Reference Values of Fetal Erythrocytes in Maternal Blood During Pregnancy Established Using Flow Cytometry

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Abstract

The aim of our study was to assess the fetal RBC count in maternal blood during uncomplicated pregnancies from 26 weeks onward. We used a flow cytometric method specifically designed for use in a routine hematology analyzer.

Pregnant women were recruited through midwives. The participating laboratories used the FMH QuikQuant method (Trillium Diagnostics, Brewer, ME) in a CELL-DYN Sapphire hematology analyzer (Abbott Diagnostics, Santa Clara, CA). The method is based on a monoclonal antibody to hemoglobin F. Flow cytometric data were analyzed by 2 independent observers. The 95th percentile reference range was estimated according to Clinical and Laboratory Standards Institute guidelines.

A total of 236 samples were statistically analyzed. Gestational ages ranged from 21.6 to 41 weeks (mean, 32.0 weeks), and the fetal RBC count in maternal blood ranged from 0.00% to 0.50% (median, 0.025%).

The fetal RBC count in maternal blood shows no correlation with gestational age. The established reference range during normal pregnancy is less than 0.125%.

Traditionally, the Kleihauer-Betke (K-B) assay has been the method of choice for quantifying fetomaternal hemorrhage (FMH).1 In recent years, flow cytometric methods gradually replaced the K-B method because the K-B method has some inherent inaccuracies and drawbacks, which can largely be eliminated by flow cytometry. The first flow cytometric methods for FMH detection were based on the use of anti-D antibodies.2-4 Methods using monoclonal antibodies to hemoglobin F (HbF) were developed later.5,6 All flow cytometric methods have better precision than the K-B technique, and, moreover, the HbF-based methods allow objective discrimination between fetal RBCs and adult RBCs with an increased content of HbF, so-called F cells.5,7,8 In the K-B method, F cells are often difficult to distinguish from fetal RBCs. Therefore, HbF-based flow cytometric methods can prevent false-positive results in patients with increased F-cell counts due to various hematologic diseases.9-11

The flow cytometric fetal RBC assay used is a combination of the FMH QuikQuant kit (Trillium Diagnostics, Brewer, ME) and the CELL-DYN Sapphire analyzer (Abbott Diagnostics, Santa Clara, CA).12,13 This is a sophisticated routine hematology analyzer that also has flow cytometric capabilities.14 It is equipped with a 488-nm solid-state blue laser, 4 detectors for scattered light, and 3 fluorescence detectors. The analyzer offers 3 additional open modes for fluorescence assays on RBCs, WBCs, and platelets, respectively, allowing users to develop cellular assays using fluorescence-labeled antibodies, similar to a flow cytometer. The advantage of this approach is that laboratories using this analyzer are able to run special cellular assays without a flow cytometer and without the expertise needed for operating a flow cytometer. Moreover,
fetal RBCs in maternal blood and the volume of fetal RBCs increases as pregnancy progresses, in particular in the third trimester.\textsuperscript{15,16} Surprisingly, there are only a few reports of reference values for flow cytometric fetal RBC counts, and some of these are not representative of pregnant women because they were established in males and nonpregnant females\textsuperscript{5} or in healthy female blood donors.\textsuperscript{17} Another recent study included pregnant women, but mainly in the last month of gestation.\textsuperscript{18} Therefore, the aim of our study was to determine reference ranges of fetal RBCs in maternal blood during the last trimester of an uncomplicated pregnancy.

