

TROPHOBLAST SAMPLING IN FIRST TRIMESTER
PREGNANCIES FOR EARLIER ANTENATAL DIAGNOSIS

by

A

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A Thesis submitted for the Degree of
Doctor of Philosophy
in the
University of London

May 1982

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ABSTRACT

The aim of this research was to explore the potential of trophoblast sampling as a source of fetal cells for antenatal diagnosis.

For this purpose, endocervical aspirates were obtained from women who were to have therapeutic terminations in the first trimester of pregnancy. Histological and cytological studies were carried out to establish whether trophoblast was present in the endocervical samples. The aspirates were found to contain both decidua and trophoblast.

The aspirates were grown in vitro and cytogenetic and tissue typing studies carried out on the cultured cells. It was found that trophoblast cells were overgrown by the maternal cells in vitro due to the slow growth of intact trophoblast villi in culture.

A technique was developed to obtain rapidly growing cultures of trophoblast cells. Using suction curettage material as a source of trophoblast villi for these experiments, a method of trypsinisation and filtration was developed, which destroyed the outermost syncytial cells of the trophoblast villi and separated the cytotrophoblast layer from the mesenchyme core of the villi. A cell suspension of mesenchyme cores was obtained by further trypsinisation and cultures were prepared. Rapid growth of the mesenchyme cells was observed. Cytogenetic studies and HLA typing were also carried out on these cells.

This technique of trypsinisation and filtration was then applied to a further series of 25 endocervical aspirates obtained from first trimester pregnancies and pure cultures of mesenchyme cells, suitable for chromosome and tissue typing studies were obtained within 18 days.

These findings suggest that trophoblast sampling could have a place as a method of diagnosing fetal abnormality in early pregnancy. Further studies of the safety and reliability of the technique are in progress at this hospital.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr D V Coleman who enabled me to carry out this research project and am deeply grateful for her invaluable help and advice during the period of research.

I would like to express my thanks to Mr F E Loeffler, Consultant Gynaecologist at St Mary's Hospital for generously supplying me with the trophoblast material and fetal tissues.

My sincere thanks are also due to Professor K Porter, Department of Pathology at St Mary's Hospital and Professor R Williamson, Department of Biochemistry at St Mary's Hospital for their help during the period of my study.

I am also grateful to Dr B Hartley, Senior Registrar in Histopathology at St Mary's Hospital for his help with the histological studies.

Lastly I would like to thank my colleague, Richard Girdler, for his co-operation and encouragement, without which I could not have completed this thesis.

STATEMENT OF PROBLEM AND AIM
OF THE RESEARCH

Several large studies have shown that in skilled hands, the technique of mid-trimester amniocentesis is a safe and reliable method of obtaining fetal cells for the prenatal diagnosis of congenital abnormality. One of the disadvantages of the technique however, is that it cannot safely be carried out before the 16th week of pregnancy, so that if termination is indicated as a result of the tests on the fetal cells, it cannot be performed until the pregnancy is well advanced into the second trimester. Late termination, particularly when it is carried out after the 20th week of pregnancy, is a cause of considerable mental stress to the mother and increases the risks of complication of termination such as haemorrhage and sepsis. For these reasons alternative methods of obtaining fetal cells earlier in pregnancy have been sought.

One approach that has been explored has been the collection of placental villi from first trimester pregnancies by endocervical aspiration. It has been claimed that fetal cells can be successfully cultured from the aspirates and antenatal diagnosis can be achieved earlier in pregnancy by this method. The aim of this research is to evaluate endocervical aspiration in first trimester pregnancies as a source of fetal cells and to determine whether pure cultures of fetal cells can be obtained from the aspirated material. A further objective is to investigate the potential value of this approach as a method of antenatal diagnosis in early pregnancy.

CHAPTER I

METHODS OF ANTENATAL DIAGNOSIS

SECTION A

INTRODUCTION

During the past few years a number of methods of diagnosing fetal abnormality in utero have been developed. Some of the techniques used to obtain information on the unborn fetus are invasive, ie. they necessitate crossing or biopsing fetal membranes to obtain the material for study. Others are non-invasive methods, eg. ultrasound scan and fetal cell sorting in the maternal blood. Some of the techniques which have been developed, such as amniocentesis, are now applied routinely in clinical practice, whereas methods such as fetal cell sorting are in their experimental stages. In this review, only techniques which involve the analysis of fetal cells are discussed.

SECTION B

AMNIOCENTESIS

1. Historical Perspective

In his History of Animals, Aristotle, referred to the possibility of predicting the sex of the unborn child by such criteria as the side on which fetal movements are felt or even the general condition of the mother (Emery, 1973). In fact only recently have precise techniques been developed for obtaining information about the sex, or genetic constitution or general health of the human fetus in utero. Withdrawal of liquor amnii

after puncture of the amniotic sac has been used therapeutically in polyhydramnios for more than a century. The use of amniocentesis for diagnostic purposes is more recent and its potential value has become apparent only during the last two decades.

One of the earliest studies was carried out by Bevis (1952) who made a systematic attempt to study the amniotic fluid when he reported on the constituents of the fluid in a case of erythroblastosis fetalis at the 28th week of pregnancy. The studies were repeated at fortnightly intervals until delivery.

Other early studies were carried out by Makowski et al (1956) and Shettles (1956), who reported almost simultaneously that fetal sex could be determined by examination of sex chromatin in amniotic fluid cells. These studies were made on fluid obtained at term either during caesarean section or when the membranes were ruptured for induction of labour. Sex chromatin investigation for the determination of the sex of the fetus for antenatal diagnosis was first reported by Riis and Fuchs (1960), on 2 pregnant mothers with family histories of haemophilia. Both patients were in the 16th week of pregnancy when 12 ml of clear yellow amniotic fluid was withdrawn by fine needle aspiration through the anterior vaginal fornix. This fluid was replaced by a similar quantity of sterile 0.9% saline. The patients were given a sedative but no anaesthesia was used and progesterone 100 mg was given in oil intramuscularly daily. In both these patients the majority of the cells were sex chromatin positive, and at term female babies were delivered.

Later Steele and Breg (1966) obtained samples of amniotic fluid by abdominal amniocentesis from patients with a suspected erythroblastic fetus, at 20 - 37 weeks gestation. These authors

were able to show that it was possible to determine the sex of the fetus from the cells present in the fluid. They also showed that amniotic fluid contained viable cells, which were fetal in origin and could be grown in tissue culture in sufficient quantity to be karyotyped. Transabdominal amniocentesis was also reported by Jacobson and Barter (1967) in a series of 85 patients who had a high risk of carrying a fetus with genetic disorders. Fifty six of the pregnancies were less than 20 weeks duration. They described a systematic culture procedure for the cells obtained from these amniotic fluids and claimed that tissue culture was successful in nearly 83% of the cases. These studies encouraged other workers to investigate the problems associated with amniocentesis, and to determine the optimum timing for the procedure and other aspects of the technique. Nadler and Gerbie (1970) found that amniocentesis was best performed between the 14th and 16th weeks of pregnancy. They found that aspiration of amniotic fluid was more difficult and probably more hazardous before the 14th week of pregnancy. At 16 weeks gestation there are about 1.2×10^4 cells/ml of amniotic fluid, (Hahnemann, 1972). It has been estimated that only 1 in 2,000 cells ^{is} viable, (Felix et al, 1974). The number of viable cells appears to increase slightly as the pregnancy progresses; but before the 15th week, there are comparatively fewer viable cells in the fluid and cultures attempted in these early specimens are less likely to be successful. Samples obtained after 16 weeks lead to termination late in the second trimester and limited the opportunity for taking repeat samples if this was required. Milunsky (1973) recommended that 10 - 20 ml of amniotic fluid was the optimum

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volume which could safely be aspirated for genetic studies at 16 weeks. According to Fuchs (1966) the amniotic fluid volume at 16 weeks gestation was about 200 ml. He noted an increase in volume of about 25 ml per week from the 11th week of pregnancy, and thereafter to the 15th week the volume continued to increase at a rate of 50 ml per week from the 10th to the 28th week (Wagner and Fuchs, 1962). Total liquor accumulation in utero during pregnancy was found to be about 4 litres (Seeds, 1974).

As experience with amniocentesis increased the problems and pitfalls of the technique became more apparent. Although rare, errors of diagnosis were detected. These were due to 3 main causes - contamination of the culture by maternal cells or mycoplasmas, culture failure due to infection or blood or scanty cell content and errors of laboratory interpretation. The exact origin of maternal cells found in amniotic fluid cell cultures is uncertain but one possibility may be the poorly fitting stylet in the amniocentesis needle will allow tiny fragments of maternal tissue to enter the space between stylet and the needle. Ferguson-Smith (1976) reported misdiagnosis of 3 of 517 amniotic samples as a result of maternal contamination and Milunsky (1979) reported 2 cases in 2,000 where the cells in the culture were of maternal origin and the progeny were males. Another source of error in diagnosis is the mycoplasma contamination. A survey of established cell strains from different laboratories in 1973 revealed that up to 60% of cultures were contaminated with mycoplasma despite the most meticulous attention to prevent contamination (Hayflick, 1965). The problem is compounded by the fact that infected cells may grow well and even appear to have normal morphology by light microscopy yet chromosome abnormalities may

result from this infection. Chromosome abnormalities due to mycoplasma may be suspected on the grounds of their bizarre nature. Abnormalities caused by mycoplasma infections are mostly composed of inconsistent chromosome breaks (Allison and Paton, 1966). Another possible cause of erroneous diagnosis is that of spontaneous mosaicism of the cell culture. The exclusion of true mosaicism can be extremely difficult when a hypo or hyperdiploidy is found in a few cells in culture. We have encountered mosaicism in the amniotic fluid cells grown in cultures from a 41 year old patient. Amniocentesis was carried out at 17 weeks gestation and a slow, epithelial type of cell growth was obtained. No chromosome spreads were obtained in the first 2 harvestings, but a Turner mosaic 45,X0/46,XX pattern was observed in 55% of the cells studied from a third harvesting after a prolonged culture. The patient eventually decided to continue with the pregnancy.

As accounts of successful diagnostic amniocentesis were reported from many countries throughout the world, further advances in this field came with the development of ultrasonography. In 1972, Curtis et al reported localizing the placenta prior to amniocentesis with the aid of ultrasound. They performed transabdominal amniocentesis on 39 patients at gestation periods ranging from 22 - 40 weeks and the placenta was localized in 95% of the cases. Later Harrison et al (1975) carried out a comparative study to evaluate the effects of amniocentesis at various stages of pregnancy with or without prior placental localisation by ultrasound. They performed blind transabdominal amniocentesis on 131 patients and performed ultrasound scan prior to amniocentesis on 107 patients. All

these patients were in the mid or late stages of pregnancy. Their results showed that the use of ultrasound considerably decreased the number of attempts made to obtain the amniotic fluid, thereby reducing the incidence of complications. Furthermore, ultrasound permitted measurements of the distance from maternal skin to the pool of liquor amnii thereby allowing the selection of an appropriate needle for obtaining the sample. There was also a significant reduction in the number of blood-stained taps in the patients who received ultrasound scan prior to amniocentesis. Bloody taps were obtained in 4% of the ultrasound group, compared to 25% in the control group, which demonstrated that prior to amniocentesis, placental localization by ultrasound significantly reduced the chances of fetal haemorrhage. In this series the use of ultrasound also helped to decrease the incidence of fetomaternal transfusions.

Against this background of successful studies and evidence that complications following second trimester amniocentesis were rare, 3 major national collaborative studies were undertaken to evaluate the risks and accuracy of second trimester amniocentesis. The first complete study was reported from the United States in 1977. The second study was carried out by the Medical Research Council in Canada. The findings of the Canadian study group were reported in detail in 1977. The third study was performed by the working party on amniocentesis sponsored by the Medical Research Council of Great Britain. Their report was published in 1978. The U.S. study investigated 1,040 pregnant women who had amniocentesis and 992 matched controls. The main aims of this study were to determine the stage of gestation when most amniocenteses were performed and the effect of the procedure on the fetus.

They also investigated whether adverse effects could be detected in infancy. This study revealed no statistically significant difference between the amniocentesis group and controls in the rate of fetal loss, ie. 3.5% for the subjects and 3.2% for the controls; and no adverse effects were noted on the fetus or neonate. The Canadian study investigated 1,020 subjects who had had amniocentesis. This study did not include a control group but compared the results in the study group with the results of other large studies. Their follow up of the cases ceased after the neonatal examination. They reported the fetal loss rate to be 3.3% which included spontaneous abortions and still-births, a figure remarkably similar to that in the U.S. study.

The British study made an assessment of the effects of amniocentesis on the mother and fetus comparing the course of pregnancy, labour, delivery and the progress of the fetus in a group of 2,428 women who had an amniocentesis with a group of 2,428 who did not. Amniocentesis in the study group was performed before the 20th week of pregnancy. Cases where fetal abnormality was detected as a result of amniocentesis were excluded from the study. The cases and controls were matched for age, parity and obstetric history. No evidence was found of any systematic bias between matched subjects and controls which could explain the excess incidence of certain abnormalities such as respiratory difficulties at birth and orthopaedic postural deformities among the amniocentesis subjects. The incidence of most complications of pregnancy and labour was similar in the subjects and in the matched controls. This study estimated that the fetal loss in the amniocentesis group was about 1.0 to 1.5%.

2. Use of Amniotic Fluid Cells for Antenatal Diagnosis

In the mid 1950's several laboratories reported almost simultaneously that fetal sex could be determined by examination of sex chromatin in amniotic fluid cells. Some 10 years later, Klinger in 1965 (communication with Steele and Breg, 1965) showed that it was possible to culture amniotic fluid cells and study their chromosome complement. Later Nadler (1968) showed that cultured amniotic fluid cells could also be used for the antenatal diagnosis of certain biochemical disorders. Currently, amniotic fluid cells are used for cytogenetic studies, enzyme assays, tissue typing and DNA analysis of the fetus. Antenatal diagnosis based on nuclear sexing is rarely carried out now.

a) Nuclear Sexing and Morphology

In 1949 Barr and Bertram noted the morphological difference between the neurons of male and female cats. They described a distinctive chromocentre, which they called the nucleolar satellite, in the nuclei of neurons of female cats but not in the nuclei of male cats. This was termed the sex chromatin or Barr body. In 1953, Moore et al were able to demonstrate sex chromatin in human skin sections. Later Sachs et al (1956) demonstrated the sex chromatin in smears prepared directly from the amniotic fluid.

Floyd et al (1969) modified the method of studying sex chromatin by centrifuging the amniotic fluid to obtain a concentrated cell button. Ferguson-Smith et al (1971) improved the preparations by filtration of the liquor and fixation of the trapped cells on the filter. One of the drawbacks to the simple and quick examination of the nuclei

of amniotic fluid cells for sex chromatin bodies is the small number of cells in the fluid in early pregnancy. In addition there are anucleate cells and cells with pyknotic nuclei in which nuclear details cannot be seen. Emery, (1973) has pointed out that fetal sexing from amniotic fluid cells could be unreliable and it is no longer widely used for antenatal sexing of the fetus. In addition to nuclear sexing, antenatal diagnosis has also been attempted on the basis of the morphology of the amniotic fluid cells and their growth patterns in vitro. Gosden et al (1977) described several different cell types in the amniotic fluid, which include long bipolar cells, giant multinucleate cells with prominent vacuoles and mononuclear cells with multiple filamentous pseudopodia. These amniotic fluids were obtained from pregnancies where the fetus was anencephalic. They observed that these cells adhered rapidly when inoculated into tissue culture flasks. Gosden et al (1977) also showed that these rapidly adhering cells were very similar to those found in smears prepared from the brain or spinal cord and suggested that they had a neural origin. They suggested that the presence of these rapidly adhering cells in amniotic fluid was an indication of neural tube defect in the fetus.

Bobrow et al (1978) suggested that false positive reports of neural tube defect had been given using rapidly adhering cells as a marker of fetal abnormality, as rapidly adhering cells were present in amniotic fluid from normal pregnancies. Chapman et al (1981) attempted to identify the rapidly adhering cells described by Gosden. They prepared cytocentrifuge preparations of the cells in amniotic

fluid obtained from fetuses with neural tube defects. The smears were fixed in isopropanol and stained by the Papanicolaou method. They observed numerous nucleated cells in the smears which showed morphological features consistent with a neural origin. They also prepared frozen and paraffin embedded sections of tissue from the surface of the fetal neural tube and observed that the cells in these sections were similar to those obtained in the cytopsin preparations. They attempted to confirm the neural origin of the cells in the amniotic fluid by glial stains but were unable to do so. They recorded that the cells seen in amniotic fluids from pregnancies with fetuses with neural tube defects could not always be differentiated from nucleated red blood cells.

b) Tissue Culture and Karyotyping of Amniotic Fluid Cells

After the first successful attempt to culture and karyotype the amniotic fluid cells by Klinger (1965) and Steele and Breg (1966), amniocentesis for cytogenetic studies soon became an accepted method for the diagnosis and management of certain high risk pregnancies. One of the first reports of cytogenetic analysis of amniotic fluid for diagnostic purposes was prepared by Nadler (1971), who karyotyped amniotic fluid cells from 255 patients. Nadler detected 16 Down's Syndrome and 5 other chromosome anomalies in those 255 high risk cases. Polani (1971) carried out cytogenetic studies on amniotic fluid cells from 159 high risk pregnancies and 13 cases of Down's Syndrome were detected. Ferguson-Smith et al (1971) reported the results

obtained from amniotic cell cultures in 30 patients referred for genetic counselling. In their series, each of the ~~was~~ ^{was} patients ~~were~~ extremely anxious about the outcome of ~~their~~ ^{her} ~~pregnancy~~ ^{her} as several patients had initially requested termination of pregnancy. The overall effect of amniocentesis was to save the pregnancies so that termination was indicated in only one case. In this case the mother was a carrier of X-linked granulomatous disease where the fetus was found to be male with a 50% risk of being affected. Subsequently the fetus was found also to have Down's Syndrome. These authors thus confirmed that prenatal genetic studies had an important role in genetic counselling.

c) DNA Analysis of the Amniotic Fluid Cells

The last decade has seen rapid progress in the development of reliable methods for the identification of genetic disorders in the human fetus by direct gene analysis. This progress has resulted from the work of Kan and Dozy (1978) who identified DNA polymorphism in a beta gene that was linked to the sickle gene. The identification of these polymorphic restriction sites in the beta gene cluster made possible the antenatal diagnosis of sickle cell anaemia and beta thalassaemia by linkage analysis studied in selected families. The gene analysis can be carried ^d out on DNA ^g ^{ad} samples of 10 g or less and has been successfully applied to amniotic fluid cells. It is probable that other single gene disorders will be detected by linkage analysis once the appropriate DNA probes are found.

d) Biochemical Studies for the Prenatal Diagnosis of Inborn Errors of Metabolism

Technical advances in somatic cell genetics during the 1960's and 1970's permitted the prenatal diagnosis of a number of different inborn errors of metabolism (Milunsky and Littlefield, 1972; Milunsky, 1973). This is mainly due to the ability of the culture of fibroblasts to retain their biochemical phenotype through successive subcultures. This facilitates the demonstration of diminished or absent enzyme activity or the detection of excess amounts of stored metabolites which is characteristic of a particular disease.

Fresh amniotic fluid cells are usually not available in sufficient numbers for such studies. Cultured amniotic fluid cells constitute the most reliable source of cells for prenatal diagnosis. Almost all biochemical disorders diagnosable in utero are autosomal recessive and currently it is possible to make the prenatal diagnosis of over 100 different biochemical genetic disorders (Milunsky, 1979).

e) Tissue Typing of Amniotic Fluid Cells for the Detection of HLA Antigens

The HLA phenotype is a readily identifiable genetic marker which can be detected on all nucleated cells and there-

fore could be useful in prenatal diagnosis. Several studies have established the clear expression of HLA antigens on amniotic fluid cells and have suggested its clinical usefulness. Jonasson et al (1972) reported using cytotoxic method for the detection of HLA antigens on cultured amniotic fluid cells. Mattiuz et al (1974) carried out HLA typing of cultured amniotic fluid cells using the fluorochromasia technique described by Bodmer et al (1967). Pollack et al (1979) used a cytotoxic method for HLA typing as a primary test for the prenatal diagnosis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Linkage between the inherited abnormality and the HLA provided a method of detecting an affected fetus antenatally. HLA typing of amniotic fluid cells has also been used for the prenatal diagnosis of another HLA linked disease, complement C4 deficiency (Pollack et al, 1980). Furthermore, because of the genetic polymorphism of the HLA system, tissue typing of amniotic fluid cells has also been used for prenatal paternal ascertainment (Jonasson et al, 1972). It has also been suggested that tissue typing of cultured amniotic fluid cells could be used in routine cytogenetic laboratories in those cases where maternal contamination of the amniotic fluid is suspected (Niazi et al, 1979).

Although all these reports established the clear expression of HLA antigens on amniotic fluid cells Pollack et al (1981) have recently reported on the technical and theoretical difficulties in the application of this technique. The variable expression of HLA B locus antigens on some amniotic cell cultures makes it difficult to obtain

complete haplotypes in some cases. Moreover, since HLA B locus antigens are found in significantly increased frequencies among patients with 21-hydroxylase deficiency there is a relatively high incidence of HLA B homozygosity among the patients. Akle et al (1981) reported that the cultured epithelial cells in human amniotic fluid cells do not express HLA antigens, although cultured amniotic fibroblasts do. They suggested that the variable expression of HLA antigens noted by Pollack et al on cultures of amniotic fluid cells may be due to mixed cell populations.

SECTION C

FETOSCOPY

1. Historical Perspective

Antenatal diagnosis by direct visualisation of the fetus was first attempted by Westin (1954). He introduced an instrument (which he termed a hysteroscope with a diameter of 10 mm) through the endocervical canal of the pregnant uterus in patients who were to have therapeutic abortion, in order to visualise parts of the live fetus. This technique required dilatation of the cervix and rupture of the membranes prior to insertion of the instrument. In 1967 direct visualisation of the fetus advanced a step when Mandelbaum et al attempted fetoscopy by a percutaneous transabdominal route. These workers used a fibroptic amnioscope with a 2 site entry into the amniotic cavity, one site for illumination and one site for viewing. Further advances in transabdominal fetoscopy were made by Valenti (1972) and Scrimgeour (1973) who applied the technique

to antenatal diagnosis. Both these workers exposed the uterus by an abdominal incision in patients undergoing abortive hysterectomy at 15 - 20 weeks gestation. Valenti (1972) used a 6 mm diameter modified cystoscope provided with biopsy forceps and obtained skin biopsies under direct vision from deltoid and gluteal areas in 6 pregnancies prior to termination.

Scrimgeour (1973) also attempted antenatal diagnosis in women who were at risk of having fetuses with severe central nervous system defects. Six women were investigated using a fiberoptic endoscope, 7.2 mm diameter. Of these 6 cases, one fetus could not be seen and the pregnancy was terminated. A second pregnancy aborted 2 days after fetoscopy. In the third pregnancy the fetus was only partly seen and at birth was proved to have a small spina bifida. The other 3 fetoscopies were uncomplicated and the babies were normal. Since these pioneer studies, the technique of fetoscopy has been widely explored and optimal conditions for fetoscopy have been clearly defined. Milunsky (1979) has summarised the advances made in the last 7 - 10 years. A fetoscope usually consists of a rigid endoscope 1.7 mm in diameter and 15 - 20 cm long. It has a depth of focus of about 2 cm and an angle of visualisation of about 70°. Accurate ultrasound information is very important for successful fetoscopy. Prior to the insertion of the endoscope, static or real-time sonography is used to identify the gestational age of the fetus, the dimensions and site of the placenta, the umbilical cord insertion on the placenta, the position and lie of the fetus and the pockets of amniotic fluid, which will allow safe entry into the amniotic cavity. The possible presence of twins, fibroid tumours or other unsuspected

anatomical variations that could complicate the procedure are defined.

For the safety of the fetus, fetoscopy is rarely attempted before the 15th week of pregnancy as the uterus and the amniotic fluid volume are small. Between 15 - 18 weeks the fetus remains relatively small compared to the surrounding amniotic fluid volume and the amniotic fluid is usually very clear. This period is ideal for visualising major surface landmarks of the fetus.

