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MINIREVIEW

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Fetal cells in transcervical samples at an early stage of gestation

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Abstract Several investigations are in progress with the aim of performing prenatal diagnosis of inherited disorders by noninvasive or minimally invasive techniques. The most important approaches are based on the detection of fetal nucleated cells in maternal blood, the analysis of fetal DNA present in maternal plasma, and the identification and isolation of fetal trophoblastic cellular elements shed into the uterine cavity and the endocervical canal. In this review, we discuss the methods that have been employed for the collection of the transcervical samples at an early stage of gestation and the techniques used for the identification of fetal cells. We also report the results of using endocervical cells for the detection of fetal chromosomal disorders by fluorescent in-situ hybridization and for performing prenatal diagnosis of fetal Rh(D) phenotypes. Recent investigations have also shown that — after the isolation of trophoblastic cells from maternal contaminants by micromanipulation — transcervical samples can be employed for the prenatal diagnosis of single gene defects, such as those causing thalassemia and sickle cell anemia. Although the present results are promising, further investigations are required to demonstrate the feasibility of performing accurate diagnosis of fetal diseases by this minimally invasive approach in all transcervical samples retrieved at an early stage of gestation.

Key words Fetal cells · Non invasive prenatal diagnosis · Transcervical cells · Small tandem repeats (STRs) · Quantitative fluorescent PCR

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Introduction

During the past 10 years, several investigations have been performed aimed at devising minimally invasive procedures for the collection, detection, isolation, and analysis of trophoblastic cells present in the endocervical canal of pregnant women between 5–7 and 13–15 weeks of gestation (Adinolfi 1996; Adinolfi and Sherlock 1997; Adinolfi and Rodeck 1999).

In many of these studies, trophoblastic cellular elements have been detected in the transcervical cell (TCC) samples with variable incidences, depending on the skill of the operator and the methods used for the collection (Rodeck et al. 1995). High rates of success (ranging from 70% to 97%) have been reported using aspiration, lavage, or a cytobrush (Fig. 1) (Adinolfi et al. 1995a,b; Ishai et al. 1995; Kingdom et al. 1995; Adinolfi 1996; Adinolfi and Rodeck 1999; Amiel, personal communication, 2000).

Thus, the hypothesis, first advanced by Shettles, in 1971, that trophoblastic cellular elements are shed not only into the uterine vein — as previously shown by Schmorl (1893) — but also into the uterine cavity, and from there to the endocervical canal, has been fully vindicated.

The shedding of fetal cells into the maternal circulation and endocervical canal raises interesting biological questions. At the same time, their presence in TCC samples opens the possibility of performing prenatal diagnostic tests by minimally invasive procedures.

Recently, two extensive reviews on this topic have been published (Adinolfi and Sherlock 1997; Adinolfi and Rodeck 1999). Here, some of the most salient aspects of using TCCs for prenatal diagnostic tests will be analyzed.

On the shedding of trophoblastic cells

Trophoblastic cellular elements present in the endocervical canal must be derived from the uterine cavity, which persists until about 13–15 weeks of gestation. How these cells

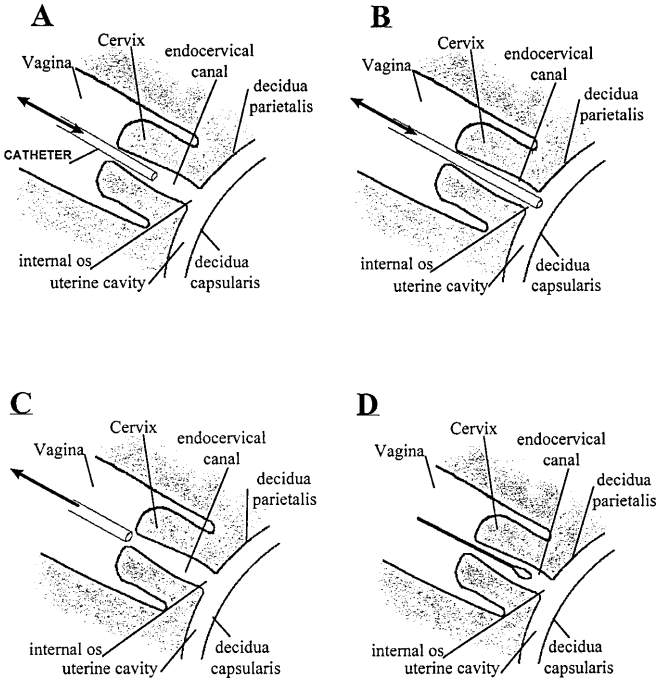

Fig. 1A–D. Methods for the collection of transcervical cell (TCC) samples: **A** by lavage of the endocervical canal; **B** by lavage of the intrauterine cavity; **C** by aspiration of the cervical mucus, and **D** using a cotton swab or cytobrush. *Arrows indicate* instillation and aspiration of saline solution

