Flow cytometry in diagnosis and management of large fetomaternal haemorrhage

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Abstract

Aims—To evaluate an indirect immunofluorescence flow cytometry technique in a series of patients with large fetomaternal haemorrhage (FMH).

Methods—Patient samples identified by Kleihauer testing in local laboratories as having FMH >4 ml were sent for flow cytometric analysis. In a proportion of cases the mothers received anti-D immunoglobulin prophylaxis according to the flow cytometer estimate of FMH volume.

Results—Forty three cases of FMH were studied prospectively. The correlation between Kleihauer and flow cytometry results was poor. In 38 (88%) cases the size of FMH quantitated by flow cytometry was lower than that estimated using the Kleihauer technique. In 13 (30%) cases no Rh D immunoglobulin positive cells were detected by flow cytometry. Centralised review of the original Kleihauer films using a calibrated microscope resulted in improved, but still suboptimal correlation with flow cytometry results. In 15 cases anti-D immunoglobulin was given according to the flow cytometer estimation of FMH size, resulting in a 58% reduction in the amount of anti-D immunoglobulin given. None of the patients were immunised when tested six months later.

Conclusions—Flow cytometry is helpful for the accurate quantitation and management of patients with large FMH and in cases where the presence of maternal haemoglobin F containing cells renders the Kleihauer technique inaccurate. Worthwhile reductions in the use of anti-D immunoglobulin can be achieved.

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The Kleihauer acid elution test is the standard method for the detection and quantitation of fetomaternal haemorrhage (FMH) in the UK. This test identifies fetal cells in the maternal circulation by the relative resistance of cells containing haemoglobin F to acid elution.¹ While the Kleihauer test is sensitive, its accuracy in quantifying FMH is open to question because of numerous sources of error including variations in the thickness of blood films, the number of red cells in a low power microscope field, the fact that some fetal cells do not stain, and the difficulty of classifying cells of intermediate staining. Furthermore, the Kleihauer

test is unable to differentiate between fetal red cells and adult haemoglobin F containing cells which may be present in women with sickle cell and thalassaemic syndromes and in up to 25% of normal women in the second trimester of pregnancy.² Descriptions of the Kleihauer test method vary between sources and therefore it is not surprising that recent audits have revealed wide variations between laboratories.^{3,4}

The present policy in the UK is that all Rh D negative women giving birth to a Rh D positive child receive 500 IU of anti-D immunoglobulin which is sufficient to neutralise up to 4 ml of fetal red cells.^{5,6} A Kleihauer screening test is performed in all such cases to detect the 1% of women who have a bleed larger than 4 ml.⁷ In these cases the Kleihauer test is used to quantify the haemorrhage and determine the additional dose of anti-D immunoglobulin. Cases with large bleeds are monitored for the disappearance of haemoglobin F containing cells using the Kleihauer test and for the presence of free anti-D in maternal serum.⁵

We have evaluated a flow cytometry technique for quantitating FMH in which fetal cells are detected by their expression of the Rh D antigen. The method has been evaluated in a series of large FMH and compared with the Kleihauer technique.

Methods

A two stage technique reported previously^{8,9} was modified as follows: red cells from the maternal EDTA sample were washed four times in normal saline then centrifuged at 3000 rpm for two minutes. Packed cells (5 µl) were incubated with 100 µl anti-D serum (70 IU/ml) for 30 minutes at 37°C. The cells were washed four times in normal saline and were then incubated with 100 µl goat anti-human Fab IgG immunoglobulin (diluted 1 in 50 and labelled with fluorescein isothiocyanate (FITC)) (Sera-lab, Crawley Down, Sussex, UK) for 30 minutes at 37°C. The cells were washed twice in normal saline, re-suspended in 2 ml normal saline and analysed for green fluorescence using a Cytoron Absolute flow cytometer (Ortho Diagnostics, High Wycombe, UK), with 65 000 cells being counted.

As a positive control, mixtures of Rh D positive cells in Rh D negative blood at varying percentages were included with each analysis. For negative controls, Rh D positive and negative red cells were incubated with AB serum instead of anti-D in the first incubation.

