Detection of fetal erythrocytes in maternal blood post partum with the fluorescence-activated cell sorter

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A study was made of the frequency and amount of fetal hemorrhage into maternal blood during labor and delivery as evidenced by the number of fetal cells present in the maternal circulation immediately after spontaneous vaginal delivery. A sensitive, indirect immunofluorescence was used with fluorescence-activated cell sorter analysis of erythrocytes. All of the 16 Rh-negative mothers studied after vaginal delivery of Rh-positive infants had circulating Rh-positive cells. The mean Rh-positive to Rh-negative erythrocyte ratio was 1:14,100 in maternal blood, which corresponds to a mean fetal hemorrhage of 156 μl. The test described is sufficiently sensitive to be used for the study of primary Rh isoimmunization and could be clinically applicable for antepartum screening to determine which patients require Rh immune globulin treatment before delivery. (AM. J. OBSTET. GYNECOL. 148:290, 1984.)

Transplacental hemorrhage that leads to fetal erythrocytes in the maternal circulation was first demonstrated by Chown in 1954.[1] Attempts to document the frequency of occurrence and the quantity of fetal erythrocytes in the maternal circulation were made possible with the introduction by Kleihauer and associates,[2] in 1957, of the acid elution method of differential staining of fetal cells. The detection of extremely small numbers of fetal erythrocytes in a large population of adult erythrocytes has been possible by fluorescence antibody methods.[3] Serologic tests, such as fluorescence-labeled antihemoglobin F,[4] mixed agglutination methods,[5] and indirect antiglobulin tests,[6] have also been used successfully to quantify fetal cells. Studies in which these various methods were used have disclosed the presence of fetal cells in postpartum maternal blood in 15% to 55% of postpartum women.[7-9] This range probably reflects the spectrum of sensitivity of the tests. Even with highly specific fetal erythrocyte markers, techniques that require manual scanning of unenriched samples of maternal blood have practical limits of accuracy when the frequency of the minor cell population is only one in several thousand.

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Supported by the Santa Clara Perinatal Project, the California Department of Health, Becton Dickinson and Company, Mountain View, California, Spectra Biologicals, Los Angeles, California, Shared Cell Sorter Facility, Stanford University, and National Institutes of Health Grant GM 17367 CA04681.

Presented at the Twenty-seventh Annual Meeting of the Society for Gynecologic Investigation, Denver, Colorado, March 19-22, 1980.

Received for publication July 16, 1982.

Revised August 10, 1983.

Accepted September 1, 1983.

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The fluorescence-activated cell sorter developed by Bonner and associates,[10] and described by Herzenberg and associates,[11] has the ability to analyze or sort fluorescence-labeled cells according to their size and fluorescence intensity. Counting and enrichment of a minor, antigen-positive cell population within a large population of antigen-negative cells are possible with the use of the fluorescence-activated cell sorter. Prior unpublished work by Jan and Herzenberg[12] in this laboratory identified fetal to maternal hemorrhage in all patients studied.

The purpose of this study was to use the fluorescence-activated cell sorter to accurately analyze small numbers of Rh-positive fetal erythrocytes in Rh-negative maternal blood obtained after delivery in otherwise normal pregnancies.

Material and method

Instrumentation. The fluorescence-activated cell sorter (Fig. 1) analyzes cells and can physically separate different populations on the basis of individual cell size and intensity of fluorescence. In the fluorescence-activated cell sorter, a narrow stream that consists mostly of cell-free sheath fluid is emitted from a nozzle and passes through the focused beam of an argon ion laser. The supervision of fluorescent antibody-treated cells forms a small core within the stream. As a cell passes through the laser beam, dye molecules that it carries are excited and emit fluorescent light which is collected to give an electronic signal proportional to the amount of dye on the cell. Laser light scattered by the cell in the forward direction generates another signal which is primarily dependent on cell size. The fluorescence and light scatter signals can be analyzed on a computer and can also be used to select cells for sorting.

