Obstetric outcome after RhD and Kell testing

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The study was conducted to report on the use of molecular biology methods and pregnancy outcome in women sensitized to either Rhesus D (RhD) or Kell 1 (K1) antigens. Paternal RhD genotype was determined by DNA amplification of an RhD-specific sequence from single sperm cells. Paternal Kell phenotype was determined by serologic assays using peripheral blood samples, and the fetal RhD or Kell-type status was established by the polymerase chain reaction (PCR) with amniotic cells. Thirteen women (14 pregnancies, one with twins) sensitized to RhD and four sensitized to K1 antigens comprised the study group. All had paternal heterozygosity to either D or K1 antigens. Nine fetuses were RhD positive and five were RhD negative. An additional woman underwent early spontaneous abortion. The nine RhD-positive fetuses underwent a total of 41 invasive procedures. One fetus with hydrops fetalis died in utero after intrauterine blood transfusion. All the remaining RhD-positive fetuses were delivered after 33 weeks gestation, and all those who were RhD negative were delivered at term. Four women were sensitized to the K1 antigen; in three, the fetus was found to be K1 negative, and in one, K1 positive, necessitating intrauterine blood transfusion. In all cases, the results of RhD or K1 genotype analyses from amniotic fluid were compatible with fetal/neonatal red blood cell RhD or Kell phenotypes. In conclusion, the use of molecular biology techniques represents a major advance in the clinical management of RhD and Kell alloimmunization.

Key words: fetal haemolytic disease/Kell/RhD

Introduction

The incidence of Rhesus (Rh) isoimmunization has dramatically reduced over the last 30 years, since the introduction of routine, passive immunization for Rhesus-negative women. Despite this, Chavez et al. (1991) stated that immunoglobulin prophylaxis had not eradicated haemolytic disease of the fetus and the newborn. They estimated that the incidence of Rh haemolytic disease in the USA was ~10.6 per 10 000 live births; thus Rh isoimmunization still contributes to the neonatal morbidity and mortality in the Western world. This is a result of non-immunization, under-immunization, and false Rh typing in rare cases (Holburn and Prior, 1986; Holburn et al., 1988).

When maternal sensitization to the D antigen is present, it is very important to establish the paternal zygosity. In the white population, the incidence of heterozygosity for the D antigen is ~56% (Race and Sanger, 1975). In such cases of paternal heterozygosity, only 50% of the fetuses will be potentially at risk for isoimmunization. Therefore, by establishing the paternal zygosity, improved counselling of couples concerning risks and treatment options can be provided. Furthermore, if paternal homozygosity is confirmed, the need for invasive procedures to diagnose fetal blood type may be obviated.

At present, RhD genotype can only be estimated by: (i) inference from the frequencies with which the individual Rh haploid gene complexes occur in the general population, combined with the number of previous RhD positive offspring (Kanter, 1992; Walker, 1993), or (ii) by RhD genotype determination using DNA amplification of an RhD specific sequence from single sperm cells (Reubinoff et al., 1996). It is anticipated that all sperm cells of a homozygous individual will contain the RhD gene, as opposed to half the cells of a heterozygous individual.

Paternal zygosity can be determined in women sensitized to Kell 1 (K1) antigen, by using peripheral blood. K and k are codominant autosomal alleles; ~8–10% of individuals are K1-positive, most of whom are heterozygous (Kk), in which case there will be a 50% likelihood of the fetus being K1-negative.

Until recently, the common practice for prenatal determination of fetal blood type was achieved by cordocentesis. This invasive procedure has a 1–2% risk of pregnancy loss, and is invariably technically feasible from 20 weeks gestation. New techniques, using polymerase chain reaction (PCR) with amniotic cells, for prenatal determination of RhD or Kell type, have been recently reported (Bowman et al., 1992, 1996; Bennet et al., 1993; Spence et al., 1995; Lighten et al., 1995; Lee et al., 1995, 1996; Dildy et al., 1996; Bowman et al., 1996; Lee et al., 1996; Van den Veyver and Moise, 1996). The use of this technique has the advantage of being less invasive and may be performed at an earlier stage of pregnancy. Chromosomal analysis may also be performed at this time. If an RhD-negative or K1-negative fetus has been diagnosed, no further work-up is required. However, if the fetus is RhD- or K1-positive, sufficient time remains to plan further follow-up and treatment.
This study presents our experience with women who have been sensitized to either RhD or K1 antigens, and who have heterozygous partners. The paternal RhD genotype was determined by single sperm cell analysis. Fetal RhD or Kell type was determined by the PCR with amniotic cells.

