfi M. Breaking the blood barrier. *Nature Genet* 1992; **1:** 316–18.

Local treatment of thrombocytopenic mucosal haemorrhage

SIR—The management of patients with epistaxis or diffuse gut bleeding due to thrombocytopenia is difficult since platelet transfusion is often ineffective because of platelet antibodies. We tried local application of platelet concentrates in 12 consecutive patients with leukaemia (9) or lymphoma (3) who had severe mucosal haemorrhages due to thrombocytopenia. 9 had epistaxis, 2 had a haemorrhagic gastritis, and 1 had diffuse colonic bleeding.

The local applications were random-donor concentrates which contained about 10^{11} platelets in 50 mL plasma. For patients with epistaxis, 10 mL of concentrate was applied to both nostrils in 1 mL doses with a syringe. In the patient with diffuse gut bleeding, 50 mL of platelet concentrate was diluted in 500 mL 0·9% NaCl and administered as an enema. The gastritis patients were given 50 mL of platelet concentrate at gastroscopy. The haemorrhage was successfully treated and prolonged freedom from bleeding achieved in all patients except 1 whose haemorrhage was caused by diffuse gastric infiltration with chronic lymphocytic leukaemia and who had only short-term relief.

Local application of platelet concentrates shows potential in treating diffuse mucosal haemorrhage due to thrombocytopenia.

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Halofantrine sensitivity

SIR—Brasseur and colleagues (April 3, p 901) investigated the in-vitro and in-vivo sensitivity of *Plasmodium falciparum* to halofantrine in the Congo and in Cameroon in 1992, and in the Congo recorded a high frequency of resistance. In Gabon halofantrine came on the market three years ago and has since been widely used. In 1992 we studied the response of *P falciparum* according to WHO criteria. For the in-vitro studies halofantrine was precoated on microtitre plates, resulting in final concentrations of 0·3, 1, 3, and 10 nmol/L blood/medium mixture. Isolates were obtained and successfully grown in vitro from 40 patients. The EC_{50}, EC_{90}, and EC_{99} were 0·4091, 0·9400, and 1·8523 nmol/L, respectively, indicating very good sensitivity of *P falciparum* to halofantrine in vitro.

In a clinical trial 30 children with uncomplicated falciparum malaria were included, distinct from those of the in-vitro study. They had not received antimalarial treatment within the preceding 7 days and were 4–14 years old. Their parents gave informed consent. A micronised formulation of halofantrine with a two-fold increase in absorption was used. The patients received three 8 mg/kg doses of halofantrine 6 hourly. Parasitaemia on thick blood films and symptoms were recorded daily for the first week and subsequently every week until 28 days after therapy. The median parasitaemia on admission was 21 000 asexual parasites per μL (range 200–140 000). The parasitaemia was cleared within 48 h in 4 patients and within 72 h in 26. The patients were free of symptoms in 24–72 h. Thick blood films of 28 patients remained free of parasites until day 28 of follow-up; in the other 2 patients symptomless parasitaemia of 15 and 50/μL was detected on day 28.

Our findings indicate pronounced sensitivity of *P falciparum* to halofantrine in Gabon in 1992 despite its widespread use there over the 3 previous years.[1] This experience is very different from that of Brasseur and colleagues in neighbouring Congo. The EC_{50} in the Congo was 30 times higher. This could be due to the notorious solubility problems with halofantrine which will be especially prominent in in-vitro systems with drug in solution. Almost half the Congo patients still had detectable parasitaemia on day 4; in Gabon all patients had a negative thick blood film on day 3, and this may be explained by the use of a new formulation of halofantrine.

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1 Richard-Lenoble D, Kombila M, Martz M, et al. Efficacy, safety and acceptability of halofantrine in the treatment of acute *Plasmodium falciparum* malaria in African children (Gabon). *Parasitol Today* 1989; suppl: 59–63.

Authors' reply

SIR—The history of resistance to antimalarial drugs has been accompanied by a parallel move of resistance of some researchers to admit that it may be so. This happened with early reports of resistance to chloroquine, quinine, and mefloquine,[1,2] and it now seems to be happening with halofantrine (May 15, p 1282, and the above letter).

persisted despite treatment with steroids. In October, 1986, she developed raised DNA binding and low complement C3 and C4 concentrations. In 1991 she presented with migraine, and four months later a vasculitic rash in a butterfly distribution typical of lupus; biopsy showed non-specific inflammatory changes. In May, 1993, she developed flitting polyarthralgia affecting her knees, hips, ankles, and wrists, leucopenia, thrombocytopenia, and renal impairment (1·5 g per day proteinuria, glomerular filtration rate 55 mL/min per 1·73 m^2). Renal biopsy showed mild mesangial proliferative glomerulonephritis with granular localisation of IgG, IgM, IgA, C1q, C3, and C4, consistent with lupus nephritis. She is now well and off medication, but has DNA binding of 10·1 mg/L (normal range 0–4) and depressed complement concentrations.

A 26-year-old man was referred in 1981 with a 5-year history of arterial and venous thromboembolism leading to amputation of part of one foot. ESR was 34 mm in the first hour and ANF diffusely weak positive. Anticardiolipin antibody (aCL) was detected, but complement concentrations, autoantibody screen, and DNA binding were repeatedly normal. In 1985 he had raised DNA binding and persistently low complement concentrations, but remained symptom-free. In 1991, however, he developed symmetrical polyarthropathy affecting the metacarpophalangeal joints, wrists, elbows, shoulders, and knees, with ANF strongly positive and raised DNA binding. He also had persistent leucopenia and thrombocytopenia. His arthralgias responded to oral prednisolone but returned when the dose was reduced to below 7·5 mg per day; indices of lupus activity have remained high.

At presentation, neither of these cases had raised DNA binding, satisfied standard criteria for the diagnosis of SLE,[3] or for the loosely-defined "lupus-like" syndrome.[4] Both were diagnosed as APS on the basis of the clinical history together with an equivocal ANF and raised aCL/aPL antibody. However, each developed lupus after an interval of 8 and 10 years, respectively, with both clinical and serological markers of disease activity. We suggest that primary APS may be a feature of early lupus in some patients and that regular serological and clinical review is indicated.

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1 Mackworth-Young CG, Loizou S, Walport MJ. Primary antiphospholipid syndrome: features of patients with raised anticardiolipin antibodies and no other disorder. *Ann Rheum Dis* 1989; **48:** 362–67.
2 Frampton G, Cameron JS, Thom M, et al. Successful removal of antiphospholipid antibody during pregnancy using plasma exchange and low-dose prednisolone. *Lancet* 1987; ii: 1023–24.
3 Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25:** 1271–77.
4 Asherson RA, Baguley E, Pal C, Hughes GRV. Antiphospholipid syndrome: five year follow up. *Ann Rheum Dis* 1991; **50:** 805–10.

Anticardiolipin antibodies in ischaemic heart disease

SIR—In their commentary (July 24, p 192) Cortellano and colleagues conclude that antiphospholipid antibodies (aPL) are not important prognostic indicators of further events in survivors of myocardial infarction. Anticardiolipin antibodies (aCL) are members of a group of aPL. We measured aCL in 249 consecutive admissions to the coronary care unit of a district general hospital. 159 patients (139 male, 20 female, mean age 58·2 [SD 9·9]) had an acute myocardial infarction, confirmed by a significant rise in cardiac enzymes, and 90 (64 male, 16 female; mean age 56·4 [9·8]) had unstable angina. 38 patients were aged less than 45 years (mean 42·1 [2·4]), of whom 21 had an acute myocardial infarction and 17 unstable angina. The controls were 299 consecutive patients (149 male, 150 female; mean age 47·3 [11·2]) attending a health screening clinic. Measurement of aCL was done with an enzyme-linked immunosorbent assay ELISA validated against international workshop standards.[1,2] Results above 25 gPL (proposed unit[2] for IgG aCL) or 25 mPL (for IgM aCL) were regarded as abnormal. The frequency of aCL in patients with ischaemic heart disease was not higher than that in controls. Raised aCL values (either IgG or IgM) were shown in 5 patients with acute myocardial infarction, 4 patients with unstable angina, and 8 controls. In patients aged less than 45, none had raised aCL values.

What are the prevalence and importance of aCL in patients with coronary artery disease? Hamsten[1] reported aCL in 21% of 62 survivors of acute myocardial infarction who were aged less than 45;[3] during follow-up there was a significantly higher incidence of further thrombotic events in the group with raised aCL. In patients after coronary artery bypass grafting who had raised preoperative aCL titres, the rate of late graft occlusion was higher than in those who had no aCL,[4] whereas others showed that an increased frequency of aCL in patients following myocardial infarction was not an independent risk factor for further thrombotic events. By contrast, other studies[5,6] showed no significant difference in aCL between patients and healthy controls; and no rise in aCL in young patients with previous myocardial infarction.

Our results suggest that aCL do not have a part in acute myocardial infarction, or unstable angina, in most patients with ischaemic heart disease in the absence of connective tissue disease.

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1 McHugh NJ, Maymo J, Skinner RP, James I, Maddison PJ. Anticardiolipin antibodies, livedo reticularis and major cerebrovascular and renal disease in systemic lupus erythematosus. *Ann Rheum Dis* 1988; **42:** 110–15.
2 Harris EN. The Second International Anti-Cardiolipin Standardization Workshop/The Kingston Anti-Phospholipid Antibody Study (KAPS) Group. *Am J Clin Pathol* 1990; **94:** 476–84.
3 Hamsten A, Norberg R, Björkholm M, De Faire U, Holm, G. Antibodies to cardiolipin in young survivors of myocardial infarction: an association with recurrent cardiovascular events. *Lancet* 1986; ii: 113–15.
4 Morton KE, Gavaghan TP, Krilis SA, et al. Coronary artery bypass graft failure—an autoimmune phenomenon. *Lancet* 1986; ii: 1353–57.
5 de Caterina R, d'Arcanio A, Marzone A, et al. Prevalence of anticardiolipin antibodies in coronary artery disease. *Am J Cardiol* 1990; **65:** 922–24.
6 Phadke KV, Phillips RA, Clarke DTR, Jones M, Naish P, Carson P. Anticardiolipin antibodies in ischaemic heart disease: marker or myth *Br Heart J* 1993; **69:** 391–94.

Transcervical flushing and antenatal diagnosis

SIR—The advantages of interphase cytogenetics with earlier and less invasive antenatal diagnostic tests are indisputable, but in their analysis Adinolfi and colleagues (Aug 14, p 403) do not estimate the total number of fetally-derived cells that might be obtained by this method or say how many cells they analysed from each sample. From their data the proportion of fetal-specific cells is very small, the range is very large, and there is considerable overlap between the percentage of cells bearing a Y-signal in male and female pregnancies.

Calculations on such small numbers is far from satisfactory. However, the mean (1SD) is 20·7% (12·1), which gives, as they

propose, a lower cut-off limit for a positive diagnosis of 9% of cells. This finding suggests a sensitivity of 85·7%, a rather high false negative rate of 14·3%, a 0% false positive rate, and a positive predictive value of 100%. Alternatively, if 2 SD is considered then the respective figures become 100%, 0%, and a very poor 75% and 70% respectively.

Trisomy 18 was diagnosed by transcervical chorionic villus sampling before their test, so many of the apparent fetal cells might have been introduced iatrogenically in the lower uterine pole. Adinolfi and colleagues do not mention the hybridisation efficiency of their probe sets, though with pure fetal tissue and the chromosome 18 probe it is only 65%. In a recent large study by Ward et al,[1] such a low hybridisation rate would have been classed as uninformative and no definitive result achievable.

One view of the future of antenatal diagnosis might include a panel of chromosome-specific probes to diagnose rapidly the more common aneuploidies[1] in interphase nuclei. With any screening test there is a trade-off between what is absolutely achievable and what is achievable (and acceptable) within available resources. The method clearly needs further evaluation. However, extrapolating from these data, it follows that if the total number of fetal cells is very small or the hybridisations unreliable, or both, there will be many false-positive diagnoses resulting in an overwhelming number of pregnancies undergoing definitive antenatal diagnostic testing. Alternatively, many chromosomally abnormal pregnancies will be missed—in a test which is far from non-invasive.

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1 Ward BE, Gersen SL, Carelli MP, et al. Rapid prenatal diagnosis of chromosomal abnormalities by fluorescence *in situ* hybridisation: Clinical experience with 4500 specimens. *Am J Hum Genet* 1993; **52:** 854–65.

Authors' reply

SIR—Gaudoin's comments offer us the opportunity to clarify our findings. Our pilot study was planned to solve the controversy raised by the demonstration that Y-derived sequences could be detected by the polymerase chain reaction (PCR) in transcervical cells (TCC)[1] and the denial published in the accompanying editorial.[2] The detection by fluorescence in-situ hybridisation (FISH) of the Y chromosome in some TCC samples when the conceptus was male; the presence of cells with trisomy 18 derived from an affected fetus; and the morphological and immunological demonstration of syncytioblastic and trophoblastic cellular elements in these samples seem to us clear evidence that placental cells are shed into the endocervical canal between 8 and 13 weeks of gestation.

Because of their great heterogeneity, the total number of cells present in each sample does not bear any relation to the number of fetal cells. We are now investigating the proportion of the various types of cells present in TCC samples with several monoclonal antibodies (MAbs). Between 50 and 100 nuclei were analysed in each slide tested by FISH. The hybridisation efficiency of the chromosome 18 probe is over 90%, but we suspect that maternal cells contaminate the placental samples from the aborted fetus, thus reducing the percentage of trisomic cells, since conventional cytogenetic tests showed that the fetus was not a mosaic.

The diagnostic value of the use of TCC samples for prenatal diagnosis can only be established by appropriate trials. Before starting one, we are investigating the possibility of obtaining the cells by aspiration, thus avoiding flushing, which could potentially spread infection. Swabs and flushed and aspirated samples are obtained and the results compared. We are also testing different procedures for the isolation of the fetal cells and extending the range of MAbs used to analyse the proportion of the various subpopulations of cells. Once this information is available we plan to start a trial in which TCC and chorionic samples will be tested simultaneously.

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1 Griffith-Jones MD, Miller D, Lilford RJ, Scott J. Detection of fetal DNA in transcervical swabs from first trimester pregnancies by gene amplification: a new route to prenatal diagnosis? *Br J Obstet Gynaecol* 1992; **99:** 508–11.

2 Morris N, Williamson R. Non-invasive first trimester antenatal diagnosis. *Br J Obstet Gynaecol* 1992; **99:** 446–48.

Diaphragmatic paralysis preceding amyotrophic lateral sclerosis

SIR—Diaphragmatic paralysis is uncommon because appropriate tests to detect it are not done. Usually the diaphragm is only one of several weak muscles, directing the clinician to the diagnosis of diaphragmatic paralysis.[1] Its most serious consequence is respiratory failure.[2] We report a case of isolated bilateral diaphragmatic paralysis with severe acute respiratory failure that revealed lateral amyotrophic sclerosis.

A 46-year-old, previously healthy man was admitted in June, 1989, to intensive care for acute respiratory failure. Heart rate 110/min, systolic blood pressure 160 mm Hg, respiratory rate 40/min, and temperature 38·5°C. The patient was unconscious and had cyanotic extremities. Blood gases revealed severe respiratory acidosis (pH 7·10 and $PaCO_2$ 105 mm Hg) and hypoxaemia (PaO_2 20 mm Hg)). He was urgently intubated and underwent mechanical ventilatory support. An hour later, he fully recovered consciousness and clinical examination was normal. The chest radiograph indicated bilateral atelectasis lines. Other tests were normal and alcohol and narcotics were not detected. The electrocardiogram, 2D-echocardiogram, and pulmonary angiogram were normal. Over the following days, despite normal clinical examination and arterial blood gases, every attempt to wean the patient from the ventilator failed. During weaning, he had paradoxical abdominal respiration that drew our attention to a possible diaphragmatic weakness. Electromyography of the limbs and of the diaphragm revealed (in both) fasciculation potentials in muscles, reduced number and increased size of motor-unit action potentials, and normal excitability and conduction velocity of the remaining fibres of motor nerves. The respiratory distress was considered to be due to bilateral diaphragmatic paralysis from motorneurone disease. Cerebrospinal fluid examination revealed normal protein levels and no cells. Serum creatine kinase was normal. The patient underwent tracheostomy and was discharged from hospital under continuous mechanical ventilation, 7 weeks after admission.