For sorting, the jet-forming nozzle is vibrated at

about 35,000 Hz to stabilize the breakup of the stream into that number of droplets per second. To sort a selected cell, the droplet that contains it is electrostatically charged at the moment that it is formed from the stream. Charged droplets falling between charged deflection plates are pulled away from the stream of uncharged drops into a collection chamber. In the work described here, the charged cells deflected into a collector were visually counted by means of an inverted fluorescence microscope.

Sample collection. Samples of maternal venous blood were obtained between 2 and 8 hours post partum from 16 unsensitized Rh-negative mothers who had been delivered of ABO-compatible, Rh-positive, live-born infants. The samples were stored at 4° C in acid citrate dextrose anticoagulant and were analyzed within 2 weeks of collection.

Fluorescence labeling. In preparation for labeling, an aliquot of 25 μl of whole blood was washed in balanced salt solution.* After being washed, the cells were suspended in balanced salt solution and incubated for 30 minutes at room temperature with the first antibody, high-titer anti-Rh_0 (D) serum from type AB, Rh-negative patients (Spectra Biologicals, Los Angeles, California). After being washed three times in balanced salt solution, the cells were suspended in balanced salt solution and treated for 30 minutes with the second antibody, fluorescenated goat antihuman IgG (Microbiological Research Corporation, Bountiful, Utah). The cells were finally washed three times in balanced salt solution prior to resuspension for the analysis by fluorescence-activated cell sorting.

The amounts of the first and second antibodies that yielded the brightest possible Rh-positive cells with the least amount of background staining on Rh-negative cells were determined experimentally.

Control samples on each individual were prepared by using the second-step antibody alone for discriminating patients with endogenous IgG present on their erythrocytes.

Fluorescence-activated cell sorting analysis. For each sample, 5,000,000 cells were analyzed at an approximate rate of 10,000 cells per second. Erythrocytes were selected for inclusion in the fluorescence analysis on the basis of their characteristic forward light scatter signal. This criterion excluded leukocytes and most artifacts. If a positively stained cell population was found on analysis, as was the usual case when the ratio of the Rh-positive cells was at least 1 : 10,000, its presence was marked by a small positive shoulder on the fluorescence histogram. The computer linked to the fluorescence-activated cell sorter calculated the number of fetal (Rh-positive) erythrocytes that gave rise to this fluorescence peak. The calculation was based on the number of cells in the sample treated with both antibodies minus the control count obtained after use of the second antibody alone. The total number of cells analyzed was recorded by the fluorescence-activated cell sorter and was the basis for determining the ratio of brightly fluorescent, Rh-positive (fetal) to Rh-negative (maternal) cells. Finally, the volume of transplacental hemorrhage was calculated by means of a formula derived by Mollison[13]: A volume of fetal erythrocytes = 2,200/ratio of fetal-to-maternal erythrocytes. This formula is based on the assumptions that maternal red cell volume is 1,800 ml and that fetal red cells are 22% larger than maternal cells.

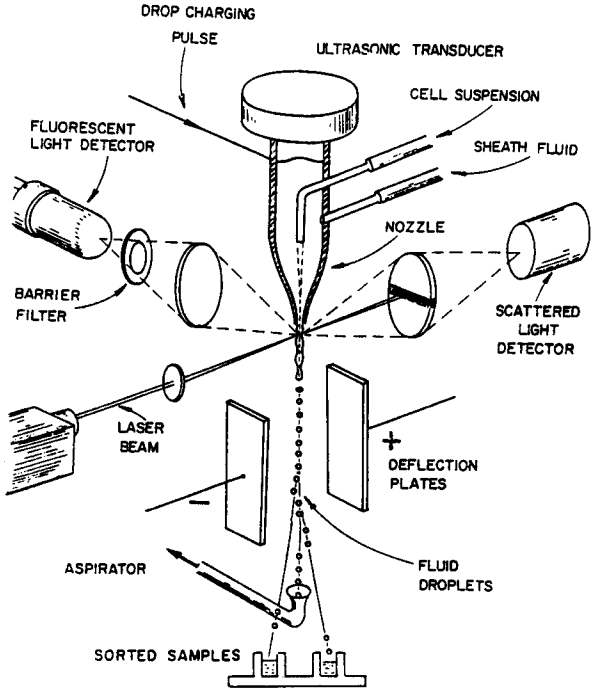

Fig. 1. Schematic diagram of the fluorescence-activated cell sorter.