Once a site has been chosen the cannula containing a sharp trocar is pushed through the abdominal and uterine walls into the amniotic cavity. The trocar is removed, a sample of amniotic fluid is withdrawn, and the fetoscope is introduced through the cannula. At present most fetoscopy is being done with local anaesthesia only, at the entry site of the instrument.

Fetoscopy can be used for antenatal diagnosis. Not only physical examination of the fetus is possible by this method; it is also possible to obtain fetal blood and fetal skin. One of the most recent evaluations of the scope of this method of antenatal diagnosis has been reported by Rodeck (1980) who sampled pure fetal blood and fetal skin and performed fetal examination by fetoscopy on 145 patients for diagnostic purposes. Fetal examination was performed between 15th - 17th week of pregnancy and blood samples were obtained from fetuses of 18 - 20 weeks gestation. Using real-time and beta-scan ultrasound facilities and a particle size analyser (Coulter channelyzer) the author claimed to have obtained pure fetal blood in 95% of the cases. He also reported a fetal loss rate of 3.7% due to fetoscopy.

2. Antenatal Diagnosis of Fetal Abnormality Using Fetoscopy

a) Phenotypic Studies

Benzie (1977), Perry (1978) and Milunsky (1979) have independently investigated a total of approximately 250 fetuses between 15 - 20 weeks gestation. All these groups of investigators used a 1.7 mm diameter rigid endoscope and claimed to have identified specific parts of the fetus but failed to examine the total surface anatomy. Limbs, joints and digits were most easily recognised. Well-developed fingernails, toenails, details of skin surface including dermal ridges, hair follicles and sweat pores could be well identified. External genitalia were often seen but sex determination was difficult. Total visualisation of the back or the entire length of the spine was found to be difficult because only a small area could be scanned, although certain fetal behaviours were witnessed through the fetoscope. Benzie (1977) reported thumb sucking and opening and closing of the mouth. Milunsky (1979) reported many quick limb movements of the hands and fingers.

Mahoney and Hobbins (1977) reported the first antenatal diagnosis based on anatomical observation. They succeeded in identifying a fetus with Ellis-van-Crevald Syndrome. The diagnosis was suspected from ultrasound measurements of limbs and bones and then confirmed by seeing a sixth digit. Benzie (1978) used the fetoscope prior to planned abortion of an abnormal fetus diagnosed to be trisomy 13 and was able to identify polydactyly and cleft palate. Rodeck and Campbell (1978) have reported seeing low spina bifida defects in 3 pregnancies in which high alpha-fetoprotein levels and ultrasound abnormalities had been detected.

b) Fetal Blood Sampling

Valenti (1973) was among the first to attempt fetal blood sampling for antenatal diagnosis during the second trimester of pregnancy using a fetoscope. He used a 27 gauge needle and aspirated 0.1 - 0.2 ml umbilical vein blood to demonstrate the potential for diagnosing fetal haemoglobinopathies. The next advance in fetal blood sampling came with the development of a much smaller instrument or needlescope with a diameter of 1.7 mm (Patrick et al, 1974). These authors used the instrument on 7 patients who were undergoing therapeutic termination between the 16 - 20 weeks of pregnancy. With the help of ultrasonography the placenta was located and a suitable site was selected on the anterior abdominal wall. Under local anaesthesia the cannula was inserted first, then the fetoscope was introduced and 1 - 3 ml of blood was aspirated from the veins coursing over the fetal surface of the placenta. The blood was studied for hexosaminidase A and B and no attempt was made to carry out cytogenetic analysis.

Later Hobbins and Mahoney (1975) were able to obtain fetal blood samples suitable for studies of haemoglobin synthesis from 23 fetuses of 15 - 22 weeks gestation. The mothers were candidates for termination of pregnancy. These authors reported inserting a sharp cannula with trocar in place under local anaesthesia using ultrasound control. They also reported to have observed 14 patients in whom abortion was delayed for 24 hours. None of these patients exhibited any evidence of labour and in none did the membranes rupture or vaginal bleeding occur.

The first antenatal diagnosis of beta-thalassaemia was reported by Alter et al (1976) in 2 fetuses of 23 and 19 weeks gestation. These diagnoses were made on fetal blood obtained at fetoscopy. The first fetus appeared to have beta-thalassaemia trait and the second fetus was diagnosed to be normal. Both fetuses reached term without complications in pregnancy.

With the development of fetal blood sampling techniques, antenatal diagnosis for many of the major haemoglobinopathies, including beta-thalassaemia and sickle cell anaemia has become an established procedure (Mahoney and Hobbins, 1978; Fairweather and Modell, 1978; Golbus and Kan, 1978).

c) Skin Biopsy

Samples of skin have also been successfully taken with the aid of the fetoscope.

Hobbins and Mahoney (1976) were able to obtain skin biopsies from fetuses from patients who were candidates for therapeutic termination. The fetuses were of 18 - 20 weeks gestation. They claimed that the technique was simple to perform and rapid growth of tissue was obtained within 5 - 8 days. Adequate cells were obtained for karyotyping and enzyme essays.

Mahoney and Hobbins (1978) reported a case where a skin biopsy was obtained from a 17 week old fetus at risk for the autosomal recessive harlequin type of congenital ichthyosis. After skin and nails were judged to be normal by direct visualisation, skin biopsy was obtained from the flank of the fetus. Electron microscopy of the 1 mm fragment

showed normal skin structure. The infant was normal at birth.

SECTION D

THE IDENTIFICATION OF FETAL CELLS IN THE MATERNAL CIRCULATION

1. Historical Perspective

Although the maternal fetal barrier is generally considered to be effective in limiting cell transfer from fetus to mother, there have been several reports during the last decade which suggest that blood samples from pregnant women contain cells derived from the fetus. Three types of fetal cells have been identified in the maternal circulation; erythrocytes, leucocytes and trophoblast cells.

Kleihauer et al (1957) showed that fetal erythrocytes occurred in the maternal blood stream during pregnancy and after delivery. Fetal erythrocytes have been found in the maternal blood stream during the first trimester in the ratio of 1 fetal cell for every 50,000 maternal cells. The frequency of the fetal cells increases slightly during the second and third trimester (Schroder, 1975).

Nucleated fetal cells thought to be leucocytes have been shown to occur in the circulation of pregnant women expecting a male child. Walkowska et al (1969) obtained blood samples from 30 pregnant women and prepared chromosome spreads from PHA* stimulated lymphocytes. They analysed 1,200 chromosome spreads from each case. In 21 cases they observed at least 2 spreads with 5 small acrocentric chromosomes which they assumed were derived from male fetuses. Similarly Schindler et al (1972)

*Phytohaemagglutinin

prepared chromosome spreads from 16 pregnant women between 12 - 36 weeks gestation. These authors analysed 600 mitotic spreads from lymphocyte cultures in each case. In 9 of these cases the presence of 5 small acrocentric chromosomes in a number of cells indicated that the cells were derived from male fetuses but at term only 5 of these mothers gave birth to male infants. In another 5 cases, all 600 mitoses analysed revealed a female chromosome complement and at term male babies were delivered. These results showed that not only was the amount of work involved in the analysis very tedious but also that a cytogenetic analysis was subject to errors. It has been suggested that the frequent erroneous finding of cells with apparently 5 acrocentric chromosomes may have been due to the presence of fetal cells from a previous pregnancy or abortion.

Zilliacus et al (1975) used brightly fluorescent Y-chromatin as a marker to detect lymphocytes from male fetuses in maternal blood at 4, 6 and 8 months of gestation. They scored nearly 330,000 uncultured interphase lymphocytes from 18 primigravidae. A brightly fluorescent spot interpreted as Y-chromatin occurred in 0.16% in 8 of the 18 women studied. Of these 8 women, 7 gave birth to a boy and one to a girl. The Y-chromatin positive result in the 8th case was a misinterpretation of autosomal fluorescence in the mother's own chromosomes.

Thus this method has a serious limitation in that bright autosomal fluorescence of the mother's chromosomes could mimic a Y-body leading to false positive results. Grosset et al (1974) cultured PHA stimulated lymphocytes in a series of 86 pregnant mothers previously shown to be carrying male fetuses by amniocentesis. Their aim was to obtain a culture of enriched fetal

cells for cytogenetic analysis. They were able to obtain accurate diagnoses on nearly 90% of their cases. These mothers were studied between the 14th - 18th weeks of pregnancy and the percentage of male cells in the blood of these women was observed to be between 1 - 2%.

Stites et al (1975) have calculated that the total number of circulating lymphocytes in the adult female is around 1×10^{10} . If 2% of these are of fetal origin, it would mean that 2×10^8 fetal lymphocytes circulate in the mother's blood. This would be an amount equal to about 200 ml of fetal blood in the maternal circulation between the 14th and 18th weeks of pregnancy, the stage when the fetus has very few circulating lymphocytes itself. Stites suggests that these calculations make it unlikely that the observations of Grosset et al were correct. On the other hand, in vitro culture of maternal and fetal cells may not accurately reflect in vivo findings.

Zilliacus et al (1975) showed that fetal cells in the maternal blood stream do not respond to PHA.

2. Fluorescence Activated Cell Sorter

It has been suggested that fetal leucocytes isolated from maternal blood samples could be used for prenatal diagnosis (Milunsky, 1979). Prenatal diagnosis could be carried out on the cells isolated from a simple blood sample from a pregnant woman and would have many advantages over current invasive procedures. The main problem is the tedious procedure of identifying the fetal cells in the blood sample and isolating them for further study. This has been overcome with the development of the fluorescence-activated cell sorter (FACS).

By means of the FACS, different cell populations can be successfully separated from each other by differences in cellular fluorescence or by differences in light scattering characteristics based on cell type and size etc. or by a combination of these 2 variables. An excellent description of the procedure is given by Hulett (1973) and Herzenberg et al (1976)

"The basic procedure involved in using the cell sorter is that the cells are forced through a micronozzle under pressure in a liquid suspension into the centre of a cell-free fluid and out through an effluent nozzle. In this way a coaxial flow is created which keeps the cells near the axis of the effluent jet. The nozzle is vibrated axially, breaking the jet into 40,000 uniform droplets and the stream is illuminated by an argon ion laser operating at a wavelength selected to excite fluorescence in cells tagged with the appropriate fluorescent material. Some of the fluorescent light, filtered to remove the exciting wavelength, is focused onto a photomultiplier tubule which generates an electrical signal proportional to the number of fluorescent molecules on each cell. A second signal, related to the volume of the cell is generated by detecting the light scattered forward out of the illuminating beam by the passing cell. Signals produced in the scatter and fluorescent channels are processed, delayed and combined as required to produce electrical pulses which are used to charge the liquid stream at the time the droplet containing the desired cells is forming. Further downstream, the droplets pass through an electric field between two charged plates. Droplets that carry a charge are deflected, while uncharged droplets continue their original course. To ensure that the droplet carrying a desired cell is charged and thus deflected, the charging pulse lasts for the period of 3 droplets, centred at the time when the cells are expected to enter a droplet. In this way the cells with the fluorescence or scatter properties will be deflected and collected separately from the uncharged cells".

Later Herzenberg et al (1979) and Iverson et al (1981) successfully isolated fetal cells from 12 maternal blood samples collected in the 15th week of pregnancy. Using the FACS, the fetal cells were distinguished from maternal peripheral leucocytes using an indirect immunofluorescence technique. The primary antiserum was raised in rabbits and directed against paternal HLA antigens absent in the mother's cells. Labelling

of fetal cells was achieved using a fluorescein-conjugated goat antirabbit immunoglobulin. Using an antiserum raised in rabbits against HLA 2 studies were carried out on blood samples of 12 HLA 2 negative mothers carrying male fetuses whose fathers were thought to be HLA 2 positive. (The fetal sex was ascertained through amniocentesis in all 12 cases as the mothers selected for this study were at a risk of having genetically abnormal fetuses). In 5 of the cases, male cells (Y-chromatin positive) could be found in frequencies of 0.3 - 1% of the enriched population. All these mothers subsequently delivered HLA 2 positive boys. In the 7 cases where no enrichment of Y-chromatin positive cells were seen, the male children were HLA 2 negative.

3. Deported Trophoblast in Maternal Blood

There have been many reports on the pulmonary emboli caused by trophoblast-like cells in the lungs of patients with pregnancies complicated by eclampsia. Bardawill and Toy (1959) examined the autopsy material from a total of 109 women in an attempt to establish the frequency of the trophoblastic emboli. They examined lungs, uterus, and cervix in each case, 57 of these cases showed definite trophoblastic embolism in these organs. Histological sections of 2 of these cases showed the deportation of entire villi to the lungs.

Douglas et al (1959) confirmed the presence of trophoblastic tissue in the maternal venous circulation. These authors prepared smears from blood samples of pregnant women and demonstrated that syncytiotrophoblast was present at various stages of pregnancy, ie, from the 18th week of gestation to term.

Raafat et al (1975) designed a series of experiments to evaluate the possibility of utilizing deported trophoblast cells for first trimester prenatal diagnosis. They prepared artificial mixtures of abortus villus tissue obtained from first trimester pregnancy and maternal blood. They found that the trophoblast tissue could be separated from the maternal blood by sedimentation and the cells could be cultured and karyotyped.

SECTION E

TROPHOBLAST SAMPLING

1. Introduction

The trophoblast is the first recognisable human tissue in the developing embryo. It forms the epithelial wall, the trophoectoderm of the blastocyst before implantation. The extra-embryonic layers, (the amnion, the chorion and the placenta) which surround the embryo throughout the pregnancy are developed from the trophoectoderm; therefore, they are not part of the fetus itself. Trophoblast tissue, cannot strictly be classified as fetal or embryonic in origin but trophoblast cells are mitotic derivatives of the zygote and their DNA accurately reflects the DNA of the fetus. It is possible that chromosomal or biochemical analysis of the trophoblast could be used for prenatal diagnosis of the fetus. It has been suggested that if early placental villi could be obtained without interrupting the pregnancy, it could be used for antenatal diagnosis (Milunsky, 1979).

Embryological studies have shown that as the pregnancy proceeds, the villi surrounding the embryo develop at different rates. Those at the site of implantation proliferate to form

the placenta, whereas villi on the side opposite the placenta under the decidua capsularis do not continue to proliferate and by the 14th - 15th weeks they degenerate and disappear. For this reason it has been suggested that these villi under the decidua capsularis could be biopsied early in pregnancy without harming the pregnancy (Milunsky, 1979).

2. Methods of Obtaining Early Placental Tissue

A number of different methods have been used to obtain early placental villi for antenatal diagnosis. These methods include transabdominal needle biopsy, transcervical hysteroscopic biopsy, cervical cytology and blind endocervical aspiration. A review of these methods is presented below.

a) Transcervical Hysteroscopic Biopsy under Direct Vision

Hahnemann and Mohr (1968) described a new hysteroscopic device for obtaining first trimester samples of extra-embryonic membranes for prenatal diagnosis. These specimens were taken prior to induced abortion during the first trimester by introducing a hysteroscope with a barrel 6 mm in diameter through the dilated cervix. The specimen was obtained by vacuum extraction and trophoblastic tissue was obtained in 7 of the 8 cases investigated. The same authors reported another study on 63 patients in the 9th and 10th weeks of pregnancy and successfully obtained specimens in all but one of the patients investigated. One biopsy failed when the amnion was punctured during insertion of the hysteroscope. Tissue culture was successful in 34 cases, but chromosomes were studied in only 12 cases. Kullander and Sandahl (1973) obtained villus biopsies from

39 patients between the 8th and 20th week of pregnancy before therapeutic termination of pregnancy. These authors used a hysteroscope 5 mm in diameter and a fibre optic light source. They obtained successful growth for karyotype analysis in 20 cases. These karyotypes were normal and agreed with the karyotypes obtained from the fetal cells. Nineteen of these patients were observed for 7 - 43 days following the biopsy, during which 2 patients developed infection and subsequently aborted.

Hahnemann (1974) described hysteroscopic biopsies on a further series of 96 patients, 88 of whom were in the first trimester of pregnancy. Sixty seven of these biopsies were taken immediately prior to therapeutic termination and the other 28 were performed in the out-patient clinic with no anaesthesia with at least an 8 day interval between biopsy and abortion. Chorionic villi were obtained in 60% of the samples and specimens were successfully karyotyped in 35% of the cases. Fetal tissues were identified from abortion products in 24 cases and tissue cultures were prepared for karyotyping. The karyotypes of the cells cultured from the fetal tissue corresponded with the karyotypes obtained from the cells cultured from the placental biopsy material.

b) Exfoliative Cytology of Cervix

Shettles (1971) was curious about the fate of the villi which disappear from the chorion between the 10th and 14th weeks of pregnancy and speculated that they were simply exfoliated. He obtained endocervical smears with

a cotton swab from endocervical mucus from 30 patients in the first, second and third trimesters of pregnancy, and analysed the cells with a fluorescent stain. In 18 cases, the fluorescent stain revealed a single fluorescent spot in a proportion of the interphase chorionic nuclei, which he assumed was a Y-chromatin material derived from a male conceptus. In the other 12 cases, no fluorescent spot was observed. Shettles assumed that in these cases the conceptuses were female and used these findings to predict the sex of the fetus at birth. However, in only a few cases was the sex of the fetus ascertained at the time of birth. Despite the lack of confirmation, he claimed that the sex of the fetus could be predicted accurately in all 3 trimesters.

Warren et al (1972) confirmed Shettles' claim and reported no errors in fetal sex determination in a study on 50 patients. In all the cases in the study the sex of the fetus was ascertained at birth. He used Shettles' method of obtaining the material and scored 300 cells from each patient and found at least 10 cells which were Y-body positive in 28 cases. They concluded that the test was extremely easy to perform and a very accurate method of predicting the sex of the fetus.

Rhine et al (1975) also attempted to predict the fetal sex by the detection of Y-body in cells in smears prepared from endocervical swabs. They reasoned that exfoliated trophoblastic tissue accumulates behind the mucus plug in the cervix and an effort was made to obtain the endocervical samples from the area of the internal cervical os with a

cotton swab. These authors studied a series of 36 patients from all 3 trimesters of pregnancy and found 18 cases where a Y-body was present in at least 3% of the cells. In all 18 cases male infants were delivered at term. In 5 cases a female was predicted but a male infant was delivered. This error was presumed to be the result of the samples being obtained from below the area of the trophoblastic cell accumulation so that the maternal cells from the mid-cervix were sampled and scored. Rhine et al concluded that endocervical smears can be a reliable indicator of fetal sex if the sample is obtained from the area of the trophoblastic cell accumulation at the internal os.

c) Blind Endocervical Aspiration

Transcervical aspiration procedure was reported from the department of Obstetrics and Gynaecology, Tietung Hospital, Anshan, China (1975). This study was performed on 100 women during the 6th - 14th weeks of pregnancy for prenatal sex determination as a part of their family planning programme. They scored the sex chromatin content of cells aspirated from the cervix. The Chinese used a rigid metal cannula, 3 mm in diameter with a blunt tip which could be extended 1 cm beyond the distal end of the cannula. Aspiration of the chorionic villus tissue was accomplished by attaching a 5 ml syringe to the cannula. The cannula was inserted into the cervix until soft resistance was felt to the tip of the instrument. The inner tube was pushed forward 0.5 - 1.0 cm and the aspiration was performed and the villi were sucked into the cannula. The aspirated

material was expressed onto a slide and smears made and stained with Papanicolaou's stain. Chorionic villi cells were differentiated from maternal cells on morphological grounds and cells of these chorionic villi were scored for the presence of Barr bodies. Aspirations were repeated in 27 cases because of the doubtfulness of the presence of chorionic villi in smears. Fetal sex was accurately predicted in 94 cases. The false predictions in 6 cases were thought to be due to the lack of experience and failure to observe the criteria of countable nuclei and X-chromatin. Spontaneous abortion occurred in 4 cases following the procedure. No attempts to prepare tissue culture or to karyotype the cells were reported.

Rhine et al (1975) developed a device named the Antenatal Cell Extractor (ACE) to obtain a sterile sample of tissue from the internal os for tissue culture. This instrument consisted of a clear plastic tube, about 15 cm long and 2.5 cm in diameter, with an attached plastic plunger and a 3.5 mm diameter plastic bulb which was secured to the tip of the plunger. The ectocervix was cleaned and the distance between the internal os and flexion of the uterus was determined. Enough saline was expressed from the syringe to fill the tube of the ACE and then the distal tip was inserted into the area of the internal os. When the bulb passed the constriction at the os, the ACE was opened by pushing the plunger forward and the sterile saline was expelled into the area just above the internal os. The saline was immediately drawn back into the tube and syringe, the bulb was pulled back

to close the tube and the ACE was withdrawn from the cervix.

Rhine et al (1977) published a study of their experience with this sampler on 3 groups of patients, which consisted of 66 women who were candidates for therapeutic termination of pregnancy during the first trimester.

For the development and modification of this newly designed instrument, no anaesthesia was given and no dilatation was performed. Tissue culture was attempted on aspirates obtained in all cases and successful cultures were obtained in 23 cases. Chromosome analysis was possible in 17 cases. In these 17 cases, chromosome preparations were also prepared from maternal lymphocytes. The chromosomes from the aspirates and from the maternal lymphocytes were scored for Q-band polymorphism. Polymorphisms observed in the aspirated samples which were not observed in the maternal lymphocytes ^{were} ~~was~~ taken to mean that the aspirated cells were derived from the fetus. In 2 specimens, chromosome polymorphism was observed in some chromosome spreads from the ACE material but not in others. This was interpreted to mean that maternal decidual contamination had occurred in these samples. These authors reported encountering a number of obstacles, one of the major ones being the poor quality of chromosome spreads which made the scoring of polymorphic variation very difficult.

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CHAPTER II

E A R L Y P L A C E N T A L D E V E L O P M E N T

SECTION A

INTRODUCTION

In this chapter a short account of the development of the placenta during early pregnancy is given, and the histology of the early placental villi is described. The HLA antigenic status of mouse and human trophoblast is also reviewed.

SECTION B

EMBRYOLOGY

After the union of male and female gametes, the fertilised egg undergoes a series of mitotic cell divisions which provide the building units for the future organism. As a result of this cleavage a solid ball of cells is formed (the morula) which eventually forms a hollow sphere (the blastula).

The fertilised human egg is believed to enter the uterine cavity early on the fourth day after fertilisation. In man the period spent in the free morula and blastula stages is 2 to 3 days. During this period the fertilised egg is transported to the site where attachment and implantation occurs. The process of implantation includes the attachment of the blastocyst to the epithelial lining of the uterus, its penetration through the epithelium and its invasion of and embedding in the compact layer of the endometrium. In the region of contact, the outermost layer of the blastocyst which is termed the

trophoblast, thickens as a result of proliferation and its more superficial cells lose their boundaries and thus become a syncytium. At this time the cells of the uterine epithelium in the area of the attachment begin to break down. The injured cells are taken up and digested by trophoblast. As a result of this erosion of the uterine wall, the invading trophoblast advances and comes into contact with the connective tissue of the endometrium. Whereas the original wall of the blastocyst was composed of distinct cells, the rapid growth of the trophoblast has now produced a thick spongy shell, composed of an inner layer of single cells, the cytotrophoblast which lines the cavity of the blastocyst, and an outer layer of syncytiotrophoblast which is in direct contact with the uterine wall. A layer of mesoderm is formed on the inner surface of the trophoblast. The entire capsule of mesoderm and trophoblast can now be called the chorion.

A prominent feature of the syncytiotrophoblast is the appearance within it of vacuoles which merge and produce irregular cavities or lacunae. This system of channels, which gives the trophoblast its spongy texture, is the beginning of the future intervillous space of the placenta and is well developed in the week following implantation. The cytotrophoblast of the differentiating chorionic sac tends to be single layered, except where proliferation has produced local masses. These masses extend a short distance into the plates and strands of syncytiotrophoblast and results in the formation of the primary villi. The mesoderm subsequently forms a core of vascular connective tissue in the developing villi. As the cytotrophoblast proliferates and perforates the shell of syncytiotrophoblast and sends cell columns outwards, the axial connective tissue extends progressively into them; continued growth and branching bring ~~a~~ tuft-like (chorionic) villi  into being. All such early tufts lack blood vessels and are known

as secondary villi. During the fourth week, the young villi become well formed and vascularized. They are then known as tertiary villi.