3 months later, he was admitted to intensive care for general fatigue and difficulty swallowing. At that time he had amyotrophic extremities, decreased peripheral and axial muscle strength, evidence of muscle fasciculation and fibrillation, and hyperactive reflexes without clonus and without Babinsky's sign. He was bed-bound, and unable to talk and swallow. He went home on mechanical ventilation and with a nasogastric tube, and died in March, 1991, from severe bronchopneumonia.

Diaphragmatic paralysis should not be overlooked in acute respiratory failure. Its diagnosis may be difficult in the absence

aims in our new curriculum. Firstly, in relation to communication skills it addresses the development of students' skills in self awareness and reflective learning. Secondly, the workbook encourages the students to understand their patient as a complex person whose health and wellbeing depend on more than biomedical considerations. The depth and extent of this understanding have been shown in some work of exceptional quality and insight.

We believe that the integrated workbook assignment embodies the ideals of our curriculum and, in particular, has considerable potential for continuing the General Medical Council's recommended strands of ethics, law, behavioural science, and communication skills throughout the clinical course.

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1 Lowry S. Assessment of students. *BMJ* 1993;306:51-4. (2 January.)

Harvard's "new pathway"

EDITOR,—As one who taught on Harvard's "new pathway" during the two pilot years,[1] as well as on its traditional courses, I would like to make some comments.

The success of any programme rests on the faculty's enthusiasm and support. Teaching well takes time and often yields little tangible reward. Harvard's new pathway got through its pilot years relying on the motivated staff and fellows. This staff may not be available at many medical schools.

The greatest change in the curriculum produced by courses based on the new pathway is seen in the preclinical faculty. Preclinical staff usually have busy schedules and may not be particularly well oriented to clinical matters. For example, teaching, say, the pharmacology of tetracycline in the traditional way is usually fairly easy for a preclinical pharmacologist with a related scientific interest. Less easy for (and possibly of less interest to) preclinical staff is dealing with a case study for the new pathway; such a case might start with the pharmacology of tetracyclines, pass through their therapeutic use in general, and end on a debate about whether oxytetracycline should be used as prophylaxis for traveller's diarrhoea in Mexico. Team teaching, with both preclinical and clinical staff present at each session, may be a feasible alternative, given the staff available at most medical schools.

It is true that the new pathway was oversubscribed in both pilot years. During the first pilot year, however, there was a sense among the "traditional" class that their colleagues in the new pathway were taking an extraordinary gamble with their medical education. During the second year this feeling persisted, but less strongly. I do not agree with Stella Lowry that "special arrangements that had been made for the new pathway students had caused resentment among other students, who felt that they were being treated like second class citizens."

Lastly, the success rate at Harvard in the national board examinations has always been extremely high (as it is at most American medical schools). These examinations are probably a poor instrument for measuring the quality of medical education because they concentrate on factual retention.

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1 Lowry S. Making change happen. *BMJ* 1993;306:320-2. (30 January.)

Community based medical education

EDITOR,—Dr Nigel Oswald, cited by Stella Lowry,[1] is correct in implying that learning skills in clinical decision making requires seeing large numbers of patients in a short space of time. This, however, is an argument against rather than for community based learning.

This is illustrated by an example from our practice. An average general practice of 10 000 patients refers 34 patients a year for assessment of breast lumps. A student attending a well directed breast clinic may personally see this number of patients in less than a month and be taught to make an accurate clinical assessment. She or he would have to spend a year in general practice to have the opportunity to acquire similar skills. To paraphrase Oswald, "It is more important to see 30 patients who might have breast cancer than five who do (but it is useful and likely that you will see them too)."

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1 Lowry S. Trends in health care and their effects on medical education. *BMJ* 1993;306:255-8. (23 January.)

Teaching how to elicit and interpret physical signs

EDITOR,—John R Hampton may be right to lament the decline in doctors' abilities to elicit and interpret physical signs, but I believe that he is wrong to conclude that training in the setting of general practice will sound the death knell of these skills.[1]

My memories of cardiac teaching rounds are of a dozen students queueing to listen to a murmur while the registrar stood at the end of the bed swinging a stethoscope and staring out of the window. Aware of restive colleagues, one listened hurriedly and joined the whisper going round the group: "What did you hear?" Coming back later on one's own was rarely useful: even if the relatives weren't round the bed there was rarely a doctor prepared to give guidance. "We don't spoonfeed you here" was one of the less excusable reasons given for declining to help floundering students.

Traditionally, doctors were trained by being apprenticed to established physicians. In hospitals the system has broken down under the pressure of numbers and new teaching methods are only slowly being found, but teaching in general practice has remained close to the tradition in which older generations of doctors learnt their skills.

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1 Hampton JR. Path to clinical confidence. *BMJ* 1993;306:595. (27 February.)

Move a medical school to Milton Keynes

EDITOR,—Why not move one of London's medical schools to Milton Keynes? Designated to receive most of its population from London, the city could now adopt one of its medical schools as well. There are precedents for such a move: during the second world war some students and staff from University College Hospital, London, relocated to Cardiff.

Milton Keynes has its own hospital; consultants and senior staff could move there with the medical school. Even the name of the medical school could be retained with just the postcode changed.

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Antenatal diagnosis of Down's syndrome

EDITOR,—The increase in antenatal diagnoses of Down's syndrome suggests a more widespread use of biochemical screening. This, and analysis of the results by David E Mutton and colleagues,[1] is to be encouraged but raises some interesting points which have potential implications for resource allocation. The two main reasons for antenatal screening are (*a*) to plan the most appropriate place and mode of delivery to minimise the hazard to neonatal life and (*b*) to offer termination of pregnancy if the diagnosis is made before 24 weeks' gestation (previously 28 weeks').

From Mutton and colleagues' raw data, assay of serum α fetoprotein concentration detected 21% of the detected cases in women under 35 while triple testing detected only 17%. This might suggest that assay of α fetoprotein concentration alone is better at detecting Down's syndrome than triple testing. The converse, however, is the case, and the difference can probably be explained simply by the more widespread use of assay of α fetoprotein concentration during the period studied.

Although the total proportion of diagnoses seems to be rising, the rise is steepest in those who historically have fallen into a high risk group—that is, woman aged 35 and older. This is not surprising as the algorithm to assign risk is weighted in favour of such cases. Unfortunately, around three quarters of cases of Down's syndrome occur in fetuses of women under this age, and in 1991 biochemical testing detected only 6·5% of all cases of the syndrome. From the analysis we do not know the proportion of pregnant women who participated in this form of screening, but it seems that around 48% of cases might be detected if triple screening was universal.[2]

Detailed ultrasound scanning detected 7·2% of all cases of the syndrome, though, again, the same rules apply—that is, what proportion of all antenatal patients underwent detailed scanning? Recently, however, Luck reported that in an unselected population detailed ultrasound scanning detected all of the cases of Down's syndrome when a physical abnormality was present.[3] At least half of all fetuses with the syndrome have a congenital heart defect, and many others have bowel atresias. Perhaps of greater importance, however, is that only half of liveborn infants with aneuploidies have Down's syndrome. Many of the other common aneuploidies (such as trisomy 13, trisomy 18, and Turner's syndrome-XO) are associated with physical abnormalities that are more readily appreciated on ultrasound scanning than the subtle ones associated with Down's syndrome.[4] Furthermore, ultrasound scanning detects other physical anomalies, of which some are associated with genetic abnormality. Many represent a hazard to neonatal life. If these anomalies are detected in good time the parents can receive counselling and the subsequent management of the pregnancy can be planned, so reducing the national perinatal mortality rate.[5]

Detailed ultrasound scanning has been shown to be cost effective as it detects most cases of Down's syndrome as well as other life threatening conditions.[5] Perhaps its wider implementation in early pregnancy should be an aim of all obstetric departments.

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1 Hutton DE, Ide R, Alberman E, Bobrow M. Analysis of maternal serum alpha fetoprotein screening for Down's syndrome in England and Wales: trends in prenatal diagnosis, 1989-91. *BMJ* 1993;306:830-2. (13 February.)

2 Wald NJ, Barnard A, Densem JW, Cuckle HS, Chard T, Butler L. Antenatal maternal serum screening for Down's syndrome: results of demonstration project. *BMJ* 1992;305:391-8.

3 Jack CA. Value of routine ultrasound scanning at 19 weeks: a four year study of 8849 deliveries. *BMJ* 1992;304:1167-8.

4 Nicolaides KH, Snijders RJM, Gosden CM, Berry C, Campbell S. Ultrasonographically detectable markers of fetal chromosomal abnormalities. *Lancet* 1992;340:704-7.

5 Royal College of Obstetricians and Gynaecologists. *Working party report on routine ultrasound examination in pregnancy*. London: RCOG, 1984.

Sexually transmitted diseases and HIV infection among homosexual men

EDITOR,—B G Evans and colleagues conclude the discussion of their paper by stating, "Because of the high background prevalence of HIV-1 infection risks to homosexual men practising unsafe sex are greatest in London."[1] They further suggest that "safe sex information aimed at . . . homosexual men in London needs special emphasis."

This statement might be misunderstood by readers to imply that unsafe sex elsewhere is somehow less risky than it is in London. This is certainly untrue of Brighton, where the high prevalence of HIV-1 infection in homosexual men is similar to that in London. Indeed, the rate of infection in Brighton is probably the highest in Britain (90 new infections in homosexual men reported in 1992 (23% of those tested)). By any statistical configuration, the concentration of HIV infected homosexual men in Brighton is higher than that in London or elsewhere in Britain. The chance of infection through unsafe sex in Brighton is therefore relatively higher.

Information on safe sex aimed at homosexual men in Brighton is needed. Statutory and voluntary organisations have recently intensified education and information programmes and increased the educational involvement of outreach groups with homosexual men in Brighton.

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1 Evans BG, Catchpole M, Heptonstall J, Mortimer J, McCarrigle C, Nicoll A, *et al*. Sexually transmitted diseases and HIV-1 infection among homosexual men in England and Wales. *BMJ* 1993;306:426-8. (13 February.)

EDITOR,—We have conducted a similar study to that of B G Evans and colleagues of sexually transmitted diseases and HIV-1 infection among homosexual men in England and Wales.[1] Our results reinforce their findings to a certain extent, but our experience in 1992 shows a further reduction in new cases of gonorrhoea in men and in the diagnosis of HIV positivity in male homosexuals.

We looked at the total incidence of gonorrhoea; the number of cases of gonorrhoea found in men overall and in homosexual men; the male to female ratio of cases of gonorrhoea; and the number of new cases of HIV infection diagnosed in homosexual men (table). Like Evans and colleagues, we found that new cases of gonorrhoea in all men and in homosexual men showed an increase in 1988-90. Our study, however, showed a reduction in 1991 and 1992. The male to female ratio of cases was lowest (1·2:1) in 1987, subsequently rising to 2·1:1 in 1990. The number of new cases of gonorrhoea in homosexuals was lowest in 1987 and then gradually increased until 1990.

Cases of newly diagnosed gonorrhoea and HIV infection, 1985-92

	1985	1986	1987	1988	1989	1990	1991	1992
Cases of gonorrhoea:								
Total	424	355	138	160	162	122	82	72
In men	268	203	76	98	96	82	52	47
In homosexual men (%)	49 (18)	13 (6)	4 (5)	8 (8)	11 (11)	24 (29)	13 (25)	6 (13)
Male:female ratio	1·7	1·3	1·2	1·6	1·5	2·1	1·7	1·9
Cases of HIV infection in homosexual men	27	24	16	17	18	18	21	14

Our study indicates that unsafe sexual practice may have increased in men from 1987. The decline in gonorrhoea and other sexually transmitted diseases in the mid-1980s may have been due to safer sex practices after health education through the mass media and various other local activities. The reduction that we found in 1992, in both gonorrhoea and HIV infection, is heartening, but vigorous and continuing health promotion will be necessary to continue this trend.

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1 Evans GB, Catchpole MA, Heptonstall J, Mortimer JY, McCarrigle CA, Nicoll AG, *et al*. Sexually transmitted diseases and HIV-1 infection among homosexual men in England and Wales. *BMJ* 1993;306:426-8. (13 February.)

EDITOR,—B G Evans and colleagues present compelling evidence that unsafe sexual behaviour and transmission of HIV have increased among gay and bisexual men in England and Wales after a decline in the 1980s.[1] They suggest that this may partly be due to a failure to sustain the successful community based health education activities of the early and mid-1980s.[2-4]

To ascertain the level of HIV prevention activity specifically targeting gay or bisexual men in Britain, staff and volunteers at the National AIDS Manual, North West Thames Regional Health AuthoriGy HIV project, the Terrence Higgins Trust, and Gay Men Fighting AIDS undertook a survey between November 1991 and April 1992.[5] Two hundred and forty organisations with a remit for HIV prevention work were identified. Answers to a standard telephone questionnaire were obtained from 226 (94%); 202 respondents were statutory organisations and 24 were voluntary agencies.

Altogether 149 respondents reported that they had never undertaken or funded any HIV prevention work specifically aimed at gay or bisexual men. Of the remaining 77, only eight had ever offered a "substantial" programme of such work; this was a relatively unexacting definition, requiring only a written needs assessment and the employment of a whole time or part time worker with a specific remit for this work. Only three agencies had ever offered a "comprehensive" package of HIV prevention work for gay and bisexual men, defined as needs assessment, the production of local health education resources, one or more public education events, staff training, and the employment of a worker.

At a time when it is increasingly popular to search for complex explanations for continuing or increasing levels of unsafe sexual behaviour among gay and bisexual men the most obvious explanation—lack of continuing education about safer sex—must not be overlooked. Evans and colleagues' concern about the failure of AIDS educators to target gay and bisexual men is well founded: it seems that those most at risk from HIV have also been the most neglected in recent years. HIV prevention workers must ensure that they prioritise their work according to epidemiologically demonstrable need if the alarming trends in surveillance data on sexually transmitted diseases and HIV infection are to be arrested or reversed.

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1 Evans BG, Catchpole MA, Heptonstall J, Mortimer JY, McCarrigle CA, Nicoll AG, *et al*. Sexually transmitted diseases and HIV-1 infection among homosexual men in England and Wales. *BMJ* 1993;306:426-8. (13 February.)

2 Coates TJ, Stall RD, Catania JA, Kegeles SM. Behavioural factors in the spread of HIV infection. *AIDS* 1988;2(suppl 1):S239-46.

3 Coutinho RA, van Griensven GJP, Moss A. Effects of preventive efforts among homosexual men. *AIDS* 1989;3(suppl 1):S53-6.

4 Weatherburn P, Hunt A, and Project SIGMA. *HIV education: the effect on the sexual behaviour of gay men*. London: Project SIGMA, 1991. (Project SIGMA working paper No 30.)

5 King E, Rooney M, Scott P. *HIV prevention for gay men: a survey of initiatives in the UK*. London: North West Thames Regional Health Authority, 1992.

EDITOR,—We recently highlighted an increase in unsafe sexual behaviour and transmission of HIV among homosexual men in England and Wales after a period of decline.[1] Our data included documented seroconversions to the end of 1991—that is, newly diagnosed HIV-1 infection in men for whom the year and month of a previous negative result of an HIV-1 test were available. The table summarises revised data, including the seroconversions reported during 1992. The number of reported seroconversions has risen steadily since 1986; the number of cases in which transmission of HIV-1 was known to have occurred during 1990-2 (157) was more than double the number recorded during 1987-9 (74).

Year of HIV-1 seroconversion in 503 homosexual men who had had negative test results, England and Wales

Year of last negative result	Year of first positive result							
	1985	1986	1987	1988	1989	1990	1991	1992
1985	16	22	11	5	4	2	3	7
1986		16	20	2	5	7	9	6
1987			15	7	13	17	10	9
1988				11	21	14	16	10
1989					7	27	15	19
1990						15	33	22
1991							21	42
1992								24
Total	16	38	46	25	50	82	107	139

Despite widespread recognition in 1991 of the unfavourable trends in sexually transmitted diseases among homosexual men[2-5] transmission of HIV during 1992 seems not to have declined but may have intensified further.

