Assessment of fetal-maternal haemorrhage in mothers with hereditary persistence of fetal haemoglobin

W N Patton, G S Nicholson, A H Sawers, I M Franklin, F A Ala, A W Simpson

Abstract

Kleihauer examination of peripheral blood cannot be used reliably to detect transplacental fetal-maternal haemorrhage in mothers with hereditary persistence of fetal haemoglobin (HPFH). In Rh(D) negative pregnancies diagnostic confusion with a large fetal-maternal haemorrhage could result in the administration of inappropriately excessive amounts of anti-D immunoglobulin, and the inability to diagnose and quantify transplacental haemorrhage in maternal HPFH by current methods could result in insufficient anti-D administration and subsequent Rh(D) sensitisation. Accordingly, a method to detect and quantify fetal-Rh(D) positive maternal haemorrhage using erythrocyte fluorescent immunocytometry was developed. An indirect immunofluorescence method with IgG anti-D immunoglobulin as the primary antibody was used, combined with quantitative analysis on a fluorescence activated cell sorter. The method was accurate, specific, and sensitive and could detect a contaminating population of 0·1 % Rh(D) positive cells in Rh(D) negative blood—a level of fetal-maternal haemorrhage well covered by a single dose of 500 IU of anti-D immunoglobulin.

Kleihauer examination of peripheral blood is used to assess the presence and extent of transplacental fetal-maternal haemorrhage, both as a diagnostic aid in detecting haemorrhage before birth and in the monitoring of pregnancies at risk of haemolytic disease of the newborn.1 The technique is ineffective when hereditary persistence of fetal haemoglobin (HPFH) is present in the mother. In the case of Rh(D) negative pregnancies diagnostic confusion with a large fetal-maternal haemorrhage could result in the administration of inappropriately large amounts of anti-D immunoglobulin, a relatively scarce resource. Secondly, the inability to diagnose and quantify transplacental haemorrhage in HPFH by current methods could result in insufficient anti-D immunoglobulin administration and subsequent Rh(D) sensitisation.

We have seen three cases of HPFH in Caucasian Rh(D) negative mothers over the past two years which prompted us to develop a prospective method to detect and quantify Rh(D) positive fetal-maternal haemorrhage using erythrocyte fluorescent immunocytometry with anti-D immunoglobulin.

Methods

A two stage technique with quantitative fluorescence cytomtery was used. All blood specimens were anticoagulated with EDTA. Erythrocytes were washed twice with filtered 0·9% saline and then centrifuged at 400 × g for 60 seconds. In the first antibody stage 25 μl of concentrated washed erythrocytes were incubated with 50 μl of 50 IU/ml human anti-D immunoglobulin (Blood Products Laboratory, Elstree) for 30 minutes at 37°C. The cells were then washed four times with filtered 0·9% saline and for the second antibody stage were incubated with 100 μl of a 1/50 dilution of 1 mg/ml fluorescein isothiocyanate (FITC) conjugated Fab anti-human IgG (Seralab Ltd, UK; SDL-3118) at room temperature for 15 minutes. After two further washes with filtered 0·9% saline the cells were resuspended in 0·9% saline at 1 × 10⁹/ml and a minimum of 20000 cells were analysed on a laser flow cytometer (Becton Dickinson 440, Becton Dickinson UK Ltd). For positive controls, artificially created mixtures of Rh(D) positive erythrocytes in Rh(D) negative blood were used. Rh(D) negative blood was used as a negative control. Additional negative controls on Rh(D) positive blood included (1) the use of normal heat inactivated non-immune human serum as a negative primary antibody and (2) the use of FITC conjugated second antibody without any primary antibody.

Haemoglobin separation was carried out by isoelectric focusing using precast polyacrylamide gels with a pH range of 5·5-8·5, supplied by Pharmacia Biotechnology Ltd. Haemoglobin A₂ was measured by elution from cellulose acetate electrophoresis strips2 and Hb F measured by alkali denaturation using a modification of Bette's method.3 Globin chain synthesis was assessed by tritiated leucine incorporation using a modification of the method of Weatherall, Clegg, and Naughton.4

Results

CASE 1

A 30 year old Caucasian Rh(D) negative primigravida delivered a normal healthy baby after 38 weeks' gestation. Examination of the cord blood showed the neonate to be Rh(D)
Assessment of fetal-maternal haemorrhage

**Summary of maternal investigations from case 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>135 g/l</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>4.10 x 10^11</td>
</tr>
<tr>
<td>Mean corpuscular volume</td>
<td>99 fl</td>
</tr>
<tr>
<td>Mean cell haemoglobin</td>
<td>32.9 pg</td>
</tr>
<tr>
<td>Mean cell haemoglobin concentration</td>
<td>331 g/l</td>
</tr>
<tr>
<td>Blood film</td>
<td>Normal</td>
</tr>
<tr>
<td>Kleihauer exam</td>
<td>Heterocellular distribution of Hb F</td>
</tr>
<tr>
<td>Haemoglobin electrophoresis</td>
<td>Hb A</td>
</tr>
<tr>
<td>Hb F</td>
<td>10.0%</td>
</tr>
<tr>
<td>Hb A</td>
<td>21.0%</td>
</tr>
<tr>
<td>Globin chain synthesis</td>
<td>non-x chain: x chain ratio = 1.04 beta chain: x chain ratio = 0.61 gamma chain: x chain ratio = 0.43</td>
</tr>
</tbody>
</table>