The mucosal lining (endometrium) of the uterus already specialised in anticipation of pregnancy and utilized as a nesting place ^{the} by ^{the} implanting embryo undergoes a number of changes and these persist throughout the gestation period. These changes occur initially in the region of implantation and then the remainder of the uterine lining becomes involved. The endometrium in which these changes occur is named the decidua. This part of the endometrium is shed with the placenta at birth. During the early stages, when the chorion lies embedded within the endometrium, 3 different regions of this thickened uterine membrane can be recognised.

1. The decidua parietalis, the general lining of the uterus exclusive of the area occupied by the embryo.
2. The decidua capsularis, a region covering the chorionic sac and interposed between the sac and the uterine cavity.
3. The decidua basalis, a region underlying the chorionic sac and situated between it and the muscular wall of the uterus.

In the early weeks, the villi cover uniformly the entire surface of the chorion and may reach 1,000 in number. With continued growth of the chorion the villi next to the stretched decidua capsularis become compressed. Their vascularity is also reduced and they subsequently atrophy. This atrophy produces a bare polar spot by 8 - 9 weeks of pregnancy. By the fourth month, the villi covering half the surface of chorion have degenerated. The area of chorionic sac lacking villi is called the chorion laeve (ie. smooth chorion).

The villi that are associated with the decidua basalis, on the other hand, persist and give the name chorion frondosum (ie. busy ^h chorion) ^h

to this part of the chorion. The area of persistent villi is normally somewhat circular in shape and is the site of the developing placenta which naturally takes the shape of a disc. The placenta continues to increase in area throughout pregnancy. During most of this time it occupies about 30% of the internal surface of the expanding uterus. The placenta is 6 times heavier than the fetus at one month, the same weight at 4 months and only one-seventh the fetal weight at birth.

SECTION C

HISTOLOGY OF THE EARLY PLACENTAL VILLI

In the first 2 months of pregnancy, the villi are relatively few in number and measure approximately 170 μm in diameter (Fox, 1978). At this stage, their outer surface is covered by 2 easily distinguishable layers of trophoblastic epithelium: an outer layer of syncytiotrophoblast and an inner layer of cytotrophoblast or Langhans' cells. The cytotrophoblast forms a complete layer and consists of cuboid or ovoid shaped cells, which have well marked cell boundaries. Their nuclei are pale staining and the cytoplasm is clear or slightly granular.

No cell boundaries are visible between the nuclei of the syncytiotrophoblast which form a layer of uniform thickness around the periphery of the villus. The nuclei of the syncytiotrophoblast are regularly spaced, smaller in size and more darkly stained than those of the cytotrophoblast. The syncytial cytoplasm is finely granular but more vacuolated and some of these vacuoles contain lipids.

The villous stroma is formed by loose mesenchymal tissue, which contains numerous Hofbauer cells. These cells may be round or ovoid,

about 25 μm in diameter, their cytoplasm is coarsely vacuolated and they have eccentrically placed nuclei. The connective tissue cores also contain fibroblasts and fetal capillaries. The fibroblasts have elongated processes, their nuclei are of various shapes and each has a distinct nucleolus.

SECTION D

EXPRESSION OF HLA ANTIGENS ON TROPHOBLAST

The placenta, like the fetus, is the mitotic derivative of the zygote and is developed for the purpose of providing the fetus with food and oxygen and removing the waste products of metabolism. It is not yet clear whether the placenta possesses and/or expresses tissue antigens. In this section, attempts to study the antigenic status of the trophoblast in both experimental animals and man are described.

1. H₂ Antigen Expression on the Mouse Trophoblast

Simmons and Russell (1962) transplanted fetal and placental tissues to homologous adult mice. They transferred the fetal and placental tissue derived from F-1 mating between 2 inbred strains of mice (and therefore, presensitised to paternal strain antigens) to adult members of the maternal strain. These authors reported that embryonic tissue grafts transplanted beneath the renal capsule of the maternal strain were rapidly destroyed, while trophoblastic grafts survived and proliferated.

Later Kirby et al (1964) and Simmons and Russell (1966) carried out histochemical and electron microscopical studies of mice placentae at 16 days gestation and found that the majority

of trophoblast cells were surrounded by an amorphous electron dense layer of mucoprotein which varied in thickness from 0.1 to 2.0 μ and was particularly thick in the trophoblast cells which were in contact with the maternal decidual cells. These authors suggested that trophoblast may contain histocompatibility antigens but these may have been masked by the mucoprotein layer, preventing the content of fetal transplantation antigens with the cells of the maternal system. Currie et al (1968) claimed that this protective barrier could be removed and when it was removed the H₂ antigens were expressed. They treated the cell suspension prepared from 7 day old trophoblast of inbred mice with neuraminidase and injected these cells into unrelated adult mice which became sensitised to the skin graft from the same strain.

Heyner (1973) used an immunofluorescence technique in an attempt to demonstrate the antigens on embryonic mouse cells at the blastocyst stage and trophoblast cells. It was reported that H₂ antigens were detectable on mouse embryonic cells but not on trophoblast.

Jenkinson and Billington (1974) applied the microcytotoxicity test to monolayers of cells cultured from 7½ day old mice placenta^o in an attempt to detect H₂ antigens on the cells. Half of these specimens were treated with neuraminidase prior to the test in an attempt to remove the mucoprotein layer described by Simmons and Russell (1966). These authors could not detect H₂ antigens on either group of specimens.

Searle et al (1976) applied the indirect immunoperoxidase technique to demonstrate H₂ antigens on trophoblast cells at different stages of gestation. They carried out the test on

embryos at the morula stage, pre-implantation and post-implantation embryos and trophoblast. These authors reported that the pre-implantation embryonic cells were densely labelled with peroxidase while the trophoblastic cells were always entirely unlabelled.

2. HLA Antigen Expression on the Human Trophoblast

Many attempts have been made in the past to demonstrate HLA antigens on human trophoblast cells but the results are conflicting.

Seigler and Metzgar (1970) investigated the HLA status of trophoblastic syncytia obtained at varying gestational ages (between 6 weeks to maturity) and monolayers of cells from tissues from corresponding fetuses. Using a panel of 22 human antisera, they were able to demonstrate HLA antigens on the fetal cell monolayer using a mixed agglutination technique to demonstrate specific haplotypes. They were unable to detect HLA antigens on trophoblast syncytia by this method.

Loke et al (1971) prepared monolayer cultures from trophoblast villi and fetal skins derived from 3 conceptuses of 12 weeks gestation and used the cytotoxic technique in an attempt to determine the HLA antigenic status of the cultured trophoblast and fetal cells. Cells cultured from both trophoblast villi and fetal cells were killed by multispecific anti-HLA serum. These authors concluded that HLA antigens were expressed on human trophoblast cells grown in the form of monolayers. There is some doubt as to whether the cultures of trophoblastic cells were uncontaminated by the presence of any other cellular components of placental tissue. There is also some doubt as to the specificity of the antisera used in this experiment, for it

has since become clear that a number of antisera of this type act against a variety of antigens, not all of which are of the histocompatibility type.

Lawler et al (1974) investigated 31 women who had a history of a first pregnancy with a hydatidiform mole. A careful history of transfusion in these patients was obtained. The HLA types of the patients and their husbands were determined and the sera of the patients were tested for cytotoxic activity against the husbands' cells and against a panel of normal donors so that the HLA specificities of the serum antibodies could be determined. A raised anti-HLA activity of paternal specificity was noted in the sera of 4 women with persistent trophoblastic disease. It was implied that the trophoblast was responsible for the immunisation of these patients.

Faulk and Temple (1976) utilized 6 week old, 13 week old and ~~full~~ term placentae from normal pregnancies for HLA studies and snap froze parts of these tissues for histological sections. Making use of beta₂ microglobulin as a specific marker for HLA antigens, they used specific antisera to demonstrate by an immunofluorescence technique, the distribution of beta₂ microglobulin in intact placental tissue. It was shown that this substance is present in villous stromal cells and in fetal endothelial cells but not in trophoblast.

Goodfellow et al (1976) assessed the levels of HLA-A and B antigens on a purified preparation of placental plasma membranes using a fluorochromatic cytotoxicity technique. Spleen lymphocytes were used as controls. Their results indicated that maternally and paternally inherited A and B antigen activities and beta₂ microglobulin are expressed to a similar extent but at

much lower levels on placental membranes than on spleen lymphocytes. The possibility that the antigens represent contamination with maternal tissue appears unlikely on the basis that maternally and paternally inherited specificities were expressed to ^a_κ similar extent and that the specificities exclusive to either parent were expressed at a much lower level.

CHAPTER III

EXPERIMENT 1

INVESTIGATION OF ENDOCERVICAL ASPIRATES FROM FIRST TRIMESTER PREGNANCIES: HISTOLOGICAL AND CYTOLOGICAL STUDIES.

A. Materials and Method

Endocervical aspirates were collected from 12 women (cases 1 - 12) immediately prior to therapeutic termination by suction curettage under general anaesthesia. The informed consent of the patient was obtained prior to the collection of the sample. Their ages varied between 14 - 25 years (mean age 18.63 years) and their gestational age (determined from the dates of the last menstrual period) ranged from 8 - 12 weeks (mean gestational age 11 weeks). The details of the patients are given in the appendix. The specimens were collected using a medicut cannula gauge 16 (length 45 mm, outer diameter 1.10 mm) attached to a 20 ml Luer lok syringe. The cervix was cleaned with Hibitane and the cannula introduced gently into the endocervical canal to a depth of nearly 1 inch, taking care not to rupture the amniotic sac.

The plunger was withdrawn and a small amount of tissue was aspirated into the cannula. The syringe was rinsed out with sterile 5 ml phosphate buffered saline (PBS) and the fragments of tissue in the syringe and cannula were expelled into a sterile universal container. The specimen was divided into 2 parts. One part was used for histological and cytological studies and the other was set aside for Experiment 2.

Approximately 2.5 ml of the specimen was centrifuged at 110 G for 10 minutes. In 3 cases the pellet of cells obtained after centrifugation was transferred onto a microscope slide and covered with a drop of aceto orcein stain. A coverslip was placed over the cells and a squash preparation of the fragments was made. Preparations were examined by light microscopy using a Leitz orthoplan microscope (Figure 1). In the remaining 9 cases, the pelleted fragments were fixed in formal saline and prepared for paraffin embedding and histological studies. The histological sections were examined by the investigator using a Leitz orthoplan microscope (Figure 2) and also reported independently by a histopathologist.

B. Results

In the histological sections, 2 types of tissues could be identified in every specimen - namely, decidua and placental villi. The latter were readily recognised by their pale staining central mesenchyme core which was surrounded by an inner cyto and outer syncytiotrophoblast layer. The decidua appeared as loose clusters of large cells with eosinophilic cytoplasm.

In the squash preparations it was possible to identify intact placental villi as long frond-like strands with a pale staining central core. Numerous syncytial buds could be seen projecting from the surface of the villi (Figure 3). Red blood cells and mononuclear giant cells were also present in the squash preparations. It was not possible to distinguish with certainty the origin of many of the single mononuclear cells in the squash preparations to determine whether they were derived

from placental villi or decidua; although few fragments of membranous tissue were seen to be present.

C. Discussion

Although endocervical aspirations have been attempted by other workers, Rhine et al (1977), Goldberg et al (1980), members of the Department of Obstetrics and Gynaecology, Tietung Hospital of Anshan Iron and Steel Company, Anshan (1975), there have been few reports of the histological and cytological appearances of cells in the aspirates.

The workers in the Department of Obstetrics and Gynaecology at Tietung Hospital in China, carried out endocervical aspiration on 100 patients for fetal sexing and claimed that "villar branches" could easily be recognised in the samples by low power microscopy. The cell borders in these "villar branches" were clearly seen and the cells contained nuclei which were irregularly arranged, over-lapping and varying in size. There was no distinct cell wall between these nuclei, although sometimes red blood cells were seen in the "villar branches". Rhine et al (1977) and Goldberg et al (1980) claim in their reports to have carried out histological and cytological studies on the endocervical aspirates but do not comment on their findings.

In our series, histological studies were used to identify the tissues from the aspirates, and these studies indicated that although endocervical aspirates appeared to be a reliable source of placental villi, the specimens were always contaminated with decidua.

Figure 1. Squash preparation of tissue in endocervical aspirate showing numerous frond-like villi (Aceto-orcein x 40)



Figure 2. Histological section of the tissues obtained by endocervical aspiration, showing the villi (v) and decidua (d)
(Haematoxylin and eosin x40)

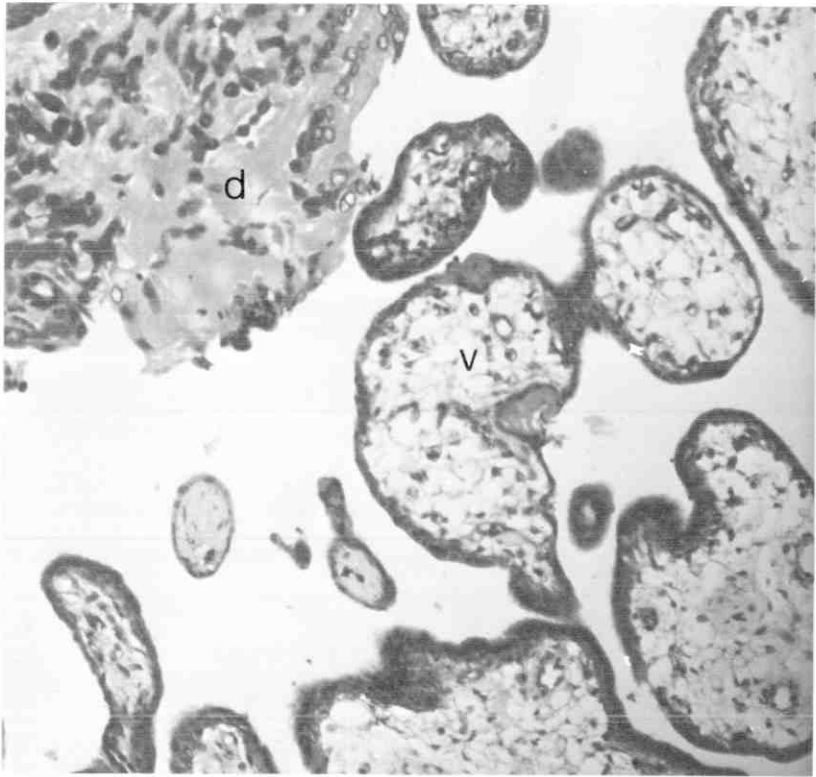
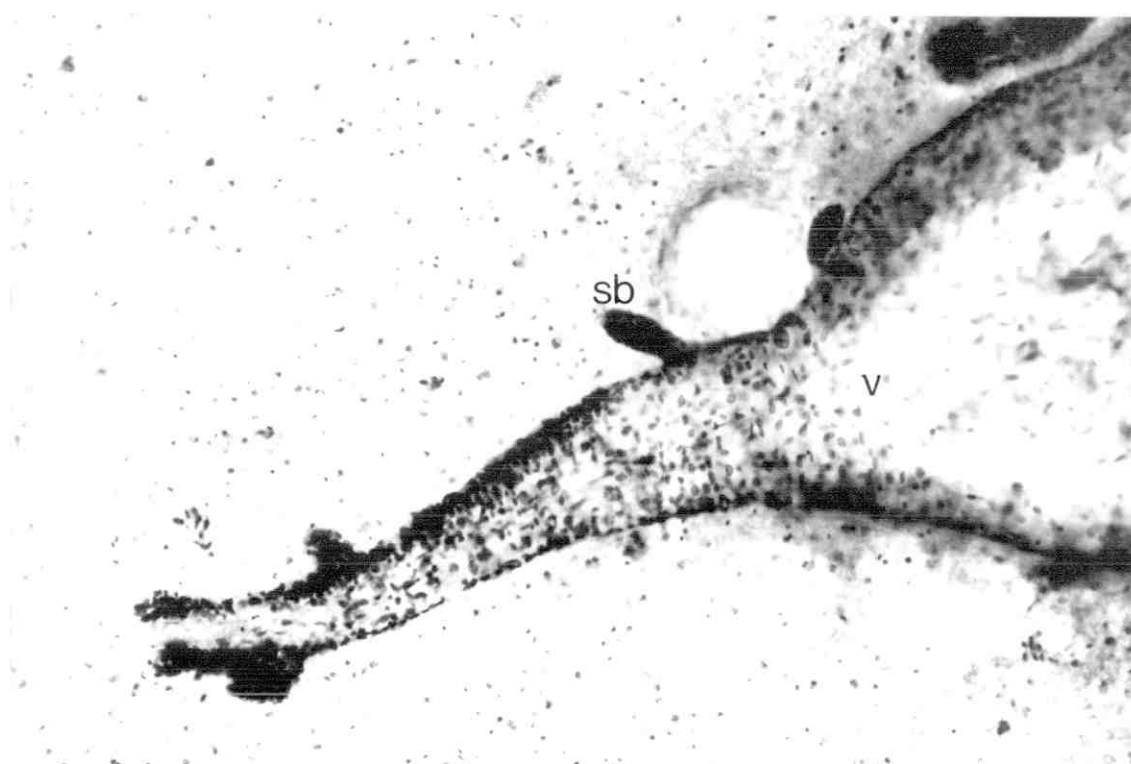


Figure 3. Squash preparation of chorionic villus (v) in endocervical aspirate showing syncytial buds (sb) (aceto-orcein x40)



EXPERIMENT 2

TISSUE CULTURE OF ENDOCERVICAL ASPIRATES FROM FIRST TRIMESTER PREGNANCIES: CYTOGENETIC AND TISSUE TYPING STUDIES TO DETERMINE THE FETAL OR MATERNAL ORIGIN OF THE CULTURED CELLS.

A. Materials and Method

i) Endocervical Aspirates

A description of the specimens used for this experiment is given in Experiment 1. Endocervical aspirates were obtained from the 12 women shown in Table I and collected into 5 ml of sterile PBS. Approximately 2.5 ml of the specimen was centrifuged at 110 G for 10 minutes. The supernatant was discarded and 10 ml of 0.5% trypsin solution in Ca^{++} and Mg^{++} free phosphate buffered saline was added to the pellet of cells. Trypsinisation was allowed to proceed for 50 minutes at 37 °C and the suspension of cells was centrifuged again at 110 G for 10 minutes. The pellet of cells thus obtained was washed with complete medium TC 199 containing 30% fetal calf serum, 1% glutamine and added antibiotics and centrifuged again. The cells were inoculated into tissue culture flasks (Falcon 25 cm²) containing 8 ml complete medium and incubated at 37 °C in 5% CO₂ - 95% air and 90% humidified atmosphere. These cultures were periodically observed under phase contrast microscopy and the medium was changed every 72 hours. The cells were subcultured after 10 days and harvested 4 - 6 days later. Chromosome studies on the harvested cells were carried out

using standard procedures (see Appendix) and tissue typing of the cultured cells was performed using a method developed by Niazi et al (1979) (see Appendix).

ii) Control Tissues

During the course of therapeutic termination which followed the endocervical aspiration obvious fetal tissues, eg. limbs or cord were also collected from 8 cases. Fragments of skin obtained from these tissues were cultured by the standard plasma clot method (see Appendix). The fibroblasts grown from the fetal skin were used as controls for tissue typing and karyotyping investigations.

Eight ml venous blood from 4 mothers were also collected in 1% tri-sodium citrate solution for tissue typing studies of peripheral lymphocytes. These were carried out at the Anthony Nolan Tissue Typing Laboratory using a microdroplet lymphocyte cytotoxicity test (Terasaki et al, 1970).

B. Results

The results of the cytogenetic and tissue typing studies are shown in Table I.

i) In Vitro Growth Patterns of the Endocervical Aspirates

Initial phase contrast studies of the culture flasks showed that the inoculum was composed of many simple nucleated cells, the origin of many of which, could not be identified with certainty. Red blood cells and fragments of tissue which were unaffected by trypsin were also present in the inoculum. The single nucleated cells

settled by the second day, while the fragments of tissue and red blood cells did not adhere to the culture flask and were discarded after the first medium change.

By the fourth day, a mixture of cell types was seen. The cultures were well-established by the tenth day and consisted of spindle-shaped fibroblasts, rounded epithelial type cells and multinucleated giant cells. In these cultures, the spindle-shaped fibroblasts grew rapidly and eventually predominated the culture. These cells were sub-cultured, and after 3 - 4 days, monolayers adequate for cytogenetic studies and tissue typing were obtained.

ii) Karyotyping

Chromosome analysis from cells grown from the aspirated material was possible in 11 of the 12 cases. In each case a normal female karyotype, 46,XX was obtained. Analysis of fetal karyotypes was possible for 8 of the fetuses (Table I).

Chromosome analysis of the fetal fibroblasts revealed that 3 of the fetuses had a normal male karyotype, 46,XY (cases 7, 11 and 12) and 5 had a normal female karyotype, 46,XX (cases 5, 6, 8, 9 and 10).

iii) Tissue Typing

Tissue typing of the cells cultured from the endo-cervical aspirates, fetal fibroblasts and maternal lymphocytes was carried out in cases 9, 10, 11 and 12. The HLA antigens identified on cells grown from the endo-cervical aspirates were identical with those identified

on the maternal lymphocytes in 3 cases (9, 10 and 11). HLA typing of fetal fibroblasts in these 3 cases revealed the presence of paternal antigens not present in the endocervical aspirates nor in the mother. In case 12, HLA typing of cells cultured from the endocervical aspirates and fetal fibroblasts was incomplete due to the non-availability of antisera at the time.

C. Discussion

In the present series of investigations of 12 endocervical aspirates, the karyotypes of the cells cultured from the aspirates were invariably 46,XX. In cases 7, 11 and 12, the karyotypes of the corresponding fetuses were consistent with those of a normal male indicating that in these 3 cases the cells harvested from the culture of the aspirates were derived from the decidua. The tissue typing studies on cases 11 and 12 further confirmed the maternal origin of the cells cultured from the endocervical aspirates because haplotypes on the aspirated cells were identical with those of the maternal lymphocytes. In cases 5, 8, 9 and 10, the fetal karyotypes were found to be female and in the absence of any demonstrable chromosome polymorphisms, we were unable to establish the origin of the aspirated cells by analysis of the karyotypes. However, tissue typing studies of the aspirated cells, maternal lymphocytes and fibroblasts from the corresponding fetuses in cases 9 and 10 indicate that in these 2 cases, as in cases 7, 11 and 12 the cells harvested from the aspirates were derived from the mother.

Our findings are in direct contrast to those of Rhine and his associates (1977) who obtained endocervical aspirates and observed growth in 12 of the 13 first trimester patients. In 11 of the 12 successful cultures, Rhine and associates obtained female karyotypes (46,XX) and they claim to have observed chromosome polymorphisms which differed from those of chromosome spreads obtained from maternal lymphocytes. This was interpreted to mean that cells from the endocervical aspirates were of fetal origin in these 11 cases. The inability of these authors to demonstrate a male karyotype among the cells obtained by endocervical aspiration casts serious doubt upon the validity of their findings, as they should have obtained at least one typically male karyotype on the basis of probability alone. By the authors own admission, the quality of the chromosome preparations was poor and this could have affected their analysis.

Goldberg et al (1980) attempted to verify Rhine's claim by comparing the chromosome preparations from aspirates with those from maternal lymphocytes, but found no conclusive chromosome polymorphic differences. They particularly concentrated on 12 cases where the fetal tissue were males, 46,XY. In these cases, the authors were positively conclusive that although villous material can be obtained by endocervical aspiration in early pregnancy, a pure growth of fetal cells cannot be obtained from the specimen. Our results indicate that the fetal cells in the aspirates may have been overgrown by rapidly proliferating maternal cells.