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1 Evans BG, Catchpole MA, Heptonstall J, Mortimer JY, McGarrigle CA, Nicoll AG, *et al*. Sexually transmitted diseases and HIV-1 infection among homosexual men in England and Wales. *BMJ* 1993;306:426-8. (13 February.)

2 Riley VC. Resurgent gonorrhoea in homosexual men. *Lancet* 1991;337:183.

3 Waugh MA. Resurgent gonorrhoea in homosexual men. *Lancet* 1991;337:375.

4 Young H, Moyes A, McKenna JG, McMillan A. Rectal gonorrhoea and unsafe sex. *Lancet* 1991;337:853.

5 Tomlinson DR, French PD, Harris JRW, Mercey DE. Does rectal gonorrhoea reflect unsafe sex? *Lancet* 1991;337:501-2.

Rapid On-Slide Location and Analysis of Enriched Fetal Nucleated Cells from Maternal Blood[a]

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Multiparameter flow-sorting is the most selective approach for isolating fetal cells from maternal blood but may not be appropriate as a routine antenatal screening tool.

We have developed a method of negative selection on artificial admixtures of fetal and adult blood. Initially, a density gradient medium is used that removes 99.94 ± 0.07% of erythrocytes. Then immunomagnetic Dynabeads coupled to anti-leukocyte monoclonal antibodies are used that remove 99.6 ± 1.4% of the remaining leukocytes. From the total starting number, 44.5 ± 11.7% of erythroblasts can be recovered. Unfortunately, negative enrichment cannot exclude those nonnucleated erythrocytes that collect with the initial nucleated cell harvest. These now become the major "contaminating" cells, and the absolute numbers present difficulties for conventional cell deposition techniques.

Nevertheless, by means of a method akin to making simple blood smears, the remaining cells can be deposited as a monolayer on two or three slides. The cells may be subjected to a modified Kleihauer-Betke test, and positive cells located by computerized automatic slide-searching using proprietary software developed in the Human Genetics Unit. Fluorescence *in situ* hybridization (FISH) can then be performed on the cells and spot-counting of hybridization signals again performed automatically.

Using this technique, we have enriched for erythroblasts from as little as one in 10^8 total cells. Initial results with maternal blood samples are very encouraging, and the outcome of the pregnancies is awaited.

The approach has advantages over others in that it is rapid and inexpensive. It does not limit selection of fetal cells to erythroblasts but also enriches for trophoblast cells, and scan criteria can be altered readily to accommodate changes in antibodies, staining, or other aspects of the selection system.

We have successfully used dual-color fluorescence with X and Y chromosome probes on enriched erythroblasts that have undergone Kleihauer treatment. This confirms that multiple probes may be used in conjunction with a cytoplasmic marker and should enable fetal cells isolated from maternal blood to be subjected to a simultaneous panel of chromosome probes directed against the more common aneuploidies.

[a]This work was supported by *Birthright,* The Royal College of Obstetricians and Gynaecologists.

Fetal Erythroblasts in Maternal Blood: Non-invasive Antenatal Diagnosis

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INTRODUCTION

Down's syndrome affects one in 800 live births and is the most common clinically significant trisomy.

The introduction of amniocentesis and subsequently chorionic villus sampling allowed a select group of women, usually over 35 years old, to be offered definitive testing. However, these invasive tests carry a small but definite risk to the pregnancy and many procedures are performed to detect one Down's syndrome fetus. Significantly, 66% of cases are actually born to women who are younger than the traditional 35-year cut-off (Figure 1) and so the majority of cases go undetected.

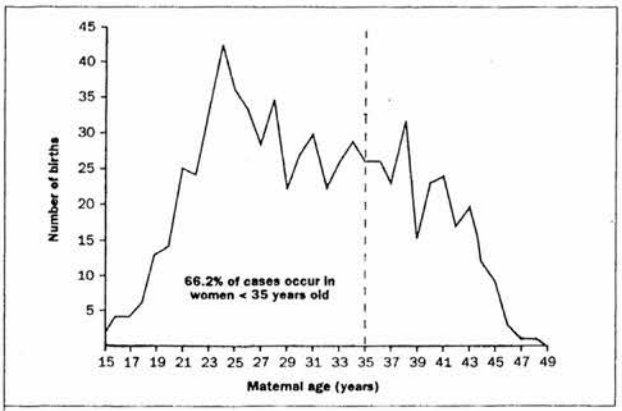

Figure 1 Maternal age distribution for the incidence of Down's syndrome in live births. (Adapted from Staples *et al.* 1991 with permission from University of Chicago Press.)

Maternal serum biochemical screening has improved detection rates of Down's syndrome to around 60%, but invasive testing must be performed to confirm the suspicion and still around 40 amniocenteses are performed to detect a single case.

Due to these inherent drawbacks of currently available screening techniques, the possibility of isolating and analysing fetal cells from maternal blood is intriguing. It offers the opportunity to screen all women with no risk to the pregnancy and has the capability of detecting most cases of Down's syndrome. As technologies develop this could be extended to the detection of other genetic abnormalities and ultimately the need for invasive tests may be obviated, as such diagnoses will be made from a simple maternal blood sample alone.

FETAL CELLS IN MATERNAL BLOOD

In 1893 a German pathologist, Schmorl, identified fetal (trophoblast) cells in the pulmonary vessels of women who had died of eclampsia, proving that fetal cells escape into maternal blood. Of course rhesus isoimmunisation in the absence of vaginal bleeding is further, and less dramatic, evidence of transplacental passage of fetal cells.

Evidence suggests that transplacental passage of cells occurs as a series of minor events and is a physiological and normal part of pregnancy. A steady state is eventually reached where fetal cells are removed at the same rate as they are added (Parks & Herzenberg 1982). If these cells can be isolated from maternal blood they might be used for antenatal diagnostic purposes.

Unfortunately, they are thought to occur in the maternal circulation in numbers approximating to 1 in 100 million maternal cells or less (Bianchi *et al.* 1990) and

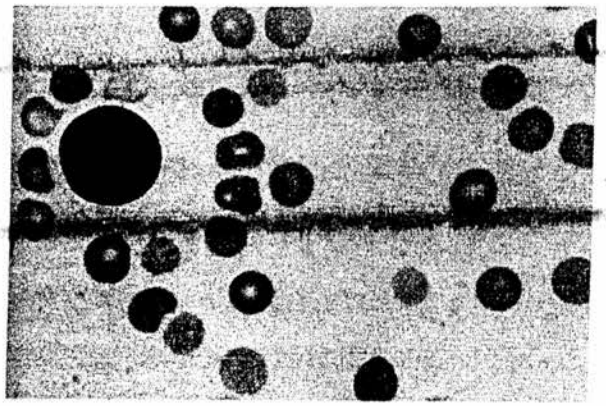

Figure 2 A single fetal erythroblast in maternal blood surrounded by non-nucleated erythrocytes. Note the large size and characteristic round, slightly eccentric nucleus.

any billions of cells from each maternal blood sample ust be analysed. This is impractical and an enrichment ocedure, to improve this ratio, is necessary.

This can be achieved using fluorescence-activated ll sorting (FACS), also known as flow sorting. Cells are belled with antigen-specific monoclonal antibodies nAbs) which have a fluorescent marker attached. When cell is detected, because it bears markers which fulfil reset conditions, it is collected. It is unlikely that a pure opulation of fetal cells from maternal blood can ever be hieved, but once they have been enriched, some form genetic analysis can be performed.

DNA amplification using polymerase chain reaction CR) has been used to show that male DNA can be und in the blood of pregnant women in early egnancy both reliably and with great accuracy (Liou al. 1993). Unfortunately, PCR depends on the fetus aring DNA unique from the mother and rarely, in is setting, could it be diagnostic for fetal genetic normalities. Furthermore, its exquisite sensitivity akes it prone to false-positive errors.

An alternative strategy is fluorescence *in situ* bridisation (FISH). This involves hybridising (binding) ecific probes to complementary DNA sequences on romosomes. The binding sites can be visualised rectly by attaching fluorescent markers (fluoro-romes) to the probe. Multiple probes can be employed multaneously by using different coloured fluoro-romes. For instance, using FISH with a probe for romosome 21 and cells from a case of Down's ndrome, three intranuclear signals would be observed.

There are a number of different types of fetal cell which ight be isolated from maternal blood: namely, ophoblast cells, lymphocytes, neutrophils and fetal ythroblasts. Each has its drawbacks but, for many asons, the last cell type appears to have the eatest potential.

FETAL ERYTHROBLASTS IN MATERNAL BLOOD

Erythroblasts are the nucleated precursor of mature, non-nucleated erythrocytes.

In 1990, Diana Bianchi and her colleagues in Boston produced an enriched mixture of fetal erythroblasts from the blood of pregnant women in early pregnancy using FACS and, utilising PCR, correctly assigned the fetal sex in 84% of cases. The enrichment of fetal erythroblasts was based on several suppositions:

1. *In early pregnancy erythroblasts are the most numerous nucleated fetal blood cell. Therefore, in any minor fetomaternal haemorrhage they would represent the greatest proportion of nucleated cells crossing the placenta.*
2. *They are large and easily differentiated from other cells (Figure 2).*
3. *In the healthy adult, including pregnant women, erythroblasts of adult origin are not expected in the peripheral circulation. So, if they are found, they should be of fetal origin.*
4. *Parks and Herzenberg (1982) demonstrated chronic leakage of fetal cells across the placenta. Thus, fetal erythroblasts should be escaping constantly into maternal blood.*
5. *It is unlikely that fetal erythroblasts persist for more than a few days in maternal blood and, consequently, persistence from one pregnancy to the next is extremely unlikely and will not confuse analyses.*
6. *Fetal erythrocytes express relatively few blood group antigens, such as rhesus, and erythroblasts fewer still. Furthermore, only one-third of susceptible women actually mount an immune response to rhesus-positive cells. Consequently, even in the cases where the blood group is incompatible, fetal erythroid cell destruction may not be as rapid as in adult-to-adult incompatible transfusions, possibly allowing substantial accumulation of erythroblasts in maternal blood.*
7. *Finally, there is growing evidence that, because of abnormal placentation, more fetal cells escape into the maternal circulation in aneuploid pregnancies such as Down's syndrome (Gänshirt-Ahlert* et al. *1993).*

Using FACS based on a number of different cell surface markers, several groups have reliably sorted fetal erythroblasts from maternal blood and diagnoses of fetal trisomy have been made (Price *et al.* 1991). These results demonstrated that common aneuploidies could be detected from a blood test alone which potentially opens the door to mass screening. However, any screening technique must be robust, reliable and inexpensive and, although FACS is the gold standard of cell enrichment, it is prohibitively expensive and time consuming. Other

groups have therefore been investigating alternative, less expensive strategies which could be applicable on a large scale.

Immunomagnetics involves specific monoclonal antibodies, bound to microscopic magnetic particles, which bind to target cells (Figure 3). Following incubation the cell / bead suspension is placed adjacent to a magnet and the beads, with attached cells, are drawn to the magnet. This approach is both rapid and inexpensive, and the incubations could be performed automatically. Using such an approach diagnoses of fetal trisomy have been made in early pregnancy (Gänshirt-Ahlert *et al.* 1993). We have also applied this latter approach prospectively in a series of 15 pregnant women in the first and early second trimesters and have successfully determined fetal sex with 86.7% accuracy ($P < 0.004$). Figure 4 shows cells enriched from maternal blood subjected to FISH using an X-chromosome probe (labelled with fluorescein isothiocyanate, FITC, which is seen as the green areas in Figure 4) and a Y-chromosome probe (labelled with Texas red). These are male (XY) cells in the blood of pregnant women in early pregnancy. We have also confirmed a case of Down's syndrome from cells in the blood of a woman at 12 weeks' gestation, diagnosed a week previously by chorionic villus sampling (Gaudoin *et al.*, in preparation). Figure 5 shows cells from this case subjected to FISH using a probe for chromosome 21 labelled with FITC. In each of these nuclei three discrete intranuclear signals can be seen clearly, demonstrating the trisomic nature of the cells.

Regardless of the enrichment technique, maternal cells will always outnumber fetal cells and it is vital that some form of cell marker is used to identify the cells positively. To date, only a Cambridge group has

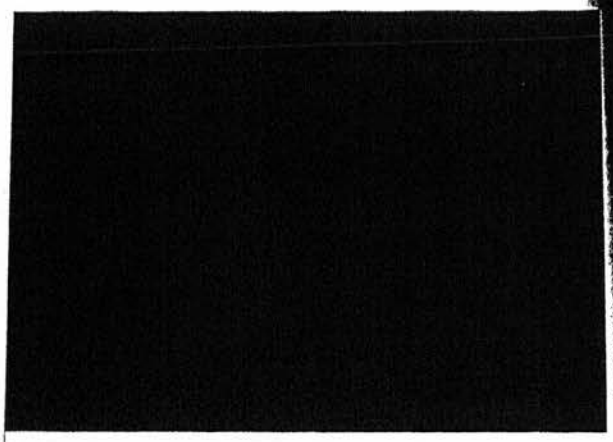

Figure 4 Male (XY) fetal cells isolated from maternal blood. The X-chromosome probe is labelled with fluorescein isothiocynate (FITC), while the Y-chromosome probe is labelled with Texas red.

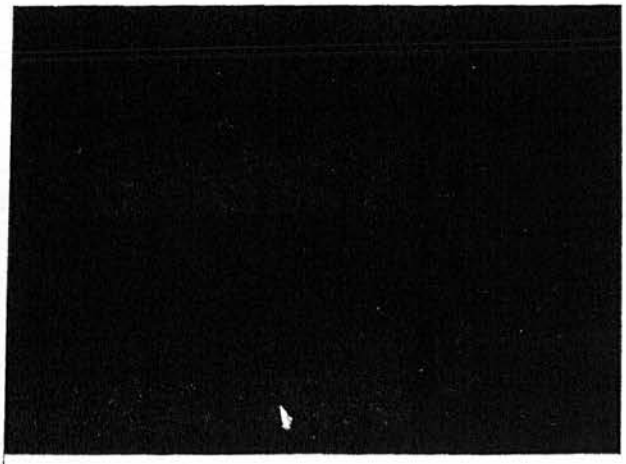

Figure 5 Trisomy 21 cells isolated from the blood of a woman at 12 weeks' gestation prior to termination of pregnancy for Down's syndrome.

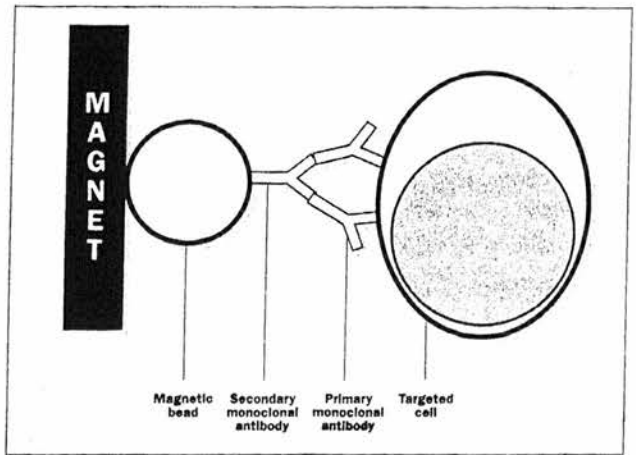

Figure 3 The principle of immunomagnetics. Specific monoclonal antibodies bind to specific cells and the beads pull the cells out of suspension when placed adjacent to a magnet.

reported a successful attempt to do this prior to FISH analysis (Zheng *et al.* 1993). They subjected the cells to immunostaining using a mAb to the γ-chain of fetal haemoglobin, recorded the position of positive cells, subjected the cells to FISH analysis and reviewed the previously γ-globin-positive cells. In five of six pregnancies, between two and 11 positive cells were identified and in all of these gender was predicted accurately.

