

## PRENATAL DETERMINATION OF FETAL RhD TYPE BY DNA AMPLIFICATION

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**Abstract Background.** An RhD-negative woman whose partner is heterozygous may have preexisting anti-RhD antibodies that may or may not affect a subsequent fetus, depending on whether it is heterozygous. A safe method of determining fetal RhD type early in pregnancy would eliminate the risks to an RhD-negative fetus of fetal-blood sampling or serial amniocenteses.

**Methods.** We determined the RhD type in 15 fetuses using the polymerase chain reaction in amniotic cells and serologic methods in fetal blood collected simultaneously. In another 15 fetuses, the RhD type determined from chorionic-villus samples was compared with that identified by typing of DNA from the fetus itself.

**Results.** RhD typing of DNA from amniotic cells correctly indicated the serologic type in every fetus. Of 10 fetuses with RhD-negative mothers, 4 were identified as

RhD-negative and 6 as RhD-positive. Of five fetuses with RhD-positive mothers, four were identified as RhD-positive and one as RhD-negative. There was also complete agreement between the results of RhD typing of DNA from chorionic-villus samples and the results of typing of DNA from fetal tissue. Eleven fetuses were RhD-positive, and four were RhD-negative. Four RhD-positive fetuses had RhD-negative mothers. Three RhD-negative fetuses had RhD-positive mothers. There was no contamination by maternal RhD-positive DNA of the samples from RhD-negative fetuses.

**Conclusions.** Determining fetal RhD type in amniotic cells without invading the fetomaternal circulation is a reliable method that will be valuable in the management of Rh alloimmunization. (*N Engl J Med* 1993;329:607-10.)

TO reduce the risk of Rh alloimmunization in a subsequent pregnancy, RhD-negative women are given anti-RhD antibodies after miscarriage, the birth of an RhD-positive baby, or any obstetrical procedure that may cause fetomaternal hemorrhage. As a result, the incidence of Rh alloimmunization has declined greatly during the past 20 years.<sup>1</sup> Maternal sensitization still occurs, however, when prophylaxis is omitted or when fetomaternal hemorrhage occurs before it is given. In the past, anemia developed in one in four fetuses sensitized to the RhD antigen, leading to hydrops, perinatal death, or both.<sup>2</sup> Recent advances in in utero diagnosis and treatment have considerably improved the outcome, with survival rates in excess of 75 percent in severely affected fetuses treated by serial ultrasound-guided intravascular transfusion.<sup>3,4</sup>

Fifty-six percent of Rh-positive whites are heterozygous for the RhD antigen.<sup>5</sup> When the father is heterozygous RhD-positive, there is a 50 percent chance that the fetus will be RhD-negative and thus will be unaffected. Currently, one of two approaches — fetal-blood sampling and serial amniocentesis — is used to identify RhD-positive fetuses. Fetal-blood sampling for serologic Rh typing is associated with a 1 to 2 percent risk of fetal loss and a 40 percent risk of fetomaternal hemorrhage, which may cause increased sensitization.<sup>6</sup> Alternatively, serial amniocenteses can be performed to measure bilirubin in amniotic fluid. This method is less accurate, cannot distinguish an

RhD-negative from a mildly affected RhD-positive fetus, and exposes the mother of an RhD-negative fetus to multiple invasive procedures.

An early and safe method of determining Rh status prenatally would be advantageous in cases in which alloimmunization is a risk factor and the father is thought to be heterozygous. It may also be useful in women with a history of fetal loss due to Rh alloimmunization, because the mother may wish to terminate the pregnancy if the fetus is RhD-positive. Rh phenotyping of fetal erythrocytes eluted from first-trimester chorionic-villus samples has been reported with use of either mixed agglutination or immunofluorescence methods.<sup>7-9</sup> These techniques have not been widely adopted in clinical practice, because they are cumbersome and time consuming and because the results may be falsely negative if an insufficient number of fetal erythrocytes is obtained.