TABLE I
RESULTS OF CYTOGENETIC AND TISSUE TYPING STUDIES ON CELLS
CULTURED FROM ENDOCERVICAL ASPIRATES

CASE NO.	PATIENTS INITIALS	AGE	PARITY (No. of children and No. of terminations)	GESTATION AGE BY LMP	HLA ANTIGENS DETECTED ON MATERNAL LYMPHOCYTES Sub locus A; Sub locus B.	HLA ANTIGENS DETECTED ON CELLS CULTURED FROM ENDO-CERVICAL ASPIRATES Sub locus A; Sub locus B.	HLA ANTIGENS DETECTED ON FETAL FIBROBLASTS Sub locus A; Sub locus B.	KARYOTYPE OF CELLS CULTURED FROM ENDOCERVICAL ASPIRATES	KARYOTYPE OF FETAL FIBROBLASTS
1	VT	26	0 + 0	12/40	ND	ND	ND	46,XX	ND
2	OT	24	0 + 0	11/40	ND	ND	ND	46,XX	ND
3	EN	32	0 + 1	10/40	ND	ND	ND	46,XX	ND
4	SB	33	3 + 0	8/40	ND	ND	ND	46,XX	ND
5	SH	18	0 + 0	10/40	ND	ND	ND	46,XX	46,XX
6	EAH	18	0 + 0	10/40	ND	ND	ND	US	46,XX
7	RJ	15	0 + 0	12/40	ND	ND	ND	46,XX	46,XY
8	YCC	23	0 + 0	11/40	ND	ND	ND	46,XX	46,XX
9	AL	20	2 + 2	12/40	A2,19; B12,35	A2,19; B12,35	A3,19; B 8,35	46,XX	46,XX
10	LH	16	0 + 1	12/40	A2,11; B13,18	A2,11; B13,18*	A1,11; B10, -	46,XX	46,XX
11	NI	14	0 + 0	12/40	A1, 3; B12,35	A1, 3; B12,35	A1, 2; B 7,12	46,XX	46,XY
12	SF	25	1 + 1	12/40	A1,29; B12,16	A1, -; B12, -	A1, 9; B 5, -	46,XX	46,XY

ND = Not done
 ~ = Antigen not detected
 * = Weak reaction
 US = Unsuitable for analysis
 NK = Not known

EXPERIMENT 3

INVESTIGATION OF THE REASONS FOR MATERNAL OVERGROWTH OF FETAL CELLS IN ENDOCERVICAL ASPIRATES: STUDIES OF EXPLANTS OF INTACT EARLY PLACENTAL VILLI.

A. Materials and Method

Placental tissue was obtained by curettage from 8 women (cases 13 - 20) during therapeutic termination in the first trimester (Table II). The tissues were washed with a sterile PBS solution to remove erythrocytes and tissue debris. The trophoblast villi were dissected out from the membranes under sterile conditions. A small fragment of tissue was fixed in formal saline for histological examination. The remaining placental fragments were set up in plasma clots (for method see Appendix) and their growth pattern observed.

B. Results

Results are shown in Table II.

Histological sections showed that these tissues were ~~comprised~~^{composed} of intact trophoblast villi only, confirming that the culture was not contaminated with maternal tissue.

Studies of the growth pattern of the placental villi showed that cells started to emerge from the clot after 8 days. This was first signified by the appearance of multinucleated knots at different areas of the surface of the explant. This was followed by the appearance of elongated spindle-shaped cells. Between the second and third week of culture, some epithelial

cell types also began to appear which later fused to form giant cells. Although elongated spindle-shaped cells were seen to be predominating the cultures, cell proliferation was very slow and an adequate number of cells for sub-culturing was obtained in only 5 cases and then only after 4 - 5 weeks of prolonged culture. Sub-culture of these cells was carried out by trypsinisation. In the sub-cultures, the group of multinucleated giant cells failed to survive and monolayers of fibroblast-like cells were formed. The cultures were maintained for 10 - 12 weeks. Monolayers of cells adequate for chromosome analysis were obtained in 5 cases after 8 - 10 weeks.

Chromosome analysis of the cells grown from trophoblast villi revealed a normal male karyotype 46,XY in cases 15 and 19 and normal female karyotypes 46,XX in cases 14, 17 and 20.

C. Discussion

Several different cell types have been described in explant cultures of intact placental villi, some of which are thought to be derived from trophoblast layers and others from the mesenchyme stroma. Jones, Gey and Gey (1943) and Stewart, Sanu and Montgomery (1948) described 3 types of cells in explants of immature placentae: the oblong cells with an oval nucleus taken to represent the syncytial cell, the round cell with a round nucleus and a halo around the nucleus which were thought to be Langhans cells, and the fibroblast type cell with a spindle-shaped appearance and which was thought to be of stromal origin.

Fox and Kharkongor (1970) described multinucleated syncytial "knots" appearing in their explant cultures after nearly 7 days. They also observed the appearance of elongated spindle-shaped

cells after the second week of culture which they thought to be derived from the stroma. Epithelial like cells were observed during the third week of the trophoblast explants in culture. Adadjem and Leuck (1981) were able to successfully suppress the growth of the spindle shaped mesenchyme stromal cells by growing cultures in anaerobic conditions, thereby allowing the morphology of the cells from the trophoblast layers to be more clearly identified. All authors appear to agree that epithelial type cells and fibroblast like cells can be distinguished in the cultures. It is assumed that the fibroblast type cells in the cultures are derived from mesenchyme core of the chorionic villi and this is in accordance with our experience bearing in mind how difficult it is to identify the origin of any cell once it is growing in culture.

The failure of placental villi to grow in the culture, especially when they were present in abundant quantities in the endocervical aspirates, prompted us to investigate this problem further. We found that intact trophoblast villi grow slowly in cultures and it took nearly 8 - 10 days for the initial cells to proliferate out of the plasma clots of the primary cultures. It was observed that the success rate of the growth of explants from intact immature placental villi was low (63% in our series). Similar results have been reported by Jones et al (1943) who used a similar approach to study the hormone production by placental cells in continuous cultures. These authors found that it took at least 8 - 10 days for the first few cells to migrate out of the primary cultures. Thiede (1960) also reported delay in the growth of intact placental tissue grown in cultures. He reported an extremely lengthy time for the cells to grow by

clot lysis and sub-cultures were unsuccessful. Fox and Kharkongor (1970) reported a period of 2 - 3 weeks before their explant cultures of placenta were established.

We have postulated that the slow growth of the placental villi in the cultures of endocervical aspirates accounted for their overgrowth by maternal cells (Experiment 2). From our experience and that of other authors it seems that cells from the mesenchyme stroma grow more rapidly and survive longer in culture than the trophoblast layers of placenta. If the growth of mesenchyme cells could be promoted and decidual elements eliminated from endocervical aspirates it should be possible to obtain pure cultures of fetal cells from these specimens for antenatal diagnosis. We, therefore, attempted to develop a method of selecting mesenchyme cells from intact placental villi and preparing cultures from them.

TABLE II

GROWTH PATTERN AND CYTOGENETIC STUDIES OF EXPLANTS OF INTACT PLACENTAL
VILLI OBTAINED FROM FIRST TRIMESTER PREGNANCIES

CASE NO.	PATIENT INDEX	AGE	PARITY	GESTATION AT TERMINATION	KARYOTYPE	DURATION OF CULTURE
13	AH	22	0 + 0	9/40	-	No growth obtained
14	BNC	20	0 + 0	10/40	46,XX	9/52
15	MCV	18	0 + 0	12/40	46,XY	8/52
16	BP	28	3 + 0	8/40	-	No growth obtained
17	JB	19	0 + 1	8/40	46,XX	8/52
18	JR	16	0 + 0	11/40	-	No growth obtained
19	KH	18	0 + 0	12/40	46,XY	10/52
20	JC	20	1 + 1	10/40	46,XX	9/52

EXPERIMENT 4

TRYPSINISATION OF PLACENTAL VILLI OBTAINED FROM FIRST TRIMESTER PREGNANCIES. EFFECT OF TRYPSIN ON TROPHOBLAST LAYERS AND MESENCHYME CORES.

A. Materials and Method

Placental tissue from 15 pregnancies of gestational age 8 - 12 weeks ^{was} ~~were~~ collected during therapeutic termination of pregnancy by suction curettage (see Appendix, cases 21 - 35, for details). The tissues were washed twice with sterile PBS to remove erythrocytes and tissue debris. The placental villi were separated from the membranes by dissection and the material was divided into 2 parts. One part was set aside for histological studies for comparison with histological sections of trypsin treated villi. The other part of the dissected villi was washed 3 more times to remove red blood cells and centrifuged in the final wash at 110 G for 10 minutes in PBS. The supernatant was discarded and the pellet of villi thus obtained was weighed. A 0.25% solution of trypsin (Difco 1:250) was prepared in Ca^{++} and Mg^{++} free phosphate buffered saline with added 2% glucose (Dextrose). The trypsin solution was added to a sterile universal container containing the pellet of trophoblast villi at a concentration of 4 ml of trypsin per one gram of tissue. This container was gently agitated in a gyratory shaker at 37 °C for 50 minutes. Ten ml complete medium TC 199 containing 30% fetal calf serum was added to this cell suspension which was then centrifuged at 110 G for 10 minutes. The pellet of trypsin treated villi thus obtained was fixed in formal

saline for paraffin embedding and light microscopy. This experiment was repeated using 4 different concentrations of trypsin (0.25%, 0.50%, 0.75% and 1%). The duration of exposure was also varied (30 minutes, 40 minutes, 50 minutes and 1 hour). The histological appearances of the trophoblast villi before and after trypsin treatment were compared by studying their appearances in the light microscope.

B. Results

Results of the exposure of placental villi to various concentrations of trypsin are shown in Table III.

i) Histology

Histological sections of the placental villi before trypsin treatment showed placental villi with outer syncytial and cytotrophoblast layers and inner mesenchyme stroma, (Figure 4). Histological analysis of the placental villi treated with 1% trypsin for 20 - 30 minutes at 37 °C revealed that this concentration of trypsin completely destroyed the trophoblast layers and also affected the mesenchyme cores (Figure 5). Paraffin embedded sections of placental villi exposed to 0.75% trypsin for 30 minutes showed similar types of changes to those obtained after treatment with 1% trypsin, although a few villi were seen in which the mesenchyme core was still intact. After exposure to 0.5% trypsin treatment, for 30 minutes, syncytial trophoblast was seen to be removed but no significant changes were noted in the cytotrophoblast layers (Figure 6). After prolonged treatment for 40 - 50 minutes at this

concentration the cytotrophoblast was completely removed. By reducing the concentration of trypsin still further to 0.25% and extending the exposure time to 50 minutes, the syncytial trophoblast was totally destroyed. The cytotrophoblast was seen to be detached in the majority of villi from the mesenchyme cores which appeared to be unaffected by the trypsin (Figure 7).

C. Discussion

Many authors in the past have reported using trypsin to obtain a suspension of cells from trophoblast tissue but few have recorded the effect of the trypsin on the tissue. Thiede (1960) used 0.25% trypsin prepared in TC 199 for 30 minutes for studies of human trophoblast in tissue culture. Soma (1961) also used 0.25% trypsin for 1½ hours for culture purposes. Fox (1970) reported using 0.5% trypsin solution in PBS to obtain monolayer cultures from trophoblast cells and to study their morphology and enzyme histochemistry. However, our results showed that 0.25% trypsin for 50 - 60 minutes duration at 37 °C completely removed the outermost layer of syncytial trophoblast. The cytotrophoblast layer was also separated, while the mesenchyme cores were still intact. We prefer to use 0.25% trypsin for removing trophoblast layers as the lower concentration was marginally less destructive and therefore permitted rapid proliferation of the cells of the mesenchyme core in subsequent tissue cultures.

Our findings are identical with those of Cotte (1980) who also examined histological sections of chorionic villi after treatment with 0.25% trypsin, although he did not study the effects at any other concentration.

Figure 4. Histological section of a placental villus showing intact outer syncytio- and cytotrophoblast layers surrounding inner mesenchyme stroma (Haematoxylin and eosin x40)

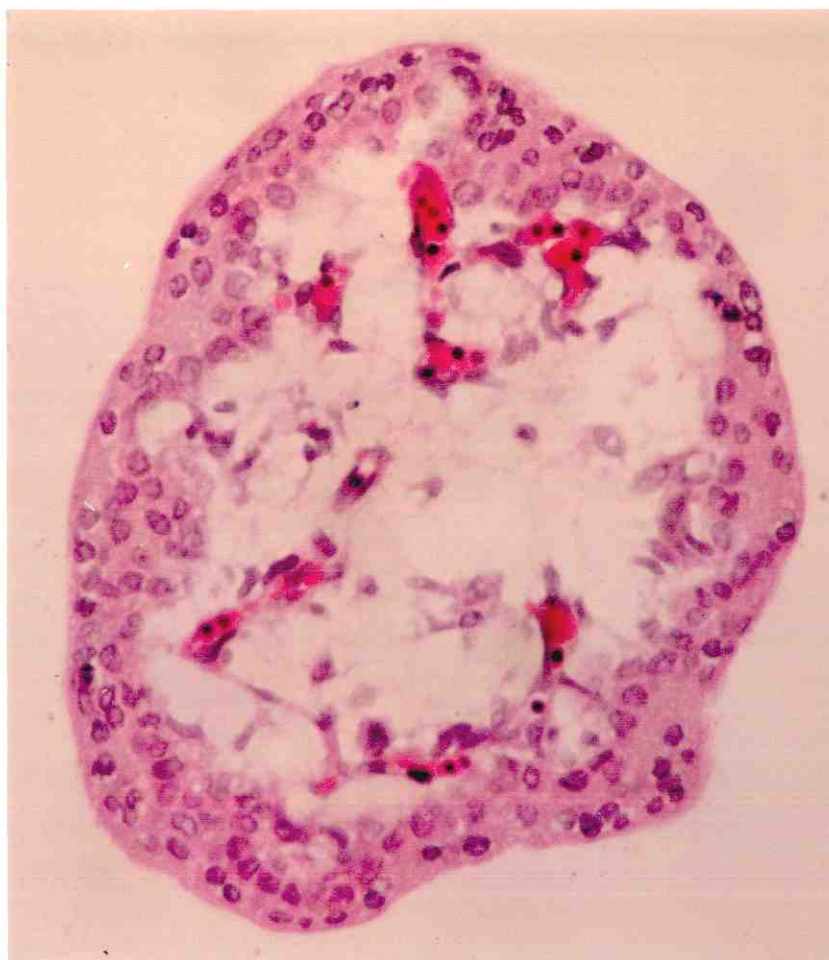


Figure 5. Histological section of the villus treated with
1% trypsin showing the complete destruction of
trophoblast layers. The mesenchyme core is also
affected by the action of trypsin
(Haematoxylin and eosin x100)

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r

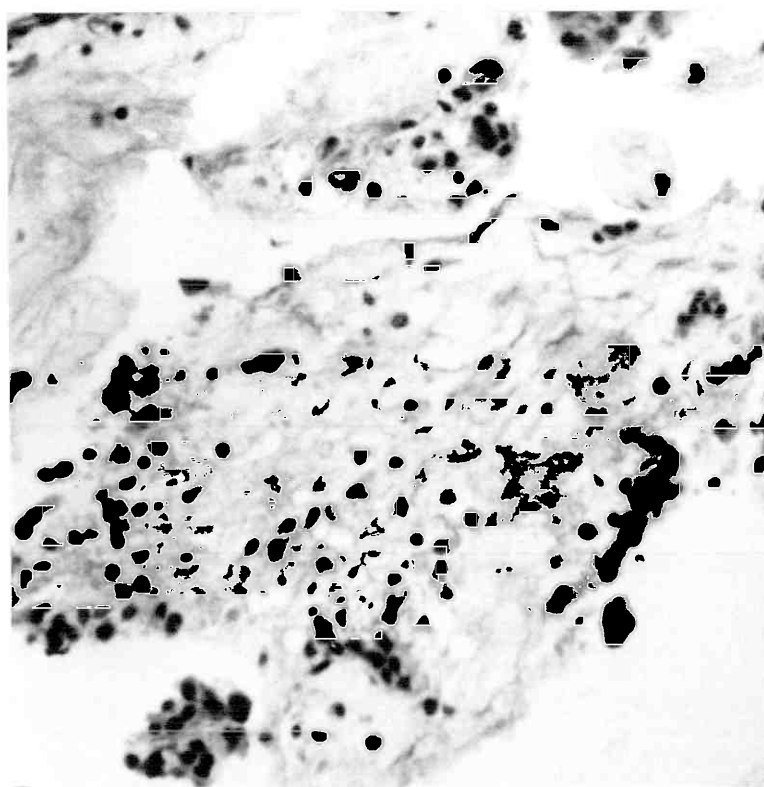


Figure 6. Histological section of chorionic villus exposed
to 0.5% trypsin showed that syncytiotrophoblast
was destroyed but most of the cytotrophoblast was
still intact
(Haematoxylin and eosin x 100)

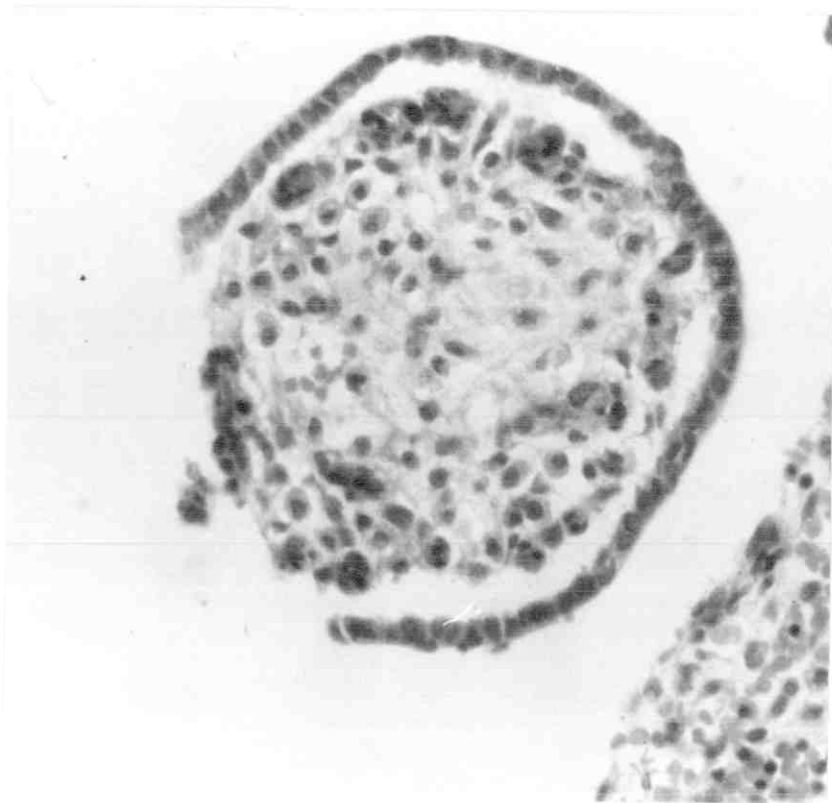


Figure 7. Histological section of the mesenchyme core collected from the top of the gauze after treatment with 0.25% trypsin for 50 minutes. The outer trophoblast layers have been removed by trypsin
(Haematoxylin and eosin x 100)

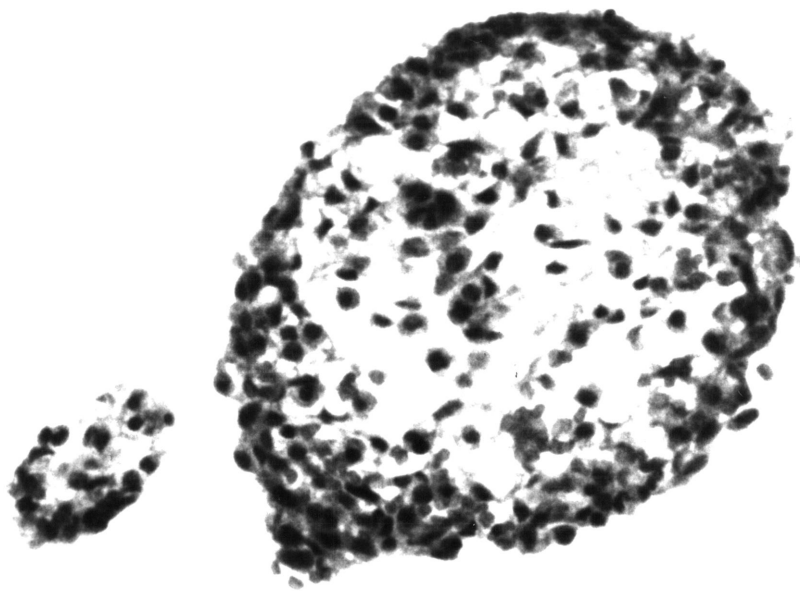
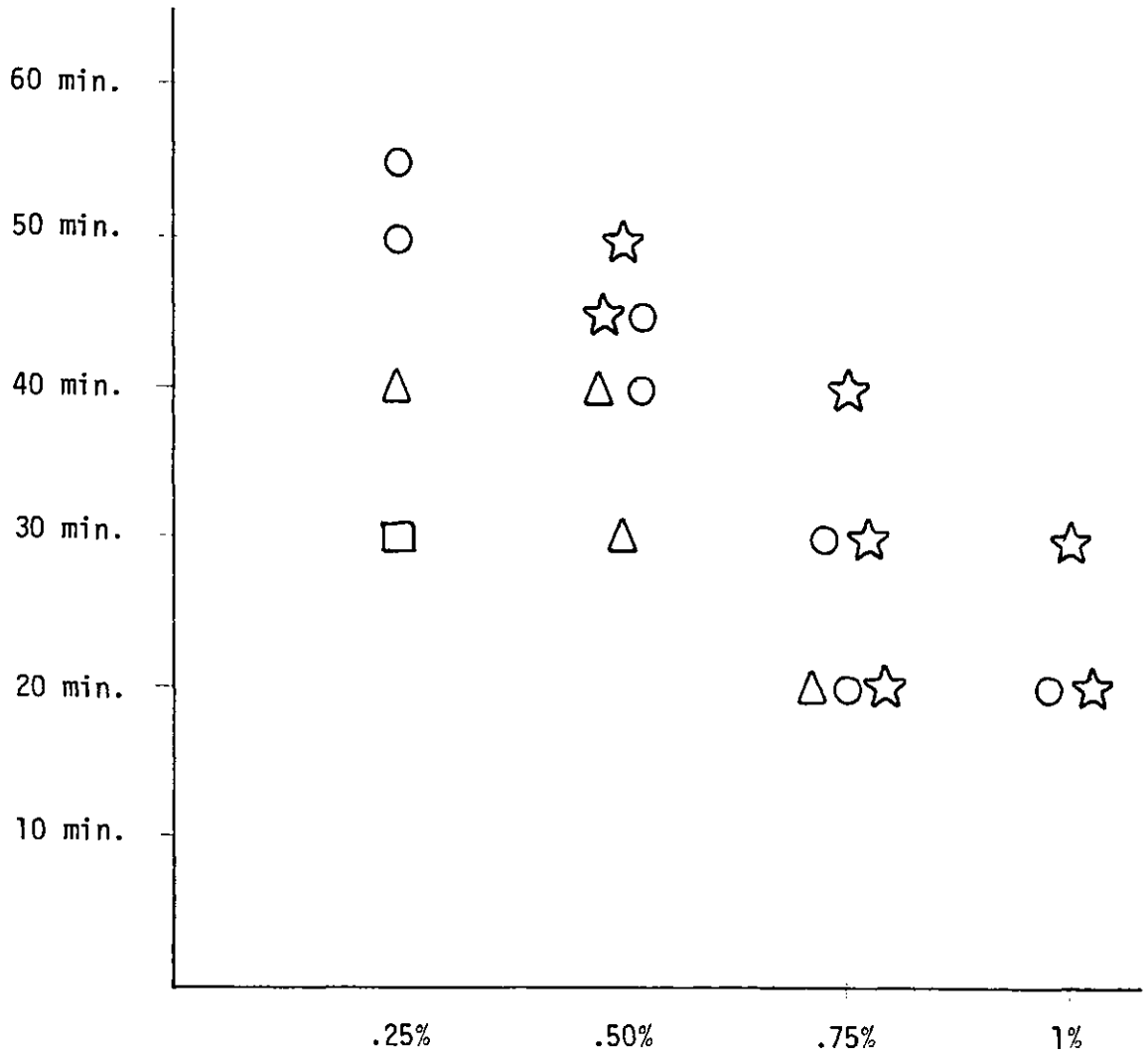


TABLE III

EXPOSURE OF PLACENTAL VILLI TO VARIOUS CONCENTRATIONS
OF TRYPSIN AT 37°C



Key to the symbols:

- Syncytial trophoblast partially removed.
Cytotrophoblast intact.
- △ Syncytial trophoblast completely removed.
Cytotrophoblast partially intact.
- Trophoblast layers removed. Mesenchyme cores intact.
- ☆ Trophoblast layers damaged. Mesenchyme cores
disintegrated.