This is a crucial step. It would mean that only fetal cells need to be analysed whilst contaminating cells could be disregarded. Furthermore, we have successfully repeated the Cambridge group's work in model systems and research continues in this area of maternal blood. Figure 6 shows three cells. Two are negative, but the cytoplasm of the third is labelled with an mAb to fetal haemoglobin and visualised with tetramethylrhodamine isothiocyanate (TRITC, red areas). The slide was then

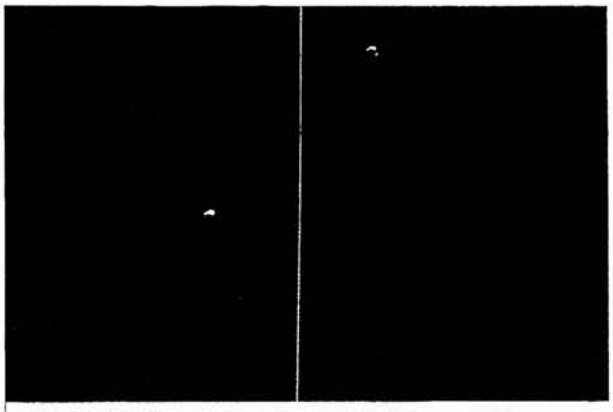

Figure 6 Immunostaining with a monoclonal antibody to fetal haemoglobin (HbF) and subsequent fluorescence *in situ* hybridisation analysis (FISH). The cytoplasms of the two cells adjacent to each other are negative for HbF, but the cytoplasm of the other is positive. All cells subsequently show two copies of chromosome 11.

subjected to FISH analysis using a probe for chromosome 11 and it can be seen that each of these cells has two copies of chromosome 11. If this or similar techniques can be devised to leave the cell cytoplasm intact and the nucleus simultaneously accessible for FISH analysis, then karyotype assignment will be made with greater confidence (particularly in the 50% of cases involving a female fetus). Moreover, prior positive identification should allow computer-driven scanners to locate positive cells so obviating the arduous task of manual searching which, in turn, would allow large numbers of samples to be processed.

CONCLUSIONS

Although erythroblasts are the most promising fetal cell type, there is a need to determine the proportion of pregnancies in which sufficient numbers can be found and the optimal gestation for sampling. As so few cells are being isolated, improvement in enrichment procedures to maximise the absolute numbers recovered is imperative. Furthermore, as the majority of cells remain maternal, it is desirable to identify the fetal cells positively.

The advantages of analysing fetal cells from maternal blood are manifold but, as with many new techniques, fetal cell retrieval may initially only be used as a complementary test, being combined with current screening methods such as maternal serum biochemical markers and ultrasonography. This could have the following wide implications for antenatal diagnosis:

1 *If the finite detection rate of Down's syndrome is maintained, then the number of invasive procedures could be reduced; or*

2 *If the rate of invasive procedures was maintained, the absolute detection rate could be improved dramatically.*

In time, however, it may be possible to make and act upon the blood sample alone, raising the possibility of a truly non-invasive, and yet definitive, diagnosis. Using multicolour FISH with a panel of chromosome probes, it is hoped that all the common trisomies will be detected. Ultimately, as techniques develop, it may be possible to diagnose any genetic disorder where the gene has been cloned and a probe will be available from a simple maternal blood test.

Given the current relatively inexpensive and automatable enrichment procedures, the continuing rapid development of molecular biological techniques and modern computer-based slide-search capability, the day of truly non-invasive, definitive antenatal diagnosis is fast approaching.

ACKNOWLEDGEMENTS

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REFERENCES

Bianchi D. W., Flint A. F., Pizzimenti M. F. *et al.* (1990) Isolation of fetal DNA from nucleated erythrocytes in maternal blood. *Proc Natl Acad Sci USA* **87**(9), 3279–3283.

Gänshirt-Ahlert D., Borjesson-Stoll R., Burschyk M. *et al.* (1993) Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting. *Am J Reprod Immunol* **30,** 194–201.

Liou J.-D., Pao C. C., Hor J. J. & Kao S.-M. (1993) Fetal cells in the maternal circulation during first trimester in pregnancies. *Hum Genet* **92,** 309–311.

Parks D. R. & Herzenberg L. A. (1982) Fetal cells from maternal blood: their selection and prospects for use in prenatal diagnosis. In *Methods in Cell Biology,* Vol. 26, Academic Press, New York, pp. 277–295.

Price J. O., Elias S., Wachtel S. S. *et al.* (1991) Prenatal diagnosis with fetal cells isolated from maternal blood by multi-parameter flow cytometry. *Am J Obstet Gynecol* **165,** 1731–1737.

Staples A. J., Sutherland G. R., Haan E. A. & Clisby S. (1991) Epidemiology of Down's syndrome in South Australia 1960–69. *Am J Hum Genet* **49,** 1014–1024.

Zheng Y.-L., Carter N. P., Price C. M. *et al.* (1993) Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting. *J Med Genet* **30,** 1051–1056.

Obstetrics and Gynaecology in the Journals: Prenatal Screening and Diagnosis

A review of some selected papers that have recently appeared in various journals.

by Marco Gaudoin and Fiona Mackenzie

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It has been less than 30 years since definitive antenatal diagnostic tests have become routine clinical practice. Over this time different techniques have been introduced with attempts to reduce the gestational age at which diagnoses are made (to allow easier and less-traumatic termination if wished) and less invasive methods adopted to minimise the risk to pregnancies from the tests involved. Furthermore, the relatively recent introduction of maternal serum screening has meant that more cases of Down's syndrome, particularly in younger women, are now being detected prenatally.

The mainstays of antenatal diagnosis remain amniocentesis and chorionic villus sampling (CVS), but ultrasound scanning for structural anomalies has become increasingly important.

We review a paper which attempts to address the issues of safety and early diagnosis, two papers concerning the implications of non-invasive diagnosis of common fetal anomalies by ultrasound and a paper looking at the possibility of improving second-trimester serum screening for Down's syndrome.

Analysis of limb reduction defects in babies exposed to chorionic villus sampling.
Firth H. V., Boyd P. A., Chamberlain P. F., MacKenzie I. Z., Morriss-Kay G. M. & Huson S. M.
Lancet 1994, **343**, 1069–1071.

If a chromosomal anomaly is detected in the mid-second trimester, prostaglandin termination of pregnancy is necessary which effectively involves the woman going through labour. If the abnormality is detected in the first trimester then suction termination of pregnancy can be offered which is physically and psychologically less traumatic for the couple.

CVS was developed in the late 1960s and early 1970s to allow fetal tissue to be sampled reliably and cultured rapidly in the first trimester. However, it was only in the mid-1980s that it became a common diagnostic tool.

CVS is particularly useful for women at greater risk of having a child with a chromosomal abnormality (e.g. older women and women who have previously delivered an affected child). Unfortunately, it carries with it the well-recognised risk of an increased abortion rate of approximately 2–4%.

Oromandibular-limb hypogenesis (micrognathia and microglossia) and terminal transverse limb reduction defects have a background rate of 1.8 per 100,000 live births and Table 1 categorises limb reduction defects (LRDs) according to their severity. Following a paper by Firth *et al.* in 1991 of five cases from 289 CVSs, a number of groups also reported a possible aetiological link.

Table 1

Categories of limb reduction defects

Category	Severity of defect
1	Absent limb or defect through humerus/femur
2	Defect through radius/tibia
3	Defect through carpus/tarsus or metacarpus/metatarsus
4	Defect through digits
5	Defect through terminal phalanx/nail

Subsequent correspondence was conflicting and reports were largely anecdotal. From our own figures in the West of Scotland, there were 78,866 births and 47 reported LRDs, although none was associated with CVS. Furthermore, in the World Health Organization CVS registry there were 48 cases in 80,051 births following CVS, an incidence not different from accepted background rates.

However, the authors pooled data from all published reports and could find 75 authenticated cases of LRDs and oromandibular-limb hypogenesis (OLH) syndrome associated with CVS.

Although the gestational age at which LRDs associated with CVS occurred was broad, there was a definite trend to more severe cases (category 1) with earlier CVS (Figure 1). This trend was not absolute as a category 1 defect was associated with CVS as late as 65 days' and a category 5 lesion at 50 days' gestation. Therefore, although there were no definite rules governing gestational age, it would appear that earlier CVS was associated with greater likelihood of a more severe lesion.

The pathogenesis of LRDs and OLH syndrome is open to conjecture and comes largely from animal studies. Temporary clamping of uterine blood vessels in pregnant rats leads to dilatation of vessels in the extremities of the fetus. These vessels eventually rupture, leading to further hypoxia, necrosis and eventual resorption of the appendage, producing LRDs in the rat offspring.

In women undergoing CVS, there is a trend to increasing feto-maternal haemorrhage (manifest as higher maternal serum α-fetoprotein [AFP] levels) in cases which go on to abort spontaneously, implying that there is fetal blood vessel disruption. It is plausible that in cases of LRDs or OLH syndrome the vessel disruption, although insufficient to cause abortion, is severe enough to produce distal anoxia and eventual appendage resorption. This hypothesis would explain why the hierarchy of limb damage correlates inversely with gestational age as it corresponds with the embryological sequence of limb formation.

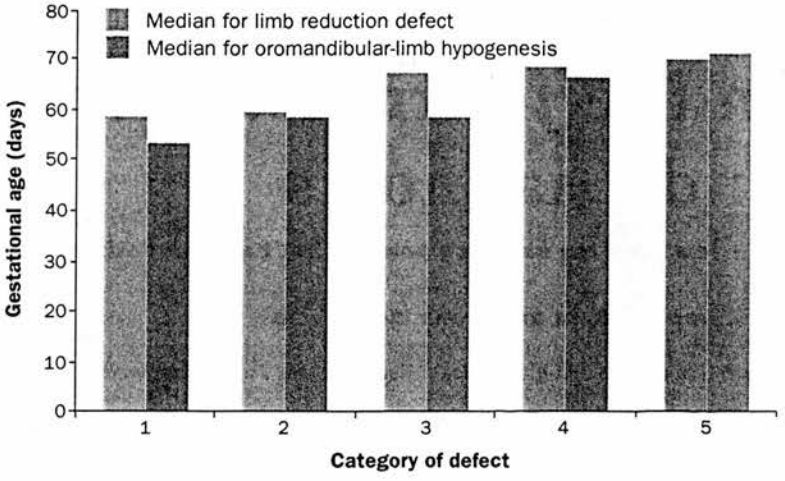

Figure 1 Mean gestational age at CVS according to level of limb reduction defect (Firth *et al.* [1994] *Lancet* **343**, 1069–1071).

From their available data it remains unclear if the absolute incidence of the syndrome is increased by CVS but, given that the pathogenic mechanism appears to be interference with fetal vascularity, this is possible. What is clear, however, is that the earlier CVS is performed, the more severe the degree of LRD and, because of this, many units, including our own, avoid CVS prior to 10 weeks' gestation.

Clinical significance of choroid plexus cysts. Gupta J. K., Cave M., Lilford R. J., Farrell T. A., Irving H. C., Mason G. & Hau C. M. *Lancet* 1995, **346**, 724–729.

Choroid plexus (CP) cysts are echo-free areas within the choroid plexus located in the posterior horn of the lateral cerebral ventricles. They can vary in size, may be bilateral and can be seen easily by ultrasound in the standard biparietal diameter view (Figure 2).

Although not themselves harmful, the management of apparently isolated CP cysts is one of the more controversial areas in obstetric ultrasound as they *may* be associated with major chromosomal abnormalities. However, to diagnose potential chromosomal abnormalities requires an invasive procedure which obviously carries a risk of miscarriage. The dilemma therefore arises in counselling prospective parents if no other abnormality is detected on detailed ultrasound.

Prospective studies to date have been too small to measure the risk of isolated CP cysts on perinatal outcome and this study, although retrospective, aimed to address this issue and perhaps offer guidelines to practising obstetricians.

The authors analysed perinatal outcome from 595 cases of known CP cysts in the Yorkshire and

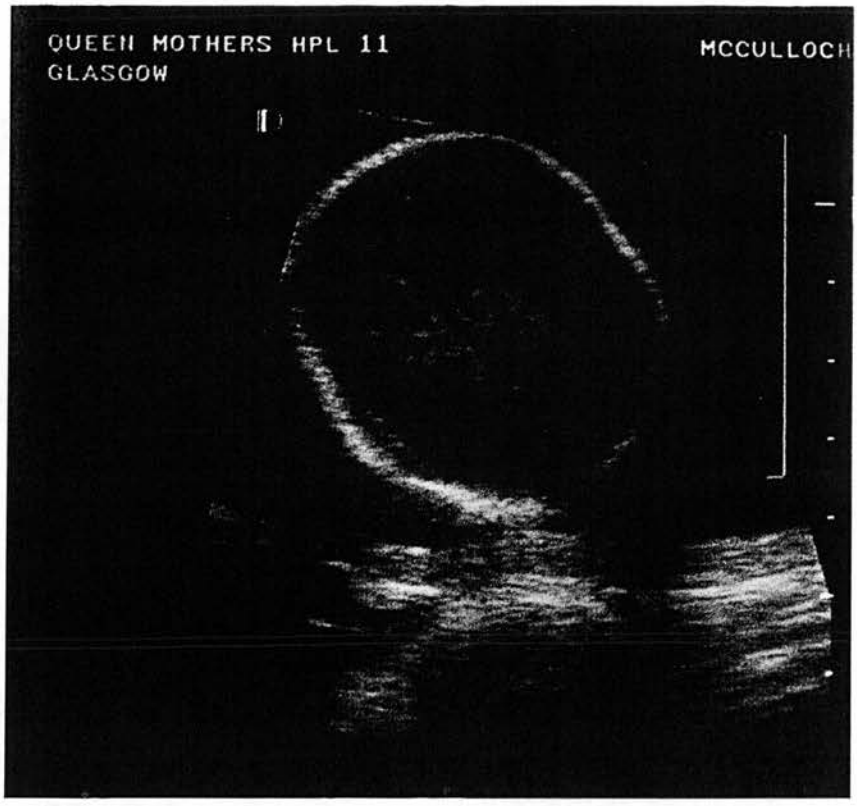

Figure 2 Bilateral choroid plexus cysts in a karyotypically normal fetus (courtesy of Dr F. Mackenzie).

Dundee regions over a three-year period and amalgamated these data with 1361 cases from prospective studies reported in the literature — a total of 1956 cases.

The reported prevalence of CP cysts ranged from 0.18% to 3.6% and overall was 0.88%. The variation was probably a reflection of the sensitivity of ultrasonic detection in different centres rather than any true difference in underlying incidence. CP cysts usually resolved by 22–26 weeks' gestation, even in the presence of chromosomal abnormality.

Of the 1956 cases there were 69 chromosomally abnormal fetuses. Overall the risk of chromosomal abnormality in the general population is approximately 0.6% (1 in 170). From their analysis, the risk of a chromosomal abnormality was 1 in 150 (12/1787) for an isolated CP cyst (95% confidence interval 1 in 85, 1 in 261). However, this rose dramatically to 1 in 3 (57/169) if there was a concomitant sonographically detectable anatomical abnormality (95% confidence interval 1 in 2.4, 1 in 3.7). Seventy-six per cent were trisomy 18 (which has characteristic ultrasound features), 17% were trisomy 21 and the remaining 7% were either triploidy or Kleinfelter's syndrome.

Most previous reports had never considered the effect of maternal age and the risk of chromosomal abnormality if a CP cyst was identified. In the authors' own series of 595 cases, they found a statistically significant increased risk of chromosomal abnormality if the mother was older than 35 years, but the numbers involved are relatively small.

There was no evidence that the size of the cysts, their location (unilateral or bilateral) or whether or not they resolved spontaneously were predictive of abnormal karyotype.

Should a woman whose unborn baby has an isolated CP cyst be informed of the presence of the cyst and the potential implications of the finding?

Isolated CP cysts may be an anatomical variant rather than an abnormality but the authors quite rightly pointed out that there is a subtle interplay between medical beneficence, autonomy and the possibility of future medico-legal action if an abnormality is not divulged to the patient and investigated further if wished.