The Rh blood-group antigens are carried by a series of at least three homologous but distinct membrane-associated proteins.<sup>10</sup> Two of these proteins have immunologically distinguishable isoforms designated C, c and E, e. The principal protein, D, has no immunologically detectable isoform d. The rhesus gene locus, on chromosome 1p34–p36,<sup>11,12</sup> consists of two adjacent homologous structural genes designated RhCcEe and RhD.<sup>13</sup> The sequences of the coding portions of the two genes are 96 percent identical, suggesting that they arose through the duplication of a single ancestral gene.<sup>13</sup> The first gene, RhCcEe, encodes both the C/c and E/e proteins, most likely by alternative splicing of a primary transcript.<sup>14</sup> The second gene, RhD, encodes the main antigen RhD and is absent on both chromosomes of RhD-negative subjects.<sup>13</sup> Therefore, the presence or the absence of the RhD gene in the genome determines the genetic basis of the polymorphisms associated with Rh positivity and Rh negativity.

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The knowledge of the organization of the rhesus gene locus in RhD-positive and RhD-negative persons<sup>13</sup> together with the cloning of the RhCcEe<sup>15,16</sup> and RhD complementary DNA (cDNA)<sup>17</sup> has provided the means to determine RhD genomic sequences with the polymerase chain reaction (PCR).<sup>17</sup> On the basis of this information we determined the fetal RhD type in DNA obtained by chorionic-villous biopsy or amniocentesis.

## METHODS

### Sampling Procedures

Amniotic fluid (2 ml) for fetal RhD typing by PCR and fetal blood (0.5 ml) for RhD typing by serologic methods were obtained from 15 fetuses (gestational age, 18 to 22 weeks) for which blood sampling and amniocentesis were clinically indicated. The samples, which had been collected with the consent of the mothers, were surplus with respect to the indications for sampling and were stored in accordance with institutional ethical guidelines. In 10 cases the procedures were performed for the management of suspected alloimmunization in RhD-negative mothers. In the other five cases the indication was for fetal karyotyping in the presence of structural markers of aneuploidy.

Ultrasound-directed biopsy of transcervical chorionic villi was performed with fine-biopsy forceps immediately before abortion in another 15 cases. Fetal tissue evacuated from the uterus was identified and recovered. These 15 pregnancies were terminated in accordance with the United Kingdom Abortion Act (1967), and consent from the mother was obtained for both the procedures and the use of fetal tissue in research in accordance with local and national ethical guidelines.

### DNA Preparation

Amniotic fluid was centrifuged at 10,000×g for 10 minutes, and the resulting pelleted cells were then washed twice in normal saline. Chorionic villi were washed in normal saline to remove contaminating maternal cells and centrifuged at 10,000×g for 10 minutes. Fetal tissue was identified and washed several times in normal saline to remove contaminating maternal cells. The tissue or cells were homogenized and centrifuged at 10,000×g for 10 minutes. The sedimented cells were then resuspended in 400 μl of buffer containing 20 mM TRIS (pH 8), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 percent polysorbate 20 (Tween 20), and 250 mg of proteinase K per milliliter. After incubation at 37°C for three hours or at 55°C for one hour, the protease was heat-inactivated and a 2-μl aliquot was used for PCR amplification. Alternatively, the genomic DNA was further purified by extraction with phenol and chloroform and precipitation with ethanol and then resuspended in 50 μl of distilled water. In this case, only a 1-μl aliquot was used for PCR.

### PCR

The following primers were used for the PCR: A1 (5'TGTGTTGTAACCGAGT3', sense primer, position 941 to 956, nucleotide position 1 taken as the first residue of the initiator AUG codon on the CcEe and D messenger RNAs [mRNAs]), A2 (5'ACATGCCATTGCCG3', antisense primer, position 1073 to 1062), A3 (5'TAAGCAAAGCATCCAA3', sense primer, position 1252 to 1268), and A4 (5'ATGGTGAGATTCTCCT3', antisense primer, position 1437 to 1422). A1, A2, and A3 encompass nucleotide sequences that are identical on both RhCcEe and RhD cDNA, whereas A4 is specific for the D sequence.<sup>15,17</sup> PCR was performed in 20-μl reaction mixtures containing 1 or 2 μl of template human DNA; 20 pmol of each of the four primers; 20 mM TRIS-hydrochloric acid (pH 8); 1.5 mM magnesium chloride; 25 mM potassium chloride; 0.05 percent Tween 20; 100 μg of bovine serum albumin per milliliter; 50 mM each of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate; and 2 units of *Taq* polymerase. Thirty cycles of denaturation for one minute at 92°C, primer an-

nealing for one minute at 49°C, and primer extension for one minute at 72°C were performed, followed by a final primer extension for nine minutes at 72°C. The reaction products were separated by electrophoresis with a 3 percent agarose gel containing 0.5 μg of ethidium bromide per milliliter and were visualized under ultraviolet light.