EXPERIMENT 5

SEPARATION OF TROPHOBLAST LAYERS FROM THE MESENCHYME CORES OF EARLY PLACENTAL VILLI USING A TECHNIQUE OF SEQUENTIAL TRYPSINISATION AND FILTRATION: CONFIRMATION BY HISTOLOGICAL AND CYTOLOGICAL STUDIES.

A. Materials and Method

Obvious placental tissue from 10 pregnancies of 8 - 12 weeks gestation was collected during therapeutic abortion by suction curettage (cases 36 - 45, see Appendix for details). The tissues were washed twice with phosphate buffered saline (PBS) to remove erythrocytes and tissue debris. The placental villi were separated from the membranes by dissection, washed 3 more times and centrifuged at 110 G for 10 minutes. The pellet of villi thus obtained was transferred to a universal container and weighed. A 0.25% solution of trypsin (Difco) was prepared in Ca^{++} and Mg^{++} free phosphate buffered saline with added 2% glucose. The trypsin solution was added to the pellet of villi at a concentration of 4 ml of trypsin solution per one gram of tissue. The container was gently agitated in a gyratory shaker at 37 °C for 50 minutes. Ten ml complete medium, TC 199 containing 30% fetal calf serum was added to this cell suspension which was then centrifuged at 110 G for 10 minutes.

The pellet was resuspended in complete medium and washed twice to remove all traces of trypsin. The cell suspension was then aspirated into a 10 ml syringe and gently filtered through a Swinnex filter apparatus containing 2 layers of sterile wire mesh gauze (mesh count 500 apertures per linear inch and aperture size 0.026 mm). Fragments of villi trapped on the

surface of the wire gauze were gently washed from the gauze with PBS into a sterile container. The filtrate was collected separately.

The filtrate was centrifuged and the supernatant decanted. The cell sediment was resuspended in 2 ml of PBS. The resulting cell suspension was layered onto a gradient of 40 ml of TC 199 containing 40% fetal calf serum and allowed to stand at room temperature for 1 hour. The cells in the surface layers and the cells from the base of the gradient were collected separately into centrifuge tubes containing PBS.

Cell samples were collected at various stages of the cell separation technique for morphological studies in order to establish the effect of trypsinisation and filtration on the placental villi and to identify the cells trapped on the surface of the gauze and the cells which collected at the top and base of the gradient.

B. Results

Histological examination of paraffin embedded sections of placental tissue prior to trypsinisation showed that the villi were intact, each being composed of a central core of loose mesenchymal tissue covered by the 2 layers of the trophoblast (Figure 4).

Histological examination of the villi after trypsinisation showed that the syncytio and cytotrophoblast layers had been stripped from the mesenchyme (Figure 7). The material trapped on the surface of the gauze was composed entirely of mesenchyme cores and no syncytiotrophoblast or cytotrophoblast was identified in any of the sections prepared from this tissue.

Cytological studies of Papanicolaou stained smears prepared from the cells from the base of the gradient showed that they contained numerous strips of cytotrophoblast cells (Figures 8 and 9). Single cells were seen in cytological smears prepared from the surface of the gradient and of these some red blood cells were recognisable but single cytotrophoblast cells could not be distinguished from single mesenchyme cells.

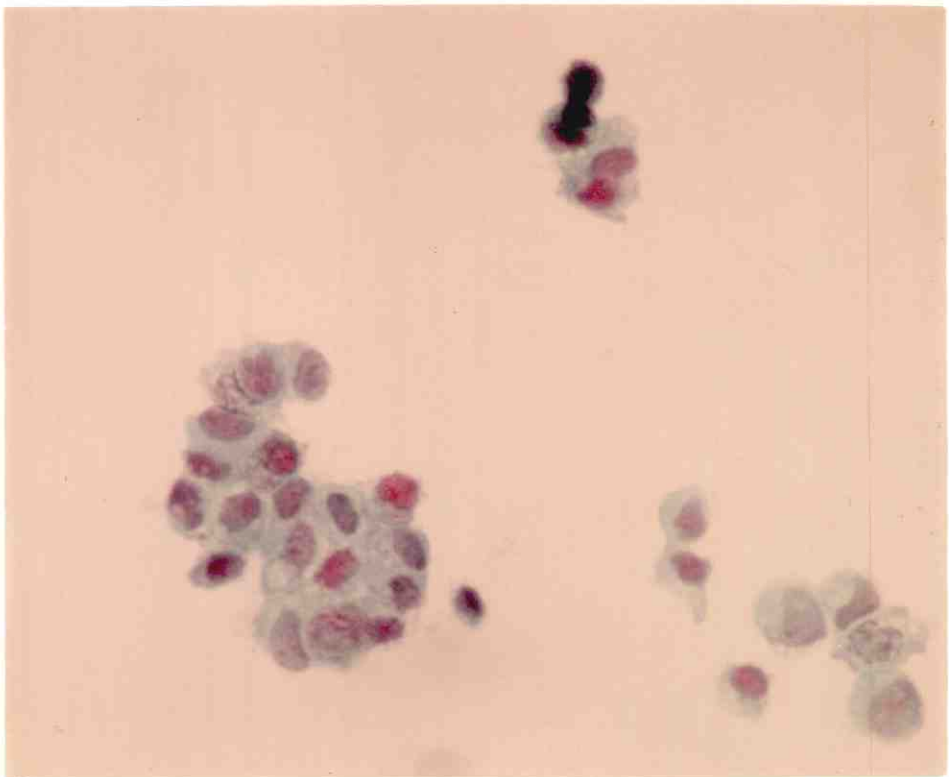
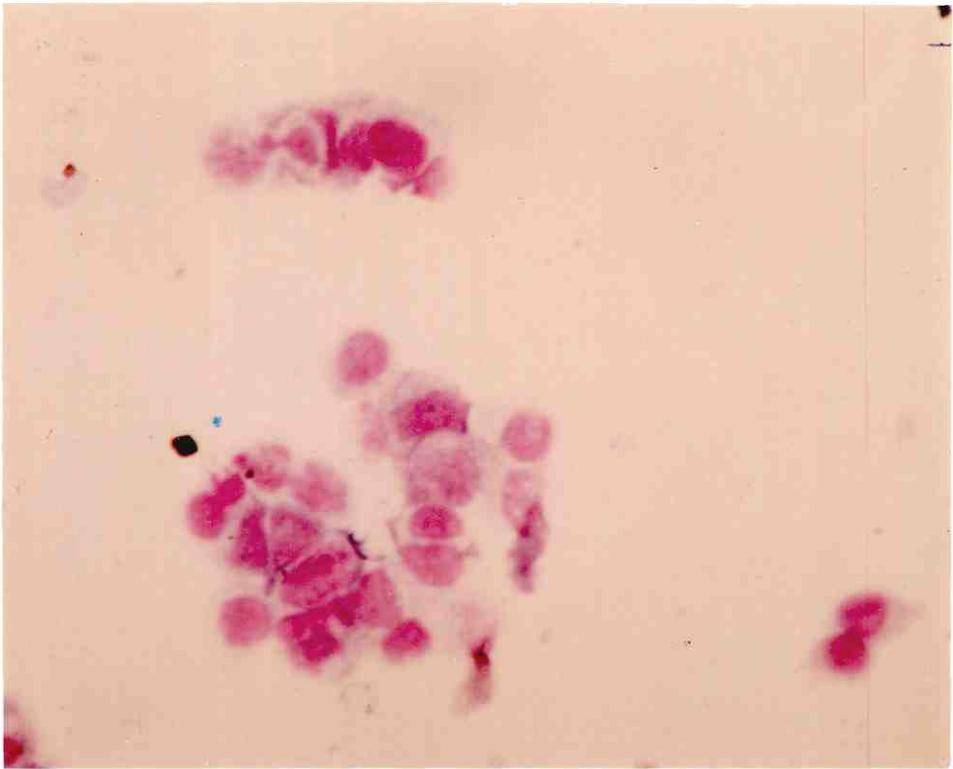
C. Discussion

By sequential trypsinisation and filtration technique we were able to separate ^{the} cytotrophoblast layer from ~~that of~~ ^{the} mesenchyme while the syncytial trophoblast was destroyed during the procedure. This method provided a reliable way of obtaining these 2 types of cells for further studies of their biological properties. Cotte (1980) used a similar technique to separate cytotrophoblast from mesenchyme by trypsinisation but used a different filtration method. He filtered the trypsinised villi through cotton gauze fitted in a 20 ml syringe for this purpose. He then proceeded to separate small strips of cytotrophoblast from the rest of the cell suspension using the gradient system similar to ours, but made no attempt to examine the cells trapped on the cotton gauze.

We tried to use cotton gauze for separation of mesenchyme cores after trypsinisation, but found it unsatisfactory.

Figure 8. Cytological smear of cytotrophoblast strips
collected from the base of the gradient
(Haematoxylin and eosin x100)

Figure 9. Cytological smear of cytotrophoblast strips
collected from the base of the gradient
(Papanicolaus stain x100)



EXPERIMENT 6

SEPARATION OF TROPHOBLAST LAYERS FROM THE MESENCHYME CORES OF EARLY PLACENTAL VILLI USING A TECHNIQUE OF SEQUENTIAL TRYPSINISATION AND FILTRATION: STUDIES OF IN VITRO GROWTH PATTERNS OF CYTOTROPHOBLAST

A. Materials and Method

Obvious placental tissue from 15 pregnancies of 8 - 12 weeks gestation was collected during therapeutic abortion by suction curettage (cases 46 - 60, detailed in the Appendix). The tissue was washed twice with PBS and the placental villi were dissected from the membranes, washed 3 more times and centrifuged at 110 G for 10 minutes. The pellet of villi was trypsinised and filtered through wire mesh gauze as described in Experiment 5.

The mesenchyme cores of villi trapped on the surface of the wire gauze were transferred into PBS in a sterile container and were trypsinised again using the Ca^{++} and Mg^{++} free 0.25% trypsin solution as before. A part of the cell suspension thus obtained was smeared onto a slide and stained with Papanicolaou stain (Figure 10). The rest of the cell suspension was centrifuged and the pellet of cells was resuspended in 2 ml complete medium. A sterile tissue culture flask (Falcon 25 cm²) containing 8 ml complete medium was inoculated with the cell suspension and incubated at 37 °C in 5% CO₂ - 95% air and 90% humidified atmosphere.

The filtrate was centrifuged and the supernatant decanted. The cell sediment was resuspended in 2 ml of PBS. The resulting cell suspension was layered onto a gradient of 40 ml of TC 199 containing 40% fetal calf serum and allowed to stand at room

temperature for 1 hour. The cells from the top of the gradient and the base of the gradient were collected separately into sterile centrifuge tubes containing PBS and inoculated into tissue culture flasks.

B. Results

Tissue culture studies of the trypsinised mesenchyme cores from the surface of the gauze showed that the mesenchyme cells settled within the first 2 days and started proliferating rapidly in the form of fibroblasts and confluence was noted by the fifth to tenth day. On average these cells were subcultured by the seventh day and monolayers suitable for chromosome preparations or tissue typing studies were obtained within the next 6 days (Figure 11).

Tissue culture studies of the cytotrophoblast cells from the base of the gradient showed that the cells settled within 48 hours and at first appeared under phase contrast as a confluent, flattened, epithelial type of growth. After 4 days a number of multinucleated giant cells were observed in the culture and there were large spaces devoid of cells in the tissue culture flask. Six days later the majority of cells appeared to have undergone fusion giving rise to a population of giant multinucleated cells which eventually disintegrated (Figures 12 and 13).

Studies of the cultures of the cells from the top of the gradient showed that some single cells settled and formed a flattened, epithelial type of growth. Multinucleate cells were apparent after 4 days and disintegrated within the next 10 days. Some single cells which were thought to be derived from the

mesenchyme grew in the form of fibroblasts. After 14 days these were the predominant type of cells in the culture.

C. Discussion

The process of trypsinisation and filtration of early placental villi enabled us to separate mesenchyme cells from cytotrophoblast layers and study their growth potential. The mesenchyme cells grew rapidly whereas the cytotrophoblast cells grew slowly and had a limited potential for growth. Cotte (1980) also studied cytotrophoblast in culture and observed a similar pattern of limited growth.

Our studies have shown that it was possible to obtain rapidly growing cultures of fetal cells from early placental villi and our results indicated that the technique of trypsinisation and filtration may be appropriate for the investigation of endocervical aspirates in order to obtain cultures of fetal cells from these specimens. We proceeded to investigate endocervical aspirates by this technique (Experiment 8). If cells grown from early placental villi are to be used for antenatal diagnosis other than chromosome analysis it is essential that they should express the biological properties which are of diagnostic value. As an initial step toward establishing the biological properties of these cells we carried out some preliminary studies on the HLA status of the mesenchyme cells (Experiment 7).

Figure 10. Smear of mesenchyme cells obtained after
trypsinisation of mesenchyme cores
(Papanicolaou stain x40)

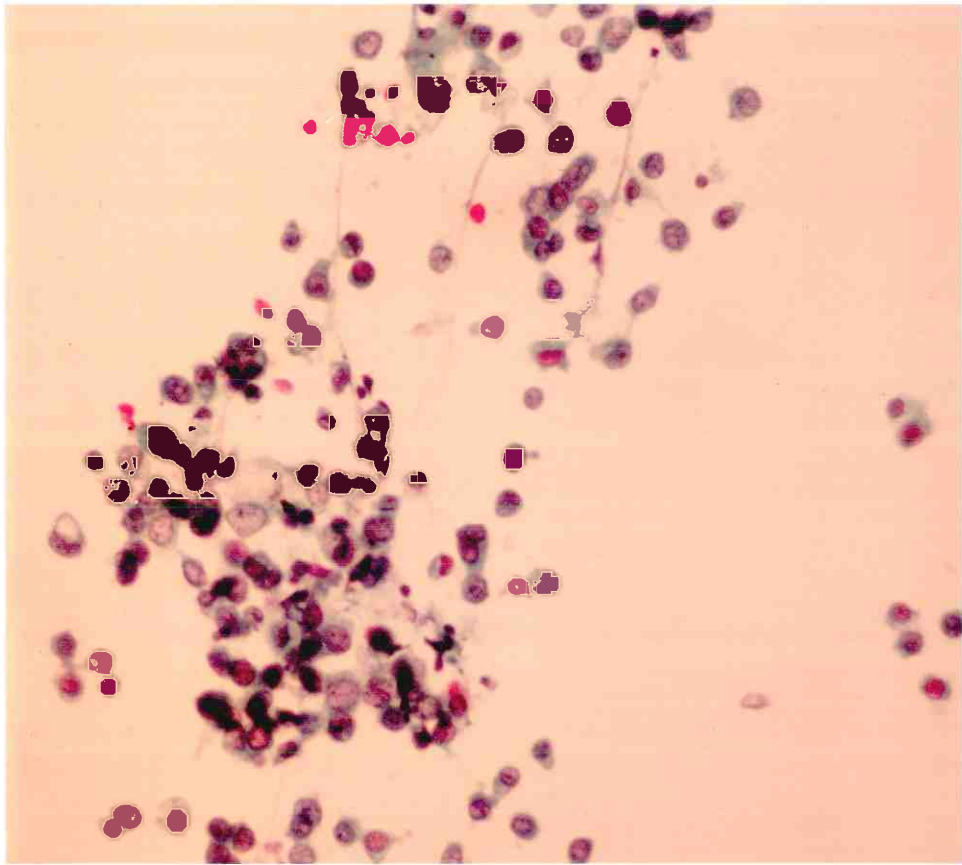
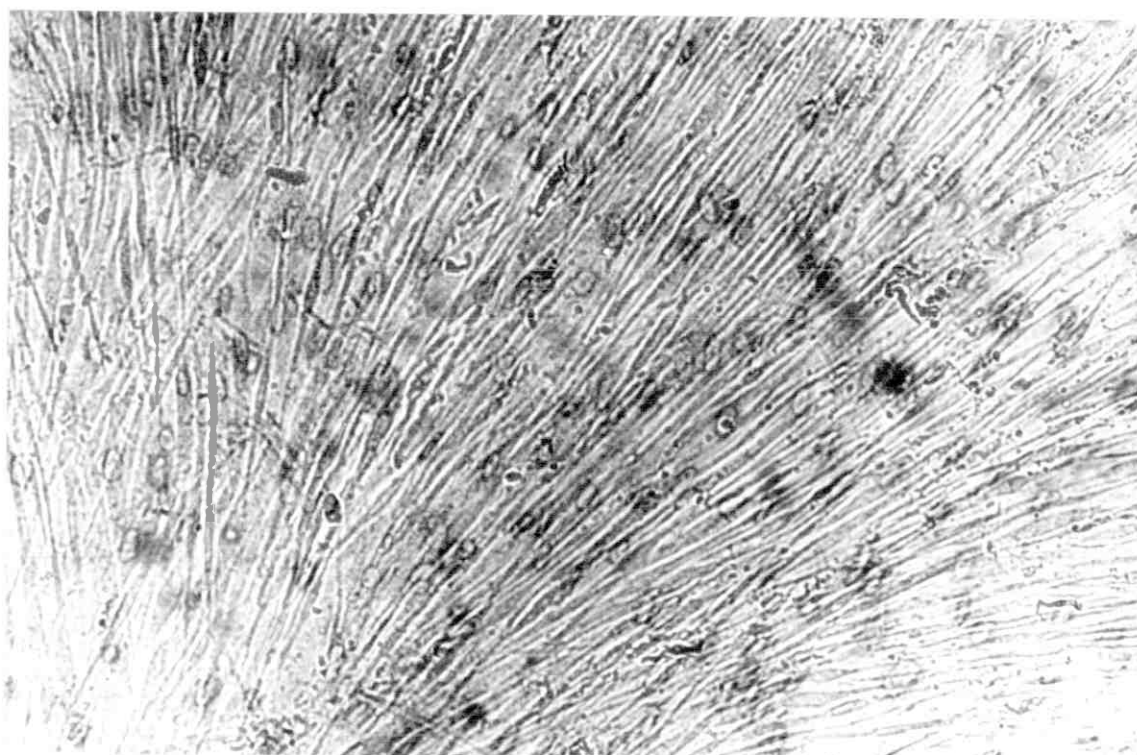
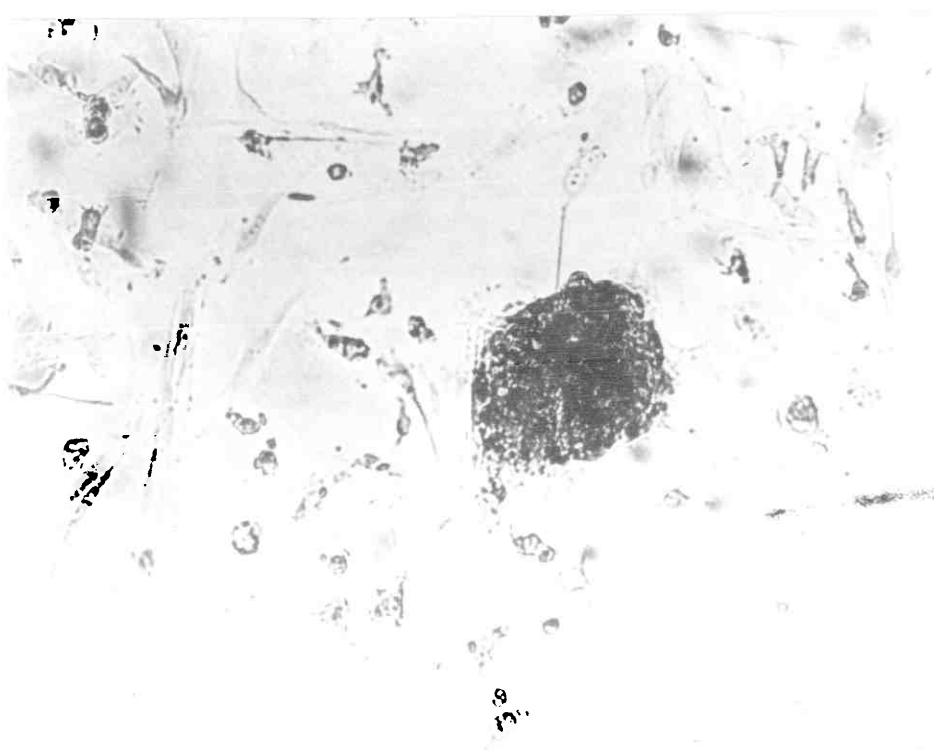
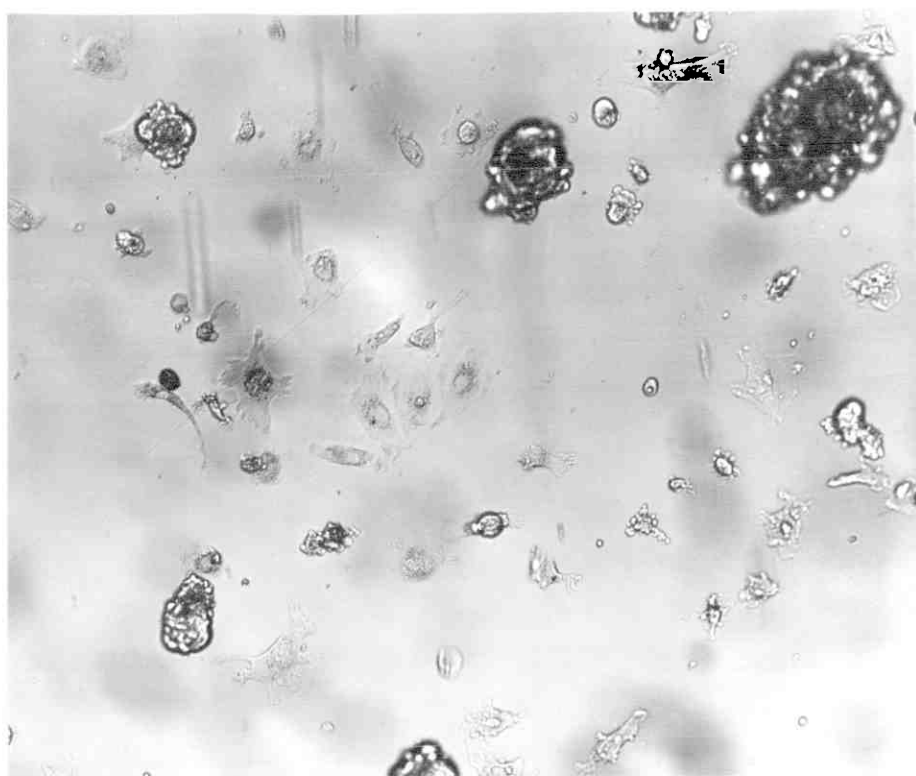


Figure 11. Monolayer of cultured mesenchyme cells after
7 days
(Phase contrast x100)



Figures 12 and 13 Cultures of cytotrophoblast cells after 10 days.
The single cells have fused to form syncytial knots.
(Phase contrast x100)



EXPERIMENT 7

SEPARATION OF TROPHOBLAST LAYERS FROM THE MESENCHYME CORES OF EARLY PLACENTAL VILLI USING A TECHNIQUE OF SEQUENTIAL TRYPSINISATION AND FILTRATION: CYTOGENETIC AND TISSUE TYPING STUDIES OF MESENCHYME CELLS.