The authors therefore outlined a course of management should a CP cyst be identified (Figure 3). The discovery of a CP cyst should prompt a detailed fetal anomaly scan in the first instance but further investigation requires an invasive procedure and risks of such a procedure must be balanced against that of finding a chromosomal abnormality. The authors recommended that *only* if the mother is over 35 years old, or if she falls into a high-risk group because of the results of maternal serum biochemical screening, or if any other anatomical abnormality is detected at ultrasound, should she be offered an invasive antenatal diagnostic test to determine the fetal karyotype.

Although these recommendations have yet to be evaluated prospectively, from current knowledge these data should assist obstetricians when counselling prospective parents as there appears to be little increased risk of chromosomal abnormality in cases of isolated CP cysts when there are no other risk factors for fetal abnormality.

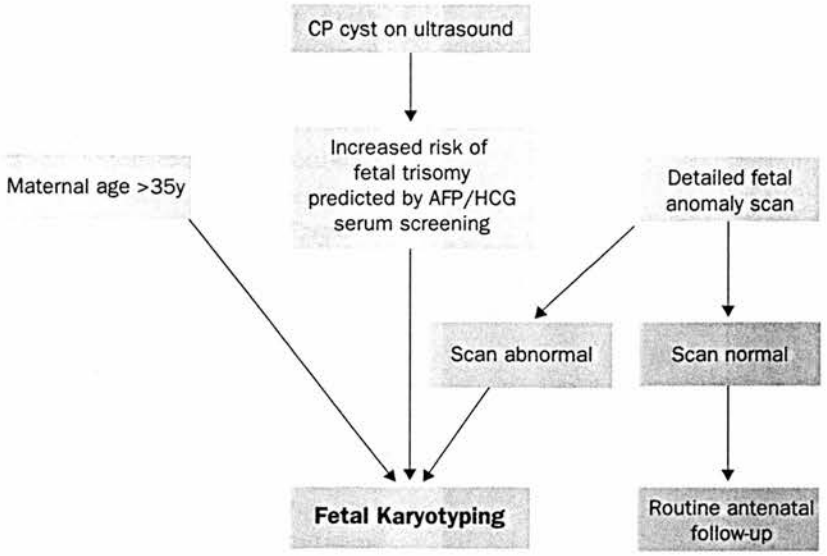

Figure 3 Suggested management of choroid plexus cysts detected by ultrasound scan (from Gupta *et al.* [1995] *Lancet* **346,** 724–729).

The implementation of first-trimester scanning at 10–13 weeks' gestation and the measurement of fetal nuchal translucency thickness in two maternity units. Pandya P. P., Goldberg H., Walton B., Riddle A., Shelley S., Snijders R. J. M. & Nicolaides K. H. *Ultrasound Obstet Gynecol,* 1995, **5,** 20–25.

The term incidence of the clinically most important aneuploidy, Down's syndrome, is approximately 1 in 800 deliveries. Although the risk rises with advancing maternal age, the majority of Down's syndrome births occur to women less than the traditional age threshold of 35 years and an invasive test (which carries a risk to the pregnancy) is still required to achieve a definitive diagnosis.

An increase in fetal nuchal translucency during the first trimester is associated with an increased risk of fetal chromosomal abnormalities. The actual pathophysiology remains undetermined but it is particularly associated with Down's syndrome (Figure 4). The potential to screen for this 'marker' in the first trimester by ultrasound in a non-invasive manner is therefore extremely appealing.

This paper, which involved a prospective study based at two district general hospitals and a coordinating tertiary referral centre, examined the feasibility of screening all pregnancies based on fetal nuchal translucency thickness.

From August 1993 first-trimester ultrasound was offered to all women to determine fetal viability, gestational dating and measurement of nuchal translucency thickness. Prior to August 1993 scans were only performed at 18–20 weeks' gestation and 70% of women took advantage of this earlier assessment.

Scans were performed by trained ultrasonographers but, importantly, implementation of the programme did not require an increase in staff or the purchase of extra (expensive) machines.

Ultrasound scans were performed between 10 and 13 weeks' gestation on 1763 fetuses (1673 singleton and 45 twin pregnancies) in which the maximum thickness of the subcutaneous translucency between the skin and the soft tissue overlying the cervical spine was measured.

It was found that nuchal translucency thickness increased with advancing gestation and overall 3.6% (63) of the fetuses demonstrated a nuchal translucency thickness of 2.5 mm or more. All but one of these women were offered CVS, of whom 49 accepted. The exception was a woman carrying monochorionic, monoamniotic twins where one of the twins had a nuchal translucency thickness of 6.0 mm whilst the other was 2.0 mm; this pregnancy ended spontaneously at 20 weeks' gestation with evidence of twin–twin transfusion. Of the 49 who underwent CVS, 45 showed a normal karyotype whilst four were abnormal: three cases of Down's syndrome and 1 case of Turner's syndrome (45,XO). The other 13 cases which did not have CVS performed went on to deliver phenotypically normal infants.

Fifty-five women who had a negative first-trimester screening ultrasound underwent definitive antenatal diagnostic testing (usually by amniocentesis) for other reasons, 50 of them either because of advanced maternal age or because of increased risk based on maternal serum biochemical screening. Of these, there was one case of trisomy 21 in a 42-year-old woman where nuchal translucency thickness had been 2.2 mm.

If we consider only Down's syndrome, of 1763 fetuses there were four cases. Three of these might have been detected by

increased nuchal translucency thickness (sensitivity, 75%). Similarly, of the 63 positive scans, three were true positives (positive predictive value, 4.8%; false positive rate, 3.4%). Likewise, the specificity was 96.6% (1699/1759) with a false negative rate of 25% (1/4).

The numbers involved in this study are relatively small, there being four Down's fetuses. The data, however, compare very favourably with detection rates from maternal serum α-fetoprotein and human chorionic gonadotrophin assays which also require invasive testing to confirm the suspicion of fetal aneuploidy. Furthermore, biochemical assays for Down's syndrome are not as reliable for twin pregnancies compared with singleton pregnancies.

The authors found that the total rate of fetal karyotyping rose from 4.3% the previous year to 5.1% during the study period. Although this rise was not statistically significant, it is somewhat inevitable, as younger women who would not otherwise have undergone fetal karyotyping were being offered CVS because of the ultrasound findings.

Measurement of distances on an ultrasound screen is dependent on the quality of the image and the plane at which the image is taken. It is, therefore, a subjective assessment and concerns have been expressed about the reproducibility of such a technique with regard to both inter-observer and intra-observer variation.

Furthermore, there also appears to be a higher pregnancy loss rate associated with this phenomenon, possibly because of *pre-mortem* development of hydrops. Therefore, although the test may identify pregnancies which were in any case destined to abort, it might falsely

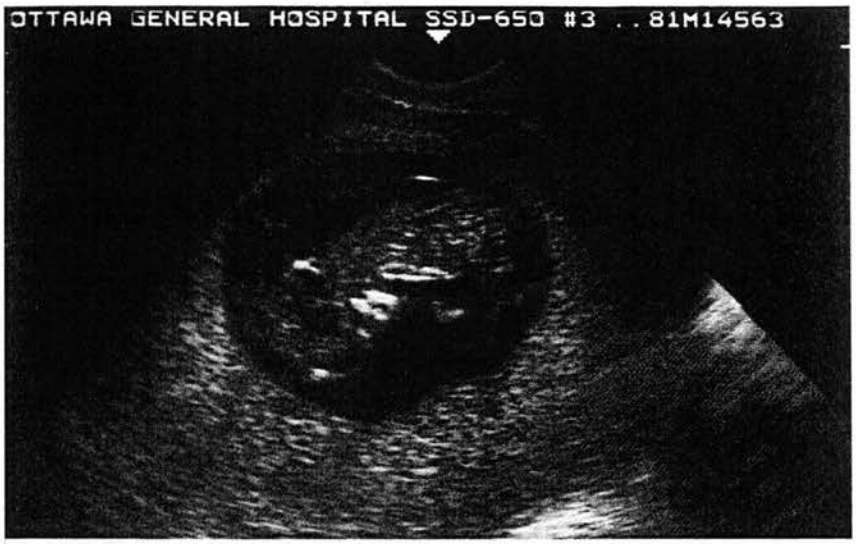

Figure 4 Nuchal oedema in a Down's syndrome fetus detected at 11 weeks' gestation by transvaginal ultrasound scan (courtesy of Dr F. Mackenzie).

improve its predictive value as a diagnostic test.

There is other evidence that as nuchal translucency thickness increases, the chance of aneuploidy also rises. Furthermore, there might be a greater chance of anatomical abnormality as well which has prompted suggestions that such women should be offered second-trimester detailed anomaly ultrasound.

These last points illustrate one of the difficulties of mass screening for relatively rare conditions from what is essentially a 'well' population. A large, prospective, multicentre study in a low-risk population would be required to build up a database of results which could then be analysed to address these issues.

In this way it may be possible to combine nuchal translucency thickness and first-trimester biochemical assays (possibly also taking into account maternal age) to improve the sensitivity and predictive values of testing compared with either test being used singly. This would require considerable reorganisation of antenatal care as women would need to attend their general practitioners at an earlier stage and be referred promptly for booking so that first-trimester screening could be performed.

The authors reported that many older women 'were reassured by the decreased risk for trisomies associated with nuchal translucency < 2.5 mm and decided against invasive testing'. However, until larger datasets have been evaluated, we must exercise caution in adopting such enthusiasm prematurely or, in the long term, we run the risk of jeopardising the implementation of what is an extremely promising development in the field.

Dimeric Inhibin A as a marker for Down's syndrome in early pregnancy. Aitken D. A., Wallace E. U., Crossley J. A., Swanston I. A., van Pareren Y., van Maarle M., Groome N. P., Macri J. N. & Connor J. M. *N Engl J Med*, 1996, **334**, 1231–1236.

Women over the age of 35 constitute approximately 5% of the pregnant population, but only one-

third of cases of Down's syndrome are born to women in this group. If we consider that, on age alone, around 120 amniocenteses are performed to detect one case of Down's syndrome, this equates to a false positive rate of approximately 5% as well. Clearly, maternal age alone is a less than adequate indicator of trisomy risk.

Although the exact mechanisms remain unclear, maternal serum α-fetoprotein (αFP) is reduced in Down's syndrome compared with unaffected pregnancies, whilst human chorionic gonadotrophin (hCG) is increased. Provided the distributions of a single variable are known for both unaffected and affected pregnancies, the median and multiples of the median (MoMs) can be calculated. However, because the serum levels of individual markers overlap considerably between the two populations, using a single parameter gives only a very poor predictive risk. More complex mathematical analysis is required as the number of variables increases but this allows the development of algorithms ('decision trees').

αFP, hCG and other markers have been incorporated into a variety of algorithms to predict the chance (risk) of a pregnancy being affected (Figure 5). In this example, if 5% of unaffected pregnancies carried a risk of Down's syndrome greater than a particular threshold (say, 1 in 220) and were offered amniocentesis, perhaps 60% of affected pregnancies might be detected. The limitations to the complexity of these algorithms is the inevitable trade-off in sensitivity, specificity and predictive values of the test (i.e. as the specificity increases, the sensitivity may decrease) and hence an acceptable balance must be struck.

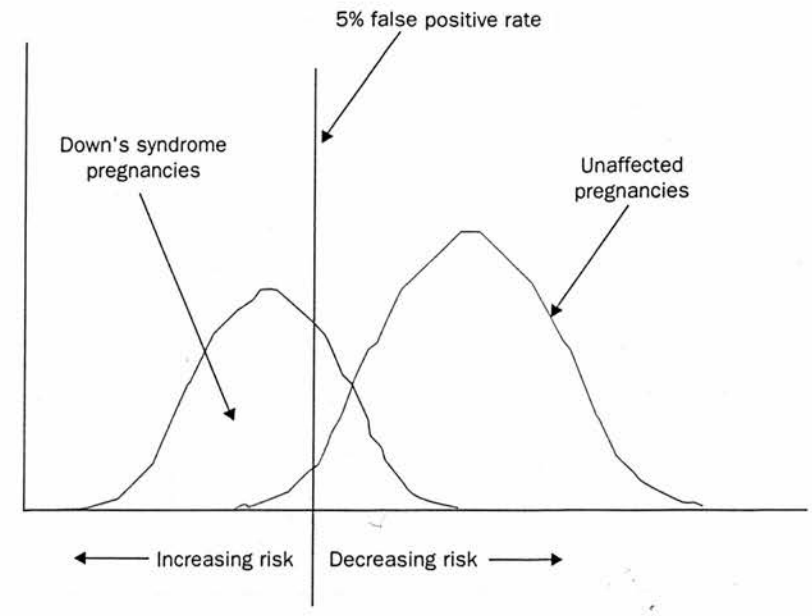

Figure 5 Schematic representation based on serum screening of the risk of having a baby with Down's syndrome.

An amniocentesis rate of 5% is usually considered to be such an acceptable level because of the traditional maternal age factor.

Inhibin is a heterodimer of an α- and a β-subunit. The β-subunit may be either a βA- or a βB-subunit (inhibin A and inhibin B, respectively).

Maternal serum inhibin is raised in Down's syndrome pregnancies compared with unaffected pregnancies and studies have suggested that inhibin A might be used as a first- and second-trimester marker.

The authors of this paper set out to determine if this was the case and specifically evaluated the possibility of incorporating maternal age, αFP, intact hCG or free βhCG (FβhCG) and inhibin A to see if they might detect more cases of Down's syndrome than currently available algorithms.

They analysed stored serum collected between 1987 and 1994 and obtained from 528 pregnant women at 7–18 weeks' gestation in the West of Scotland. Of these, 58 were Down's syndrome pregnancies (eight at 7–12 weeks' gestation and fifty at 13–18 weeks' gestation), 32 were trisomy 18 samples and 438 were control samples. If an ultrasound scan suggested greater than one week's difference compared with menstrual dates, the ultrasound was used to date the pregnancy.

Serum dimeric inhibin A levels were measured using a two-site enzyme-linked immunosorbent assay (ELISA) utilising first a monoclonal antibody directed against the βA-subunit of inhibin and then a further monoclonal antibody directed against the α-subunit of inhibin.

Median and multiples of the median values are already well established for intact hCG and αFP in the West of Scotland population. For inhibin A, the median values and multiples of the median were

calculated from the serum of the control and the study groups.

The authors estimated the detection rates and 95% confidence intervals for Down's syndrome at a fixed 5% false positive rate for combinations of the individual variables (Table 2).

Although inhibin A levels are reduced in trisomy 18 pregnancies, they were not statistically different from the control group.

Inhibin levels in Down's syndrome pregnancies are no different from unaffected pregnancies in the first trimester and the authors of this study could find no difference in inhibin A levels between first-trimester groups either. It was only after 13 weeks' gestation that inhibin A levels in affected pregnancies were significantly greater than controls. If two multiples of the median were used as a cut-off, using inhibin A levels alone (i.e. without age or other markers), 32 of the 50 cases of Down's syndrome might have been detected, although the confidence interval would have been wide.

Currently in the West of Scotland, risk scoring is based on αFP, intact hCG and maternal age. Table 2 demonstrates that the addition of inhibin A might improve detection rates by as much as 20% whilst maintaining a 5% amniocentesis rate.

The incorporation of inhibin A will require large prospective studies to determine if this new marker is as promising as these initial retrospective data suggest. If so, although a test suggesting an 'increased risk' would still require further investigation by amniocentesis, it will be a substantial step forward in prenatal serum screening.

Table 2

Detection rates and 95% confidence intervals (95% CI) for Down's syndrome at a fixed 5% false positive rate.