cross the decidua capsularis is not yet clear, because it is claimed that the surface of this complex structure is intact. An earlier view, that trophoblastic cells "drop off" by degeneration and exfoliation from the extraplacental villi during the process in which the chorion frondosum becomes the chorion laeve, does not take into consideration the fact that the villi are covered by this membrane. However, small areas of ulcerations have been noticed, and it is through them that fetal cells may gain access to the uterine cavity (see Adinolfi and Rodeck 1999).

Thus, the window for the possible collection of TCC samples starts at about 5–7 weeks and it is over at around 13–15 weeks, when the uterine cavity disappears.

Collection of TCC samples

Several methods have been used to collect TCC samples, including cotton swabs, cytobrush, aspiration of the mucus, with various types of cannulae, and lavage of the endocervical canal, using 5–10 ml of physiological saline solution (Fig. 1) (Adinolfi 1996; Adinolfi and Rodeck 1999).

In 1971, Shettles retrieved TCCs with cotton swabs and tested them for the presence of the Y chromosome, using a fluorescent dye (quinacrine mustard). At the time of publishing his short report, he had correctly diagnosed the sex in 10 out of 18 pregnancies investigated. Several short notes were soon published, some confirming the presence of fetal cells in samples retrieved with cotton swabs, while other papers reported a high incidence of false-positive or false-negative results (see Adinolfi 1996).

On the assumption that trophoblastic cells shed from the placenta would accumulate behind the cervical mucus, Rhine and collaborators (1975, 1976) flushed the endocervical canal with sterile saline with a device termed a "prenatal cell extractor". Not only did they collect samples containing fetal cells but they were also able to culture them in vitro in 14 of 34 cases investigated. Fetal cells were identified from the banding patterns of their chromosomes, which were different from those present in maternal cells in metaphase.

However, using the same procedure, Goldberg et al. (1980) detected only cells in metaphase with maternal chromosome banding patterns, although they had observed trophoblastic cellular elements in the original samples.

For over 10 years this topic was abandoned, until Griffith-Jones et al. (1992) tested TCC samples retrieved with cotton swabs or lavage and correctly detected the sex of male fetuses in a proportion of the samples tested, using the polymerase chain reaction (PCR) for the detection of Y-derived DNA sequences, but false-positive and false-negative results were also recorded.

However, doubts about the validity of these results were expressed by Morris and Williamson (1992), who suggested that the detection by PCR of Y-derived DNA was caused by contamination with spermatozoa from male partners.

To avoid this possibility, fluorescent in situ hybridization (FISH) assays were employed to determine conclusively whether trophoblastic cells could be found in TCC samples (Adinolfi et al. 1993). The advantages of FISH are that the morphology of the "positive" nuclei can be established and that, by using X and Y chromosome probes, it should be possible to prevent sperm contamination from affecting the results.

Frequencies of fetal cells in TCC samples

It was the detection of nuclei containing Y fluorescent signals — when the fetus was male — and the detection of a case of trisomy 18 among the first group of mothers tested (Adinolfi et al. 1993) that prompted further investigations aimed at improving the methods used for the collection of the TCC samples. Thus, in subsequent studies (performed at the Galton Laboratory, University College London [UCL]), cytobrush, aspiration, or lavage were employed (Pertl et al. 1994; Kingdom et al. 1995; Rodeck et al. 1995; Adinolfi et al. 1995a,b; Ruangvutilert et al. 1997; Sherlock et al. 1997a,b; also see Adinolfi and Rodeck 1999).

