Quantitating fetomaternal hemorrhages of D+ red cells using an FITC-conjugated IgG monoclonal anti-D by flow cytometry: a case report

A. Lubenko, R. Collier, M. Williams, D. Hindmarch, S. Wilson, and J. Pluck

Several methods for quantitating fetomaternal hemorrhages (FMHs) have been described; these include the Kleihauer-Betke and red cell rosetting tests, and flow cytometry that uses an indirect antiglobulin technique, employing either FITC-conjugated IgG/unlabeled anti-D or streptavidin conjugates with biotinylated anti-D to enumerate D+ red cells in maternal blood. We have used a recently described directly conjugated FITC anti-D for direct flow cytometric (direct FC) quantitation of FMH in a patient who presented with a large fetal bleed (approx. 80mL) as determined using the Kleihauer method. We compared the efficacy of the direct FC technique to the rosetting and Kleihauer tests in estimating the quantity of Rh immunoglobulin (RhIg) to be administered to the mother to suppress Rh alloimmunization. Both the Kleihauer and the direct FC gave precise estimates of 80mL for the size of bleed, whereas the rosetting test failed to be as precise. The former tests predicted that a 10,000iu dose (2,000μg) of RhIg would be adequate; the lack of alloanti-D in a maternal follow-up sample obtained 9 months after delivery supported this prediction and underlined the reliability of the direct FC method as an alternative to the Kleihauer method for quantitating large FMHs. Immunohematology 1997;13:12–14.

Rh immunoglobulin (RhIg) should be routinely administered to D– mothers recently delivered of D+ infants. Calculation of the dose of RhIg needed requires an accurate estimation of the volume of the fetomaternal hemorrhage (FMH). To this end, acid elution of fetal hemoglobin (Hb F) is routinely performed using the Kleihauer-Betke test. However, in recent years, alternative testing strategies have been developed. These include the rosette screening test as well as immunofluorescence flow cytometry (IFC). Several alternative strategies for flow cytometric measurements of FMHs of fetal D+ red blood cells (RBCs) in D– mothers have been described. These include (1) indirect staining, generally using an FITC-conjugated Fab preparation of anti-human IgG to detect RBC-bound anti-D after incubating the maternal RBC sample with IgG anti-D; indirect staining with biotinylated anti-D followed by PE-conjugated streptavidin has also been applied; and (2) more recently, direct staining using FITC-conjugated IgG monoclonal anti-D to detect fetal D+ RBCs in the maternal blood sample has also been described.

The flow cytometric direct staining technique offers a faster and theoretically more reproducible detection method than indirect staining. However, little information exists in regard to the comparability of flow cytometry with the Kleihauer and rosetting tests in the detection of FMHs over a wide range of fetal bleeds. A patient with a large FMH, as initially determined using the Kleihauer test, presented the opportunity for us to draw samples over a period of time to compare all three methods as the volume of fetal RBCs in her circulation declined following the administration of a single dose of RhIg. The volume of the fetal bleed, determined by flow cytometry, agreed with the volume suggested by the Kleihauer result. The effectiveness of the dose given was assessed by screening maternal serum for alloanti-D some 9 months after delivery.

Materials and Methods

The Kleihauer and rosetting tests were performed using the procedure of Kleihauer and Betke as modified by Sebring in 1984. For direct staining with FITC conjugated anti-D, the following method was used: quadruplicate 20 percent RBC suspensions from maternal samples and control D+ and D– RBC samples were incubated with 100μL of FITC-labeled IgG anti-D (BRAD 3) at a 1 in 10 dilution for 30 minutes at 37° C. Likewise, 20 percent suspensions of mixtures of D+/D– RBCs, pre-
pared in order to provide a calibration curve, were also incubated with FITC-BRAD 3. In these mixtures, the proportion of D+ RBCs varied from 10 percent to 0.125 percent. In parallel, 20 percent suspensions of maternal RBCs and the control D+ and D– RBC samples were incubated with phosphate buffered saline (PBS) instead of the conjugate in order to allow an assessment of background signals recorded by the flow cytometer to be made.

All samples were then washed manually 3×, taking care to avoid RBC loss, and were then analyzed on an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Loudwater, Buckinghamshire, UK) for the intensity of bound fluorescence (see Fig. 1).

RBCs were identified on the basis of their forward angle and orthogonal light scatter patterns. A total of 65,000 events were counted for each sample, and each of the four maternal replicates was counted twice to give an estimate of the number of fetal RBCs based on a minimum of 520,000 (8 × 65,000) cells in the maternal sample being analyzed in order to minimize the coefficient of variation encountered in rare event analyses.

Maternal samples were reassessed (as above) for residual D+ RBCs following the administration of 10,000 iu (2,000 μg) of RhD Immunoglobulin (BPL [Bio-Products Laboratory], Dagger Lane, Elstree, Hertfordshire, UK). The concentration of free anti-D in the maternal serum after RhIg administration was determined using the AutoAnalyzer™.

