The role of preimplantation genetic diagnosis in the management of severe rhesus alloimmunization: first unaffected pregnancy: Case report


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Rhesus (Rh) D alloimmunization may cause haemolytic disease of the fetus and newborn if the fetal Rh blood type is positive. Although the incidence of severe RhD alloimmunization has decreased with prophylactic anti-D immunoglobulin administration during and after pregnancy, sensitization still occurs in a small group of women. In such women, Rh disease will continue to be significant problem and for their babies who may be affected. Preimplantation genetic diagnosis (PGD) may be utilized to avoid materno-fetal blood group incompatibility in an RhD-sensitized woman. Biopsy of a single cell from early cleavage-stage embryos screening for RhD-negative embryos allows the transfer of only RhD-negative embryo(s) into the uterus. This avoids any complications related to haemolytic disease of the fetus and newborn. This article describes the first reported case of an unaffected pregnancy using PGD for Rh disease. IVF and embryo transfer resulted in a clinical pregnancy and the birth of a healthy girl confirmed to be blood type RhD negative. PGD in couples with a heterozygous RhD-positive male partner provides an option for avoiding haemolytic disease of the newborn in RhD alloimmunized mothers.

Key words: blastomere biopsy/haemolytic disease of the newborn/PCR/preimplantation genetic diagnosis/rhesus alloimmunization

Introduction

Rhesus (Rh) D alloimmunization manifesting in haemolytic disease of the fetus and newborn has the potential to cause perinatal morbidity, mortality and long-term disability. An RhD-negative woman may develop anti-D antibodies when exposed to an RhD-positive fetus during or after pregnancy. Although various red cell antigens have been implicated in haemolytic disease of the newborn, RhD antigen is the most common and immunogenic (NHMRC, 1999; Avent and Reid, 2000). As a result of red blood cell destruction, the fetus develops haemolytic anaemia, which, when severe, leads to hydrops fetalis, intrapartum fetal demise or both.

The Rh blood group system is highly polymorphic, consisting of ≥45 independent antigens. D antigen expression is by the RhD protein while the RhCE protein expresses either C or c antigens together with E or e antigens on the same protein (Mouro et al., 1993; Blunt et al., 1994; Avent et al., 1996; Smythe et al., 1996). Although the RhD and RhCE proteins have a high degree of homology, the RhD protein does not express C/c or E/e antigens and the RhCE protein does not express the D antigen.

The RhD and RhCE proteins are encoded by two highly homologous genes, RHD and RHCE respectively, which have been mapped to chromosomal position 1p34.3–1p36.13 (Cherif-Zahar et al., 1991; MacGeoch et al., 1992). The two genes are each composed of 10 exons that in tandem encompass 69 kilobases of DNA (Avent and Reid, 2000). The gene that encodes the D polypeptide is present in Rh-positive persons and is absent in Rh-negative subjects (Colin et al., 1991).

Approximately 57% of the RhD-positive Caucasian population is heterozygous for the presence of the RhD gene (Lewis et al., 1971). Approximately 83% of Caucasian women are positive for RhD, meaning that ~17% of pregnant women will be RhD negative. In these RhD-negative women, ~60% will have an RhD-positive baby in their first pregnancy (NHMRC, 1999). Should a woman become sensitized in her first pregnancy, all subsequent RhD-positive babies will be at risk of haemolytic disease of the fetus and newborn. A fetus has a 50% chance of being heterozygous Rh positive and a 50% chance of being Rh negative if an Rh-negative woman becomes pregnant to a heterozygous Rh-positive father. In the latter case the fetus will avoid any potential adverse sequelae from maternal RhD alloimmunization.

Severe RhD alloimmunization is uncommonly encountered today largely due to the development of anti-D immunoglobulin and its utilization in clinical practice. Antepartum and postpartum prophylaxis with anti-D immunoglobulin are recommended (NHMRC, 2003). In spite of the clear reduction in affected women with anti-D’s availability and utilization, RhD alloimmunization still occurs (Bowman and Pollock,
1987; Crowther and Middleton, 2004). In these sensitized women, Rh haemolytic disease will continue to be a significant problem and for their babies who are affected.

In women who have suffered repeated pregnancy losses, invasive interventions such as serial intrauterine blood transfusions or an affected fetus or neonate, the prospect of having another affected pregnancy with all its complications may seem too great. In such women, rather than risk having another baby with haemolytic disease of the newborn, they may opt to avoid further pregnancies. Even with close monitoring of sensitized pregnant women for the early detection of fetal anaemia and the instituting of intrauterine transfusion at the appropriate time, there is a significant degree of fetal mortality (Abdalla et al., 2004). Although the risk of fetal death from cordocentesis is relatively low, procedure-related fetal loss has been documented (Daffos et al., 1985). The procedures are also associated with the risk of potentially increasing maternal RhD antibody production through secondary feto-maternal haemorrhage (Nicolini et al., 1988; MacGregor et al., 1991).