A. Materials and Method

Placental specimens were obtained from 23 women (cases 60 - 83, see Appendix). Twenty-one of these were collected immediately prior to suction termination of pregnancy during the first trimester. Placental tissue from case 76 was obtained after termination of pregnancy at the 22nd week of gestation for chromosomal abnormality. A diagnosis of Down's Syndrome was made from cultured amniotic fluid cells. Chorionic villi were collected from case 77 after a missed abortion during the 13th week of pregnancy. Cultures of mesenchyme cores were obtained by the process of trypsinisation and filtration described in Experiments 5 and 6.

Tissue typing was performed in 6 cases (78 - 83) and chromosome preparations were made from cultures of mesenchyme cells in 17 cases (61 - 77). Methods for chromosome preparation and tissue typing are described in the Appendix.

i) Control tissues

Fetal tissues, eg. limbs, cord etc. were collected from 10 cases in this series. These tissues were cultured using the plasma clot method and cultured cells were harvested and tissue typed using the procedures described in the

Appendix. Tissue typing studies were carried out in 2 cases and cytogenetic studies were made in 8 cases.

Maternal venous blood was collected in cases 78 - 83 and tissue typing studies were carried out on maternal lymphocytes at the Anthony Nolan Laboratory, Westminster Hospital, London.

B. Results

i) Tissue Typing Studies

Tissue type results are shown in Table IV. Tissue typing studies of cases 78 - 83 showed that HLA antigens at the 2 major loci were expressed and detectable on cells grown from mesenchyme cores. Tissue typing studies of cultured fetal cells in 2 cases (cases 82 and 83) were identical with those of the cultured mesenchyme cells from the same cases. Comparison of the tissue types of the mesenchyme cells cultured from cases 78 - 83 with those of the mother showed that mesenchyme cells expressed one haplotype which was present in the mother and another haplotype which was not detected on maternal lymphocytes.

ii) Cytogenetic Studies

Chromosome results are shown in Table V. Chromosome studies on cells grown from mesenchyme cores revealed normal female karyotypes 46,XX in 9 cases and normal male karyotypes, 46,XY in 6 cases. The karyotype from case 76 was that of Down's Syndrome, 47,XX + 21. In case 77 chromosome preparation from the mesenchyme cores of the chorionic villi showed a modal number of 69 chromosomes

and Y-chromosome fluorescence was positive. A karyotype of 69 XXY was obtained (Figure 14). Chromosome results from the mesenchyme cells of the chorionic villi were obtained between 15 - 24 days (mean 19 days).

C. Discussion

Several groups of workers have studied the expression of HLA antigens on chorionic villi. Loke, Joysey and Borland (1971) demonstrated the expression of HLA antigens on cells cultured from trypsinised suspensions of chorionic villi obtained from 3 conceptuses of 12 weeks gestation. These authors used a cytotoxic technique to determine the HLA antigens of the cultured trophoblast and fetal cells. Cells cultured from both trophoblast villi and fetal cells were killed in the presence of multi-specific anti-HLA serum. Therefore, it was concluded that HLA antigens were expressed on human trophoblast cells grown in the form of monolayers. However, as there were both cytotrophoblast and mesenchyme cells present in their cultures, it is not clear from the experiment which types of cells were expressing the antigens. Moreover, there is some doubt as to the specificity of antisera used in this experiment, for it has since become clear that a number of antisera of this type act against a variety of antigens, not all of which are of the histocompatibility type (Fox, 1978).

Faulk and Temple (1976) used an indirect immunofluorescence technique to study the distribution of beta₂ microglobulin on trophoblast cells. Since HLA has been shown to be invariably associated with beta₂ microglobulin (Nilsson et al, 1974), the presence of beta₂ microglobulin was used as a marker for HLA.

They used specific antisera which were supplied by Dr A Sanderson, Department of Biochemistry, Guy's Hospital Medical School, London to demonstrate the distribution of β_2 microglobulin in intact placental tissues. They obtained specific fluorescence of villous stromal cells and of fetal endothelial cells but not of trophoblast or trophoblast basement membrane. Treatment of cryostat sections of early placental villi with various enzymes (Hyaluronidase, collagenase or trypsin) failed to reveal β_2 microglobulin on trophoblast or trophoblast basement membrane by immunofluorescence. They also repeated the test using specific antisera against the HLA antigens, A1, A2, B7 and B12 raised in rabbits and obtained similar results. Our study of cultures of mesenchyme cells also indicate that HLA antigens are present in these cells and thus confirm Faulk and Temple's findings. We were unable to study HLA expression on isolated trophoblast cells. We attempted to repeat the experiment reported by Faulk and Temple (1976). We obtained specific antisera raised in mice and chicken against β_2 microglobulin from Dr Sanderson, and attempted to demonstrate the antigens on frozen sections of early placental villi using an indirect immunoperoxidase technique, but our results were inconclusive.

Cytogenetic studies of cultured mesenchyme cells showed the practical application of the cell separation technique. Using this technique it was possible to obtain chromosome results within a period of 3 weeks, which is particularly useful as our own experience (Experiment 3) and those of other authors (Jones, Gey and Gey, 1943; Thiede, 1960; Fox and Kharkonger, 1970) had shown that growth of intact placental villi is slow and 8 weeks may elapse before adequate cells for chromosome studies are

obtained. It was also possible to detect abnormal karyotypes when they were present. In case 76, the diagnosis was made from cells cultured from the amniotic fluid and similar results were obtained from placental cells. In case 77 a diagnosis of triploid fetus was made from rapidly growing cultures of mesenchyme cells.

Figure 14. A triploid karyotype from cells grown from material obtained from missed abortion. Cultures prepared from villous mesenchyme after trypsinisation and filtration of the abortus tissue (C-banded preparation)

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CYTOGENETIC UNIT

GH & Co.

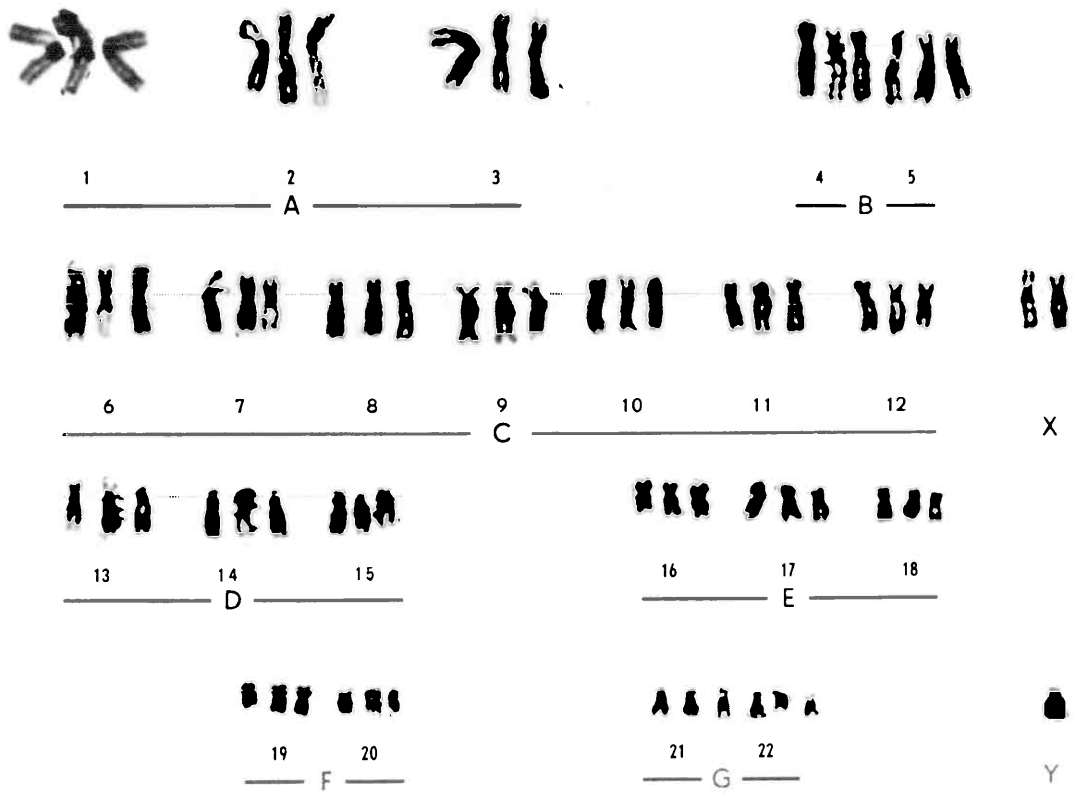


TABLE IV

TISSUE TYPING OF MESENCHYME CELLS

CASE NO.	PATIENT INDEX	AGE	GESTATION	PARITY	TISSUE TYPE OF MATERNAL LYMPHOCYTES Sub loci A & B	TISSUE TYPE OF FETAL CELL Sub loci A & B	TISSUE TYPE OF MESENCHYME CELLS Sub loci A & B
78	CC	20	11/40	0 + 0	A1,11; B -, 3	ND	A1, 2; B 8,12
79	CM	18	10/40	0 + 0	A2, -; B 7,12	ND	A2, 3; B 7, 5
80	BE	26	12/40	2 + 0	A2, 9; B -, 7	ND	A1, 9; B -, 7
81	KL	17	10/40	0 + 1	A3, -; B ;W22	ND	A3,*1; B -; 8
82	NF	19	12/40	0 + 0	A11,-; B12,14	A1,11; B18,12	A1,11; B18,12
83	MO'S	24	10/40	1 + 1	A10,9; B -; 5	A3, 9; B -; 5	A3, 9; B -; 5

* weak reaction

TABLE V
KARYOTYPING OF MESENCHYME CELLS

CASE NO.	PATIENT INDEX	AGE	GESTATIONAL AGE (by LMP)	PARITY	KARYOTYPE OF FETAL FIBROBLASTS	KARYOTYPE OF MESENCHYME CELLS	DURATION OF CULTURE OF MESENCHYME CELLS
61	ER	20	10/40	NK	ND	46,XX	18 days
62	RG	18	12/40	NK	ND	46,XX	20 days
63	YCC	20	9/40	0 + 1	46,XX	46,XX	20 days
64	AL	24	8/40	0 + 0	ND	46,XX	16 days
65	RG	29	14/40	0 + 0	ND	46,XX	15 days
66	LH	29	12/40	1 + 2	46,XX	46,XX	19 days
67	NL	36	?11/40	1 + 4	ND	46,XY	18 days
68	AW	23	10/40	0 + 3	46,XY	46,XY	20 days
69	SF	20	14/40	0 + 0	46,XX	46,XX	20 days
70	SH	19	12/40	0 + 0	46,XY	46,XY	20 days
71	JR	18	10/40	0 + 0	46,XY	46,XY	20 days
72	JB	25	?13/40 by size	1 + 0	46,XX	46,XX	18 days
73	BP	25	12/40	0 + 0	ND	46,XX	18 days
74	NCB	23	10/40	0 + 2	ND	46,XY	20 days
75	AH	19	9/40	0 + 0	46,XY	46,XY	24 days
76	CM	41	23/40	0 + 0	47,XX + 21	47,XX + 21	21 days
77	HSG	31	13/40	0 + 1	ND	69,CP-,XXY	24 days

ND = Not done
Mean duration of culture of mesenchyme cells = 20 days

EXPERIMENT 8

INVESTIGATION OF ENDOCERVICAL ASPIRATION AS A METHOD OF OBTAINING FETAL CELLS FROM FIRST TRIMESTER PREGNANCIES FOR EARLY ANTENATAL DIAGNOSIS.

A. Materials and Method

This study was performed on 25 patients who were admitted for therapeutic termination of pregnancy at the St Mary's Hospital. Their ages varied from 14 - 40 years (mean 22 years); 12 of these patients were multiparous and the rest were primigravidae. Their gestational age varied between 6 - 13 weeks (mean gestational age at the time of termination of pregnancy was 9.5 weeks). The details of these patients (cases 83 - 108) are shown in the Appendix. The informed consent of the patient was obtained at least a day before the scheduled abortion. Endocervical aspirations were obtained immediately before termination in all cases. Trophoblast samples were obtained using a medicut cannula (gauge 16) attached to a 20 ml syringe as in Experiment 1 (Figure 15). The patient was anaesthetised and placed in the lithotomy position for termination of pregnancy and the obstetrician determined the size and the position of the uterus by bimanual examination. The ectocervix was then cleaned with Hibitane solution and the anterior lip of the cervix was held firmly with 2 pairs of volsellum forceps. The medicut cannula attached to the syringe was then inserted into the cervical canal and the plunger of the syringe was withdrawn 4 - 5 cm thus aspirating the tissues into the cannula and the

syringe. After aspiration the syringe was withdrawn and the aspirate was collected in 10 ml of TC 199 into a sterile universal container.

After the aspirate had been obtained, termination of the pregnancy was carried out by suction curettage and the products of conception, including obvious fetal tissues, were collected separately into TC 199. All samples were immediately transported to the Cytogenetics Laboratory at St Mary's Hospital. Upon arrival at the laboratory, the endocervical aspirates were weighed and the contents of the container were poured into the sterile petri dish. The contents of the dish were examined macroscopically and under phase in a Leitz diavert microscope, magnification x40, for the presence of villous tissue. In 16 cases, samples were prepared for histological examination and in 5 cases chorionic villi were dissected out under phase contrast microscopy and treated by the trypsinisation-filtration technique for the culture of cells.

B. Results

The results of endocervical aspirates are shown in Table VI.

i) Macroscopic and Phase Contrast Examination

This was carried out on all 25 cases. Chorionic villi were identified macroscopically as delicate frond-like structures floating freely in the culture medium. These coral like structures were readily distinguished from any other tissues (decidua and membranes) that were present in the aspirates. On phase microscopy the chorionic villi were recognised as finger-like structures with numerous syncytial buds (Figures 16 and 17). The

results of macroscopic and phase contrast studies showed that 5 specimens contained chorionic villi only and varying traces of blood or mucus were present. Six specimens contained chorionic villi along with other tissues, ie. decidua and membranes.

In 10 specimens no chorionic villi were observed. They only consisted of fragments of tissues which were mostly decidua. Four specimens were composed of blood only, and no other recognisable tissue was observed.

ii) Histology

Histological examination was carried out in 16 cases. These studies confirmed macroscopic and phase contrast findings. Seven of these cases were seen to contain chorionic villi. One of these cases contained villous material only and in the other 6 cases both villous tissue and decidual tissue were seen to be present. No villous material was seen in the histological sections from the other 9 cases.

iii) Tissue Culture

Tissue culture was carried out in 5 cases, only one of which was also examined histologically. However, all 5 cases appeared macroscopically to contain chorionic villi. Two cultures failed to grow due to infection with *Aspergillus niger*, a common atmospheric spore. Cultures were successful in the remaining 3 cases and karyotyping studies of the cultured cells revealed a normal male chromosome complement in 2 cases and normal female complement in one case (Figures 18 and 19). This last

result correlated with cytogenetic studies in cells grown from the corresponding fetus which was also found to have a normal female karyotype. The time taken to grow adequate cells for cytogenetic studies was 16, 20 and 19 days respectively.

iv) Weights of Aspirated Tissue

Endocervical aspirates were weighed in 18 cases. This varied between 0.2 to 1 gram (mean weight, 0.6 gram). It was possible to dissect out the chorionic villi in 9 cases and their weights varied between 0.08 - 0.7 gram (mean weight 0.39 gram).

C. Discussion

This study demonstrated that it was possible to aspirate chorionic villi during early pregnancy without prior cervical dilatation. The phase contrast examination of the aspirated tissue followed by the separation of readily recognisable frond-like placental villi from the decidua made it possible to trypsinise the villi and filter the cells. By doing this we stripped the villi of their covering syncytiotrophoblast and cytotrophoblast and thus promoted the growth of mesenchyme cells in culture. In this way we were able to suppress the growth of maternal decidual cells which normally swamp intact (or unstripped) placental villi in tissue culture, and obtain a pure growth of fetal cells.

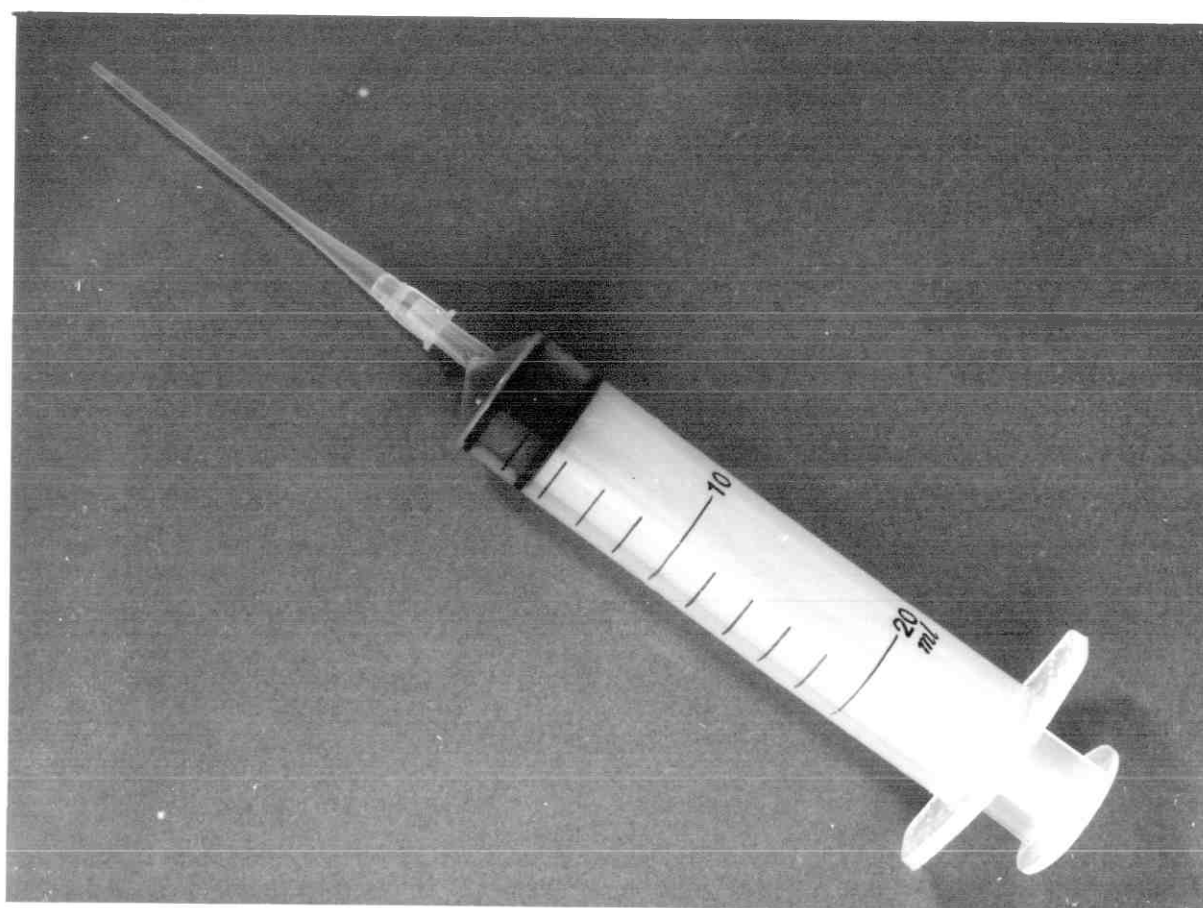
Endocervical aspiration was considered successful if it included chorionic villi. In this series of 25 patients villous material was present in 44% of the aspirates. In a further

series of 15 cases, villous tissue was seen to be present in 4 cases. Our overall success rate of obtaining villi is 37.5%.

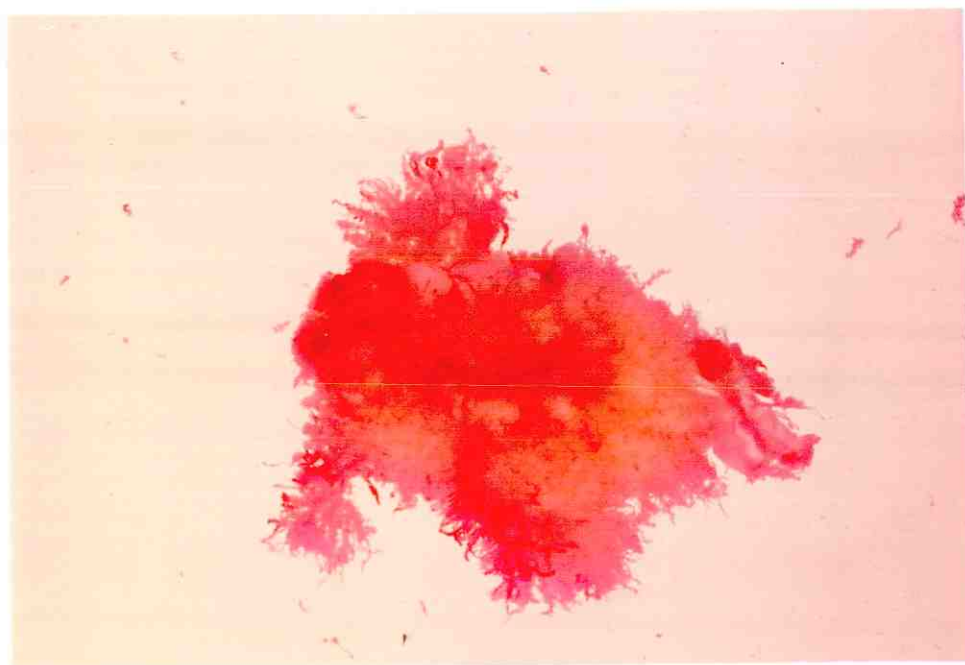
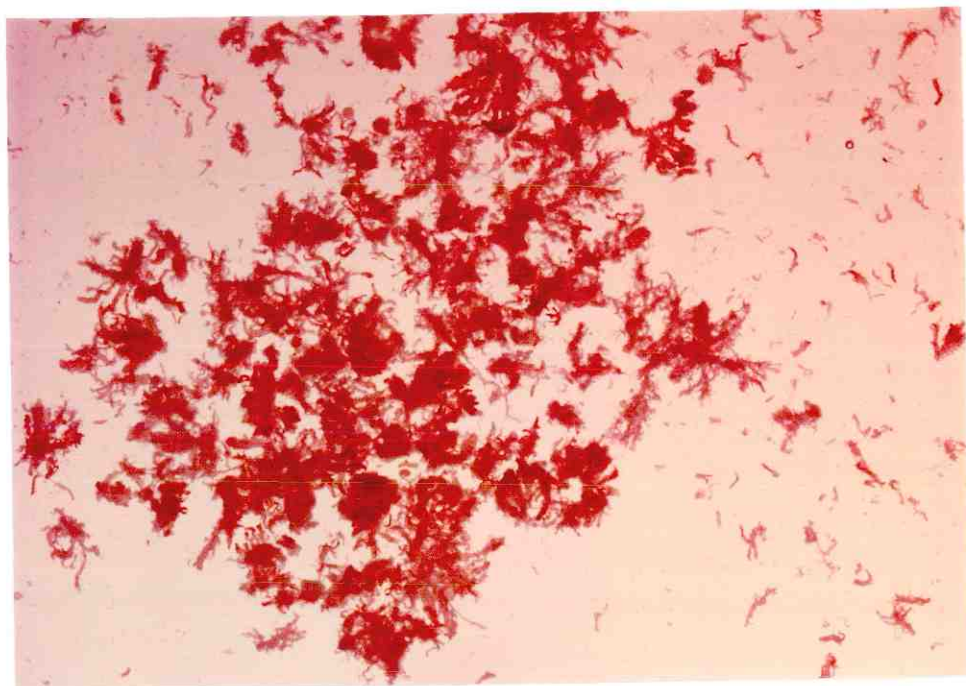
The minimum amount of villous material from which a successful culture was obtained in this series was 0.4 grams. We have now successfully cultured specimens from 3 further cases which weighed less than 0.4 grams. These latter cultures took 18, 15 and 20 days to grow successfully.

We attempted to evaluate the optimum gestational period for obtaining the specimen. For this purpose we related the gestational age of the pregnancy to our ability to obtain chorionic villi successfully (Table VII). Pure villous material, free of decidua was obtained from women whose mean gestational age was 7.9 weeks. In the second and third groups where chorionic villi were found together with decidua and membranes the mean gestational age was 9.5 and 9.8 weeks respectively. In those specimens where blood was aspirated, the pregnancies varied between 10 - 12 weeks gestation. We were more successful in obtaining villi in earlier pregnancies than in later pregnancies. This trend was confirmed by our further series of 15 cases (not included in this thesis).