Fluorescence-activated cell sorting for analysis of very minor Rh-positive erythrocyte populations. Samples of maternal blood that failed to show a positive fluorescence peak on computer analysis were generally those with Rh-positive (fetal) cells at ratios lower than 1 : 10,000 Rh-negative (maternal) cells. Accurate analysis of these samples could not be done by direct computer methods but instead required physical sorting of the cells into a shallow well that contained balanced salt solution in order to enrich the fluorescence-labeled Rh-positive cell population. By enriching Rh-positive cells from a known total population of 5,000,000 cells

*A liter of balanced salt solution contains the following: 80 gm of NaCl, 0.4 gm of KCl, 0.186 gm of $CaCl_2$, 0.2 gm of $MgCl_2 \cdot 6H_2O$, 0.2 gm of $MgSO_4 \cdot 7H_2O$, 1.0 gm of anhydrous dextrose, 0.06 gm of KN_2PO_4, plus 0.189 gm of NA_2HPO4.

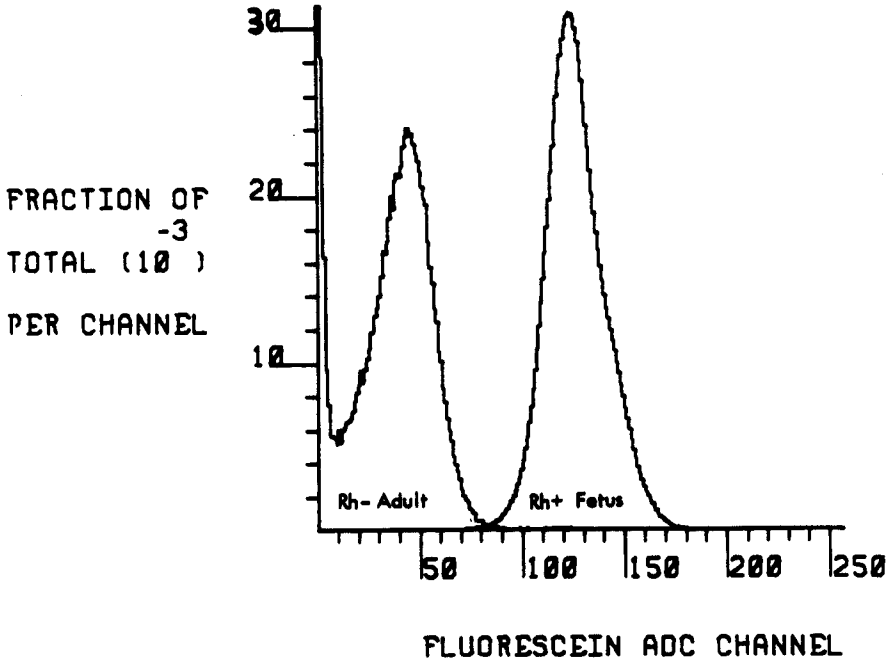

Fig. 2. Analysis by fluorescence-activated cell sorting of Rh-negative adult blood and Rh-positive umbilical cord blood with the use of anti-D serum and fluoresceinated anti-IgG. The curves were superimposed after separate staining and analysis of samples. The fluorescence scale is logarithmic with 56 channels per decade. Thus, a typical Rh-positive cell at channel 125 is 27 times as bright as an Rh-negative cell at channel 45.

and then visually scanning on an inverted fluorescence microscope, and counting all fluorescent cells with erythrocyte morphologic features, we determined the original ratio of positive-to-negative cells. The sensitivity of this method depends on the background count of fluorescent erythrocytes (second antibody alone) for each sample. Since a few cells with fluorescence were found in the second antibody controls, we concluded that Rh-positive cells were present only when the sample treated with first and second antibodies had twice the number of fluorescent erythrocytes found with the second antibody incubation alone. Background counts were generally on the order of 20 cells per five million cells sorted and analyzed. The sensitivity by this fluorescence-activated cell sorting and microscopic scanning technique was in the range of 1:250,000 erythrocytes, but varied with individual patients. The measured ratio of cells was converted into the corresponding volume of transplacental hemorrhage by the above-mentioned formula. Thus, the volume of fetal to maternal hemorrhage is 2,200 divided by the number of maternal cells counted for each fetal cell—a ratio of 1:220,000 represents a fetal to maternal hemorrhage of 0.01 ml or 10 μl of fetal erythrocytes.