### Serologic RhD Typing

RhD serotyping was performed with two monoclonal anti-RhD antiserum preparations, MAD2 (BPL Diagnostics, London) and Seraclon (Biotest Diagnostics, Munich, Germany). The samples were incubated on microtiter plates for 15 minutes at room temperature. The plates were read manually, and negative results were checked microscopically.

## RESULTS

Primers A1, A2, A3, and A4 were designed to characterize the transcription products specific to the RhCcEe and RhD genes.<sup>17</sup> The first pair of primers (A1 and A2) amplify a 136-base-pair (bp) region common to the RhCcEe and RhD genes (exon 7) (Chérif-Zahar B, et al.: unpublished data). The second pair of primers (A3 and A4) amplify a 186-bp region specific to the 3' untranslated sequence (exon 10) of the RhD gene. The two amplification reactions are performed in the same tube (multiplex PCR). Only the 136-bp product is amplified from RhD-negative DNA, whereas both the 136-bp and 186-bp products are amplified from RhD-positive DNA (Fig. 1 and 2).

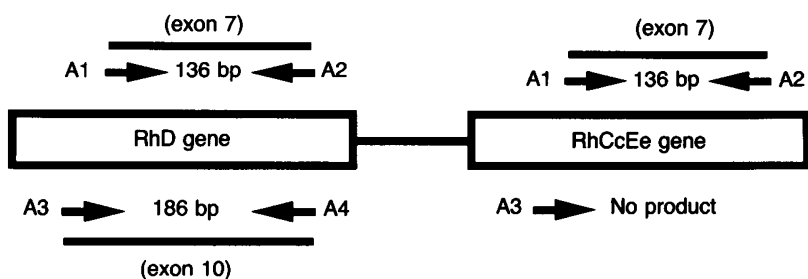
Analysis of the RhD genotype in amniotic-fluid cells from 15 fetuses and chorionic-villous-biopsy samples from 15 fetuses was performed simultaneously in London and Paris; the investigators at each site were unaware both of the results from the other site and of the serologic results. There was complete agreement on the results of DNA typing between the two laboratories. Four fetuses studied for RhD alloimmunization were identified by both PCR and serologic techniques as RhD-negative, and six were identified by both methods as RhD-positive. The mothers of all 10 fetuses were Rh-negative. Of the remaining five fetuses, whose mothers were RhD-positive, four were identified as RhD-positive and one as RhD-negative by both PCR and serologic methods. There was 100 percent agreement between the results of PCR and the results of serologic typing. One RhD-negative fetus had an RhD-positive mother.

There was complete correlation between the results of PCR RhD typing of chorionic-villous tissue and the results of typing of fetal tissue. Eleven fetuses were RhD-positive, and four were RhD-negative. Four mothers who were RhD-negative had RhD-positive fetuses. Three mothers who were RhD-positive had RhD-negative fetuses. There was no evidence of contamination of the PCR by maternal DNA in any of these studies.

## DISCUSSION

We found that the fetal RhD genotype can be reliably and rapidly determined in amniotic-fluid and chorionic-villous cells using DNA amplification. The

## RhD Positive



## RhD Negative

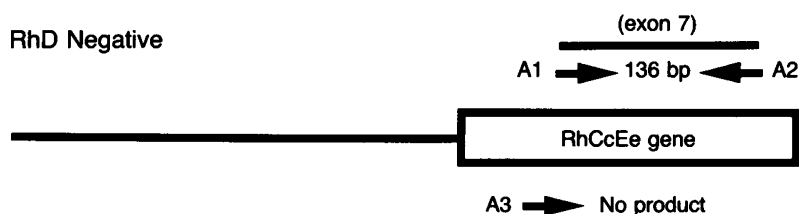


Figure 1. Principle of the Amplification of the RhD-Specific Region and the Combined RhD and RhCcEe Regions of the Rhesus Gene Locus.