Figure 15. Medicut cannula and syringe used for endocervical aspiration
(Actual size)



Figures 16 and 17. Macroscopic appearance of chorionic villi
obtained from the endocervical aspirates.



Figures 18 and 19. Normal male and female chromosome spreads
(G-banded preparation) obtained from
cultured mesenchyme cells after sequential
trypsinisation and filtration technique



TABLE VI

STUDIES OF ENDOCERVICAL ASPIRATES

CASE NO.	PATIENT INDEX	AGE	GESTATION	PARITY	WEIGHT OF THE ASPIRATED TISSUE	WEIGHT OF VILLI AFTER DISSECTION	PHASE CONTRAST MICROSCOPY	HISTOLOGY OF THE ASPIRATED TISSUE	CULTURE
84	SB	25	12/40	1 + 0	ND	ND	Fragments of tissue and villi	Placental villi and decidua present	ND
85	SM	18	11/40	0 + 0	ND	ND	Fragments of tissue, mucus, blood and villi.	Placental villi. Decidua and membranes present	ND
86	KC	23	8/40	0 + 0	1 gram	.2 gram	Membrane tissue, mucus and villi	Placental villi and decidua present	ND
87	DH	14	10/40	0 + 0	.5 gram	.08 gram	Fragments of tissue. Few villi present.	Placental villi and decidua present	ND
88	EL	18	12/40 by size	0 + 0	ND	ND	Few fragments of tissue. No villi observed	No villi observed Decidua present	ND
89	CS	20	11/40	1 + 0	ND	ND	12 ml blood only	ND	ND
90	MJ	15	9½/40	1 + 1	.5 gram	.5 gram	Placental villi only slightly blood stained	ND	Culture infected
91	AA	23	12/40	1 + 1	ND	ND	10 ml blood only. No tissue observed	ND	ND

Continued.....

TABLE VI (Continued)

CASE NO.	PATIENT INDEX	AGE	GESTATION	PARITY	WEIGHT OF THE ASPIRATED TISSUE	WEIGHT OF VILLI AFTER DISSECTION	PHASE CONTRAST MICROSCOPY	HISTOLOGY OF THE ASPIRATED TISSUE	CULTURE
92	CW	16	6/40	0 + 1	.9 gram	.5 gram	Placental villi. Some mucus present	Placental villi present. No decidua observed	Culture infected
93	MM	23	9½/40	2 + 0	.8 gram	.4 gram	Mucus and few villi	Placental villi present. No decidua observed	ND
94	KT	20	9/40	0 + 0	.6 gram	0.1 gram	Fragments of tissue. Few villi observed	Placental villi and decidua present	ND
95	SA	23	7/40	0 + 0	.8 gram	0.4 gram	Mostly villous tissue, some membranous tissue present	ND	Culture successful
96	NM	20	11/40	2 + 2	.4 gram	ND	Fragments of membrane and blood	No villi observed. Decidua present	ND
97	NA	22	10/40	2 + 0	.6 gram	ND	Membranous tissue. Fragments of clotted blood	No villi observed. Decidua present	ND
98	ST	25	7½/40	3 + 1	.7 gram	0.7 gram	All villous material	ND	Culture successful
99	TA	26	10/40	2 + 1	ND	ND	14 ml blood only	ND	ND
100	PW	22	12/40	0 + 0	ND	ND	10 ml blood only	ND	ND

Continued

TABLE VI (Continued)

CASE NO.	PATIENT INDEX	AGE	GESTATION	PARITY	WEIGHT OF THE ASPIRATED TISSUE	WEIGHT OF VILLI AFTER DISSECTION	PHASE CONTRAST MICROSCOPY	HISTOLOGY OF THE ASPIRATED TISSUE	CULTURE
101	KH	20	7/40	2 + 0	.6 gram	0.6 gram	Placental villi some blood	ND	Culture successful confirmed by karyotyping fetal cells
102	EH	21	13/40	1 + 1	.5 gram	ND	6 ml blood and unrecognisable tissue	ND	ND
103	NF	25	10/40	0 + 0	.2 gram	ND	Fragments of tissue	No villi observed. Decidua present	ND
104	KL	25	10½/40	0 + 0	.4 gram	ND	Fragments of tissue	No villi observed. Decidua present	ND
105	MW	40	6/40	0 + 0	.5 gram	ND	Fragments of tissue and mucus	No villi observed. Decidua present	ND
106	SL	24	8/40	0 + 0	.6 gram	ND	Endocervical material and mucus only	No trophoblast seen. Decidua present	ND
107	MA	23	9/40	0 + 0	.6 gram	ND	Mucus and one fragment of cervical tissue	No trophoblast seen. Decidua present	ND
108	SK	15	8/40	0 + 0	.4 gram	ND	Blood and fragments of tissue	No Trophoblast seen. Decidua present	ND

ND - Not done

TABLE VII

STUDY OF ENDOCERVICAL ASPIRATES FROM 25 PATIENTS

Specimen	Chorionic villi only with small or no traces of blood	Chorionic villi with decidua, membranes, mucus and blood	Decidua and membranes with small or no traces of blood	Blood only, no recognisable tissue
Number	5	6	10	4
Percentage	20	24	40	16
Range of maternal age	15 - 25 years	14 - 25 years	15 - 40 years	20 - 26 years
Mean maternal age	19.8 years	20.5 years	23.3 years	22.8 years
Range of gestation	6 - 9.5 weeks	7 - 12 weeks	6 - 13 weeks	10 - 12 weeks
Mean gestation period	7.9 weeks	9.5 weeks	9.8 weeks	11.3 weeks

CHAPTER IV

GENERAL DISCUSSION

A. Disadvantages of Current Methods of Antenatal Diagnosis and Potential Advantages of Trophoblast Sampling

The disadvantage of the current methods of antenatal diagnosis ie. amniocentesis and fetal blood sampling, is that they are carried out after the 16th week of pregnancy, so that if therapeutic termination is indicated it is carried out late in pregnancy. This problem is particularly acute when the diagnosis depends upon the analysis of cultured cells. Polani et al (1979) published a report on patients attending the antenatal clinic at Guy's Hospital for diagnostic amniocentesis. The patients under investigation were at risk of having an abnormal fetus with a chromosomal disorder, neural tube defect or an inborn error of metabolism. According to this report, the majority of terminations for all reasons were carried out after the 20th week of pregnancy. Terminations for neural tube defects were carried out slightly earlier in the pregnancy than terminations carried out for conditions such as Down's Syndrome. This can be explained by the fact that the assay of the alphafetoprotein content of amniotic fluid can be carried out in a shorter time than cytogenetic studies which depended on culture of large numbers of cells.

Late terminations cause a great deal of mental stress both for the mother and the caring hospital staff. The delay in decision making about the diagnostic outcome of the pregnancy in its advanced stages is a cause of anxiety to both parents.

It is also unacceptable to some couples to have late terminations, particularly in the Asian community. Recently Modell et al (1980) have published a report on 22 East African Asian couples who were at risk of producing a child with thalassaemia major. These authors reported that 9 of these couples refused the opportunity of antenatal diagnosis. Some did so because of their previous experience of late termination. Others did so for religious or family reasons. The Sunna sect of Islamic religion believe that according to Holy Quran the soul enters into the developing human fetus at 16 weeks gestation and therefore it is considered sinful to induce termination of the developing human fetus after this stage of pregnancy. Moreover, a history of a genetically abnormal pregnancy can prove disadvantageous in a community where arranged marriages are the rule. Any history of genetic disease in the family prejudices a woman's chances of marriage. A late termination for genetic disease cannot be concealed from the community and this may have been a factor influencing the couples against antenatal diagnosis.

Other consequences of late termination are the increased risks of haemorrhage, sepsis and cervical laceration for women whose pregnancy has been terminated in the second trimester compared with women whose pregnancy has been terminated in the first trimester (Brash, 1978). Trichopoulos et al (1976) also showed that there was evidence that the risks of secondary infertility and spontaneous abortion in subsequent pregnancies were higher after later termination of one pregnancy. These increased risks of medical complication are due to the methods used to induce the abortion. Suction curettage, which is a safer method of terminating pregnancies, is not appropriate for termination of pregnancies of 14 weeks gestation or more. For termination of later pregnancies intra-amniotic prostaglandins

or intra-amniotic saline infusion are used. These techniques are more likely to be followed by clinical complications.

For these reasons there is real need for developing methods of earlier antenatal diagnosis. A method which could provide fetal cells for diagnostic purposes earlier in pregnancy would be useful as it will not only reduce the risk of medical complication associated with late termination but it will also reduce the anxiety of those unfortunate couples who are at risk of having genetically abnormal children. Trophoblast sampling would offer such an approach at least for those abnormalities where the diagnosis of genetic abnormality is based on analysis of the fetal cells.

B. Future Prospects and Further Areas of Investigation in Trophoblast Sampling

i) Selection of Patients for Clinical Trials

The selection of patients for a clinical trial of trophoblast sampling is complicated by the ethical problem involved in developing a new technique for which the risks have not yet been ascertained. One group of women who may be suitable for clinical trials are those patients who have a high risk of producing a genetically abnormal fetus and who are refusing current methods of antenatal diagnosis, and in whom the diagnosis of the genetic abnormality can be made on fetal cells.

Dr Modell at University College has identified at least 70 such couples who already have had the experience of late termination for a thalassaemia fetus and would only contemplate a further pregnancy if a method of earlier

antenatal diagnosis was offered. In all these families the diagnosis of thalassaemia could be made by studies of familial DNA polymorphisms.

Another group of women who may be suitable for clinical trials are those who present for termination before the 8th week of pregnancy. Trophoblast sampling could then be performed at 6 - 8 weeks of gestation, and pregnancy could be allowed to continue for another 2 weeks before the suction termination of pregnancy is carried out. This approach may be particularly appropriate for women who request sterilisation following termination as this could minimise problems which may arise if trophoblast sampling is found to produce an ascending infection of the uterus and fallopian tubes.

ii) Biological Studies of Trophoblast Cells

The cell separation technique that we have developed permits analysis of the syncytiotrophoblast, cytotrophoblast and mesenchyme cells independently of each other which has not been possible previously. We have shown that monolayers of mesenchyme cells are suitable for chromosome studies and tissue typing. Other biological studies such as enzyme and hormone assays for enzyme defects and inborn errors or metabolism could be carried out on the separated cells.

We have also observed under phase contrast microscopy that the chorionic villi in the endocervical aspirates are permeated by fetal capillaries and we are currently exploring the possibility of extracting the

fetal red blood cells from these samples for early antenatal diagnosis of thalassaemia. Dr Modell has developed a technique for detecting abnormal protein synthesis on very small samples of fetal red blood cells.

iii) Genetic Studies on DNA of Chorionic Villi

Recent developments in molecular biology indicate that techniques involving analysis of DNA and identification of gene sequences will be widely applied to antenatal diagnosis within the next few years. Little et al (1980) have demonstrated polymorphisms of DNA restriction sites and their linkage to the human fetal globin genes in individuals who were heterozygous for beta thalassaemia. These polymorphisms have already been used for the antenatal diagnosis of beta thalassaemia using fibroblasts grown from the amniotic fluids. These linked polymorphisms could be applied to the antenatal diagnosis of any other single gene defect for which there is a DNA probe linked to an unaffected locus. Recently Williamson et al (1981) have shown that there is enough DNA in one trophoblast villus for this purpose and have demonstrated that accurate globin gene analysis is possible with the DNA obtained from these samples.

C. Problems Encountered with Trophoblast Sampling and their Possible Solutions

There appear to be several major problems which would need to be resolved before trophoblast sampling becomes an acceptable form of antenatal diagnosis. One of these is the lack of success in obtaining a specimen from every case. Previous workers who

have attempted endocervical aspiration have considered the aspiration to be successful in 60% of cases. In none of these studies ^{was} ~~were~~ the contents of the aspirates analysed consistently prior to culture. It was assumed that they all contained trophoblast. In our series an endocervical aspiration was considered successful if chorionic villi were identified in the sample macroscopically and microscopically prior to culture. Our results indicate that chorionic villi can be obtained in 37% of the aspirates. It is possible that this rate of obtaining the chorionic villi could be improved by carrying out the endocervical aspiration under ultrasound control, an approach which is currently being explored at the University College Hospital. Our preliminary results indicate that aspiration is more likely to be successful if the developing placenta is anteriorly placed, but we are still unable to obtain trophoblast material in every case.

A second problem is that of lack of success in culturing cells from every specimen. Kullander and Sandahl (1973) reported to have karyotyped only 12 of 39 specimens obtained by endocervicoscopy. Hahnemann (1974) was able to successfully karyotype 35 of 68 samples which were obtained by hysteroscopic biopsy. Our experience suggests that the failure of trophoblast biopsies or aspirates to grow was probably due to the inhibiting effect of the outer syncytiotrophoblast layers in intact villous material in the culture. This has also been demonstrated by other authors. We were able to overcome this problem by developing a technique of trypsinisation and filtration for the removal of syncytiotrophoblast and cytotrophoblast layers from chorionic villi, which made it possible to obtain rapid growth

of villous mesenchyme cells in vitro. Our recent experience indicates that this technique is successful for most specimens.

Another major problem that needs assessment is the reliability with which fetal cells can be grown from the aspirates. We and other authors have identified maternal cell contamination in the aspirated tissues. Goldberg et al (1980) showed that in 12 cases the fetal tissues were karyotyped as males 46,XY while karyotypes from the cultured cells from the aspirated tissues were those of females 46,XX. We were able to show that decidua was present in the endocervical aspirates by histological studies and also by karyotyping and tissue typing studies. We have successfully resolved this problem by separating the chorionic villi in the aspirate under phase microscopy. They are clearly recognisable frond-like structures which can be distinguished and dissected free from decidua and membranes. We have also shown that maternal cells that contaminate the samples were also eliminated by sequential trypsinisation and filtration technique. We have found the sampling techniques to be most successful in 7 - 9 weeks pregnancies. Women at genetic risks would have to be encouraged to seek medical advice for their pregnancy after missing their first period.

Perhaps the most serious potential complications of trophoblast sampling are those of sepsis and abortion. These risks can only be assessed by clinical trials. Only 2 groups of workers have embarked on clinical trials in which pregnancy is allowed to proceed after trophoblast sampling (Kullander and Sandahl, 1973 and members of the Department of Obstetrics and Gynaecology, Tietung Hospital, Anshan, China). In the series of hysteroscopic biopsies carried out by Kullander and Sandahl

(1973) 67 cases were allowed to proceed to term after biopsy. Abortion occurred within 8 days of sampling 3 cases (4%), of which 2 had developed sepsis. The Chinese reported an abortion rate of 4% following endocervical aspiration. It is difficult to evaluate this figure as 1 in 12 pregnancies abort spontaneously in the first trimester (Rushton, 1981). We have not yet explored this problem but it is possible that sampling under ultrasound control would minimise the risk. We would be in a position to determine accurately the rate of abortion associated with the sampling procedure by carrying out chromosome studies on the aborted pregnancies. We could expect spontaneous abortion to occur in those cases which show the abnormal karyotypes.

Recent reviews of the techniques currently used for antenatal diagnosis - namely amniocentesis and fetal blood sampling - shows that in their developmental stages the techniques were also associated with a high risk of abortion and other complications of pregnancy. The risks diminished with improved technology and experience (although the risk of abortion following fetoscopy is still in the region of 5%). It is reasonable to assume that the development of trophoblast sampling will follow the same pattern and the potential risks associated with the procedure will be reduced to an acceptable level.

A P P E N D I XA. PATIENT INDEX

<u>Case No.</u>	<u>Patients initials</u>	<u>Age</u>	<u>Gestation</u> (At the time of termination)	<u>Parity</u> (No. of children and No. of terminations)
1	VT	26	12/40	0 + 0
2	OT	24	11/40	0 + 0
3	EN	32	10/40	0 + 1
4	SB	33	8/40	3 + 0
5	SH	18	10/40	0 + 0
6	EAH	18	10/40	0 + 0
7	RJ	15	12/40	0 + 0
8	YCC	23	11/40	0 + 0
9	AL	20	12/40	2 + 2
10	LH	16	12/40	0 + 1
11	NI	14	12/40	0 + 0
12	SF	25	12/40	1 + 1
13	AH	22	9/40	0 + 0
14	BNC	20	10/40	0 + 0
15	MCV	18	12/40	0 + 0
16	BP	28	8/40	3 + 0
17	JB	19	8/40	0 + 1
18	JR	16	11/40	0 + 0
19	KH	18	12/40	0 + 0
20	JC	20	10/40	1 + 1
21	AA	36	10/40	3 + 0
22	GD	19	9/40	0 + 0
23	ST	20	9/40	0 + 0
24	GR	28	10/40	2 + 0

Continued

<u>Case No.</u>	<u>Patients initials</u>	<u>Age</u>	<u>Gestation</u> (At the time of termination)	<u>Parity</u> (No. of children and No. of terminations)
25	VT	30	8/40	0 + 0
26	EN	32	9/40	3 + 0
27	CM	25	8/40	0 + 0
28	ML	20	12/40	0 + 0
29	JM	23	12/40	0 + 0
30	SF	24	10/40	0 + 0
31	LH	29	10/40	1 + 0
32	EM	18	12/40	0 + 0
33	DM	19	9/40	0 + 0
34	VE	18	8/40	0 + 0
35	CV	20	22/40	0 + 0
36	CP	23	10/40	0 + 1
37	BA	20	9/40	0 + 0
38	NT	23	12/40	0 + 0
39	AM	18	8/40	0 + 0
40	JA	35	11/40	2 + 0
41	MB	24	8/40	2 + 1
42	JA	15	7/40	0 + 0
43	AW	19	8/40	0 + 0
44	PM	22	10/40	0 + 0
45	AB	28	9/40	0 + 0
46	PB	20	8/40	0 + 0
47	CA	16	7/40	0 + 0
48	BS	18	10/40	0 + 0
49	SW	14	6/40	0 + 0
50	RJ	25	9/40	0 + 0

Continued

<u>Case No.</u>	<u>Patients initials</u>	<u>Age</u>	<u>Gestation</u> (At the time of termination)	<u>Parity</u> (No. of children and No. of terminations)
51	MC	34	11/40	2 + 0
52	BN	28	10/40	0 + 1
53	AH	23	12/40	0 + 0
54	BC	19	8/40	0 + 0
55	MT	22	10/40	1 + 1
56	PS	17	9/40	0 + 0
57	RP	15	8/40	0 + 0
58	CW	32	10/40	0 + 1
59	AA	30	8/40	3 + 1
60	MJ	27	8/40	0 + 0
61	ER	20	10/40	NK
62	RG	18	12/40	NK
63	YCC	20	9/40	0 + 1
64	AL	24	8/40	0 + 0
65	RG	29	14/40	0 + 0
66	LH	29	12/40	1 + 2
67	NL	36	?11/40	1 + 4
68	AW	23	10/40	0 + 3
69	SF	20	14/40	0 + 0
70	SH	19	12/40	0 + 0
71	JR	18	10/40	0 + 0
72	JB	25	?13/40 by size	1 + 0
73	BP	25	12/40	0 + 0
74	NCB	23	10/40	0 + 2
75	AH	19	9/40	0 + 0
76	CM	41	23/40	0 + 0
77	HSG	31	13/40	0 + 1

Continued

<u>Case No.</u>	<u>Patients initials</u>	<u>Age</u>	<u>Gestation</u> (At the time of termination)	<u>Parity</u> (No. of children and No. of terminations)
78	CC	20	11/40	0 + 0
79	CM	18	10/40	0 + 0
80	BE	26	12/40	2 + 0
81	KL	17	10/40	0 + 1
82	NF	19	12/40	0 + 0
83	MO'S	24	10/40	1 + 1
84	SB	25	12/40	1 + 0
85	SM	18	11/40	0 + 0
86	KC	23	8/40	0 + 0
87	DH	14	10/40	0 + 0
88	EL	18	12/40 by size	0 + 0
89	CS	20	11/40	1 + 0
90	MJ	15	9½/40	1 + 1
91	AA	23	12/40	1 + 1
92	CW	16	6/40	0 + 1
93	MM	23	9½/40	2 + 0
94	KT	20	9/40	0 + 0
95	SA	23	7/40	0 + 0
96	NM	20	11/40	2 + 2
97	NA	22	10/40	2 + 0
98	ST	25	7½/40	3 + 1
99	TA	26	10/40	2 + 1
100	PW	22	12/40	0 + 0
101	KH	20	7/40	2 + 0
102	EH	21	13/40	1 + 1
103	NF	25	10/40	0 + 0
104	KL	25	10½/40	0 + 0

Continued

<u>Case No.</u>	<u>Patients initials</u>	<u>Age</u>	<u>Gestation</u> (At the time of termination)	<u>Parity</u> (No. of children and No. of terminations)
105	MW	40	6/40	0 + 0
106	SL	24	8/40	0 + 0
107	MA	23	9/40	0 + 0
108	SK	15	8/40	0 + 0

B. Methodology

This section includes some of the methods which are used routinely in the Cytogenetics Laboratory at St Mary's Hospital and have been applied in the present research work.

1. Fibroblast Culture

Plasma clot method for primary culture (sterile conditions throughout)

1. The tissue to be cultured is placed in a petri dish containing TC medium 199 and cut into small fragments (approximately 1 mm square).
2. These tissue fragments are picked up with a pasteur pipette or a syringe and needle with as little fluid as possible and transferred to a tissue culture flask (Falcon 25 cm³). Six fragments are placed in two rows in the flask.
3. A drop of chicken embryo extract and a drop of chicken plasma are mixed and placed over each fragment of tissue in the culture flask. The neck of the flask is then flamed and the cap is replaced.
4. This mixture clots very rapidly in the incubator at 37 °C for 20 minutes.
5. When the plasma clot is formed, 8 ml complete medium* is gently added into the flask.
6. The tissue culture flasks are gassed with 5% CO₂, 95% air mixture and incubated at 37 °C.
7. The medium is changed every third day.

* Complete medium: complete medium is prepared as follows:-

TC medium 199 (with Earl's modified salts)	= 70 ml
Fetal bovine serum (Mycoplasma tested)	= 30 ml
Glutamine	= 1 ml
Penicillin	= 200 units/ml
Streptomycin	= 100 µg/ml
NaHCO ₃	= 1.1 g/litre

2. Subculturing (Sterile Conditions Throughout)

When cells emerge from the periphery of the clots, they are subcultured.

1. The medium from the tissue culture flask is discarded and 8 ml of 1% trypsin in PBS is added. The flask is incubated at 37 °C for 5 - 10 minutes to allow trypsin to act on the cells attached to the flask.
2. After the cells start to detach from the flask and appear rounded, the flasks are shaken gently and the contents are transferred into sterile conical centrifuge tubes with a screw cap. The tube is centrifuged at 110 G for 5 minutes.
3. The supernatant is discarded and the cells are resuspended in 2 ml complete medium. This suspension of cells is then added to a flask containing 8 ml freshly prepared complete medium, and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ 95% air mixture.
4. When the cells have formed a monolayer, they are trypsinised as before and transferred to 2, 4 or 8 flasks according to the requirements of tests to be performed.

3. Termination of Culture (Harvesting)

If the cultured cells are to be used for chromosome analysis, the cell division is arrested at metaphase by the addition of colchicine. The optimum time for adding colchicine is when the monolayer is not completely confluent but a number of rounded cells are identified in the culture flask. This indicates that the cells are actively proliferating.

Harvesting procedure can be described in the following steps:-

1. Three hours before harvesting add 0.20 ml of colchicine solution (10 mg/25 ml) per 10 ml of tissue culture medium into the tissue culture flask. From this point sterility is no longer necessary.
2. After 3 hours, the culture medium is discarded and 8 ml of 1% trypsin in PBS is added and the flask is incubated for 10 minutes at 37 °C.
3. The flask is examined under inverted phase (magnification x100) and if the cells are detached, the contents of the flask are transferred into conical centrifuge tubes.
4. The tube is centrifuged at 110 G for 5 minutes.
5. The supernatant is discarded and the cell button is resuspended in 8 ml of 0.075 M Kcl and incubated for 15 minutes at 37 °C.
6. The tube is centrifuged at 110 G for 5 minutes.
7. The supernatant is discarded and the cell button is resuspended in fixative (1 part glacial acetic acid: 3 parts of absolute alcohol). Steps 6 and 7 are repeated.