Results

Initial evaluation of the fluorescence-activated cell sorting analysis technique consisted of comparisons of pure samples of Rh-negative (adult, nonpregnant) erythrocytes to samples of Rh-positive umbilical cord blood. Analysis of the staining characteristics was done separately, and then artificial dilutions at known ratios were prepared for analysis. Fig. 2 shows the results of double-antibody fluorescence staining and fluorescence-activated cell sorting analysis of an Rh-negative adult and a sample of Rh-positive cord blood, known to be heterozygous for Rh_0 (D) because the mother was Rh-negative. The two samples were analyzed separately and their fluorescence curves were superimposed. Separation on the histogram of the Rh-negative and heterozygous Rh-positive cells shows that the anti-D serum used as the first antibody had the ability to distinguish and therefore enumerate Rh-positive erythrocytes in a mixture. The ordinate of the histogram in Fig. 2 indicates the fraction of the total cells analyzed, and the abscissa represents increasing fluorescence intensity in log units.

To further document the applicability and accuracy of the proposed method of analysis in clinical situations, artificial mixtures of heterozygous Rh-positive cord blood and Rh-negative adult blood were prepared and analyzed. Fig. 3 shows a histogram of an analysis of a 1:5,000 mixture. A positive signal peak appears at the intensity rate of fluorescence at which positive cells are expected to appear. Computer analysis of the number of cells represented by this peak first involves

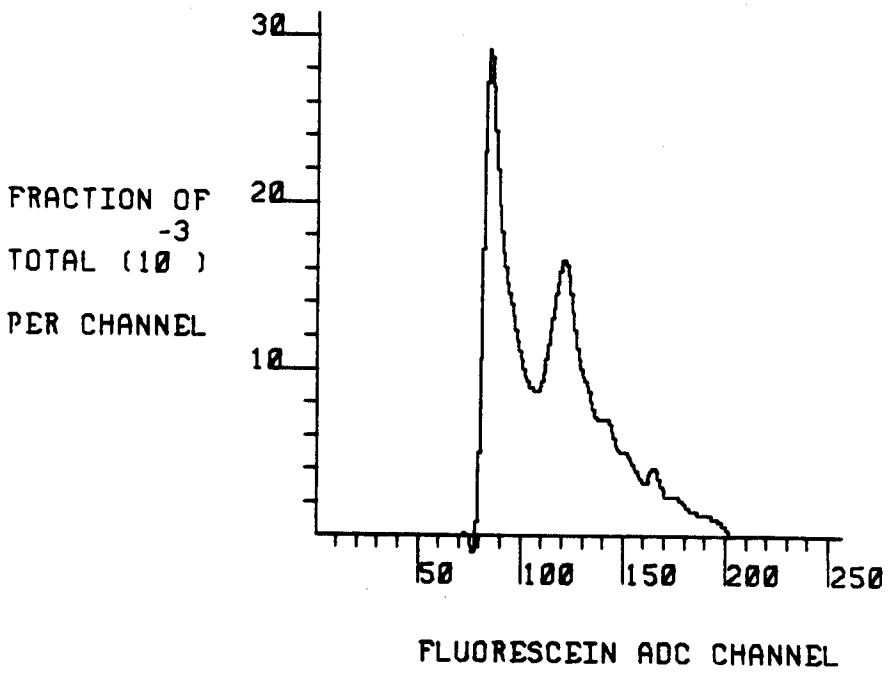

Fig. 3. Artificial mixture of 1/5,000 Rh-positive to Rh-negative erythrocytes. The 99.8% cells below channel 80 were excluded to give this magnified display of the brighter cells. The fluorescence scale is logarithmic with 56 channels per decade.