Variable	Detection rate	95% CI
αFP+Age	*33*	*19–48*
Intact hCG+Age	*41*	*26–47*
FβhCG+Age	*47*	*32–63*
Inhibin A+Age	*48*	*32–63*
αFP+intact hCG+Age	*54*	*38–69*
αFP+FβhCG+Age	*53*	*37–68*
αFP+inhibin A+Age	*57*	*41–72*
Intact hCG+inhibin A+Age	*57*	*41–72*
FβhCG+inhibin A+Age	*68*	*52–81*
Intact hCG+FβhCG+Age	*40*	*25–56*
αFP+intact hCG+inhibin A+Age72	*57–84*	
αFP+FβhCG+inhibin A+Age	*75*	*60–87*
αFP+intact hCG+FβhCG+Age	*52*	*36–67*

Conclusions

1. *Early CVS may be associated with an increased risk of OLH and LRDs and should be avoided prior to 10 weeks' gestation.*
2. *There is little increased risk of chromosomal abnormality in cases of isolated choroid plexus cysts. However, there is much greater risk if the CP cyst is associated with an anatomical abnormality, if the mother is greater than 35 years old or maternal serum screening places the woman at increased risk of carrying a trisomic fetus.*
3. *Screening first-trimester fetuses for increased nuchal translucency associated with Down's syndrome has enormous potential, particularly if it could be combined with first-trimester biochemical screening.*
4. *The introduction of inhibin A into maternal serum screening algorithms has the potential to improve the second-trimester detection of Down's syndrome by 20% whilst maintaining the current rate of invasive prenatal investigations.*

Erratum

The use of lavender oil in pregnancy

In the March 1997 issue of *The Diplomate* we published an article on complementary medicine and childbearing in which a phrase was added in error to the second paragraph on aromatherapy and the use of essential oils.

The author has asked us to point out that lavender oil is classified as an emmenagogue and therefore should be avoided during pregnancy until the last few weeks, and then only used with caution.

References

Adinolfi M., Camporesi C. and Carr T.
Gene amplification to detect fetal nucleated cells in pregnant women
Lancet 1989; **5**: 328-329

Adinolfi M. (a)
Fetal nucleated cells in the maternal circulation.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Adinolfi M. (b)
Breaking the Blood Barrier
Nature Genet 1992; **1**: 316-318

Adinolfi M., Davies A., Sharif S., Soothill P. and Rodeck C.
Detection of trisomy 18 and Y-derived sequences in fetal nucleated cells obtained by transcervical flushing
Lancet 1993; **342**: 403-404

Adinolfi M., Sherlock J., Tutschek B., Halder A., Delhanty J. and Rodeck C.
Detection of fetal cells in transcervical samples and prenatal diagnosis of chromosomal abnormalities.
Prenatal Diagn 1995; **15**(10):943-9

Aitken D.A., Wallace E.U., Crossley J.A., Swanston I.A., van Pareren Y., van Maarle M., Groome N.P., Macri J.N. and Connor J.M.
Dimeric Inhibin A as a marker for Down's Syndrome in early pregnancy
New Eng J Med 1996; **334**: 1231-6

Allen C., Weereratne H., Gale R., Beverley P.C.L. and Huehns E.R.
The use of mAb UCHβ and UCHγ for the antenatal diagnosis of β-thalassaemia
Br J Haematol 1987; **65**: 199-203

Al-Mufti R., Hambley H., Farzaneh F. and Nicolaides K.H.
Investigation of maternal blood enriched for fetal cells: role in screening and diagnosis of fetal trisomies.
Am. J. Med. Genet. 1999; **85**(1):66-75

Anonymous
Randomised trial to assess safety and fetal outcome of early and midtrimester amniocentesis. The Canadian Early and Mid-trimester Amniocentesis Trial (CEMAT) Group.
Lancet 1998; **351**: 242-7

Attwood H.O and Park W.W.
Embolism to the lungs by trophoblast
J Obstet Gynaecol Br Commonw 1961; **68**: 611-17

Bahado-Singh R.O., Kliman H., Feng T.Y., Hobbins J., Copel J.A. and Mahoney M.J.
First-trimester endocervical irrigation: feasibility of obtaining trophoblast cells for prenatal diagnosis.
Obstet. Gynecol. 1995; **85**(3):461-4

Battye F.L. and Shortman K.
Flow cytometry and cell separation procedures
Curr Opin Immunol 1991; **3**: 238-241

Berkowitz R.L.
Should every pregnant woman undergo ultrasonography?
N Eng J Med 1993; **329**: 874-7

Bertoncello I., Bradley T.R. and Watt S.M.
An improved negative immunomagnetic selection strategy for the purification of primitive hemopoietic cells from normal bone marrow
Exp Hematol 1991; **19** (2): 3232-3237

Beverley P.C.L., Linch D. and Delia D.
Isolation of human haematopoietic progenitor cells using monoclonal antibodies
Nature 1980; **287**: 332-333

Bhat N.M., Bieber M.M. and Teng N.N.H.
One step separation of human fetal lymphocytes from NRBCs
J Immunol Methods 1990; **131**: 147-149

Bianchi D.W., Flint A.F., Pizzimenti M.F., Knoll J.H. and Latt S.A.
Isolation of Fetal DNA from Nucleated Erythrocytes in Maternal Blood
Proc. Natl. Acad. Sci. USA 1990; **87** (9): 3279-3283

Bianchi D.W., Stewart J.E., Garber M.F., Lucotte G. and Flint A.F.
Possible effect of gestational age on the detection of fetal nucleated erythrocytes in maternal blood
Prenat Diagn 1991; **11**: 523-528

Bianchi D.W, Mahr A., Zickwolf G.K., Houseal T.W., Flint A.F. and Klinger K.W.
Detection of fetal cells with 47,XY,+21 karyotype in maternal peripheral blood
Hum Genet 1992; **90**: 368-370

Bianchi D.W., Zickwolf G.K., Yih M.C., Flint A.F., Geifman O.H., Erikson M.S. and Williams J.M.
Erythroid-specific antibodies enhance detection of fetal nucleated erythrocytes in maternal blood
Prenat Diagn 1993; **13**: 293-300

Bianchi D.W., Zickwolf G.K., Weil G.J., Sylvester S. and DeMaria M.A.
Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum.
Proc. Nat. Acad. Sci. USA 1996; **93**(2):705-8

Bianchi D.W., Williams J.M., Sullivan L.M., Hanson F.W., Klinger K.W. and Shuber A.P.
PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies.
Am J Hum Genet 1997; **61**(4):822-9

Bischoff F.Z., Lewis D.E., Nguyen D.D., Murrell S., Schober W., Scott J., Simpson J.L. and Elias S.
Prenatal diagnosis with use of fetal cells isolated from maternal blood: five-color fluorescent in situ hybridization analysis on flow-sorted cells for chromosomes X, Y, 13, 18, and 21.
Am J Obstet Gynecol. 1998; **179**(1): 203-9

Bowman J.M. and Pollock J.M.
Transplacental fetal haemorrhage after amniocentesis
Obstet Gynecol 1985; **66**: 749-754

Boyer S.H., Noyes A.N. and Boyer M.L.
Enrichment of erythrocytes of fetal origin from Adult-fetal Blood mixtures via selective hemolysis of adult blood cells: An aid to Antenatal diagnosis of Hemoglobinopathies
Blood 1976; **47** (6): 883-897

Boyer S.H., Siegel S. and Noyes A.N.
Developmental changes in human erythrocyte carbonic anhydrase levels: co-ordinate expression in adult hemoglobin.
Dev Biol 1983; **97**: 250-253

Bøyum A.
Separation of White blood cells
Nature 1964; **204**:793-794

Brock D.J.H.
Early diagnosis of fetal defects.
Current reviews in Obstetrics and Gynaecology, Vol. 2.
In: Brock D.J.H. (ed)
Edinburgh: Churchill Livingstone, 1982

Bruch J.F., Metezeau P., Garcia-Fonknechten N., Richards Y., Tricottet V., Hsi B-L., Kitzis A., Julien C. and Papiernik E.
Trophoblast-like cells sorted from peripheral maternal blood using flow cytometry: A multiparametric study involving transmission electron microscopy and fetal DNA amplification
Prenat Diagn 1991; **11**: 787-798

Brun A., Gaudernack G. and Sandberg S.
A New Method for Isolation of Reticulocytes: Positive Selection of Human Reticulocytes by Immunomagnetic Separation
Blood 1990; **76** (11): 2397-2403

Bryndorf T., Christensen B., Vad M., Parner J., Carelli M.P., Ward B.E., Klinger K.W., Bang J. and Philip J.
Prenatal detection of chromosome aneuploidies in uncultured chorionic villus samples by FISH.
Am. J. Hum. Genet. 1996; **59**(4):918-26

Bulmer J.N., Rodeck C. and Adinolfi M.
Immunohistochemical characterization of cells retrieved by transcervical sampling in early pregnancy.
Prenat Diagn 1995; **15**(12):1143-53

Cacheux V., Milesi-Fluet C., Tachdjian G., Druart L., Bruch J.F., Hsi B.L., Uzan S. and Nessman C.
Detection of 47,XYY trophoblast fetal cells in maternal blood by fluorescence in situ hybridisation after using immunomagnetic lymphocyte depletion and flow cytometry sorting
Fetal Diagn Ther 1992; **7**: 190-194

Camaschella C., Alfarno A., Gattardi E., Travi M., Primignani P., Cappio F.C. and Saglio G.
Prenatal Diagnosis of Fetal Hemoglobin Lepore-Boston Disease on Maternal Peripheral Blood
Blood 1990; **75** (11): 2102-2106

Campagnoli C., Multhaupt H.A., Ludomirski A., Haut M.J. and Warhol M.J.
Noninvasive prenatal diagnosis. Use of density gradient centrifugation, magnetically activated cell sorting and in situ hybridization.
J. Reprod. Med. 1997; **42**(4):193-9

Canadian collaborative CVS-Amniocentesis clinical trial group
Multicentre randomised clinical trial of chorion villus sampling and amniocentesis
Lancet 1989; **1**: 1-6

Chang S.D., Lin S.L., Chu K.K. and Hsi B.L.
Minimally-invasive early prenatal diagnosis using fluorescence in situ hybridization on samples from uterine lavage.
Prenatal Diagn 1997; **17**(11):1019-25

Cheung M.C., Goldberg J.D. and Kan Y.W.
Prenatal diagnosis of sickle cell anaemia and thalassaemia by analysis of fetal cells in maternal blood
Nature Genet 1996; **14**: 264-8

Chitty L.S., Hunt G.H., Moore J. and Lobb M.O.
Effectiveness of routine ultrasonography in detecting fetal structural abnormalities in a low risk population
BMJ 1991; **303**: 165-1169

Chueh J. and Golbus M.S.
The search for fetal cells in the maternal circulation
J Perinat Med 1991; **19**: 411-420

Ciaranfi A., Curchod A. and Odartchenko N.
Survie de lymphocytes foetaux dans le sang maternel pospartum
Schweiz Med Wochenschr 1977; **107**: 134-138

Cohen F., Zuelzer W.W., Gustafson D.C. and Evans M.M.
Mechanisms of Isoimmunization. I. The Transplacental Passage of Fetal Erythrocytes in Homospecific Pregnancies
Blood 1964; **23**: 621-646

Cooke H.J., Schmidtke J. and Gosden J.R.
Characterisation of a human Y chromosome repeated sequence and related sequences in higher primates
Chromosoma 1982; **87**: 491-502

Covone A.E., Mutton D., Johnson P.M. and Adinolfi M.
Trophoblast cells in peripheral blood from pregnant women
Lancet 1984; **1**: 841-843

Covone A.E., Kozma R., Johnson P.M., Latt S.A. and Adinolfi M.
Analysis of peripheral maternal blood samples for the presence of placenta-derived cells using Y-specific probes and McAb H315
Prenat Diagn 1988; **8**: 591-607

Crane J.P.
Genetic amniocentesis.
Progress in Obstetrics and Gynaecology
In: Studd J. (ed)
Edinburgh: Churchill Livingstone, 1983

Davenport D.M. and Macri J.N.
The clinical significance of low maternal serum alpha-fetoprotein
Am J Obstet Gynecol 1983; **146**: 657-661

de Graaf I.M., Jakobs M.E., Leschot N.J., Ravkin I., Goldbard S. and Hoovers J.M.
Enrichment, identification and analysis of fetal cells from maternal blood: evaluation of a prenatal diagnosis system.
Prenatal Diagn 1999; **19**(7):648-52

DeMaria M.A., Zheng Y.L., Zhen D., Weinschenk N.M., Vadnais T.J. and Bianchi D.W.
Improved fetal nucleated erythrocyte sorting purity using intracellular antifetal hemoglobin and Hoechst 33342
Cytometry 1996; **25**: 37-45

Douglas G.W., Thomas L., Carr M., Cullen N.M. and Morris R.
Trophoblast in the circulating blood during pregnancy
Am J Obstet Gynecol 1959; **78**: 960-973

Down J. L. Haydon
Observations on an ethnic classification of idiots
Clin Lect & Rep London Hosp 1866; **3**: 259-263

Durrant L.G., McDowell K.M., Holmes R.A. and Liu D.T.Y.
Screening of monoclonal antibodies recognizing oncofetal antigens for isolation of trophoblasts from maternal blood
Prenat Diagn 1994; **14**: 131-140

Durrant L., McDowell K., Holmes R. and Liu D.
Non-invasive prenatal diagnosis by isolation of both trophoblasts and fetal nucleated red blood cells from peripheral blood of pregnant women
Br J Obstet Gynaecol 1996; **103**: 219-22

Edward R., Dynal UK Ltd.
Personal communication

Eiben B., Trawicki W., Hammans W., Goebel R. and Epplen J.T.
A prospective comparative study on fluorescence in situ hybridization (FISH) of uncultured amniocytes and standard karyotype analysis.
Prenatal Diagn. 1998; **18**(9):901-6

Eiben B., Trawicki W., Hammans W., Goebel R., Pruggmayer M. and Epplen J.T.
Rapid prenatal diagnosis of aneuploidies in uncultured amniocytes by fluorescence in situ hybridization. Evaluation of >3,000 cases.
Fetal Diagn. Ther. 1999; **14**(4):193-7

Elias S., Price J., Dockter M., Wachtel S., Tharapel A., Simpson J.L. and Klinger K.
First trimester prenatal diagnosis of trisomy 21 in fetal cells from maternal blood
Lancet 1992; **340**: 1033

Erslev A.J.
Production of erythrocytes.
Hematology, 4th edition
In: Williams W.J., Beutler E., Erslev A.M. and Lichtman M.A. (eds)
New York: McGraw-Hill, 1990

Evans M.I, Klinger K.W., Isada N.B., Shook D., Holzgreve W., McGuire N. and Johnson M.
Rapid prenatal diagnosis by fluorescent in situ hybridisation of chorionic villi: An adjunct to long-term culture and karyotype
Am J Obstet Gynecol 1992; **167**: 1522-5

Ewigman B.G., Crane J.P., Frigoletto F.D., Lefevre M.I., Bain R.P. and McNellis D.
The effect of prenatal screening on perinatal outcome. RADIUS study group
New Eng J Med 1993; **329** (12): 821-7

Falcinelli C., Battafarano S., Neri C., Mazza V., Ranzi A. and Forabosco A.
Analysis of fetal sex in TCC sample DNA: a contribution to the validation of this approach.
Prenatal Diagn 1998; **18**(11):1109-16

Ferguson-Smith M.A. and Yates J.R.W.
Maternal age-specific rates for chromosome aberrations and factors influencing them: Report of a collaborative European study on 52,965 amniocenteses
Prenat Diagn 1984; **4**: 5-44

Findlay I., Atkinson G., Chambers M., Quirke P., Campbell J. and Rutherford A.
Rapid genetic diagnosis at 7-9 weeks' gestation: diagnosis of sex, single gene defects and DNA fingerprint from coelomic samples
Hum Reprod 1996; **11**: 2548-53

Firth H.V., Boyd P.A., Chamberlain P., MacKenzie I.Z., Lindenbaum R.H. and Huson S.M.
Severe limb abnormalities after chorion villus sampling at 56-66 days' gestation
Lancet 1991; **337**: 762-63

Firth H.V., Boyd P.A., Chamberlain P.F., MacKenzie I.Z., Morriss-Kay G.M. and Huson S.M.
Analysis of limb reduction defects in babies exposed to chorionic villus sampling
Lancet 1994; **343**: 1069-1071