These studies, as well as those carried out by other investigators (Kawamura et al. 1995; Massari et al. 1996; Bahado-Singh et al. 1995; Briggs et al. 1995; Ishai et al. 1995; Maggi et al. 1996; Daryani et al. 1997) were all performed on small numbers of samples collected at about 10–12 weeks of gestation, just before termination of pregnancy. The presence of cells from male fetuses was documented using FISH, conventional PCR, or quantitative fluorescent PCR (QF-

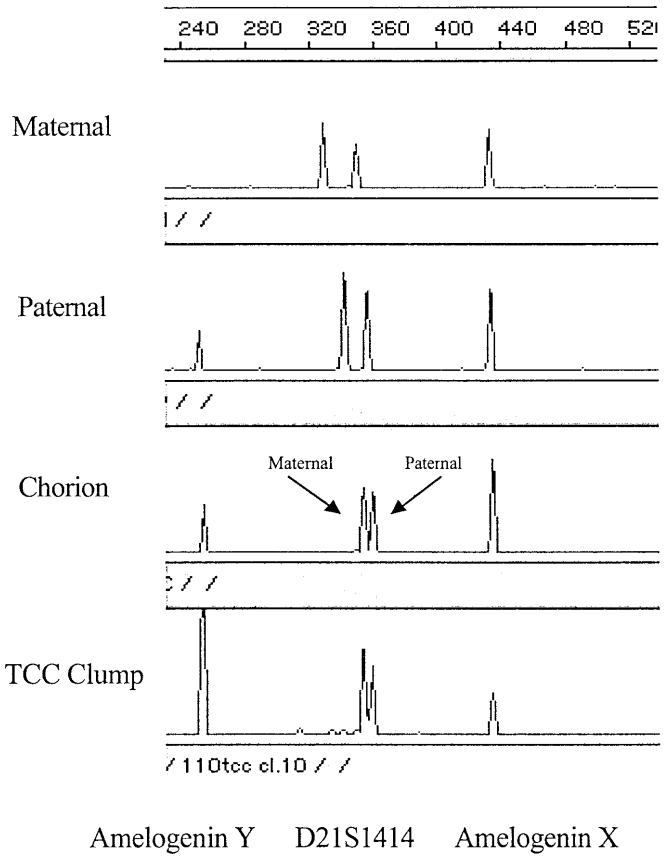

Fig. 2. Electrophoretogram of the quantitative fluorescent polymerase chain reaction (QF-PCR) products of maternal, paternal, and chorionic samples, and a TCC clump tested using the amelogenin X/Y (AMXY) and D21S1414 markers. Note, in the TCC clump, the maternal X and the paternal chromosome Y marker, as seen in the chorionic biopsy. The paternal chromosome 21 marker is also present in these two samples. These findings document the presence of fetal cells in TCC

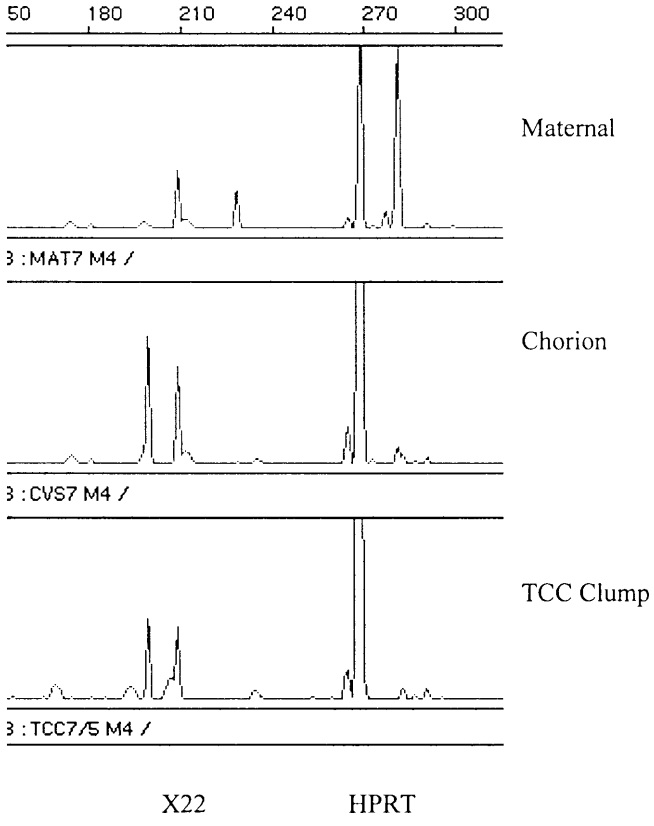

Fig. 3. Electrophoretogram of the QF-PCR products of the X22 (see text for definition) and hypoxanthine-guanine phospho-ribosyltransferase (HPRT) markers tested using maternal samples, chorionic samples, and a clump of cells retrieved from a TCC sample. The paternal X22 marker is present in the TCC sample, thus documenting the presence of fetal cells

PCR) assays (Fig. 2) (Pertl et al. 1994; Sherlock et al. 1997a,c) with DNA markers specific for chromosome Y.