Materials and methods

Pregnant women with evidence of sensitization to RhD or K1 antigen were referred to the Fetal Medicine Unit at The Chaim Sheba Medical Center. The study group comprised only couples where the male partner was RhD or K1 positive and was found to be heterozygotic. The Kell phenotype was determined by conventional assays using a peripheral blood sample. The RhD genotype of the male partner was determined by single sperm cell analysis. Fresh semen samples were liquefied at 37°C for at least 20 min, layered on an 80% Percoll column (Pharmacia, Inc., Uppsala, Sweden), washed, and diluted to 1 × 10^6 motile cells per ml. The sampling of a single spermatozoon was carried out with an intracytoplasmic sperm injection micropipette (Cooke, Queensland, Australia) attached to an IM-6 (Narishige, Tokyo, Japan) pico-injector under a ×200 magnification of a diaperted (Nikon, Tokyo) microscope equipped with micromanipulators (Narishige, Tokyo, Japan). A small amount of the diluted suspension was displaced into a 5 µl drop of polyvinylpyrrolidone, allowing motile cells to migrate to the periphery. Morphologically-normal single sperm cells were aspirated by gentle suction into a micropipette and transferred to a 5 µl drop of phosphate-buffered saline solution. After microscopic examination of the droplet to verify that only one spermatozoon was present, the droplet was dispensed by a fire-pulled Pasteur pipette into a siliconized, PCR tube. Tubes containing single cells were frozen in liquid nitrogen and stored at −80°C until use. Cell lysis was carried out. After three cycles of freeze, boil, and spin down, samples were incubated overnight at 37°C in 1.7 µmol/l sodium dodecyl sulphate, 20 mmol/l diithiothreitol, and 0.05 mg/ml proteinase K and then heat inactivated for 10 min before amplification.

Four sets of primers were designed for the final amplification of two DNA fragments of different lengths. One fragment (145 bp long) was RhD specific (external primers: D3:5'-TAAGCAAAAAGCATCTCCAA starting at nucleotide 1252 and D4:5'-ATGGTGAGATCTCTCTT starting at nucleotide 1437; internal primers: N3:5'-AAAAACAGGCC-TGTTCAAA starting at nucleotide 1272 and N4:5'-AGTGCGAGAAGGAAGGAT starting at nucleotide 1416). The second fragment (94 bp long) was shared by both genes, RhD and RhCcez, and was used as an internal control (external primers: D1:5'-TGTTGTTGAACCGAGTAT starting at nucleotide 941 and D2:5'-ACATGCCATTTGCGGAT starting at nucleotide 1075; internal primers: N1:5'-AGTGCCTGGGGATTTC starting at nucleotide 954 and N2:5'-AAGCACCAGCAGCA starting at nucleotide 1047). The D1–D4 primer set was equivalent to the dual amplification was performed by a two-round PCR procedure (Avner et al., 1993). The dual amplification was performed under the same conditions except for annealing, which was performed at 50°C for 30 s. Nested products (15 µl) were analysed by electrophoresis on a 2% agarose–ethidium bromide stained gel. Genomic DNA of D-positive and D-negative persons, as well as deionized water, was used as a positive and negative control in all amplification experiments. For each male partner, between 10 and 20 single sperm cells were analysed.

Strict measures were taken to minimize the possibility for carryover contamination. All steps from the isolation of a single sperm cell up to the end of the first PCR round were performed in the same tube. In addition, only positive displacement pipettes and filter barrier, aerosol-resistant tips, were used. Polymerase chain reactions and post-reaction steps were all performed in separate rooms and handled with different sets of pipettes (Reubinoff et al., 1996).

The women underwent amniocentesis at 16–18 weeks gestation, for prenatal determination of RhD or K1 status using the PCR with amniotic cells (Bennet et al., 1993). Amniotic fluid (10 ml) for RhD or K1 typing was used, and 20 ml was used for chromosomal analysis. There were certainly enough cells from the amniotic fluid for the PCR reaction. Multiplex reaction was necessary to obtain an internal control of whether the cells were RhD positive or negative. The fetal Kell type status was determined at the Genetics and IVF Institute, Fairfax, Virginia, USA.