Fisk N. and Bower S.
Fetal blood sampling in retreat
BMJ 1993; **307**: 143-144

Frazier J.L., Caskey J.H., Yoffe M. and Seligman P.A.
Studies of Transferrin Receptor on both Human Reticulocytes and Nucleated Human Cells in culture
J Clin Invest 1982; **69**: 853-865

Gänshirt-Ahlert D. Burschyk M., Garritsen H.S.P., Helmer L., Miny P.Horst J., Schneider H.P.G. and Holzgreve W.
Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood
Am J Obstet Gynecol 1992; **166** (5): 1350-1355

Gänshirt-Ahlert D. Borjesson-Stoll R., Burschyk M., Dohr A., Garritsen H.S.P., Helmer E., Miny P., Velasco M., Walde C., Patterson D., Teng N., Bhat N.M., Bieber M.M. and Holzgreve W.
Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting
Am J Reproduct Immunol 1993; **30**: 194-201

Gänshirt D, Garritsen H., Miny P. and Holzgreve W.
Fetal cells in maternal circulation throughout gestation
Lancet 1994; **343**: 1038-39

Gaudoin M.R. (a)
Non-invasive antenatal diagnosis
Lancet 1993; **342**: 54-55

Gaudoin M.R. (b)
Transcervical flushing and antenatal diagnosis
Lancet 1993; **342**: 989-990

Gaudoin M.R. (c)
Antenatal diagnosis of Down's syndrome
BMJ 1993; 306: 791-2

Gaudoin M.R., Rutovitz D., Piper. J., van Heyningen V., Fantes J., Poole I., Eason P., Perry P. and Hill A.E.
Rapid on-slide location of fetal cells after enrichment from maternal blood
Ann. NY. Acad. Sci. 1994; **731**: 136-7

Gaudoin M.R.
Fetal erythroblasts in maternal blood: Non-invasive antenatal diagnosis
Diplomate 1995; **2** (1): 20-23

Gaudoin M. and Mackenzie F.
Prenatal Diagnosis and Screening
Diplomate 1997; **4** (2): 145-151

Gee A.P., Mansour V.H. and Weiler M.B.
Effects of target antigen density on the efficacy of immunomagnetic cell separation
J Immunol Methods 1991; **142**: 127-136

Goldstein H.
One year of health and social services for adolescents with Down's Syndrome
Soc Psychiatry Psychiatr Epidemiol 1989; **24**: 30-34

Gosden J. and Scopes G.
Uncultured blood cells can be labelled by PRINS and ready for chromosome enumeration analysis one hour after collection
Biotechniques 1996; **21**: 88-91

Greenough A., Yuksel B., Naik S., Cheeseman P. and Nicolaides KH.
First trimester invasive procedures: effects on symptom status and lung volume in very young children.
Pediatric Pulmonology 1997; **24**(6): 415-22

Griffith-Jones M.D., Miller D., Lilford R.J., Scott J. and Bulmer J.
Detection of fetal DNA in transcervical swabs from first trimester pregnancies by gene amplification: a new route to prenatal diagnosis?
Br J Obstet Gynaecol 1992; **99**: 508-511

Grosveld F., Dillon N. and Higgs D.
The regulation of human globin gene expression
Baillière's Clinical Haematology
In: Higgs D.R. and Weatherall D.J. (eds)
London: Baillière Tindall, 1993

Guy K., van Heyningen V., Cohen B.B., Deane D.L. and Steel C.M.
Differential expression and serologically distinct subpopulations of human Ia antigens with monoclonal antibodies to Ia α and β chains.
Eur J Immunol 1982; **12**: 942-948

Hahn S., Kiefer V., Brombacher V., Troeger C. and Holzgreve W.
Fetal cells in maternal blood. An update from Basel.
Eur. J. Obstet. Gynecology. Reprod. Biol. 1999; **85**(1):101-4

Hahnemann N. and Mohr J.
Genetic diagnosis in the embryo by means of biopsy from extraembryonic membranes
Bull Europ Soc Hum Genet 1968; **2**: 23

Hamada H., Arinami T., Kubo Takeshi., Hamaguchi H. and Iwasaki H.
Fetal Nucleated cells in maternal peripheral blood: frequency and relationship to gestational age
Hum Genet 1993; **91**: 427-432

Harper T.A.
The Peripheral Blood Film
London: Butterworths & Co. Ltd., 1968

Hengstschlager M. and Bernaschek G.
Fetal cells in the peripheral blood of pregnant women express thymidine kinase: a new marker for detection
FEBS Lett 1997; **404**: 299-302

Herzenberg L.A., Bianchi D.W., Schröder J., Cann H.M. and Iverson G.M.
Fetal cells in the blood of pregnant women: Detection and enrichment by fluorescence-activated cell sorting
Proc Natl Acad Sci USA 1979; **76** (3): 1453-1455

Holcomb W.L., Gunderson E. and Petrie R.H.
Clinical use of the Kleihauer-Betke Test
J Perinat Med 1990; **18**: 331-337

Holzgreve W., Troeger C., Schatt S., Vial Y., Louwen F., Gloning K. and Hahn S.
Prenatal diagnosis with fetal cells in maternal blood: report of experiences in Basal
Schweize Medizin Wochenschrift 1998;**128**(43):1641-5

Hook E.
Prevalence, risk and recurrence.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Huber K., Wolf H., van Lindern M., Worofkass., Rosen A., Hafner E., Beug H., Philipp K. and Bauer K.
Development of a rapid means of estimating the haemoglobin content of candidate fetal cells isolated from maternal blood using HPLC
Prenat Diagn 1996; **16**: 1011-1019

Iverson G.M., Bianchi D.W., Cann H.M. and Herzenberg L.A.
Detection and isolation of fetal cells from maternal blood using the fluorescence-activated cell sorter (FACS)
Prenat Diagn 1981; **1**: 61-73

Jackson L.G., Wapner R.J. and Brambati B.
Limb abnormalities and chorionic villus sampling
Lancet 1991; **337**: 1423

Jackson L.G., Zachary J.M., Fowler S.E., Desnick R.J., Golbus M.S., Ledbetter D.H., Mahoney M.J., Pergament E., Simpson J.L., Black S. and Wapner R.J.
A randomized comparison of transcervical and transabdominal chorionic-villus sampling
N Eng J Med 1992; **327**: 594-8

Jansen M.W., Brandenburg H., Wildschut H.I., Martens A.C., Hagenaars A.M., Wladimiroff J.W. and Veld P.A.
The effect of chorionic villus sampling on the number of fetal cells isolated from maternal blood and on maternal serum alpha-fetoprotein levels.
Prenatal Diagn. 1997; **17**(10):953-9

Jonasson J. A.
Analysis and interpretation of human chromosome preparations
Human Genetics. A practical approach
In: Rooney D.E. and Czepulkowski B.H. (eds)
Oxford: IRL Press, 1986

Jurkovic D., Jauniaux E., Campbell S., Pandya P., Cardy D.L. and Nicolaides K.H.
Coelocentesis: a new technique for early prenatal diagnosis
Lancet 1993; **341**: 1623-24

Kalousek D.K., Dill F.J., Pantzar T., McGillivray B.C., Yong S.L. and Wison R.D.
Confined chorionic mosaicism in prenatal diagnosis
Hum Genet 1987; **77**: 163-67

Kansas G.S., Muirhead M.J. and Dailey M.O.
Expression of CD11/CD18, Leukocyte Adhesion Molecule 1 and CD44 Adhesion Molecules during normal Myeloid and Erythroid Differentiation in Humans
Blood 1990; **76** (12): 2483-2492

Kennerknecht I., Baur-Aubele S., Grab D. and Terinde R.
First trimester amniocentesis between the seventh and 13th weeks: evaluation of the earliest possible genetic diagnosis
Prenat Diagn 1992; **12**: 595-601

Kirsch-Volders M., Assche E.L-V. and Susanne C.
Increase in the amount of fetal lymphocytes in maternal blood during pregnancy
J Med Genet 1980; **17**: 267-272

Kleihauer E., Braun H. and Betke K.
Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blutausstrichs
Klinische Wochenschrift 1957; **35**: 637

Klinger K.W. Landes G., Shook D., Harvey R., Lopez L., Locke P., Lerner T., Osathanondh R., Leverone B., Houseal T., Pavelka K. and Dackowski W.
Rapid Detection of Chromosome Aneuploidies in Uncultured Amniocytes by Using Fluorescence In Situ Hybridisation (FISH)
Am J Hum Genet 1992; **51**: 55-65

Kratzer P., Golbus M.S., Monroe S.E., Finkelstein D.E. and Taylor R.N.
First-trimester aneuploidy screening using serum human chorionic gonadotrophin (HCG), free αHCG and progesterone
Prenat Diagn 1991; **11**: 751-756

Kurle R.
T3 Cluster Report: CD3
Leucocyte Typing IV, White Cell Differentiation Antigens
Oxford: Oxford University Press, 1989

Langer-Safer P.R., Levine M. and Ward D.C.
Immunological method for mapping genes on Drosophila polytene chromosomes
Proc Natl Acad Sci USA 1982; **79**: 4381-4385

Langlois S. and Wilson R.D.
Non-invasive prenatal fetal testing by analysis of maternal blood
Clin Invest Med 1993; **16** (5): 333-338

Lea T., Vartdal F., Davies C and Ugelstad J.
Magnetic Monosized Polymer Particles for Fast and Specific Fractionation of Human Mononuclear Cells
Scand J Immunol 1985; **22**: 207-216

Lejeune J., Gautier M. and Turpin R.
Etude des chromosomes somatiques de neuf enfants mongoliens (Study of somatic chromosomes from nine mongoloid children)
Comptes rendus hebdomadaires des séances de l'Académie des Sciences 1959; **11**: 248

Levan A.
Chromosomes in cancer tissue
Ann N Y Acad Sci 1956; **63** (5): 774-92

Li H., Gyllensten U.B., Cui X., Saiki R.K., Erlich H.A. and Arnheim N.
Amplification and analysis of DNA sequences in single human sperm and diploid cells
Nature 1988; **335**: 414-417

Liou J-D., Pao C.C., Hor J.J. and Kao S-M
Fetal cells in the maternal circulation during first trimester in pregnancies
Hum Genet 1993; **92**: 309-311

Little M.T., Langlois S., Wilson R.D. and Lansdorp P.M.
Frequency of fetal cells in sorted subpopulations of nucleated erythroid and CD34+ hematopoietic progenitor cells from maternal peripheral blood.
Blood 1997; **89**(7):2347-58

Lo Y-M. D., Wainscoat J.S., Gillmer M.D., Patel P., Sampietro M. and Fleming K.A.
Prenatal sex determination by DNA amplification from maternal peripheral blood
Lancet 1989; **2**: 1363-1365

Lo Y-M. D., Patel P., Sampietro M., Gillmer M.D.G, Fleming K.A. and Wainscoat J.S.
Detection of single-copy fetal DNA sequence from maternal blood
Lancet 1990; **335**:1463-1464

Lo Y-M. D., Bowell P.J., Selinger M., Mackenzie I.Z., Chamberlain P., Gillmer M.D.G., Littlewood T.J., Fleming K.A. and Wainscoat J.S.
Prenatal determination of fetal RhD status by analysis of peripheral blood of rhesus negative mothers.
Lancet 1993; **341**: 1147-48

Lo Y-M. D., Moray A.L., Wainscoat J.S. and Fleming K.A.
Culture of fetal erythroid cells from maternal peripheral blood
Lancet 1994; **344**: 264-265

Lockwood D.H. and Neu R.L.
Cytogenetic analysis of 1375 amniotic fluid specimens from pregnancies with gestational age less than 14 weeks
Prenat Diagn 1993; **13**: 801-805

Loken M.R., Shah V.O., Dattilio K.L. and Civin C.I.
Flow Cytometric Analysis of Human Bone Marrow: I. Normal Erythroid Development
Blood 1987; **69** (1): 255-263

Luck C.A.
Value of routine ultrasound scanning at 19 weeks: a four year study of 8849 deliveries
BMJ 1992; **304**: 1474-8

MacKenzie I.Z.
Termination of pregnancy.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

MacLachlan N.A.
Amniocentesis.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Makrydimas G., Georgiou I., Kranas V., Zikopoulos K. and Lolis D.
Prenatal diagnosis of beta-thalassaemia by coelocentesis.
Molec. Hum. Reprod. 1997; **3**(8): 729-31

Manyonda I.T., Soltys A.J. and Hay F.C.
A critical evaluation of the magnetic cell sorter and its use in the positive and negative selection of CD45RO+ cells
J Immunol Methods 1992; **149**: 1-10

Mavrou A., Colialexi A., Tsangaris G.T., Antsaklis A., Panagiotopoulou P., Tsenghi C. and Metaxotoy C.
Fetal cells in maternal blood: isolation by magnetic cell sorting and confirmation by immunophenotyping and FISH.
In Vivo. 1998; **12**(2):195-200

Merkatz I.R., Nitowsky H.M., Macri J.N. and Johnson W.E.
An association between low maternal serum alpha-fetoprotein and fetal chromosome abnormality
Am J Obstet Gynecol 1984; **148**: 886-891

Mesker W.E., Ouwerkerk-van Velzen M.C., Oosterwijk J.C., Bernini L.F., Golbus M.S., Kanhai H.H., Van Ommen G.J. and Tanke HJ.
Two-colour immunocytochemical staining of γ- and ε-type haemoglobin in fetal red cells.
Prenatal Diagn. 1998; **18**(11):1131-7

Meuer S.C.
T2 Cluster Report
Leucocyte Typing IV, White Cell Differentiation Antigens
Oxford: Oxford University Press, 1989

Mikkelson M. and Brøndum-Nielsen K.
Karyotype analysis and chromosome disorders.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Millar D.S., Davis L.R., Rodeck C.H., Nicolaides K.H. and Mibashan R.S.
Normal Blood Values in the Early Mid-Trimester Fetus
Prenat Diagn 1985; **5**: 367-373

Miny P., Holzgreve W., Horst J. and Lenz W.
Limb abnormalities and chorionic villus sampling
Lancet 1991; **337**: 1423-1424

Modell B. C.
Screening as public policy.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Mueller U.W., Hawes C.S., Wright A.E., Petropoulos A., DeBoni E., Firgairi F.A., Morley A.A., Turner D.R. and Jones W.R.
Isolation of Fetal Trophoblast Cells from the peripheral blood of Pregnant Women
Lancet 1990; **336**: 197-200

Mutton D.E., Ide R., Alberman E. and Bobrow M.
Analysis of national register of Down's syndrome in England and Wales: trends in prenatal diagnosis, 1989-91
BMJ 1993; **306**: 431-2

Nakagome Y., Nagafuchi S. and Nakahori Y.
Prenatal sex determination
Lancet 1990; **335**: 291

Nakagome Y., Seki S., Nagafuchi S., Nakahori Y. and Sato K.
Absence of Fetal Cells in the Maternal Circulation at a Level of 1 in 25,000
Am J Med Genet 1991; **40**: 506-508

Newnham J.P., Evans S.F., Michael C.A., Stanley F.J. and Landau L.I.
Effects of frequent ultrasound during pregnancy: a randomised controlled trial
Lancet 1993; **343**: 887-891

Nicolaides K.H., Snijders R.J.M., Thorpe Beeston J.G., Van den Hof M.C., Gosden C.M. and Bellingham A.J.
Mean red cell volume in normal, anemic, small, trisomic and triploid fetuses
Fetal Ther 1989; **4**: 1-13

Nicolaides K.H., Snijders R.M.J., Gosden C.M., Berry C. and Campbell S.
Ultrasonographically detectable markers of fetal chromosomal abnormalities
Lancet 1992; **340**: 704-707

Nicolini U. and Rodeck C.H.
Fetal blood and tissue sampling.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Northern Regional Survey Steering Group
Fetal abnormality: an audit of its recognition and management
Arch Dis Child 1992; **67**: 770-774

Nyberg D.A., Resta R.G., Luthy D.A., Hickok D.E., Mahony B.S. and Hirsch J.A.
Prenatal sonographic findings of Down syndrome: review of 94 cases
Obstet Gynecol 1990; **76**: 370-377