Materials and Methods

Recruitment of Subjects and Blood Sample Collection

Pregnant women were recruited for this study through their midwives. Women were eligible if they had an uncomplicated singleton or twin pregnancy of 26 weeks or more. Exclusion criteria were triplet pregnancy, recent abdominal trauma, abdominal surgery or invasive prenatal diagnostic procedures (all within 8 weeks from blood collection), and blood transfusion or intrauterine blood transfusion during the current pregnancy. In the Netherlands, practically all pregnant women participate in the national program for prevention of Rh(D) antagonism, which comprises blood collection in week 30 for all Rh(D)-negative women and women with relevant irregular antibodies. To avoid too many blood collections for prophylaxis of Rh(D) antagonism. In other clinical conditions, such assays are always available, which is nearly impossible using a regular flow cytometry facility. As for the FMH assay, the fetal RBC assay on the CELL-DYN Sapphire represents such an assay, in accordance with clinical needs and compatible with routine laboratory operations.

By far the most frequent application of any FMH test is to determine the dosage of anti-D needed for the immune prophylaxis of Rh(D) antagonism. In other clinical conditions, a reliable estimate of FMH may also be necessary, for example, after abdominal trauma, prenatal invasive tests (chorionic villous biopsy, amniocentesis, cordocentesis), and stillbirth. For these indications, reference values are required to correctly interpret the FMH test result. It has been suggested that the presence of fetal RBCs in maternal blood and the volume of fetal RBCs increases as pregnancy progresses, in particular in the third trimester.\textsuperscript{5,16} Another study indicated that the women included in the study were representative of pregnant women because they were established in males and nonpregnant females\textsuperscript{5} or in healthy female blood donors.\textsuperscript{17} The aim of our study was to determine the reference range of fetal RBCs in maternal blood during the last trimester of an uncomplicated pregnancy.

Analytic Procedure

All participants used the FMH QuikQuant kit on a CELL-DYN Sapphire hematology analyzer, following the assay protocol provided by the kit manufacturer. In brief, 25 μL of blood is treated with glutaraldehyde solution (0.3 g/L in saline) for 10 minutes for fixing the RBC. Then, 2 mL of permeabilization solution is added and incubated for 10 minutes. After a single washing step, 25 μL of antibody mixture is added and incubated for 10 minutes in the dark; this mixture contains fluorescein isothiocyanate–labeled monoclonal anti-HbF and propidium iodide for staining WBC nuclei. Subsequently, the reagent mixture is centrifuged, the supernatant decanted, and 2 mL of saline added for resuspending the cells. The labeled cells are then ready to be run on the CELL-DYN Sapphire.

Samples were processed in the CELL-DYN Sapphire RBC Flow mode; in this mode, up to 50,000 cellular events are analyzed within 1 minute, and light scatter and fluorescence signals are recorded. Raw data are stored in an FCS list mode file and retrieved for off-line analysis using standard flow cytometry software. For the present study, the FCS files were analyzed centrally by 2 observers (Lybrich Schakel and J.J.M.L.H.); one used WinList (Verity Software House, Topsham, ME) and the other used FCS Express (De Novo Software, Los Angeles, CA). Results of the 2 observers were averaged; discrepancies were solved by consensus.

The flow cytometric method used on CELL-DYN Sapphire has a sensitivity of less than 0.05% fetal cells and precision of less than 15% (as the coefficient of variation) in the very low range (<0.50%) and less than 5% for higher values.\textsuperscript{12,13}

Quality Control

Before sample collection started, 3 fresh blood samples were sent to all participating laboratories for external quality assessment. A reference laboratory estimated the fetal RBC count using a flow cytometric method, also using anti-HbF antibody. Internal quality control was performed using FETALtrol, stabilized control material for FMH assays (Trillium Diagnostics), in every run.

Statistics

Standard statistical methods, including analysis of variance (ANOVA), were used for data analysis using the MedCalc software package (MedCalc, Mariakerke, Belgium).\textsuperscript{19} The reference range, derived from the 95th percentiles, was determined according to Clinical and Laboratory Standards Institute guidelines.\textsuperscript{20}
Results

In total, blood samples from 284 pregnant women were included in the study in 4 laboratories [Table 1], and after analysis of the FCS files, 236 were suitable for statistical analysis. Of the 48 excluded samples, the vast majority showed unusually high background fluorescence; 2 samples had to be excluded because the gestational age was missing (Table 1). The results of the external quality assessment exercise are shown in [Table 2]. For the internal quality control samples, the coefficients of variation varied between 6.3% and 31.2% in the 4 laboratories.