The volume of the FMH was calculated by multiplying the percentage of Rh D positive

Table 1 Results of flow cytometric analysis of prepared dilutions of Rh D positive cells in Rh D negative red cells

Percentage Rh D positive	Flow cytometer result				
	Mean	Maximum	Minimum	Median	SD
100	98.0	99.9	92.0	99.0	2.154
50	52.2	63.4	43.4	53.6	6.058
10	10.5	14.8	8.3	10.2	1.732
5	5.9	8.0	4.1	5.7	1.054
1	1.2	2.0	0.8	1.2	0.259
0.5	0.6	0.9	0.3	0.7	0.108
0.4	0.5	0.7	0.3	0.5	0.109
0.3	0.4	0.5	0.3	0.4	0.075
0	0.04	0.1	0	0	0.050

0.3% corresponds to a FMH of 5.4 fetal red cells. $r=0.998$; $r^2=0.996$.

cells detected by flow cytometry by 1800 (assuming a red cell volume of 1800 ml in a pregnant woman at term).¹⁰

Haematology laboratories in the northwest region of England were invited to send maternal EDTA samples in cases where the Kleihauer test suggested a FMH of greater than 4 ml. Anti-D immunoglobulin was administered according to the FMH volume, estimated by flow cytometry, with 125 IU recommended for each ml of fetal red cells. Subsequent monitoring was carried out according to local laboratory practice and further anti-D immunoglobulin was given if follow up Kleihauer tests at 48 hours showed the presence of haemoglobin F containing cells in the absence of free anti-D immunoglobulin in the maternal serum.

Results

Excellent differentiation was achieved between Rh D positive and negative red cells. Dilutions of Rh D positive cells in Rh D negative cells revealed the limit of sensitivity of the technique to be 0.1%, corresponding to a FMH of 1.8 ml. The results of varying dilutions of Rh D positive red cells in separately prepared dilutions are shown in table 1. To ensure that fetal Rh D positive red cells were detected as reliably as adult cells, similar dilutions were made using fetal Rh D positive cells in adult Rh D negative cells with identical results (not shown).

Over a 12 month period, 43 maternal samples were received from 14 laboratories in which the Kleihauer test suggested a FMH in excess of 4 ml fetal red cells. Kleihauer results ranged from 4 to 254 ml with a mean of 35.5 ml and median of 12 ml. The correlation between the Kleihauer test and the flow cytometry results ($r=0.714$) is illustrated in fig 1. To compare results, the Kleihauer test and the flow

Figure 1 Correlation between results obtained using the original and reviewed Kleihauer tests, and on flow cytometry. The dotted lines indicate 50 and 200% of the Kleihauer result. Original Kleihauer test, $r=0.714$; $r^2=0.509$. Reviewed Kleihauer test, $r=0.915$; $r^2=0.837$.

Figure 2 Correlation between the original and reviewed Kleihauer test results. The dotted lines indicate 50 and 200% of the original Kleihauer test result; $r=0.810$; $r^2=0.656$.

cytometry results were regarded as being in agreement when the flow cytometry results were within 50–200% of the Kleihauer test, as shown by the dashed lines in fig 1. Despite this rather liberal definition, only 26% (11/43) of samples were in agreement. In one case the result on flow cytometry was larger than that estimated by the Kleihauer test and in 31 (72%) cases the Kleihauer test result was larger than that obtained on flow cytometry. In 13 (30%) cases no Rh D positive cells were detected by flow cytometry.

In view of the wide variation in results, a survey of Kleihauer methods in 17 hospitals in the region was performed and compared with the guidelines produced by the Association of Clinical Pathologists.¹¹ Wide variability in practice was observed, as shown in table 2.

Table 2 Survey of the procedures for performing the Kleihauer test

Procedure	Number (n=17)
Use of a standard operating procedure	14
Use of a microscope with calibrated field size	2
Objective lens power	
× 10	10
× 40	5
Other	2
Use of positive control preparation	17
Use of negative control preparation	8
Quantitation of FMH in large bleeds	
by fetal:adult ratio	3
by count of positive cells in 50 low power fields	14
Formula for calculation of anti-D dosage	
cells/50 low power fields × 2.5	10
cells/50 low power fields × 3	6
cells/50 low power fields × 5	1

Table 3 Patients dosed according to flow cytometer estimate of FMH volume ($n=15$)

Patient number	FMH volume (original Kleihauer)	FMH volume (flow cytometry)	Vol. treated by anti-D given (ml)	Free anti-D at 48 hours	Anti-D at six months	Volume untreated (ml)*
1	254	130	144	Y		110
2	100	0	20	Y	Neg	80
3	95	83	80	Y	Neg	15
4	90	0	4	Y	Neg	86
5	40	5.4	8	Y	Neg	32
6	30	0	4	Y	Neg	26
7	29	0	8	Y	Neg	21
8	28	0	4	Y	Neg	24
9	20	0	4	Y	Neg	16
10	20	0	4	Y	Neg	16
11	19	15.3	16	N**	Neg	3
12	14	6.3	8	Y	Neg	6
13	12	2.9	4	Y		8
14	8	1.8	4	Y	Neg	4
15	5	1.8	4	Y	Neg	1

* Relative to original Kleihauer estimate.

