Detection of Trace Amounts of Fetal RhD-Positive Red Cells with Flow Cytometry after Multiple Rh-Negative Intrauterine Transfusions and RhD-Gene Determination with PCR

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Summary
In 2 cases of RhD-sensitized women, hemolytic disease of the newborns (hdn) was mitigated by intrauterine transfusions of O Rh-negative donor blood during pregnancy. In both cases preterm delivery was performed by cesarean section in week 32 of pregnancy. With cord blood the direct antiglobulin test (DAT) was negative using the gel test. The blood groups corresponded to the transfused donor blood. With flow cytometry (FC), 2% of IgG-coated red cells were found in the cord blood in case 1 and 5% in case 2. Using FC, these cells were RhD positive. Polymerase chain reaction (PCR) analyses showed the presence of RhD-gene sequences in both cases. Therefore, FC and PCR seem to be suitable methods for the diagnosis and the monitoring in cases of imminent hemolytic disease due to anti-D alloimmunization. With further transfusions, both children developed well after delivery.

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Schlüsselwörter
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Zusammenfassung
In zwei Fällen Rh-negativer Mütter mit Anti-D-Antikörpern wurde durch intrauterine Transfusionen von O Rh-negativem Spenderblut ein Morbus haemolyticus abgewendet. Die Kinder wurden durch Sectio caesarea in der 32. Schwangerschaftswoche entbunden. Mit dem Geltest war der direkte Coombs-Test in beiden Fällen am Nabelschnurblut negativ. Die Blutgruppe entsprach der transfundierten Spenderblutgruppe. Durchflusszytometrisch ließen sich 2% (Fall 1) bzw. 5% (Fall 2) IgG-beladene Erythrozyten im Nabelschnurblut finden. Diese Zellen waren mittels Durchflusszytometrie RhD-positiv. Die DNS-Analysen aus Amnionzellen ergaben in beiden Fällen den Befund RhD-positiv. Somit scheinen Durchflusszytometrie und Polymerase-
Kettenreaktion in Fällen eines drohenden Morbus haemolyticus bei Anti-D-Alloimmunisierung für die Diagnostik und das Monitoring geeignet zu sein. Nach weiteren Transfusionen entwickelten sich beide Kinder gut.

We report on two cases of hemolytic disease of the newborn (HDN), caused by anti-D in case I and by anti-D + anti-s in case II. In case I, there was a history of stillbirth due to anti-D (IgG1 and IgG3) two years before. In case II the first pregnancy was uneventful, but in spite of anti-RhD prophylaxis after delivery progressively rising titers of anti-D were detected in the first trimenon of the second pregnancy. Furthermore, anti-C, anti-E and anti-s were found later on in case II. (The anti-C in this case might be proven to be anti-G. Red cells for respective tests are very rare and were not available.) Table 1 shows the mothers’ preterm results and the blood groups of the fathers.

In case I, 5 intrauterine transfusions of O Rh-negative donor red cells had been given, and 3 such transfusions had been administered in case II. In both cases the newborns were delivered at week 32 of gestation by cesarean section. Exchange transfusion were performed immediately after delivery. The blood groups in the cord blood samples were (after multiple intrauterine transfusions) exclusively O Rh negative with a negative DAT, also when using the gel test method. However, an acid eluate prepared from the cord red cells contained high amounts of anti-D (gel test) in case I, and of anti-D + anti-s in case II.

The discrepancies were further investigated by additional flow cytometric (FC) investigations with FITC-labeled sheep antibodies (Binding Site, Heidelberg, Germany) against human IgG and its subclasses, as described recently [1]. The results are illustrated in figure 1. In case I, the cord blood could be shown to contain about 2% of fluorescent red cells coated with IgG (A in fig. 1), and in case II, 5% of IgG-coated red cells were found (B in fig. 1). These cells obviously represented the source of the eluted anti-D. After incubation with monoclonal IgG-anti-D (clone BS221 from Biotest, Dreieich, Germany) the

<table>
<thead>
<tr>
<th>Case I</th>
<th>Case II</th>
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<tbody>
<tr>
<td>The mothers’ blood group (gel test)</td>
<td>The fathers’ blood groups</td>
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<tr>
<td>Antibodies (gel test)</td>
<td></td>
</tr>
<tr>
<td>O Rh negative</td>
<td>A CCD.ee</td>
</tr>
<tr>
<td>Anti-D 1:256 Anti-C 1:2</td>
<td>O ccD.Ee, Ss</td>
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<tr>
<td>A Rh negative, SS</td>
<td></td>
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<tr>
<td>Anti-D 1:1024 Anti-C 1:64 Anti-E 1:256 Anti-s 1:32</td>
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Table 1. The mothers’ preterm results and the blood groups of the fathers

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cord red cells showed a significant shift to higher fluorescence when analyzed with FITC-anti-human-IgG (case I = A2, case II = B2 in fig. 1), indicating that they were RhD positive. With further in vitro studies (not shown here) we could demonstrate serologically with FC as few as 0.1% Rh-positive red cells (ccD.Ee) in a mixture with Rh-negative cells, using the same monoclonal IgG-anti-D for coating and the FITC-labeled polyclonal sheep anti-human-IgG for detection. The RhD genotype of both cases was tested with 3 sequence-specific polymerase chain reactions (PCR). In case II the RhD genotype was determined during pregnancy from genomic DNA isolated from cultured amnion cells. Each duplex PCR contained 2 oligonucleotides specific for the Rh-D gene and 2 primers for the amplification of a 366-bp fragment from the glycoprotein IIa gene [2] as internal control. We investigated polymorphisms at pos 201 [3,4] and 307 on exon 2 (142 bp), pos 985 (115 bp) on exon 7, and pos 992,1048 (90 bp), also on exon 7 with sequence-specific primers. The results are shown in figure 2. All amplification products in case I were weaker due to weak DNA, but RhD genotyping was still possible without doubt. After the exchange transfusions, both neonates exhibited progressive anemia requiring additional transfusions of O Rh-negative donor red cells, possibly not only due to residual anti-D antibodies but also to preterm delivery. Fifteen weeks after delivery, the children were developing well, and their blood groups could be determined without doubt using agglutination tests (gel test). The blood groups were A Rh positive ccD.ee in Fig. 1. Flow cytometric investigations of cord blood after multiple intrauterine transfusions of O Rh-negative red cells. The histograms show in a log/log plot the number of red cells counted (ordinate) against the fluorescence intensity (abscissa). In case I, 2% (A1) and in case II 5% (B1) of IgG-coated red cells could be shown using FITC-labeled sheep anti-human-IgG. Fluorescence was markedly enhanced (A2 and B2) after binding of monoclonal IgG-anti-D to these red cells.

90 bp 115 bp 142 bp 366 bp
Case 1 Case 2 Case 2 D pos D neg

cord blood amnion DNA

Fig. 2. Electrophoretic patterns of DNA from cord blood after PCR amplification. With 3 different sequence-specific primers, both neonates could be shown to be RhD positive. In case II, DNA could also be used from amniotic cell cultures. Control DNA samples were prepared from Rh-positive and Rh-negative donor blood. case I and A Rh positive ccD.ee (Ss) in case II. With these new, sensitive techniques it seems possible to detect fetomaternal bleedings, trace amounts of red cells in mixed blood samples and to determine the fetal blood group from trace amounts of fetal cells.
References

Trace Amounts of Fetal Red Cells
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261