The gestational age ranged from 21.6 to 41 weeks (mean, 32.0 weeks; median, 31.0 weeks) and more than 99% of the samples were collected in week 26 or later [Figure 1]. Furthermore, it appeared that 20% of the samples were collected in pregnancy week 30, just meeting the protocol requirement. With the exception of the 30th week, the distribution of gestational ages was relatively equal for the range between 26 and 40 weeks (Figure 1).

The fetal RBC counts varied between 0.00% and 0.50% (mean, 0.047%; median, 0.025%), displaying a highly skewed, non-gaussian distribution (not shown). [Figure 2] is a scatter plot of the fetal RBC count as a function of gestational age. There was no significant correlation between the percentage of fetal RBCs and gestational age ($r = -0.096; P = .141$). One-way ANOVA also indicated that there was no relationship between these parameters ($P = .666$).

After outliers were identified by using the Tukey definition and rejected, the 95th percentile reference range for fetal RBCs in maternal blood was found to be 0.00% to 0.125% (with 90% confidence limit of the upper value, 0.115%-0.145%). In addition to fetal cells, adult F cells also were measured. These showed a slightly skewed, nonnormal distribution with a mean of 3.8% and a median of 2.9%; the F cells ranged from 0.0% to 17.0%. No significant relationship between F cells and gestational age was shown ($P = .477$; ANOVA).

Discussion

The fetal and maternal blood circulations are fully separated, but transplacental passage of fetal cells into the maternal blood is a common event during pregnancy. Some

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**Table 1**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>No. of Women Recruited</th>
<th>No. of Samples Excluded</th>
<th>No. of Samples Included</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>62</td>
<td>9*</td>
<td>53</td>
</tr>
<tr>
<td>B</td>
<td>17</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>C</td>
<td>97</td>
<td>0</td>
<td>97</td>
</tr>
<tr>
<td>D</td>
<td>108</td>
<td>37*</td>
<td>71</td>
</tr>
</tbody>
</table>

* One sample each excluded owing to missing gestational age. All other samples were excluded owing to high background fluorescence.

**Table 2**

<table>
<thead>
<tr>
<th>Fetal RBC Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference flow cytometer</td>
<td>1.38</td>
<td>0.20</td>
<td>0.38</td>
</tr>
<tr>
<td>Laboratory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.68</td>
<td>0.24</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>1.34</td>
<td>0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>C</td>
<td>1.37</td>
<td>0.17</td>
<td>0.43</td>
</tr>
<tr>
<td>D</td>
<td>1.52</td>
<td>0.15</td>
<td>0.41</td>
</tr>
<tr>
<td>Mean</td>
<td>1.458</td>
<td>0.182</td>
<td>0.42</td>
</tr>
<tr>
<td>SD</td>
<td>0.142</td>
<td>0.038</td>
<td>0.034</td>
</tr>
<tr>
<td>Coefficient of variation (%)</td>
<td>9.8</td>
<td>21.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* Data are given as percentages relative to maternal RBCs.
The vast majority of FMH tests are performed for determining the dosage of anti-D to be administered to Rh(D)-negative women. Because the women receive a standard dose of anti-D anyway, the goal of the FMH test is mainly to establish whether the FMH volume is so large that 1 or more additional anti-D doses are required. Because anti-D dosing is well described in obstetric protocols and guidelines, reference values do not have an important role in this clinical setting. However, when the FMH test is requested for another indication, it is essential that reference values are available for correctly interpreting the test results. Moreover, an anti–HbF-based method is preferred for these indications because it does not have the limitation of maternal and fetal Rh(D) blood groups, which is inherent to anti-D methods.