Although the methods employed for retrieving the endocervical mucus ranged from aspiration with different types of cannulae or lavage with variable amounts of saline solutions, the results clearly documented the presence of trophoblastic cells in a large proportion of TCC samples collected from mothers with male fetuses (Adinolfi and Rodeck 1999).

The percentages of TCC samples containing fetal cells were shown to vary not only according to the method employed for their collection but also according to the skill of the operator (Rodeck et al. 1995). Aspiration of the cervical mucus produced "positive" results in 50% to 70% of cases, while lavage could result in the detection of male cells in up to 80%–90% of TCC samples retrieved from mothers with male fetuses (Fig. 3). As already mentioned, in a small group of TCC samples collected by cytobrush, all samples obtained from mothers with male fetuses were positive for chromosome Y DNA when tested by PCR, while two-thirds showed Y signals when investigated using FISH (Kingdom et al. 1995). Recently, Amiel, personal communication, (2000) has confirmed that the great majority (97%) of TCC samples collected with a cytobrush contain fetal cells. Collection by aspiration followed by lavage also produced high percentages of TCC samples with fetal cells (see Adinolfi and Rodeck 1999).

The incidence of fetal cells in each "positive" sample tested by FISH shows great variability, from 0.5% to 40%. This probably reflects the selection of the aliquot (e.g., 50 μl) of material removed from the whole TCC sample. If, by chance, an aliquot containing a few clumps of syncytiotrophoblastic cellular elements is tested, a large number of fetal nuclei would be available for FISH analysis.

In the above mentioned studies, although false-positive results (e.g., detection of male DNA markers in TCC samples retrieved from mothers with female fetuses) were rarely observed, discordant results have been reported by Overton et al. (1996), who often misdiagnosed the sex of the fetuses. The high frequency of Y signals detected in TCC samples obtained from mothers with female fetuses was probably caused by laboratory contamination.

Convincing evidence for the shedding of trophoblastic cellular elements into the endocervical canal can also be obtained by testing TCC samples with primers for chromosomes 21, 18, and 13 and by QF-PCR (Fig. 3) (Adinolfi et al. 1993; Maggi et al. 1996; Sherlock et al. 1997a,c).

Until recently, it has been difficult to document the presence of trophoblastic cells in samples collected from mothers with female fetuses, because of the lack of highly polymorphic markers for the detection of paternally derived X chromosomes. However, recently, this problem has been solved by the use of a pentanucleotide short tandem repeat (STR), termed X22, present on the pseudoautosomal region (PARA2) of the long arm of both X and Y chromosomes (Cirigliano et al. 1999a; Adinolfi and Cirigliano 2000). The detection of the paternally derived X chromosome marker in the TCC samples can be taken as clear evidence of the presence of fetal cells in the tested TCC sample (Fig. 3).

Immunostaining of TCC samples

TCC samples contain a large variety of cells; most of them are derived from the mother and can be readily identified as monocytes, neutrophils, erythrocytes, and cells released from the mucosa of the endocervical canal.

The presence of trophoblastic cells in TCC samples obtained before termination of pregnancy has been documented using monoclonal antibodies (MAbs) against placental antigens (Bulmer et al. 1995). Occasionally, multinucleated syncytial fragments have been detected, often in association with pleomorphic cells with large hyperchromatic nuclei, which have been identified as cytotrophoblasts. These types of cells were more abundant in TCC samples collected by lavage than in those collected by aspiration. Samples obtained by cytobrush contain more maternal cellular fragments, as a result of the abrasive procedure.

TCC samples collected during pregnancy

There is still limited information about the safety of performing aspiration of cervical mucus in continuing pregnancies. In one study (Adinolfi et al. 1995a), TCC samples were collected from a group of 130 women at about 10–12 weeks of gestation prior to an invasive chorionic villus sampling (CVS) procedure. A control group of 145 pregnant women having only transcervical CVS, but not TCC sampling, was included in the follow-up study. The results showed that cervical mucus aspiration performed before CVS had no deleterious effects on the course of the pregnancy. Although limited to a small number of cases, the rate of infections or abortions was lower in the first group of women than in the second.