integration of the number of cells within the fluorescence intensity spanned by the peak, and then analysis of a parallel second antibody control sample. The difference in number of cells in this band between the sample and its control is the number of Rh-positive cells present. Reconstruction experiments with the use of artificial mixtures that ranged from 1:100,000 showed that computer analysis with the fluorescence-activated cell sorter gave results which were within 20% of true ratios so long as the mixtures were in excess of 1:10,000. Similar accuracy was extended to 1:100,000 if fluorescence-activated cell sorting and visual fluorescence microscopic scanning were combined for the more dilute mixtures. Beyond 1:100,000, accurate analysis was dependent on the frequency of positive IgG (second antibody alone) staining cells. In most cases these were present at a frequency of about 1:250,000, but were as rare as 1:1,000,000 or as common as 1:100 in one individual. Because of this extreme variability, second antibody controls were critical for analysis of each individual, and the sensitivity for each analysis was dependent on the number of erythrocytes bearing non-Rh IgG on their cell surfaces.

The study population chosen for analysis of unknown mixtures was composed of Rh-negative women who were delivered of Rh-positive ABO compatible infants. None of these women had positive Coombs tests nor had they been treated with RhIG prior to delivery or before drawing of the postpartum sample of blood for analysis by fluorescence-activated cell sorting. Sixteen postpartum patients were studied and the ratios of positive (fetal) to negative (maternal) Rh cells are shown in Table I, along with the calculated volume of fetal to maternal hemorrhage. Patient 16 was excluded in calculating the mean ratio and fetal hemorrhage

Table I. Ratio of fetal to maternal erythrocytes in unsensitized ABO compatible, Rh-incompatible postpartum women

Patient	*Ratio*	*Volume**
1	1/3,900	0.564
2	1/5,000	0.440
3	1/6,400	0.343
4	1/9,800	0.224
5	1/16,900	0.130
6	1/18,100†	0.121
7	1/18,300	0.120
8	1/25,000	0.088
9	1/29,000	0.076
10	1/38,300†	0.057
11	1/40,200	0.055
12	1/61,600	0.036
13	1/63,700	0.034
14	1/78,100	0.028
15	1/79,000	0.028
16	1/600‡	3.67

Mean volume = 156 μl. Mean ratio 1/14,100.

*Reference 13. Milliliters of fetal erythrocytes present in the maternal circulation = 2,200/maternal erythrocytes per each fetal erythrocyte.

†Twin pregnancy.

‡Postsplenectomy; not included in the mean.

Table II. Ratio of fetal to maternal erythrocytes in unsensitized Rh-negative women subsequent to delivery of an infant with Rh-positive blood

Mother	*D-positive/D-negative ratio*
1	1 : 41,000
2	1 : 43,600
3	1 : 12,100
4	1 : 14,600
5	1 : 105,000
6	1 : 5,360
7	1 : 75,200
8	1 : 163,000
9	1 : 11,900
Mean	1 : 18,100

Modified from Jan and Herzenberg.[12]

volume because of the known delay in clearance of antibody-coated erythrocytes by individuals who have had a splenectomy.[14] Data from the other 15 patients showed a mean ratio of 1 : 14,100 fetal to maternal cells, which represents a mean fetal erythrocyte volume of 156 μl in the maternal blood.

Comment

Transplacental passage of fetal erythrocytes is a recognized biologic phenomenon which, according to several reports, is most prevalent at the time of labor and delivery. With the relatively insensitive Kleihauer-Betke test, fetal-to-maternal hemorrhage has been estimated to occur in as few as 15% or as many as 55% of cases studied. The accuracy of the Kleihauer-Betke test was investigated by Mollison. Reports on fetal cell frequency in prepared mixtures of 1 : 1,000 to 1 : 10,000 were variable by tenfold among the 12 laboratories that participated.[13] Furthermore, the specificity of the Kleihauer-Betke test in clinical use is open to question, since up to 10% of adults have a few cells which have the staining characteristics of fetal erythrocytes.[8] Fluorescence-activated cell sorting offers several advantages in the study of fetal to maternal hemorrhage: the ability (1) to examine many cells rapidly, (2) to analyze for specific, unique fetal erythrocyte antigens, such as Rh_o (D), and (3) to physically sort and enrich minor cell populations to facilitate visual counting methods.