A literature search on reference values for FMH tests that we recently performed yielded surprisingly few results, despite the fact that a considerable number of reports of analytic evaluation have been published on flow cytometric methods for FMH quantification. And some of the few reports that include reference values derived them from studies in men and nonpregnant women. By doing this, the authors did not take into account that small fetomaternal transfusions are physiologic in normal pregnancies, especially in the third trimester. We therefore decided to establish reference values in the relevant group of subjects: pregnant women.

A detailed protocol was designed for this multicenter study, focusing on 3 major aspects. First, we wanted to enroll samples from pregnant women covering a wide range of gestational ages and to avoid the majority of samples being collected in week 30. Then, for collecting samples to determine reference values, we considered it essential to exclude women with complications or a high-risk pregnancy. Moreover, there is the matter of how to define a reference range. It could be anticipated that the data would not form a gaussian distribution; therefore, a nonparametric method would be necessary. Following the Clinical and Laboratory Standards Institute guideline, we chose the 95th percentile method using a large number of samples because it is generally accepted that this method gives the best results. The protocol required central assessment of the list mode files to reduce variations. Finally, all participating laboratories used the same analytic method; this approach would minimize interlaboratory variation. From the external quality exercise, it appeared that there was no correlation between fetal RBC count and gestational age. However, the number of FMH tests performed for maternal Rh(D)+ women, as the most frequent indication, is not negligible.

When analyzing the results of the list mode data files, it appeared that 44 samples showed unusually high background fluorescence, ie, the HbF– RBCs had fluorescence intensity in a position where normally only HbF+ cells are found. As a consequence, it became impossible to separate HbF– from HbF+ cells and to determine the fetal RBC count. We therefore rejected these 44 samples. The reason for high background fluorescence was found to be high variability of the fixation step in the assay. Meanwhile, the kit manufacturer has released an improved assay protocol, which practically eliminates this background issue.

Our study yielded 2 important results: the fact that there is no correlation between fetal RBC count and gestational age and the reference range itself. Although it has previously been demonstrated that the incidence and volume of FMH increases with increasing gestational age, to the best of our knowledge, a statistical correlation was never shown, and the results from the present study make it unlikely that such a correlation would exist.

The reference range determined was 0.00% to 0.125% fetal RBCs in maternal blood. These values are higher than reported by some other groups. In their study of 150 males and nonpregnant females, Davis et al found a mean of less than 0.02% (range, 0.00%-0.09%) by using an anti-HbF method that is very similar to ours. Other authors, using another anti–HbF-based kit, studied 74 nonpregnant female blood donors and reported a mean reference value of 0.007% with an upper reference limit less than 0.03%. The obvious explanation for our higher reference range is the fact that we included pregnant women in our study, in whom the higher count of fetal RBCs is the physiologic consequence...
of pregnancy. In contrast, the median fetal RBC count in pregnant women without invasive procedures was found to be less than 0.015%,²² which very well agrees with the value found in the present study.

When we compare our findings with those from older studies performed with the classical K-B test, the results are in good agreement. In about 29% of our study population, the calculated FMH volume was 0.01 mL or less, which is identical with one third of women as reported by others.¹⁵ Also, the data on higher FMH volumes are in agreement with published values: 87%, 98%, 96%, and 91% of women had less than 2.0 mL FMH in our study and studies of others, respectively.¹⁶,¹⁸,²⁷ These results demonstrate that the flow cytometric method used by our working group provides values in pregnant women that are comparable to those of the K-B test.

Because flow cytometry also allows the specific recognition of F cells, we measured F cells in our group of pregnant women. There was no significant relationship between F cells and gestational age. Because we performed a transversal study, this result is not incompatible with the observation that F cells can increase in some women during gestation.²²,²³ We have demonstrated that in an uncomplicated pregnancy, the fetal RBC count in maternal blood is not correlated with gestational age. The reference range of fetal RBCs during the second half of pregnancy is less than 0.125%.

References