Unfortunately, another clinical trial is not informative, because of the lack of appropriate controls and follow-up. In Taiwan, over a period of 2 years, about 20000 prenatal diagnoses for fetal sexing were performed in private clinics by lavage of the uterine cavity (Chang et al. 1996). Because most pregnancies with female fetuses were terminated, only incomplete data could be collected about the outcome of this diagnostic test (Hsi and Adinolfi 1997). However, it was verbally claimed by some participating obstetricians that, by testing the TCC samples with PCR, the success of detecting male fetuses was close to 95%.

Detection of single-gene disorders

Prenatal diagnoses of chromosome diseases can be performed using whole TCC samples and FISH (Maggi et al. 1996; Sherlock et al. 1997a,b,c), because the presence of contaminating maternal cells does not interfere with the tests. Freshly collected samples can also be tested for the detection of the fetal Rh (D) phenotype (Adinolfi et al. 1995c). Endocervical cells were retrieved at about 10 weeks of gestation, by aspiration, from 6 mothers, and in another 6, by lavage (Adinolfi et al. 1995c). All 12 mothers were serologically Rh(D)-negative. Using PCR and two sets of primers specific for the untranslated Rh(D) and Rh(CE) DNA regions, the fetal Rh(D) type was correctly diagnosed in most cases. Some discrepancies could be attributed to the poor quality of the primers employed. Technical improvement in Rh(D) detection by QF-PCR, as recently described by Pertl and Adinolfi (2000), should allow accurate diagnoses of the fetal Rh phenotypes.

Because of the presence of maternal cells, whole TCC samples can be used only for the diagnosis of dominant inherited conditions. For recessive and X-linked conditions it is imperative to isolate fetal cells from maternal contaminants.

Attempts using MAbs against placental antigens and iron-containing beads have not been successful (because of the stickiness of trophoblastic cells. Thus, the isolated clumps were always contaminated with maternal cells. A more efficient method for the isolation of fetal cells from TCC samples is based on the use of an inverted microscope; clumps of cellular elements with the characteristics of trophoblastic cells can be identified and removed with fine pipettes (Tutsheck et al. 1995; Adinolfi et al. 1997).

This approach has been employed in a recent study by Cirigliano et al. (1999b), who selected ten pairs of parents who were carriers of thalassemia or sickle cell anemia (HbS). TCC samples were retrieved, just before CVS, from the pregnant mothers, at about 10–12 weeks of gestation, by aspiration of the cervical mucus. After treatment with a mucolytic agent, a small aliquot of each sample was isolated under an inverted microscope and analyzed for the presence of clumps of cells with the apparent morphology of trophoblastic cells. After being washed several times in drops of phosphate-buffered saline (PBS), single clumps, containing from 5 to 20 cells, were transferred into microcentrifuge tubes, and the DNA was then prepared. The DNA extracts were separated into two aliquots; one was tested for the presence of fetal DNA, using QF-PCR assays with primers for chromosomes 21, 18, and 13, as well as amelogenin (AMXY) for sexing (Fig. 4). If this sample was shown to contain only fetal cells, the other aliquot was later analyzed for the detection of thalassemia and/or HbS mutations, using a modified amplification

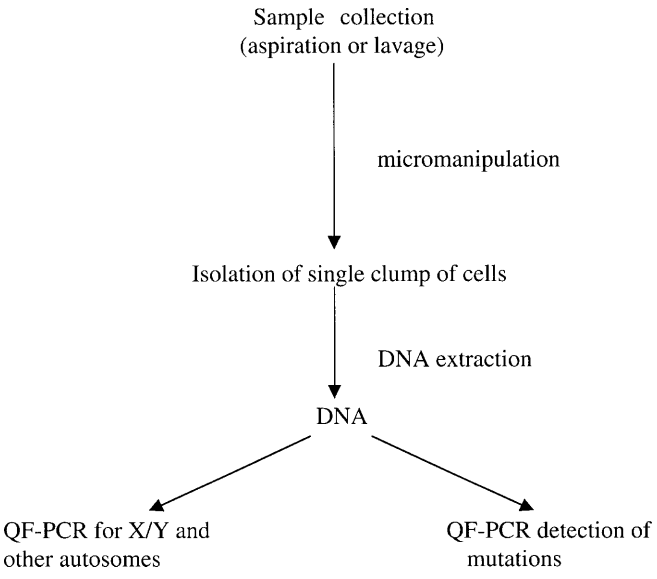

Fig. 4. Method for the detection of mutations using TCC samples. One aliquot of the isolated clump is tested for the detection of fetal cells. In the absence of maternal contaminants, the other aliquot is tested for the detection of a specific mutation (e.g., sickle-cell anemia) in the fetus

system (ARMS) method, previously described by Sherlock et al. (1998).