Results

The initial Kleihauer test indicated a total of 4,000 fetal cells per 50 low-power fields, equivalent to an FMH of 80 mL of RBCs (i.e., the day 0 value in Table 1). The same figure was obtained by direct IFC, whereas rosetting indicated that the volume of the FMH was merely > 3% of the maternal blood volume (i.e., ≥ 54 mL). Therefore, a divided dose of RhIg (two injections of 5,000 iu [1,000 μg] in each, 24 hours apart) was given to the mother.

Thereafter, sequential samples were taken at 2-day intervals for the determination of the residual volume of fetal RBCs and for the assessment of circulating free RhIg to ensure the adequacy of the dose of anti-D (see Ref. 7). A sample taken at day 2 gave similar estimates of the FMH using all three techniques. In subsequent samples, direct IFC indicated approximately twice the volume of fetal RBCs were present when compared with the Kleihauer results (rosetting was not undertaken). Calibration curves derived from testing artificial mixtures of D+ and D– RBCs indicated that direct IFC gave linear results (Fig. 1) with increasing concentrations of D+ RBCs in the range 0.125% to 10% (i.e., equivalent to bleeds of 0.18 to 180 mL) of fetal RBCs.

Free anti-D at a level of 0.27–0.39 iu/mL was detected using the AutoAnalyzer™. A follow-up sample taken

![Fig. 1. Calibration curve for fetomaternal hemorrhage quantitation obtained using FITC-conjugated monoclonal IgG anti-D (BRAD 3). Artificial mixtures of D+ red blood cells (RBCs) and D– RBCs were prepared as described and stained with FITC-conjugated BRAD 3 prior to analysis on an Ortho Cytoron Absolute flow cytometer. Results represent the mean of two determinations.](image_url)

<table>
<thead>
<tr>
<th>Day</th>
<th>Kleihauer-Betke</th>
<th>Rosetting</th>
<th>Direct IFC</th>
<th>AutoAnalyzer™</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal RBCs</td>
<td>Fetal bleed (mL)</td>
<td>% of maternal RBCs</td>
<td>% of maternal RBCs</td>
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<tr>
<td>0</td>
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<td>ND†</td>
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<td>10</td>
<td>&lt; 100</td>
<td>&lt; 2</td>
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<td>&lt; 0.1</td>
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*Red blood cells per 50 low-power fields
†Not done

**Table 1. Results of fetal blood volume determinations on successive maternal samples**
from the mother 9 months after delivery of her D+ offspring lacked detectable alloanti-D using either standard serologic techniques (i.e., two-stage enzyme tests and indirect antiglobulin tests) or the AutoAnalyzer™ method.

Discussion

The rosette test is simple to perform, but the initial large FMH could not be quantitated accurately due to the large size of the rosettes observed. Direct IFC for FMH quantitation proved to be less time-consuming and easier to perform than the standard indirect IFC but was tedious to undertake in comparison with rosetting.

Good agreement between the initial Kleihauer test results and the direct IFC method was obtained; however, in follow-up samples obtained after the administration of RhIg, direct IFC gave higher (occasionally two-fold greater) estimates of the volume of the FMH compared to the Kleihauer test. The linearity of calibration curves derived using direct IFC suggested that the latter test should have given reliable estimates of the volume of the FMHs measured over the range encountered. It is not clear, however, to what extent the presence of RBC-bound anti-D may have affected the ability of fetal D+ RBCs to lyse in the Kleihauer test.

Nevertheless, the excellent agreement between the direct IFC and Kleihauer techniques on the initial sample resulted in the administration of an adequate dose of RhIg, as reflected in the low levels of anti-D quantitated by the AutoAnalyzer™ in the immediate post-delivery period and in the complete absence of alloanti-D in maternal serum in a sample taken 9 months after delivery. Therefore, in this case, the projected dose of anti-D administered to the mother was adequate for preventing her alloimmunization to the fetus’ D antigen. Direct IFC appears to be superior to rosetting for confirming large-volume FMHs when estimated by the Kleihauer test. Its relative simplicity with respect to the indirect IFC would make the direct IFC the method of choice for quantitating FMH by flow cytometry. However, the use of calibration curves as described here makes the technique unnecessarily cumbersome. Experience with over 30 maternal samples tested since the above case indicates that the use of calibration curves in the direct IFC assay are largely irrelevant since the technique has excellent reproducibility.

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References


Anatole Lubenko, PhD, Head, Clinical Diagnostics Department, National Blood Service–Leeds, Bridle Path, Leeds LS8 7TW, West Yorkshire, UK; John Raymond Collier, FIBMS, Chief Biomedical Scientist, Huddersfield Royal Infirmary, Blood Transfusion Department, West Yorkshire, UK; Mark Williams, FIBMS, Head, Red Cell Reference Laboratory, National Blood Service–Leeds; Damien Hindmarch, Medical Laboratory Scientific Officer, National Blood Service–Leeds; Sally Rosemary Wilson, BA, Medical Laboratory Scientific Officer, National Blood Service–Leeds; and Julie Pluck, HNC, Medical Laboratory Scientific Officer, National Blood Service–Leeds, West Yorkshire, UK.

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