** Patient received a further 500 IU anti-D according to study protocol.

In 31 cases the original Kleihauer films were reviewed centrally using a calibrated microscope. The correlation between the corrected and original Kleihauer tests is shown in fig 2. Fifteen (48%) of the 31 corrected Kleihauer tests are within 50–200% of the original results ($r=0.810$). The correlation between the corrected Kleihauer test and the flow cytometry results is illustrated in fig 1. Results were in agreement in 12 (39%) of the 31 cases ($r=0.915$).

In 13 cases Rh D positive cells were not detected by flow cytometry despite having a sizeable FMH as measured by the Kleihauer test (range 5–100 ml fetal red cells). Further investigation of these cases revealed that four were due to poor Kleihauer preparations, three were due to mid-trimester maternal haemoglobin F, two resulted from adult haemoglobin F syndromes confirmed post-natally, two were cases of raised maternal haemoglobin F at delivery which resolved after six months, possibly persistent mid-trimester increases, and two were caused by probable Rh D negative fetuses.

Fifteen cases were treated according to the flow cytometer estimate of FMH volume. Results in these patients are given in table 3.

Discussion

The Kleihauer acid elution test is widely used in the UK for the detection and quantitation of FMH. While the test is very sensitive, its accuracy in quantitating larger bleeds, particularly those over 4 ml which are not adequately treated with the standard dose of anti-D immunoglobulin, is a cause for concern.³⁴ Although the test has some inherent inaccuracies,⁶ the major problems seem to be caused by a lack of consistency in the methods used by various laboratories^{16,11} and inaccurate microscope calibration. Hopefully the recent introduction of a national external quality assurance scheme for the Kleihauer test will result in improved standardisation and results.⁷

The flow cytometry technique differentiated clearly between Rh D positive and negative cells, showed good correlation in dilution experiments and had a sensitivity of 0.1% in accordance with previous studies.¹² Analysis took about 75 minutes, which could shortened

further by incorporating a one stage procedure utilising directly conjugated monoclonal anti-D reagents (for example, FITC-BRAD-1, IGBRL, UK).

While the Kleihauer test is a simple, sensitive and cost effective technique for detecting patients with a FMH below 4 ml, flow cytometry offers several advantages in patients with larger FMH; 65 000 cells are assessed individually using the flow cytometer and several sources of inherent errors in the Kleihauer test are eliminated. Raised maternal haemoglobin F concentrations render the Kleihauer test inconclusive, whereas the flow cytometer detects potentially immunising Rh D positive cells even in cases where the Rh status of the fetus is not known.^{13,14} In 88% of cases the FMH detected using flow cytometry was lower than that estimated by the Kleihauer test. This observation is in accord with the general view that the Kleihauer test frequently overestimates the size of FMH. This was clearly demonstrated in the FMH of 254 ml estimated by the Kleihauer test, which exceeds the entire red cell volume of a fetus and placenta.

In our study of 15 patients treated with anti-D immunoglobulin according to the FMH estimated by flow cytometry, significant reductions in the dose of anti-D immunoglobulin needed were achieved, with free anti-D immunoglobulin being detected at 48 hours in all but one case. In the one patient in whom anti-D immunoglobulin was not detected, implying inadequate dosing, further study showed that the anti-D immunoglobulin was administered in two doses separated by 24 hours and that the 48 hour screen for anti-D immunoglobulin was performed approximately 12 hours after the second injection. It is possible that this was too early for adequate absorption, but the study protocol required the administration of further anti-D immunoglobulin in this situation. No patients were immunised when tested six months later. Improved accuracy in the quantitation of large FMH results in the more rational use of anti-D immunoglobulin.

In conclusion, flow cytometry may be helpful for the accurate quantitation and management of patients with large FMH and in cases where the presence of maternal haemoglobin F containing cells renders the Kleihauer technique

inaccurate. While a well standardised Kleihauer test is appropriate as a screening test for FMH, studies to assess the role of flow cytometry for detecting FMH are warranted.

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