Because the carrier mutations were known, selected primers could be used to test the second aliquot, together with testing the corresponding chorion biopsies.

When DNA extracts prepared from single clumps were analyzed by QF-PCR with primers for chromosomes 21, 18, and 13, and AMXY, several were contaminated with maternal cells. However, clumps of TCCs free of maternal DNA were also observed, and prenatal diagnoses concordant with the results of testing chorion biopsies were reached in six of ten pregnancies.

Although only partially successful, these results confirmed those reported during previous investigations, based on the isolation of cell clumps and the use of a limited set of STRs (Adinolfi et al. 1997b).

In-vitro culture of TCC samples

Perhaps a higher success rate could be achieved by culturing, in vitro, whole TCC samples or selected cell clumps isolated by micromanipulation. As mentioned in the "Introduction", Rhine at al. (1977) were able to detect fetal cells in a proportion of cultured TCC samples, but these results were not confirmed by Goldberg et al. (1980), despite their having seen trophoblastic cellular elements in the original samples. The reason for this discrepancy is probably the length of culture time; the longer the cells are kept in culture, the more likely would be the presence of maternal cells. This hypothesis is based on the results of a study by Ishai et al. (1995), who, using short-term cultures, were able to detect fetal cells in metaphase in 19 of 28 TCC samples collected.

However, in our laboratory, attempts to culture fetal cell clumps isolated by micromanipulation have failed (Cirigliano and Adinolfi, personal observations). When tested with QF-PCR assays and STRs, almost all cultured cells expressed maternal markers.

Conclusions

It is now accepted that the shedding of trophoblastic cells into the uterine veins is a physiological phenomenon that occurs in the course of all normal human pregnancies (see Adinolfi 1996; Adinolfi and Rodeck, 1999). The biological significance of the release of these fetal cells is not known, although it has been suggested that they could "present" fetal antigens to the mother and modulate a state of immunological tolerance (Thomas et al. 1959).

Shettles (1971) was intrigued by the fate of the cellular elements released during the degeneration of the chorionic villi, and correctly suggested that fetal cells were also shed into the uterine cavity and then into the endocervical canal. The strong invasive property of placental cells has now been repeatedly confirmed.

Probably, trophoblastic cells are present in the endocervical canal in all pregnant women; their apparent absence in some TCC samples could be attributed to the difficulty of collecting the mucus and some technical artefacts. For example, when tested by FISH, only a few hundred nuclei are analyzed. The use of an automatic scanner to detect fluorescent signals could increase the frequency of observing fetal cells. In fact, samples apparently free of fetal cells when tested by FISH have been found to be "positive" when tested by QF-PCR and polymorphic markers for chromosomes 21, 18, 13, X, and Y. On the other hand, PCR-based assays may produce false-positive results caused by TCC contamination with spermatozoa.

So far, no trial using lavage for the collection of TCC samples in continuing pregnancies has been carried out. Because transcervical CVS biopsy, a more invasive procedure than lavage of the endocervical canal, is frequently performed in many obstetric units, there should be no ethical objections to the setting-up of trials based on the follow-up of several pregnant women.

It is difficult to establish what might be an acceptable rate of failure to retrieve fetal cells in TCC samples. If the only procedure allowed is aspiration, and if no fetal cells are detected in a sample collected at 7–10 weeks of gestation, a second sample can be collected after 1 week, because the method is rapid and not painful (Ishai et al. 1995).

The next major step is to devise better techniques for the isolation of the fetal cells in order to perform prenatal diagnosis of single-gene defects. Although micromanipulation under an inverted microscope has produced some interesting results, this procedure is hampered by the need to collect several clumps of cells to identify samples that are free of maternal contamination.

The best approach would be to employ MAbs that are highly specific for trophoblastic cell membrane antigens.

The MAbs tested so far in our unit were mostly directed against placental alkaline phosphatase and failed to produce clumps free of maternal contaminants, with the use of iron beads. Work is in progress to assess the possible advantages of using MAbs against HLA-G markers.

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