Our quantitative data on the extent of fetal to maternal hemorrhage with immediate postpartum sampling are similar to previously unpublished findings in our laboratory by Jan and Herzenberg,[12] who used a similar technique (Table II). Notably, in both series, each of the postpartum Rh-negative women studied by fluorescence-activated cell sorting analysis had circulating Rh-positive fetal erythrocytes. This consistent finding is at variance with the findings of previous reports mentioned in which fetal to maternal hemorrhage was present in 15% to 55% of cases.[7-9] On the basis of our data, we hypothesize that fetal-to-maternal hemorrhage is a universal occurrence, which can only be recognized with the use of adequately sensitive methods. Our findings also account for previous reports that Rh isoimmunization has occurred in cases with negative cell counts by other techniques.[15] Isoimmunization may result from fetal to maternal hemorrhage of fetal erythrocyte volumes less than 100 μl, which would give a fetal-to-maternal ratio of 1 : 20,000 or less.[16] The difference in mean volumes of fetal hemorrhage between the previous and the current series (122 versus 156 μl) is not statistically significant. In addition, the amounts of hemorrhage should not be compared because the initial series was not controlled for ABO incompatibility.

The sensitive technique described directs our interests toward a reassessment of primary Rh immunization. Having established that fetal to maternal hemorrhage occurs by the end of nearly all pregnancies, we have posed new questions about the occurrence of primary Rh immunization in only 15% of those at risk, without the use of prophylactic Rh immune globulin subsequent to delivery. We now have a method sufficiently sensitive to produce direct observations on minor Rh-positive erythrocyte populations, in the dose range believed to result in immunization.

With the fluorescence-activated cell sorting erythrocyte technique, we now have the capability of documenting which obstetric events, procedures, or complications represent genuine hazards for increased risk of Rh immunization. Antepartum sensitizations are rare events even after amniocentesis, which is thought to increase risk. Reports have not generally included sufficient observations to establish the degree of amniocentesis-related risk. In fact, our recent data would not suggest that amniocentesis results in an increased incidence of immunization.[17] With a surveillance technique which measures minor Rh-positive erythrocyte populations beyond the dose range of immunizations, the several conditions previously thought to represent immunization hazards[18] can now be studied for confirmation or rejection.

The fluorescence-activated cell sorting erythrocyte technique must be considered to be a research procedure because of the equipment, time, and expense needed to evaluate each sample. An individual sample plus control requires a minimum of 4 to 5 hours, and the usual time needed for the complete analysis of one to three samples is 2 hours of cell sorter time and 5 to 6 hours of technician time.

To date, cell sorters have been designed as research instruments; currently, clinically applicable cell analyzers are now being marketed. The usefulness and cost benefit for detection have yet to be determined.

Finally, with the sensitive method described in this

report, we have opened a new avenue of approach for further reduction of Rh immunization. Rather than contemplate the marginal benefits versus risks of antepartum prophylactic Rh immune globulin for all pregnant Rh-negative women, we can offer the alternative of population screening to identify specific Rh-negative mothers at risk. Women known to have circulating fetal cells would benefit from antepartum Rh immune globulin treatment at a much higher ratio. Further refinement of the methods described may lead to a clinically useful screening test for fetal-to-maternal hemorrhage and a safer and more cost-effective approach to the prevention of primary Rh immunization.

With the use of high-titer anti-D immune globulin, the fluorescence-activated cell sorter is capable of quantitatively analyzing minor populations of Rh-D–positive erythrocytes with ratios as rare as 1:10,000 in Rh-negative blood. The sensitivity can be extended to ratios of below 1:100,000 by enriching the minor population with fluorescence-activated cell sorting into a counting well and subsequent manual counting of all fluorescence-labeled erythrocytes with a fluorescence microscope. Our studies of postpartum patients have shown that fetal-to-maternal hemorrhage was a regular occurrence during labor and delivery.

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