Serial ultrasound examinations were performed every 4 weeks in fetuses with RhD- or K1-negative genotype, and no further invasive procedures were undertaken. The follow-up of those with an RhD or K1-positive genotype included serial amniocentesis or cordocentesis to determine the severity of fetal anemia, and weekly ultrasound and Doppler evaluations. When indicated, fetuses were treated with intrauterine blood transfusion. Timing of labour was scheduled after amniocentesis and documentation of fetal lung maturity, unless there were other indications for earlier termination of pregnancy, such as fetal distress.

Results

Thirteen women (14 pregnancies) sensitized to RhD, and four sensitized to K1, comprised the study group (Table I). They all had paternal heterozygosity to either D or K1 antigens. Once paternal zygosity had been determined, the women were given the option of undergoing amniocentesis to characterize the fetal D or Kell typing.

The obstetric history of the women is listed in Table I. One woman (patient 11) experienced early spontaneous abortion. In the remaining 13 RhD-negative pregnancies (including one twin pregnancy), nine fetuses were RhD positive, and five were RhD negative. The nine RhD-positive fetuses underwent a total of 41 invasive procedures: 34 amniocentesis and seven intrauterine blood transfusion. One fetus with hydrops fetalis died in utero 5 days after intrauterine blood transfusion (patient 3a), and the woman conceived again. In her next pregnancy, the fetus was RhD negative. No further invasive procedures were performed, and the mother delivered a healthy infant at term. One woman with an RhD-positive fetus developed severe pre-eclampsia at 33 weeks, necessitating immediate Cesarean section. All the remaining RhD positive fetuses were delivered after 34 weeks gestation, and the five RhD negative fetuses were delivered at term.

Four women with heterozygous partners were sensitized to the K1 antigen. In three cases, the fetus was found to be K1 negative; thus no further invasive procedure was required. In one case (patient 16), the fetus was found to be K1 positive.
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Antigen</th>
<th>Paternal genotype</th>
<th>Fetal blood type</th>
<th>Past obstetric history</th>
<th>Invasive procedures (index pregnancy)</th>
<th>Gestational age at delivery (weeks)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD positive</td>
<td>Hydrops fetalis (IUFD)</td>
<td>Amniocentesis × 2</td>
<td>35</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>2</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD positive</td>
<td>–</td>
<td>Anniocentesis × 5</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>3a</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD positive</td>
<td>Hydrops fetalis (IUFD)</td>
<td>IUT × 2</td>
<td>28</td>
<td>IUFD (hydrops fetalis)</td>
</tr>
<tr>
<td>3b</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD negative</td>
<td>Hydrops fetalis (IUFD)</td>
<td>Amniocentesis × 1</td>
<td>40</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD negative</td>
<td>Normal pregnancy (IUT)</td>
<td>Amniocentesis × 1</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>5</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD positive × 2 (both twins)</td>
<td>Normal pregnancy (IUFD)</td>
<td>Amniocentesis × 5</td>
<td>34</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>6</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD negative</td>
<td>Normal pregnancy (IUFD)</td>
<td>Amniocentesis × 3</td>
<td>35</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>7</td>
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<td>RhD positive</td>
<td>Normal pregnancy (IUFD)</td>
<td>Amniocentesis × 1</td>
<td>38</td>
<td>A&amp;W</td>
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<tr>
<td>8</td>
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<td>Normal pregnancy (IUT)</td>
<td>Amniocentesis × 1</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>9</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD negative</td>
<td>Normal pregnancy (IUT)</td>
<td>Amniocentesis × 1</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>10</td>
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<td>Normal pregnancy (IUT)</td>
<td>Amniocentesis × 1</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>11</td>
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<td>Normal pregnancy (IUT)</td>
<td>Amniocentesis × 1</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>12</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD positive</td>
<td>–</td>
<td>Amniocentesis × 5</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>13</td>
<td>D</td>
<td>Heterozygote</td>
<td>RhD positive</td>
<td>–</td>
<td>Anniocentesis × 4</td>
<td>33</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>14</td>
<td>Kell 1</td>
<td>Heterozygote</td>
<td>Kell negative</td>
<td>–</td>
<td>Amniocentesis × 1</td>
<td>40</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>15</td>
<td>Kell 1</td>
<td>Heterozygote</td>
<td>Kell negative</td>
<td>–</td>
<td>Amniocentesis × 1</td>
<td>36</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>16</td>
<td>Kell 1</td>
<td>Heterozygote</td>
<td>Kell positive</td>
<td>–</td>
<td>Amniocentesis × 1</td>
<td>37</td>
<td>A&amp;W</td>
</tr>
<tr>
<td>17</td>
<td>Kell 1</td>
<td>Heterozygote</td>
<td>Kell negative</td>
<td>–</td>
<td>Amniocentesis × 1</td>
<td>39</td>
<td>A&amp;W</td>
</tr>
</tbody>
</table>

The patient received three intrauterine blood transfusions and delivered at 37 weeks gestation.