Orlandi F., Damiani G., Jakil C.Lauricella S., Betolino O. and Maggio A.
The risks of early cordocentesis (12-21 weeks'): analysis of 500 procedures.
Prenat Diagn 1990; **10**: 425-428

Orsetti B., Lefort G., Boulot P., Andreo B. and Pellestor F.
Fetal cells in maternal blood: the use of primed in situ (PRINS) labelling technique for fetal cell detection and sex assessment.
Prenatal Diagn 1998; **18**(10):1014-22

Overton T.G., Lighten A.D., Fisk N.M. and Bennett P.R.
Prenatal diagnosis by minimally invasive first trimester transcervical sampling is unreliable
Am J Obstet Gynecol 1996; **175**: 382-7

Palomaki G.E., Knight G.J., Haddow J.E., Canick J.A., Saller D.N. and Panizza D.S.
Prospective intervention trial of a screening protocol to identify fetal trisomy 18 using maternal serum alpha-fetoprotein, unconjugated oestriol and human chorionic gonadotrophin
Prenat Diagn 1992; **12**: 925-930

Pandya P.P., Goldberg H., Walton B., Riddle A., Shelley S., Snijders R.J.M. and Nicolaides K.H.
The implementation of first trimester scanning at 10-13 weeks' gestation and the measurement of fetal nuchal translucency thickness in two maternity units
Ultrasound Obstet Gynecol 1995; **5**: 20-25

Parks D.R. and Herzenberg L.A.
Fetal Cells from Maternal Blood: Their Selection and Prospects for Use in Prenatal Diagnosis
Methods Cell Biol 1982; **26**: 277-95

Payne R.
The development of leukoagglutins in parous women
Blood 1962; **19**: 411-424

Pilling D., Kitas G.D., Salmon M. and Bacon P.A.
The kinetics of interaction between lymphocytes and magnetic polymer particles
J Immunol Methods 1989; **122**: 235-241

Piper J., Poggensee M., Hill W., Jensen R., Ji L., Poole I., Stark M. and Sudar D.
An automatic fluorescence metaphase finder speeds translocation scoring in FISH painted chromosomes
Cytometry 1994; **16** (1): 7-16

Playfair J.H.L., Wolfendale M.R. and Kay H.E.M.
The leucocytes of peripheral blood in the human foetus
Br J Haematol 1963; **9**: 336-344

Pool C., Aplin J.D., Taylor G.M. and Boyd R.D.H.
Trophoblast cells and maternal blood
Lancet 1987; **1**: 804-805

Price J.O., Elias S., Wachtel S.S., Klinger K., Dockter M., Tharapel A., Shulman L.P., Phillips O.P., Meyers C.M., Shook D. and Simpson J L.
Prenatal diagnosis with fetal cells isolated from maternal blood by multi-parameter flow cytometry
Am J Obstet Gynecol 1991; **165**: 1731-37

Rasmusson A.M., Erikstein B.K., Smeland E., Caignault L. and Funderud S.
A new method for detachment of Dynabeads from positive selected B-lymphocytes
Abstract, British Society of Immunology, 1991; April 10-12

Reading J.P., Huffman J.L., Wu J.C., Palmer F.T., Harton G.L., Sisson M.E., Keyvanfar K., Gresinger T.H., Cochrane W.J., Fallon L.A.
Nucleated erythrocytes in maternal blood: quantity and quality of fetal cells in enriched populations
Hum Reprod 1995; **10** (5): 2510-5

Rearden A. and Chiu P.
Lack of Rhesus antigen expression by human committed erythroid progenitors
Blood 1983; **61** (3): 525-529

Reynolds T.M. and Penney M.D.
The mathematical basis of multivariate risk screening: with special reference to screening for Down's syndrome associated pregnancy
Ann Clin Biochem 1989; **27**: 452-458

Rhine S.A., Cain J.L., Cleary R.E., Palmer C.G. and Thompson J.F.
Prenatal sex determination with endocervical smears: Successful results utilizing Y-body fluorescence
Am J Obstet Gynecol 1975; **122**: 155-160

Royal College of Obstetricians and Gynaecologists
Working party report on routine ultrasound examination in pregnancy.
London (1984)

Sambrook J., Fritsch E.F. and Maniatis T. (eds)
Molecular Cloning. A laboratory Manual. 2nd edition
USA: Cold Spring Harbor Laboratory Press, 1989

Schmorl G.
Pathologische-Anatomische Untersuchen uber Puerperal-eklampsie.
Vogel Leipzig,1893

Schröder J., Schröder E. and Cann H.M.
Lack of response of fetal cells in maternal blood to mitogens and mixed lymphocyte culture
Hum Genet 1977; **38**: 91-97

Sekizawa A., Farina A., Zhen D.K., Wang J.Y., Falco V.M., Elmes S. and Bianchi D.W.
Improvement of fetal cell recovery from maternal blood: suitable density gradient for FACS separation.
Fetal Diagn Ther. 1999 **14**(4):229-33

Selypes A. and Lorencz R.
A non-invasive method for determination of the sex and karyotype of the fetus
Hum Genet 1988; **79** (4): 357-9

Seres-Santamaria A., Catala V., Cuatrecasas E. and Villanueva R.
Fluorescent in-situ hybridisation and Down's syndrome
Lancet 1993; **341**: 1544

Sherlock J., Halder A., Tutschek B., Delhanty J., Rodeck C. and Adinolfi M.
Prenatal detection of fetal aneuploidies using transcervical cell samples.
J. Med. Genet. 1997; **34**(4):302-5

Shettles L.B.
Use of Y chromosome in prenatal sex determination
Nature 1971; **230**: 52-53

Siebers J.W., Knauf I. and Hillemans H.G.
Antenatal sex determination in blood from pregnant women
Humangenetik 1975; **28**: 273-280

Sieff C., Bicknell D., Caine G., Robinson J., Lam G. and Greaves M.F.
Changes in cell surface antigen expression during hemopoietic differentiation
Blood 1982; **60** (3): 703-713

Silverman N.S. and Wapner R.J.
Chorionic villus sampling.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Simpson J.L. and Elias S.
Isolating Fetal cells from maternal blood. Advances in prenatal diagnosis through molecular technology
JAMA 1993; **270**: 2357-2361

Sipes S.L., Weiner C.P., Wenstrom K.D., Williamson R.A. and Grant S.S.
The association between fetal karyotype and mean corpuscular volume
Am J Obstet Gynecol 1991; **165**: 1371-6

Slunga-Tallberg A., el-Rifai W., Keinanen M., Ylinen K., Klinger K., Ylikorkala O. and Knuutila S.
Maternal origin of nucleated erythrocytes in peripheral venous blood of pregnant women
Hum Genet 1995; **96** (1): 53-7

Smid M., Lagona F., Papasergio N., Ferrari A., Ferrari M. and Cremonesi L.
Influence of gestational age on fetal deoxyribonucleic acid retrieval in maternal peripheral blood.
Am J Obstet Gynecol 1997; **177**(6):1517-22

Smidt-Jensen S., Permin M. Philip J., Lundsteen C., Zachary J.M., Fowler S.E. and Grüning K.
Randomised comparison of amniocentesis and transabdominal and transcervical chorionic villus sampling
Lancet 1992; **340**: 1237-44

Smith S.K., Brown V.A., Dewar A.E., Stockdill G., Cohens B. and Maddy A.H.
Abnormalities in the expression of the leucocyte-common antigen in chronic lymphocytic leukaemia
Clin Exp Immunol 1985; **59**: 55-63

Sohda S., Arinami T., Hamada H., Nakauchi H., Hamaguchi H. and Kubo T.
The proportion of fetal nucleated red blood cells in maternal blood: estimation by FACS analysis.
Prenatal Diagn. 1997; **17**(8):743-52

Staples A.J., Sutherland G.R., Haan E.A. and Clisby S.
Epidemiology of Down Syndrome in South Australia, 1960-89
Am J Hum Genet 1991; **49**: 1014-1024

Suzumori K., Adachi R., Okada S., Narukawa T., Yagami Y. and Sonta S.
Fetal cells in the maternal circulation: Detection of Y-sequence by DNA gene amplification
Obstet Gynecol 1992; **80** (1): 150-154

Takeda T. and Nagai B.
Differential Hemolysis Test in the Fetal- and Adult-type Human Erythrocytes
Acta Haematol Jpn 1981; **44**: 828-832

Tanke H.J., Oosterwijk J.C., Mesker W.E., Ouwerkerk van-Velzen M.C., Knepfle C.F., Wiesmeyer C.C., van Ommen G.J., Kanhai H.H. and Vrolijk J.
Detection of 'rare event' fetal erythroblasts in maternal blood using automated microscopy.
Early Hum. Devel. 1996; **47** Suppl:S89-93

Tharapel A.T., Jaswaney V.L., Dockter M.E., Wachtel S.S., Chandler R.W., Simpson J-L., Shulman L.P., Meyers C.M., and Elias S.
Inability to detect fetal metaphases in flow-sorted lymphocyte cultures based on maternal-fetal HLA differences
Fetal Diagn Ther 1993; **8**: 95-101

Thomas M.L.
The Leukocyte Common Antigen Family
Ann Rev Immunol 1989; **7**: 339-369

Thomas M.R., Williamson R., Craft I., Yazdani N. and Rodeck C.H.
Y chromosome sequence DNA amplified from peripheral blood of women in early pregnancy
Lancet 1994; **343**: 413-414

Thomas M.R., Tutschek B., Frost A., Rodeck C.H., Yazdani N., Craft I. and Williamson R.
The time of appearance and disappearance of fetal DNA from the maternal circulation
Prenat Diagn 1995; **15**: 641-6

Tipton R.E., Tharapel A.T., Change H.T., Simpson J.L. and Elias S.
Rapid chromosome analysis using spontaneously dividing cells derived from umbilical cord blood (fetal and neonatal)
Am J Obstet Gynecol 1989; **161**: 1546-1548

Toth T., Findlay I., Papp C., Toth-Pal E., Marton T., Nagy B., Quirke P. and Papp Z.
Prenatal detection of trisomy 21 and 18 from amniotic fluid by quantitative fluorescent polymerase chain reaction.
J. Med. Genet. 1998; **35**(2):126-9

Trask B.J.
Fluorescence in situ hybridisation - applications in cytogenetics and gene mapping
Trends Genet 1991; **7** (5): 34-45

Uciechowski P. and Schmidt R.E.
N3 Cluster Report: CD18
Leucocyte Typing IV, White Cell Differentiation Antigens
Oxford: Oxford University Press, 1989

Valerio D., Aiello R., Altieri V., Malato A.P., Fortunato A. and Canazio A.
Culture of fetal erythroid progenitor cells from maternal blood for non-invasive prenatal genetic diagnosis
Prenat Diagn 1996; **16**: 1073-82

Verjeslev L.O. and Mikkelson M.
The European collaborative study on mosaicism in chorionic villus sampling: Data from 1986 to 1987
Prenat Diagn 1989; **9**: 575-588

Voak D and Williams M.A.
An explanation for the failure of direct antiglobulin test to detect erythrocyte sensitization in ABO haemolytic disease of the newborn and observations on pinocytosis of IgG anti-A antibodies by infant (cord) red cells
Br J Haematol 1971; **20**: 9-23

Wachtel S.S., Sammons D., Manley M., Wachtel G., Twitty G. Utermohlen J., Phillips O.P., Shulman L.P., Taron D.J., Muller U.R., Koeppen P., Ruffalo T.M., Addis K., Porreco R., Murato-Collins J., Parker N.B. and McGavran L.
Fetal cells in maternal blood: recovery by charge flow separation
Hum Genet 1996; **98** (2): 162-6

Wachtel S.S., Sammons D., Twitty G., Utermohlen J., Tolley E., Phillips O. and Shulman L.P.
Charge flow separation: quantification of nucleated red blood cells in maternal blood during pregnancy.
Prenatal Diagn. 1998;**18**(5):455-63

Wald N.J. and Cuckle H.S.
Recent advances for screening for neural tube defects and Down syndrome
Baillière's clinical obstetrics and gynaecology. International practice and research
In: Rodeck C. (ed)
London: Baillière Tindall, 1987

Wald N.J., Cuckle H.S., Densem J.W., Nanchahal K., Royston P., Chard T., Haddow J.E., Knight G.E., Palomaki G.E. and Canick J.A.
Maternal serum screening for Down's syndrome in early pregnancy
BMJ 1988; **297**: 883-887

Wald N.J., Kennard A., Densem J.W., Cuckle H.S., Chard T. and Butler L.
Antenatal maternal serum screening for Down's syndrome: results of a demonstration project
BMJ 1992; **305**: 391-4

Wald N. and Cuckle H.
Biochemical screening.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Walknowska J., Conte F.A. and Grumbach M.M.
Practical and theoretical implications of fetal/maternal lymphocyte transfer
Lancet 1969; **1**:1119-1122

Wang J.Y., Zhen D.K., Falco V.M., Farina A., Zheng Y.L., Delli-Bovi L.C. and Bianchi D.W.
Fetal nucleated erythrocyte recovery: fluorescence activated cell sorting-based positive selection using anti-gamma globin versus magnetic activated cell sorting using anti-CD45 depletion and anti-gamma globin positive selection.
Cytometry. 2000; **39**(3):224-30

Ward B.E., Gersen S.L., Carelli M.P., McGuire N.M., Dackowski W.R., Weinstein M., Sandlin C., Warren R. and Klinger K.W.
Rapid prenatal diagnosis of chromosomal abnormalities by fluorescence in situ hybridisation: Clinical experience with 4500 specimens
Am J Hum Genet 1993; **52**: 854-865

Waye J.S. and Willard H.F.
Chromosome-specific alpha satellite DNA: nucleotide sequence analysis of the 2.0 kilobasepair repeat from the human X chromosome
Nucleic Acids Res 1985; **13**: 2731-2743

Weatherall D.J., Pembrey M.E. and Pritchard J.
Fetal Haemoglobin
Clin Haematol 1974; **3** (2): 467-508

Wessman M., Ylinen K. and Knuutila S.
Fetal Granulocytes in maternal venous blood detected by in situ hybridisation
Prenat Diagn 1992; **12**: 993-1000

Whiteman D.A.H. and Klinger K.
Efficiency of rapid in situ hybridisation methods for prenatal diagnosis of chromosome abnormalities causing birth defects
Am J Hum Genet 1991; **49** (Suppl): A1279

Yokochi T., Brice M., Rabinovitch P.S., Papayannopoulou T. and Stamatoyannopulos G.
Monoclonal antibodies detecting antigenic determinants with restricted expression on erythroid cells: From the erythroid committed progenitor to the mature erythroblast
Blood 1984; **63** (6): 1376-1384

Young I.D.
Incidence and Genetics of Congenital Malformations.
Prenatal Diagnosis and Screening.
In: Brock D.H., Rodeck C.H. and Ferguson-Smith M.A. (eds).
Edinburgh:Churchill Livingstone, 1992

Zeitune M., Aitken D.A., Crossley J.A., Yates J.R.W., Cooke A. and Ferguson-Smith M.A.
Estimating the risk of a fetal autosomal trisomy at mid-trimester using maternal serum alpha-fetoprotein and age: a retrospective study of 142 pregnancies
Prenat Diagn 1991; **11**: 847-857

Zheng Y.L., Ferguson-Smith M.A., Warner J.P., Ferguson-Smith M.E., Sargent C.A. and Carter N.P.
Analysis of Chromosome 21 copy number in cultured amniocytes by Fluorescence in situ Hybridisation using a cosmid contig
Prenat Diagn 1992; **12**: 931-943

Zheng Y-L., Carter N.P., Price C.M., Colman S.M., Milton P.J., Hackett G.A., Greaves M.F. and Ferguson-Smith M.A.
Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting
J Med Genet 1993; **30**: 1051-6

Zilliacus R., de la Chapelle A., Schröder J., Tiilikainen A., Kohne E. and Kleihauer E.
Transplacental Passage of Foetal Blood Cells
Scand J Haematol 1975; **15**: 333-338