PGD was designed for the prevention of genetic disorders in the offspring of couples at increased risk. Since its introduction in 1990, PGD has been mainly used for detection of single-gene disorders such as cystic fibrosis or for screening of chromosomal disorders (ESHRE PGD Consortium Steering Committee, 2002). More recently, use of PGD for social sexing and HLA matching has been reported (Verlinsky et al., 2001; ESHRE PGD Consortium Steering Committee, 2002). The ethics of such use is beyond the scope of this paper and will not be discussed. PGD necessarily involves IVF. After IVF, the early embryo is screened for the disorder before the corresponding embryo is transferred into the uterus of the mother. In Rh disease this allows the transfer of Rh-negative embryos back into the RhD-alloimmunized mother, avoiding the potential complications and morbidity of haemolytic disease of the fetus and newborn.

Although the use of PGD in the management of Rh disease has been previously published, clinical pregnancy has not to date been achieved (Avner et al., 1996). However, PGD for the Kell genotype has been performed successfully to prevent severe alloimmunization occurring in an at-risk couple (Verlinsky et al., 2003). We report, to our knowledge, the first case of an unaffected RhD-negative baby being born to an RhD-alloimmunized mother using PGD. We discuss the role of PGD in the management of RhD alloimmunization in selected couples where the sensitized woman and her RhD heterozygous partner can avoid the potential morbidity and mortality associated with an RhD-positive fetus.

Case report

A 27 year old married woman sensitized with RhD antibodies sought preconception counselling regarding her options in attempting a future pregnancy. She and her husband had had two children, the second of which was affected by haemolytic disease of the newborn. Both of these pregnancies had proceeded to term and delivered vaginally.

The second child developed hyperbilirubinaemia and neonatal jaundice requiring phototherapy as well as significant haemolytic anaemia that did not require transfusion. The peak serum bilirubin level measured 425 mmol/l and the lowest haemoglobin level recorded was 75 g/l. The neonate’s blood group was ‘A’ RhD positive with a positive direct Coomb’s test at birth.

Six weeks postpartum the maternal anti-D antibody level was elevated with an anti-D antibody quantification of 157 IU/ml. The maternal anti-D antibody level remained significantly raised 6 months post delivery with a level of 70 IU/ml. The husband’s blood type was RhD positive with his serologically determined RhD phenotype being cDee. He was genotyped and found to be RhD heterozygote positive.

The pertinent issues regarding RhD alloimmunization and future potentially more severely affected pregnancies were discussed with the couple. The couple were counselled that RhD screening prior to implantation using PGD could allow the selective transfer of only RhD-negative embryos and thereby avoid any possibility of materno-fetal blood incompatibility in that pregnancy. The couple agreed to assisted reproduction and PGD for transfer of an RhD-negative embryo to the uterus.

The woman underwent routine ovarian stimulation and ICSI fertilization. Nineteen oocytes were aspirated and 17 were suitable for ICSI. On day 1, 12 were observed as fertilized. Day 3 biopsy, PCR amplification and analysis revealed nine RhD-positive embryos, two RhD-negative embryos and one with a uni-parental amplification profile. Two embryos (a 10-cell embryo and a compacting morula) were transferred on day 5. This resulted in a clinical pregnancy.

PGD was performed with IVF utilizing ICSI (to reduce extraneous DNA contamination), DNA analysis of single biopsied blastomerse from cleavage-stage embryos and multiplex PCR. Oligonucleotide primers amplifying parts of both the RHD and RHCE genes were designed by aligning the two gene sequences [GenBank accession identifications NT_030571 (RHEE gene) and NT_004391 (RHD gene)] using BioManager (ANGIS, Australia) and selecting regions where the RHCE and RHD genes differed in sequence length between identical flanking DNA sequences. A total of 12 different primer pairs targeting different regions of the alignment that would result in amplification product differing in size between RHCE and RHD genes were designed (Primer Express; Applied Biosystems, Australia). After testing, three pairs of primers gave both reliable amplification and distinguishable amplicon differences and were suitable for further use. In addition, the final multiplex included two short tandem repeat (STR) loci, one adjacent to the RHCE gene (D1S2674) and the other a little more distal (D1S199), as a monitor for extraneous DNA contamination and to confirm the gene profile (RhD positive or RhD negative) by linkage (Table I).

The PGD was performed using a direct PCR amplification of alkaline extracted/neutralized blastomerse with analysis by capillary electrophoresis of the fluorescently labelled amplicons (Cui et al., 1989). Amplification was performed in
a two-step PCR: 94°C for 30 s and 60°C for 120 s for 43 cycles.

The RhD genotypes of 12 embryos were determined. The presence of the RHD gene in an embryo resulted in a double peak for each selected amplification region; the RHD gene amplicon and the control RHCE gene amplicon (Figure 1). In combination with the STR markers, a test accuracy of >99% could be given to the patient.