Positive with a negative direct antiglobulin test and a normal full blood count with a haemoglobin concentration of 170 g/l. Subsequent Kleihauer examination of the maternal blood proved strongly positive with 33% of cells containing large amounts of haemoglobin resistant to acid elution. The maternal full blood count was normal, showing erythrocytes of a normal size distribution, and indicating that a large population of macrocytic fetal cells could not be identified. Repeat analysis of the same specimens and further correctly identified samples from the mother and baby gave the same results. As the clinical and laboratory features were inconsistent with a large fetal-maternal haemorrhage, maternal HPFH was suspected. Subsequent investigation of the mother confirmed HPFH consistent with the British heterozygote subtype (table). At birth the mother was given three doses of 500 IU anti-D immunoglobulin. The extent of initial fetal-maternal haemorrhage and its response to treatment were quantified using erythrocyte fluorescence immunocytometry with anti-D immunoglobulin; the results are shown in figs 1–5. These are expressed as dot contour plots with fluorescence on a log scale on the vertical axis and cell size on a linear scale on the horizontal axis. Rh(D) positive cells are identified in the boxed area.

**CASE 2**

A 21 year old Caucasian Rh(D) negative gravida 2 delivered a normal healthy baby after 40 uneventful weeks of gestation. Examination of the cord blood showed that the neonate was Rh(D) positive and direct antiglobulin test negative, with a haemoglobin concentration of 148 g/l. Subsequent Kleihauer examination of the maternal blood showed 450 cells containing haemoglobin resistant to acid elution per three low power fields, and these findings were verified on a repeat maternal sample. In view of the low neonatal haemoglobin concentration a large fetal-maternal haemorrhage of 90 ml was provisionally diagnosed and 10500 IU of anti-

**Figure 1** Positive control containing 0.1% Rh(D) positive cells.

**Figure 2** Positive control containing 1.0% Rh(D) positive cells.

**Figure 3** Negative control containing 100% Rh(D) negative cells.

**Figure 4** Initial post partum sample from case 1, before the administration of anti-D immunoglobulin, showing the presence of 0.2% Rh(D) positive cells.
D immunoglobulin was given intramuscularly. Erythrocyte fluorescent immunocytometry with anti-D immunoglobulin was used to confirm the extent of initial fetal-maternal haemorrhage and to monitor the response to treatment. Figure 6 shows the initial maternal sample before administration of anti-D, and fig 7 shows the maternal sample two days later. A repeat maternal Kleihauer examination showed <6 positive cells per 3 low power fields, free anti-D remained detectable in the maternal serum, and no further anti-D was given. Subsequent examination of the mother showed no evidence of HPFH and the initial maternal Hb F concentration was 1.0%. A maternal antibody screen six months later was negative for anti-D.

Discussion

Erythrocyte fluorescent immunocytometry with anti-D immunoglobulin is an accurate, precise, specific, sensitive and objective method for the detection and quantitation of fetal Rh(D) positive maternal haemorrhage in Rh(D) negative mothers. Its accuracy, precision, and objectivity have considerable advantages over the commonly used Kleihauer method, but its high sensitivity, high capital and running costs, and relative technical complexity make it both unnecessary and unsuitable for routine use. Its ability to detect at least 0.1% contaminating Rh(D) positive cells in Rh(D) negative blood is well within the threshold level of fetal-maternal haemorrhage treated by a single dose of 500 IU anti-D immunoglobulin.

Erythrocyte flow cytometry with anti-D immunoglobulin is indicated in certain situations, however, as shown by two of our cases. We suggest that it is now the method of choice to diagnose and quantify Rh(D) positive fetal-maternal haemorrhage in Rh(D) negative mothers with HPFH as it is more accurate and objective than previous methods using rosette techniques. It should also be considered in cases of large fetal-maternal haemorrhage where diagnostic confusion with maternal HPFH might exist and where the more accurate quantitation of fetal-maternal haemorrhage is an advantage. In the latter situation a more accurate estimate of the amount of anti-D immunoglobulin to be given to the patient will be produced which should prevent the administration of inappropriately excessive amounts of this relatively scarce resource. The method also permits greater accuracy in monitoring the efficacy of treatment in this more critical situation which carries a higher risk of Rh(D) sensitisation.

These situations are uncommon, but appropriate assessment of all cases should be possible as the 72 hour window for administration of anti-D immunoglobulin after giving birth should permit sufficient time for blood sample transport and analysis in units with flow cytometry facilities.

Erythrocyte fluorescent immunocytometry has many other potential applications in detecting small percentages of erythrocytes using either anti-D or antibodies to other red cell antigens. We are currently evaluating fetal Rh(D) typing following chorionic villus biopsy sampling in pregnancies at risk of haemolytic disease of the newborn and Rh(D) positive engraftment after allogeneic bone marrow transplantation of Rh(D) negative recipients with Rh(D) positive donors, using anti-D immunoglobulin. Other potential applications include chimera testing, red cell survival studies with transfused erythrocytes, the determination of antigen density on erythrocytes and the quantification of red cell bound immunoglobulin.
We are grateful to Mr R Sawers of the Birmingham Maternity Hospital for permission to report case 1.


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*J Clin Pathol* 1990 43: 728-731
doi: 10.1136/jcp.43.9.728

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