8. After the second fixation, the supernatant is discarded and the cell button is resuspended in 3 - 4 drops of fixative as above and a drop of cell suspension is poured onto a tilted slide and the smear is air dried.
9. Finally for conventional staining, the slides are stained in 10% Giemsa stain in Sorenson's buffer pH 6.8 for 5 minutes.
10. The slides are washed in buffer and mounted in DPX.

4. Tissue Typing Cultured Cells Grown in Monolayers

Tissue typing of cultured cells was carried out using the technique developed by Niazi et al (1979). Monolayers of cells to be tissue typed were prepared in tissue culture flasks (Falcon 25 cm³).

1. The cells in the flask are trypsinised using 8 ml of 1% trypsin.
2. After the cells are detached from the flask they are centrifuged. Washed. The cell button is resuspended in complete medium and a cell count is made.
3. The cells are plated into the wells of microtest plates at a concentration of 200 - 300 cells per well.
4. The plates are incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air until confluent monolayers were formed; this usually takes between 24 - 48 hours.
5. The monolayers are washed with Hanks balanced salt solution, and the plates are flooded with low density paraffin oil.
6. Two µl of each of the battery of human HLA antisera* of different specificities are added to the wells and the plates are incubated at 37 °C for one hour.
7. This is followed by the addition of 2 µl rabbit serum* as a source of complement to each well and the plates are further incubated for one hour.
8. The cells are stained with 5% eosin, fixed with formaldehyde, and the percentage of dead cells is determined using an inverted phase microscope. Cytotoxic activity of a test antiserum is measured as a

significant increased killing of cells over that in negative controls. Normal human serum AB is used as a negative control.

- * Human HLA antisera and rabbit complement were supplied by the National Tissue Typing Reference Laboratories at Bristol.

5. Trypsin-Giemsa Banding Technique

A modification of the method of Seabright (1971) was used.

1. 10 ml of sterile isotonic saline or distilled H₂O is added to a phial of 10 ml dried Bacto Trypsin.
2. 4 ml of this solution is diluted with 36 ml of isotonic saline in a Coplin jar.
3. Slides with good chromosome spreads are left on a hot plate overnight at 60 °C.
4. These preheated slides are dipped into Trypsin solution for 10 seconds.
5. Slides are immediately transferred into fetal calf serum for 10 seconds.
6. These slides are then rinsed with saline solution and stained with Giemsa, 1:20 dilution in Sorenson's buffer, pH 6.8 for 3 minutes.
7. The slides are washed in Sorenson's buffer, pH 6.8, cleared in CNP 30 and are mounted in DPX.

C. Reagents and media

Reagents and media used in this laboratory are obtained from the following sources:-

Acetic Acid (Glacial)	BDH Chemicals Limited, Poole England
Bacto Trypsin	Difco Laboratories, Detroit, Michigan, USA
Chicken embryo extract, EE100	Difco Laboratories, Detroit, Michigan, USA
Chicken plasma. Desicated.	Difco Laboratories, Detroit, Michigan, USA
CNP 30 (solvent)	Bestobell Paints Chemicals Ltd, Bassington Industrial Estate, Cramlington, Northumberland, England
Colcemid This is a 0.04% solution ie. 0.01 g (10 mg) in 25 ml sterile distilled water	BDH Chemicals Limited, Poole England
DPX	Searle Gurr Diagnostics, High Wycombe, Buckinghamshire, England
Fetal Bovine Serum Stored at -20 °C	Flow Laboratories Ltd, Victoria Park, Heather House Road Irvine KA12 8NB Ayrshire, Scotland
Giemsa stain This is filtered before use	Searle Gurr Diagnostic, High Wycombe, Buckinghamshire, England
Heparin (without preservative)	Paines and Byrine Ltd, Greenford, England
KCl (Analar) This is a 0.075 M solution ie. 5.592 g in 1 litre distilled water	Hopkin and Williams, Chadwell Heath, Essex, England
L-Glutamine, 200 mM	Flow Laboratories Ltd, Victoria Park, Heather House Road, Irvine KA12 8NB Ayrshire, Scotland

Methanol (Methyl alcohol Analar)

Searle Hopkin & Williams,
Chadwell Heath,
Essex, England

Phosphate buffered saline

Flow Laboratories Ltd,
Victoria Park,
Heather House Road
Irvine KA12 8NB
Ayrshire, Scotland

Phytohaemagglutinin (PHA)
eg. 5 ml dried (If obtained in dried
form, resuspended with sterile
distilled water).

Wellcome Reagents Ltd
Wellcome Research Laboratories,
Beckenham, England

Sorensens Buffer tablets
These must be dissolved in
distilled water

Searle Gurr Diagnostic,
High Wycombe,
Buckinghamshire, England

Sterile solution of dextrose

The Boots Company Ltd,
Nottingham, England

Tissue Culture Medium (TC 199)
Single strength with added Penicillin
200 units 1 ml, Streptomycin 100 mg/ml
Sodium bicarbonate 1.1 g/litre,
stored at 4 °C

Gibco Europe Ltd,
Cowley Mill Trading Estate,
Longbridge Way, Uxbridge,
Middlesex, England

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PUBLICATIONS

1. Niazi, M, Coleman, D V, Mowbray, J F and Blunt, S (1979)
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J. Med. Genet. 16, 21-23.
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TISSUE TYPING AMNIOTIC FLUID CELLS: POTENTIAL USE FOR
DETECTION OF CONTAMINATING MATERNAL CELLS

BY

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Reprinted from Journal of Medical Genetics
Volume 16, No. 1, pages 21-23, February, 1979

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Tissue typing amniotic fluid cells: potential use for detection of contaminating maternal cells

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SUMMARY The presence of contaminating maternal cells in amniotic fluid is an important, though infrequent, cause of error in karyotyping the fetus. A method of detecting contaminating maternal cells in amniocentesis specimens by determining the HLA phenotype of the cells of amniotic fluid and the mother is described. Tissue typing of 15 amniocentesis specimens was performed, and in 14 cases the fetal origin of the cells was established. In one case, the results of tissue typing suggested maternal cell contamination, though this had not been suspected from chromosome studies of the amniotic fluid cell cultures. Other possible uses for tissue typing of amniotic fluid specimens for prenatal diagnosis are also described.

In the majority of cases the cells cultured from amniotic fluid are fetal in origin. Occasionally, the amniotic fluid is contaminated with maternal cells which, during the culture process, overgrow the fetal cells and lead to errors in chromosome analysis. Ferguson-Smith (1975) reported the misdiagnosis of 3 of 617 amniotic samples as a result of maternal contamination. The karyotypes of the cultured cells were reported as those of a normal female, but at birth 2 of the neonates were normal males and the third was a female with Down's syndrome.

Though errors of diagnosis as a result of maternal cell contamination occur rarely (Nadler, 1972; Milunsky, 1973), they are of considerable importance and two precautionary measures have been recommended (Polani, 1971; Ferguson-Smith and Ferguson-Smith, 1976). One of these involves chromosome analysis of several primary cultures of amniotic fluid, and the other relies upon the identification of parental chromosome polymorphism in the fetal karyotype. Both techniques have their drawbacks. The first is time consuming and delays reporting, and the usefulness of the second is limited by the quality of the preparation (Lawler and Reeves, 1976). We describe here another possible method of detecting maternal cell contamination by tissue typing cells of amniotic fluid and the mother.

Materials and methods

Samples of amniotic fluid obtained between the 16th and 18th week of pregnancy for prenatal diagnosis

were cultured to provide a source of cells for cytogenetic studies and tissue typing. At least two tissue culture flasks (Falcon 3013) were prepared from each primary culture, one of which was used for chromosome analysis and the other for tissue typing. The cells in this flask were trypsinised and plated out into the wells of Falcon Terasaki microtest plates at a concentration of 300 to 400 cells per well. The plates were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide and 95% air until confluent monolayers were formed; this usually took between 24 and 48 hours. The monolayers were washed with Hank's balanced salt solution, and the plates were flooded with low density paraffin oil.

Using a battery of human antisera of different specificities, the human leucocyte antigens of the cell monolayers in the wells were determined using a modification of the cytotoxic method of Terasaki (1970). 2 µl antiserum were added to the wells and the plates were incubated at 37°C for one hour. This was followed by the addition of 2 µl rabbit serum as complement to each well and the plates were further incubated for one hour. The cells were stained with 5% eosin, fixed with formaldehyde, and the percentage of dead cells determined using an inverted phase microscope. Cytotoxic activity of a test antiserum was measured as a significant increased killing of cells over that in negative controls.

Blood samples obtained from the parents were tissue typed by the lymphocytotoxic method and the fetal origin of the amniotic fluid cells was established by showing the presence of HLA haplotypes from

Received for publication 14 June 1978

both parents on the cultured cells. In three cases the HLA antigens of amniotic cells were also compared with those detected on the lymphocytes of the cord blood. As we only encountered possible maternal contamination in one specimen, suspensions of amniotic fluid cells and maternal skin fibroblasts were prepared, mixed in equal proportions, and grown in the wells of the microtest plates to provide a model for testing the sensitivity of the method.

Results

The results of tissue typing are shown in the Table.

The presence of an HLA haplotype of each parent indicated the fetal origin of the amniotic fluid cells in 14 of the 15 amniocenteses analysed. The tissue type of the amniotic fluid cells was identical to that of the lymphocytes of the cord blood in the 3 cases investigated.

In the 3 cases where the HLA antigens on mixed cell cultures were determined, both maternal and fetal antigens were detected. The fraction of cells killed with specific antisera varied from 50 to 100%.

In case 2, where the HLA antigens on maternal lymphocytes and amniotic fluid cells were identical, the fetal origin of the amniotic fluid cells could not be established from analysis of HLA phenotypes.

In case 10, both maternal B locus antigens 12 and 14 were detected on the cells grown from amniotic fluid and a low percentage of cells were killed by antibody against B14. These findings suggest that maternal contamination may have occurred and that the cultures contained both fetal cells with the phenotype A1, —; B8, 12, and maternal cells with the phenotype A11, —; B12, 14.

Discussion

Our results show that HLA antigens are clearly expressed on cells grown from amniotic fluid at an early stage of pregnancy. We have found that the cytotoxic method of detecting these antigens is simple to perform and easy to read, provided that good monolayers are obtained in the wells of the microtest plates and the following precautions are taken. The plates should be incubated for at least 48 hours after distributing the cells in the microwells, and the monolayers should be washed thoroughly to remove all traces of fetal bovine serum before adding the specific antisera.

In the one case where the HLA phenotype of the amniotic fluid cells was indistinguishable from that of the maternal cells, the mother had only one detectable HLA antigen at each locus (A2 and B15), both of which were also present in the father. As a result, analysis of the parental HLA phenotypes could not be used to determine the source of the cells in the amniotic fluid. Though this can be interpreted as a limitation of the method, such an occurrence in unrelated parents is a rare event.

We observed varying degrees of cell killing in the mixed cell culture, which appeared to reflect the proportion of fetal and maternal cells in the culture. Analysis of our results showed that antigens common to mother and fetus were detected on all cells, while those specific to mother or fetus were only found on some cells.

In case 10, maternal cell contamination was not suspected from chromosome studies. Analysis of 15 spreads showed a normal male karyotype indicating that tissue typing may be a more sensitive technique

Table HLA antigens detected in parents, amniotic fluid, and cord blood

Case no.	Maternal				Paternal				Amniotic fluid				Cord blood				Mixed culture of maternal skin fibroblasts and amniotic fluid			
	Locus A		B		Locus A		B		Locus A		B		Locus A		B		Locus A		B	
1	1	3	8	12	1	9	27	40	1	9	12	27	1	9	12	27				
2	2		15		2		15	w22	2		15									
3	2		40		10	w32	18		2	10	18									
4	1		8	w35	2	3	w35		2		8	w35					1*	2*	8	w35
5	2		12	14	1		5	8	1	2	8	12								
6	28	11	27	w25	2	9	12		2	28	w35									
7	3	10	7	12	2	11	7	w35	2	10	7	w35	2	10	7	w35	2*	3*	10	7
8	3	29	7	12	28		12	15	3	28	7*	15*								
9	2		12	13	1	28	8	14	1	2	8	12	1	2	8	12				
10	11		12	14	1		8		1		12	14*								
11	3	9	7	12	2	11	12	15	2	3	12									
12	3	29	7	12	2	28	12	17	2	3	7	12					2*	3	29*	7
13	1		12	17	2	11	12	15	2		12	17								
14	3		7		NT				1		w35	7								
15	1	3	8	14	NT				1		8	27								

*Weak reaction; NT, not tested.

than karyotyping for detecting maternal cells in the fluid. We suggest that tissue typing of amniotic fluid cells could be used in a routine cytogenetic laboratory as an alternative to analysis of several primary cultures or demonstration of familial chromosome polymorphism in those cases where maternal contamination is suspected. Another advantage of tissue typing is that it offers the possibility of eliminating contaminating maternal cells from the culture. This could be achieved by applying to the cultured cells specific antisera directed against the maternal haplotype not present on the fetal cells.

The HLA phenotype is a readily identifiable genetic marker which can be detected on all nucleated cells and, therefore, could be useful in prenatal diagnosis. If linkage between an inherited abnormality and HLA were known, tissue typing the amniotic fluid cells would provide a method of detecting an affected fetus *in utero*. Furthermore, because of the genetic polymorphism of the HLA system, tissue typing of amniotic fluid cells could be used for prenatal paternal ascertainment. Thus, tissue typing of fetal cells has the potential for a wider application in the field of antenatal diagnosis.

We thank Mrs H. Harris and Miss S. Hollowell, St. Mary's Hospital, for technical assistance, and Dr C. C. Entwistle, Deputy Director, National Tissue

Typing Reference Laboratory, Bristol, for providing the specific antisera used in this study.

This work was supported by a grant from the Joint Standing Research Committee of St. Mary's Hospital.

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TROPHOBLAST SAMPLING IN EARLY PREGNANCY. CULTURE OF RAPIDLY DIVIDING CELLS FROM IMMATURE PLACENTAL VILLI

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Summary

Trophoblast was obtained by ordinary suction curettage and by transcervical aspiration with a medicut cannula from women having a therapeutic abortion in the first trimester of pregnancy. The decidual tissue which is invariably attached to early placental villi was separated and pure cultures obtained from the trophoblast layers and from the mesenchymal core of placental villi. Cytotrophoblast had a very limited life span in tissue culture, whereas mesenchymal cells grew rapidly and could be used for antenatal diagnosis.

TRANSABDOMINAL amniocentesis is of low risk to the mother and fetus and the accuracy of fetal karyotyping from amniotic fluid cell cultures is high (The NICHD Study Group, 1976; Working Party on Amniocentesis, 1978). However, amniocentesis cannot be done before 16 weeks and the fetal karyotype is rarely known before 19 weeks gestation. Termination, if indicated, then has to be done well into the second trimester, and the delay causes considerable maternal anxiety and increases the risk of complications of termination such as trauma, sepsis, and haemorrhage (Brash, 1978). For these reasons other methods of obtaining fetal cells for the diagnosis of fetal abnormality earlier in pregnancy would be desirable. An obvious source is the trophoblast around the amniotic sac in early pregnancy which accurately reflects the genetic constitution of the fetus.

Several workers have observed that immature placental villi grow very slowly *in vitro* (Jones *et*

al, 1943; Stewart *et al*, 1948; Thiede, 1960). Thiede (1960) showed that cell proliferation could be accelerated if the tissue was first trypsinised and filtered. We therefore decided to study such a technique on specimens of early trophoblast obtained by suction curettage or transcervical aspiration and we now report our results and their application to prenatal diagnosis of fetal abnormality in early pregnancy.

METHODS

Placental tissue from pregnancies of 8 to 12 weeks gestation was collected by suction and sponge-holding forceps from 25 patients during therapeutic abortion. The tissue was washed twice with phosphate buffered saline to remove erythrocytes and debris. The placental villi were separated from the decidua by dissection, washed three more times and centrifuged at 110 *g* for 10 minutes. The pellet of villi thus obtained was transferred to a universal con-

tainer and weighed. A 0.25% solution of trypsin (Difco) was prepared in Ca^{2+} and Mg^{2+} free phosphate buffered saline with added 2% glucose. The trypsin solution was added to the pellet of villi, 4 ml being used for each gram of tissue. The container was gently agitated in a gyratory shaker at 37°C for 50 minutes. A 10 ml volume of complete medium, TC 199, containing 30% fetal calf serum was added to this cell suspension which was then centrifuged at 110 g for 10 minutes. The pellet was resuspended in complete medium and washed twice to remove all traces of trypsin. The cell suspension was then drawn into a 10 ml syringe and gently filtered through a Swinnex filter apparatus containing two layers of sterile wire mesh gauze (500 apertures per linear inch, aperture size 0.026 mm). Fragments of villi trapped on the surface of the wire gauze were transferred into phosphate buffered saline in a sterile container. The filtrate was collected separately. The fragments of villi were trypsinised again using the Ca^{2+} and Mg^{2+} free 0.25% trypsin solution. The cell suspension thus obtained was centrifuged and the pellet of cells was resuspended in 2 ml complete medium. A sterile tissue culture flask containing 8 ml complete medium was inoculated with the cell suspension and incubated at 37°C in 5% CO_2 and 95% air at 90% humidity.

The filtrate was centrifuged and the supernatant decanted. The cell sediment was resuspended in 2 ml of phosphate buffered saline. The resulting cell suspension was layered on to a gradient of 40 ml of TC 199 containing 40% fetal calf serum and allowed to stand at room temperature for one hour. The cells from the base of the gradient were collected separately into sterile centrifuge tubes containing phosphate buffered saline and inoculated into tissue culture flasks.

Cell samples for histological studies were collected at various stages of the cell separation technique in order to establish the effect of trypsinisation and filtration. Chromosome preparations were made from the cultured cells using standard procedures and the karyotypes compared with those obtained from the corresponding fetuses.

RESULTS

Histological studies

Histological examination of the villi after

trypsinisation showed that the mesenchyme core had been stripped of syncytio and cytotrophoblast (Fig. 1). The material trapped on the surface of the gauze was composed entirely of mesenchymal cores with no syncytiotrophoblast or cytotrophoblast (Fig. 2).

Cytological studies of the smears prepared from the cells from the base of the gradient showed that they contained numerous strips of cytotrophoblast (Fig. 3).

Tissue culture

Tissue cultures of trypsinised suspensions of the mesenchymal cores from the surface of the gauze showed that the cells settled within the first two days and then started proliferating rapidly into sheets of fibroblasts by the fifth to tenth days. These cells were usually subcultured by the seventh day and monolayers were obtained within the next six days which were suitable for chromosome preparations (Figs. 4 and 5).

Nine of the 25 specimens were karyotyped and all were normal, five being male and four female. The karyotypes of the mesenchymal cells were identical with the karyotypes of the corresponding fetuses.

Tissue cultures of cytotrophoblast cells from the base of the gradient showed that the cells settled within 48 hours and at first appeared under phase contrast as confluent, flattened, epithelial cells. After four days a number of isolated multinucleated giant cells were seen. Six days later the majority of cells were giant multinucleated cells and these eventually disintegrated (Fig. 6).

Transcervical aspirates

We applied our cell separation technique to transcervical aspirates obtained from three patients immediately before a therapeutic abortion during the tenth week of pregnancy. The aspirates were collected without prior cervical dilatation by a No 20 medicut cannula connected to a 20 ml syringe. The aspirates were examined by phase contrast microscopy. The readily recognisable, frond-like placental villi (Fig. 7) were dissected out from the decidua, trypsinised and filtered. The cells from the surface of the gauze filter grew rapidly in culture

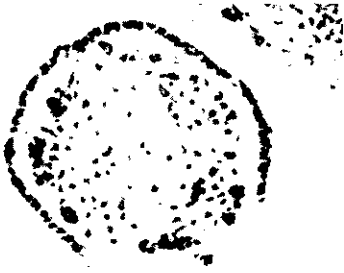


FIG. 1

Histological section of the villus after trypsinisation showing separation of trophoblast layers from mesenchyme. (Haematoxylin and eosin $\times 40$).



FIG. 2

Histological section of the mesenchyme core collected from the top of the gauze. (Haematoxylin and eosin $\times 40$).



FIG. 3

Cytological smear of cytotrophoblast strips collected from the base of the gauze. (Haematoxylin and eosin $\times 40$).



FIG. 4

Cell suspension after trypsin treatment of the mesenchyme core. (Haematoxylin and eosin $\times 40$).

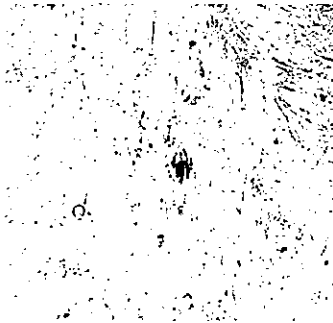


FIG. 5

Monolayer of cultured mesenchyme cells after 7 days. (Phase contrast $\times 100$).



FIG. 6

Cytotrophoblast in culture after 10 days showing syncytial knots. (Phase contrast $\times 100$).



FIG. 7

Fron-like villus structure with syncytial buds in endocervical aspirate from first trimester pregnancy. (Aceto-orcein stain $\times 40$).

and their fetal origin was confirmed by cytogenetic studies.

DISCUSSION

We have described the technique for producing a pure culture of rapidly dividing fetal cells from a few first trimester placental villi. The most important step was treatment of the villi with trypsin.

By doing this we stripped them of their covering syncytiotrophoblast and cytotrophoblast and thus promoted the growth of the exposed mesenchymal core cells. In this way we were able to suppress the growth of maternal decidual cells which normally swamp intact (or unstripped) placental villi in tissue culture (Goldberg *et al*, 1980). By eliminating maternal cells we overcame one major difficulty attending the use of placental villi for the establishment of fetal cells in tissue culture.

The number of genetic and biochemical diagnoses that can be made from such cultures is still rather limited. However, recent advances in molecular biology such as the analysis of DNA sequences in genes (Little *et al*, 1980) make it likely that a pure culture of rapidly dividing fetal cells will soon provide a vast amount of genetic and biochemical information about the developing embryo. This lends considerable importance to the perfection of methods of tissue culture for fetal cells and the earlier in pregnancy that such a culture can be established, the better.

We were able to show that a simple transcervical aspiration technique used in the first trimester of pregnancy provided sufficient villi for a fetal tissue culture. But we do not know how consistently such material can be obtained by so simple a method, nor what its complication rate might be; these are matters which we think it will be justifiable to study. Others have obtained specimens just before termination by directed biopsy using a hysteroscope (Kullander and Sandahl, 1973; Hahnemann, 1974) or by aspiration of exfoliated trophoblastic tissue in the endocervical canal (Rhine *et al*, 1977).

Only the Chinese have obtained trophoblast for fetal sexing by a blind transcervical aspiration technique (Dept Obstet Gynecol, Tietung Hospital, Anshan, 1975) and they reported an abortion rate of around 5% which is similar to the abortion rate given by Kullander and

Sandahl (1977) and similar to the abortion rate for fetoscopy with fetal blood sampling (Rodeck, 1980). This abortion rate is high when compared with that for amniocentesis and it may be possible to lower it by using ultrasound to locate the placenta or by employing transuterine rather than transcervical routes for obtaining minute samples of trophoblastic tissue. But even an abortion rate of 5% might be acceptable if it led to a diagnosis of serious genetic, biochemical or metabolic abnormality early enough to prevent the psychological and technical problems which attend mid-second trimester termination of pregnancy and which made some patients refuse an opportunity for prenatal diagnosis even though their progeny are at serious risk of congenital abnormality (Modell *et al*, 1980). It is for this reason that we believe sampling of trophoblast in early pregnancy to be a technique which deserves further attention and, in spite of obvious difficulties, it may ultimately play a role in reducing the incidence of congenital abnormality.

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Received March 2, 1981/Accepted July 16, 1981.