In all cases, results of RhD or K1 genotype analyses from amniotic fluid deoxyribonucleic acid samples, were compatible with fetal/neonatal red blood cell RhD or Kell phenotypes.

**Discussion**

In this study, the new methods of PCR for paternal RhD genotype determination by single sperm cell analysis, as well as for fetal blood group (RhD, K1) determination in amniotic cells were used. Both methods represent major progress in the clinical management of RhD and K1 alloimmunization.

The determination of RhD or Kell zygosity of the partner in couples where the woman has been sensitized is clinically important. Knowledge of paternal zygosity is essential for counselling the couple with regard to the risk of an affected fetus in future pregnancies, and their decision regarding permanent sterilization or artificial insemination (Kanter, 1992).

During early pregnancy, sensitized mothers with a history of unfavourable obstetric outcomes may elect to terminate the pregnancy. In such cases, determination of paternal homozygosity would obviate the need to determine fetal blood type during the first trimester by invasive procedures, such as chorionic villus sampling, or early amniocentesis (Kickler et al., 1992; Fisk et al., 1994). In ongoing pregnancies, accurate determination of paternal zygosity will allow the performance of invasive diagnostic procedures (such as amniocentesis or cordocentesis).
only in cases of paternal blood group heterozygosity. However, in cases of homozygosity, such procedures would be scheduled later for the evaluation of fetal anaemia, rather than as an early diagnostic procedure for the determination of fetal blood types. If preimplantation genetic diagnosis is to be considered, determination of paternal zygosity is essential. Only couples with a heterozygous partner may have RhD-negative or K1-negative embryos that may be selected for transfer (Avner et al., 1996).

The ability to determine RhD or K1 typing of the fetus early in pregnancy will facilitate decision making in couples where alloimmunization is a factor and the partner is heterozygous. If the fetus is RhD or K1 negative, the parents may be reassured early in pregnancy, and invasive procedures may later be avoided. Conversely, if a fetus is RhD or K1 positive, invasive management may be planned more rationally.

The determination of RhD genotype by single sperm cell analysis has been shown to be accurate. Since the number of sperm cells to be analysed is practically unlimited, the accuracy of diagnosis of RhD zygosity by PCR is extremely high, as long as an adequate number of cells is amplified (Reubinoff et al., 1996). However, cognisance should be taken concerning the reliability of the ‘declared’ partner in fact being the father of the fetus.

The PCR is not a quantitative method, and a positive signal will be obtained from diploid cells of homozygous, as well as heterozygous, individuals. This discrimination may be performed on haploid cells, hence the advantage of the single sperm cell analysis in the management of these very high-risk pregnancies.

Prenatal determination of fetal blood type by PCR of amniotic cells has recently become common practice in the management of a sensitized woman. The accuracy of this method has been evaluated by several groups. Van den Veyver and Moise (1996) recently reviewed articles describing the use of PCR in amniotic fluid for RhD typing. A total of 500 cases was reviewed. The sensitivity and specificity of PCR typing were 98.7% and 100% respectively, and the positive and negative predictive values were 100% and 96.9%, respectively. In five cases, an RhD-positive fetus was incorrectly diagnosed. Dildy et al. (1996) recently determined the accuracy of DNA analysis for fetal RhD status using PCR on uncultured amniocytes. In 346 of 347 cases (99.7%), the PCR results of amniotic fluid concurred with the serologic RhD blood type. The single error was an RhD negative fetus, whose amniotic fluid was contaminated with blood from an RhD positive mother. Yankowitz et al. (1995) determined RhD blood type by PCR in 765 human blood samples. The sensitivity was 99.7%, and the specificity 94.9%.

Although our study comprised only 17 sensitized pregnancies, in all cases the results of analysis of RhD or K1 genotypes from the amniotic fluid samples were compatible with the fetal/neonatal red blood cell RhD or K1 phenotypes. The use of molecular biology methods represents a major advance in the clinical management of RhD and Kell alloimmunization.

References


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