Only the 136-bp product will be amplified from RhD-negative chromosomes, whereas both the 136-bp and 186-bp products will be amplified from RhD-positive chromosomes. A1, A2, A3, and A4 are the primers used for PCR.

results of the determination of the RhD type in amniotic-cell DNA agreed completely with the results of the serologic typing of fetal blood. In the clinical context, prenatal RhD typing would be performed in an RhD-negative mother whose fetus is at risk of alloimmunization. Contamination of the amniotic fluid by maternal cells would therefore not affect the accuracy of the prenatal typing of the fetus. Nevertheless, both the clinicians concerned with the sample collection and the laboratory technicians must be aware of the extreme sensitivity of PCR and the dangers of sample contamination with RhD-positive DNA. Handling of the samples should be kept to a minimum, and the reactions should be carried out in a sterile environment separate from the area in which completed reactions are analyzed. Using these safeguards, we have not found contamination of fetal DNA with maternal DNA when the mother was RhD-positive and the fetus was RhD-negative. The inclusion of an internal control, the amplification of a portion of the RhCcEe gene present in all cells, guards against the possibility that an RhD-positive fetus will be identified as RhD-negative through the failure of the reaction itself. Control reactions containing known samples of RhD-positive and RhD-negative DNA should be performed simultaneously with the test reaction to ensure the quality of the reagents used.

The technique described here can easily be performed in any laboratory that uses basic molecular-biology techniques. The specific PCR products can be differentiated under ultraviolet light after standard agarose-gel electrophoresis, and a result can be obtained within 24 hours after sample collection.

This ability to determine the Rh type of a fetus early in pregnancy will facilitate decision making when alloimmunization is a factor and the father is heterozygous or his zygosity is unknown. If the fetus is RhD-negative, the parents can be reassured in the first trimester, and sampling later in pregnancy can be

avoided. If the fetus is RhD-positive, invasive management can be planned more rationally. Several authors have identified a subgroup of women who, after a previous pregnancy in which the fetus was severely affected, request termination of pregnancy if the fetus is RhD-positive,<sup>8,9,18</sup> either because they feel unable to cope with the multiple invasive procedures needed for management or because they do not wish to expose the fetus to the risks of serial transfusions. A further indication for first-trimester testing and possible termination of the pregnancy would be the rare case in which severe fetal anemia becomes evident before the stage at which fetal-blood sampling and transfusion can be performed.

Either chorionic-villus sampling or amniocentesis could be used for the prenatal determination of the RhD genotype. Amniocentesis might be preferred for several reasons. Chorionic-villus sampling is associated with higher risks of fetal loss and late-pregnancy



Figure 2. Reaction Products after the Amplification of Regions of Both the RhCcEe and RhD Genes (136 bp) and a Region Specific to the RhD Gene (186 bp) from DNA Prepared from Amniotic-Fluid Cells from Five Fetuses.

In samples 1 and 3 there is no amplification of the 186-bp RhD-specific region; these fetuses are RhD-negative. In samples 2, 4, and 5 amplification of the 186-bp RhD-specific region shows that these fetuses are RhD-positive.

complications<sup>19,20</sup> and has been associated with fetal limb-reduction deformities.<sup>21</sup> More important, chorionic-villus sampling results in fetomaternal hemorrhage in up to 50 percent of cases,<sup>22,23</sup> which in the presence of an RhD-positive fetus may theoretically increase antibody levels and thus the severity of disease. The use of simultaneous real-time ultrasonography allows the placenta to be avoided during amniocentesis and greatly reduces the risk of fetomaternal hemorrhage. Although amniocentesis can be safely performed at as early as 10 weeks' gestation,<sup>24,25</sup> there remains some concern about the effect of withdrawing the large volume of amniotic fluid required for karyotyping. In contrast, only a few cells are needed for DNA amplification and analysis of the fetal RhD genotype, and therefore only 1 or 2 ml of fluid needs to be aspirated. We therefore think that amniocentesis late in the first or early in the second trimester will become the procedure of choice for fetal Rh typing.

Although more studies are needed to confirm the high sensitivity and specificity of this method, the ability to determine the Rh status of the fetus early in pregnancy without invading the fetomaternal circulation should represent a major advance in the management of Rh alloimmunization. This technique may also be appropriate for the diagnosis of RhD status in embryos before implantation<sup>26</sup> and in fetal cells in the maternal circulation.<sup>27</sup>

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