Regular ultrasound examination of the fetus throughout the pregnancy failed to show any evidence of fetal anaemia or hydrops fetalis. The maternal anti-D antibody level was also monitored regularly and remained stable, although

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1S199</td>
<td>6FAM-CCTGGGTGACAGAGTGAAG</td>
<td>TTTCCCTTCTCCCCCTCC</td>
<td>~140</td>
</tr>
<tr>
<td>D1S2674</td>
<td>NED-CCAAATGGAATCTTCTG</td>
<td>TCCACGTGGGGAAACAGAAT</td>
<td>~240</td>
</tr>
<tr>
<td>Rh384465</td>
<td>6FAM-TCTGGGTGTTGGTTATG</td>
<td>GCAGTCACAGGTGGTTGGTT</td>
<td>162 (CE)/168 (D)</td>
</tr>
<tr>
<td>Rh41712</td>
<td>VIC-CCCTATGAGACTGACTG</td>
<td>CATGCTGCTGGCATCTGGTG</td>
<td>137 (CE)/140 (D)</td>
</tr>
<tr>
<td>Rh4925d</td>
<td>VIC-CAGGCGCCAGAGATTCATT</td>
<td>TCGTATTTCTCCTGTG</td>
<td>109 (D)/112 (CE)</td>
</tr>
</tbody>
</table>

*a*Indicates the amplicon size for the respective gene.

*b*Indicates the short tandem repeat locus.

*c*Indicates the position on the RhD sequence alignment of the two genes.

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**Figure 1.** Electropherogram demonstrating in: (a) the RhD-negative mother; (b) the RhD-positive father; (c) a RhD-negative embryo, and (d) a RhD-positive embryo. CE represents RHCE gene products. D represents RHD gene productd. Amplicon identifications as seen in Table 1 are indicated in each panel.
significantly raised, throughout the duration of the pregnancy (range 21.0–28.0 IU/ml). Labour was induced at 39 weeks gestation. The baby’s blood group was ‘A’ RhD negative and the direct Coomb’s test was negative. There were no complications noted in the immediate neonatal period or thereafter.

**Discussion**

The incidence of RhD alloimmunization has decreased since the introduction of anti-D prophylaxis during and after pregnancy (Bowman, 2003; Bowman and Pollock, 1987; Crowther and Middleton, 2004). Nevertheless severe RhD alloimmunization still occurs and can have serious implications in a pregnancy with haemolytic anaemia, which, when extreme, causes fetal morbidity, stillbirth or perinatal mortality. After having experienced a significantly affected pregnancy, couples in whom RhD alloimmunization is present are often faced with the dilemma of whether to attempt further pregnancies and potential adverse sequelae. The tendency for Rh disease to worsen with each subsequent Rh-incompatible pregnancy in a sensitized woman also plays a major part in the decision-making process. In the case presented above, such a scenario existed. The probability of a sensitized woman with a pregnancy to an RhD-positive heterozygote having an RhD-positive fetus is 50%. In such couples, the use of PGD for RhD typing allows the selective transfer of only RhD-negative embryo(s), thereby avoiding any complications due to materno-fetal incompatibility. The case presented is, to our knowledge, the only PGD cycle resulting in a successful pregnancy.

PGD was introduced in 1990 with the first established pregnancies in two couples known to be at risk of transmitting recessive X-linked diseases (Handyside et al., 1990). Since then it has been usually offered for three major categories of disease: sex-linked, single gene defects and chromosomal disorders. PGD has also been employed for social sexing and HLA matching by some practitioners. PGD allows at-risk couples to avoid potential complications or another unfortunate experience in future pregnancies.

PGD necessarily involves assisted reproductive techniques where ovarian stimulation and IVF are required to produce in vitro several embryos in order to select unaffected ones for transfer. This means that even couples that are fertile must undergo the processes of assisted reproduction. There are also the financial costs associated with IVF and PGD to consider. However, the economic cost for follow-up and treatment of a typical pregnancy and newborn affected by severe RhD is not inconsiderable together with the psychological and physical burden (van den Veyver et al., 1995).

This case demonstrates that PGD can be used to determine the RhD status of early cleavage-stage embryos by single cell analysis. This permits selective transfer of only RhD-negative embryos, avoiding the development of haemolytic disease in the fetus. Although at-risk pregnancies detected by prenatal diagnosis may be treated by intratertiary transfusion, potential complications including fetal death cannot always be completely prevented even after this procedure. Pregnancy termination may also be unacceptable to the couple. In fact, genetic risk and objection to termination of pregnancy are still the most important reasons for couples seeking PGD, with about one-quarter of couples having one or more affected children (ESHRE PGD Consortium Steering Committee, 2002). PGD may be seen as a preventative treatment measure in couples affected by RhD alloimmunization and a male Rh-positive heterozygote.

PGD was designed for detecting genetic defects, but, as this case demonstrates, may be used for couples at risk with RhD alloimmunization. This provides an approach for selected couples to avoid the risk of having babies affected by haemolytic disease of the fetus and newborn.

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**References**


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