#71

Fetal Erythrocytes Detected and Separated from Maternal Blood by an Electronic Fluorescent Cell Sorter

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INTRODUCTION

As the genetic and biochemical nature of many diseases becomes known, ante-natal diagnosis of infants at risk will become more frequent. At present amniocentesis is most commonly used method of obtaining fetal material for diagnosis is by amniocentesis. Yet clearly, there are fetal cells present in the maternal circulation. Given a means to separate fetal from maternal cells, simple maternal venipuncture could replace amniocentesis.

Recently in our laboratory we have developed a fluorescent cell sorter(1) which has the ability to count and to separate fluorescently labelled cells in a mixed population of stained and unstained cells. Among its numerous applications(1,2), the cell sorter has been used to separate antigen binding cells from spleen cells in mice(2). Thus, it seemed reasonable to explore its ability to separate human fetal cells from maternal cells in samples of gravidic blood. As the initial experiments along this line, we chose to develop a system utilizing our cell sorter to count a minor population of Rh positive fetal erythrocytes in a major population of Rh negative maternal cells.

Fetal cells appear in the maternal circulation usually

through transplacental hemorrhage, though there is evidence for other means of leakage as well(3,4). Besides being a possible source of material for ante-natal diagnosis, fetal cells in the maternal circulation are potentially antigenic. Immunization of the mother against, for example, a blood group antigen possessed by the fetus may well do harm to the present fetus and future fetuses. Rh hemolytic disease of the newborn is a classic example. Admirable advances in preventing this disease through anti-Rh gamma globulin treatment (5-7) have been achieved, and much research into the mechanical cause of this disease, transplacental hemorrhage, has been done.

The anti-Rh gamma globulin is customarily given shortly after the birth of the Rh positive infant because transplacental hemorrhage is largely associated with labor(8-13). Yet there still are some anti-Rh gamma globulin failures. In these cases it is thought that the mother probably sustained an immunizing transplacental hemorrhage in the course of her pregnancy or following a previous, unnoticed spontaneous abortion. Thus the detection of transplacental hemorrhage during pregnancy, following abortion, or during obstetrical maneuvers such as amniocentesis is important. Indeed, this has been

investigated by a number of groups(8-19). But the quantitation of fetal cell leakage, commonly either by the acid-elution method of Kleihauer-Betke(20) or by fluorescent-antibody methods(18) generally has required the manual counting of positive cells. With very small transplacental bleeds only a few fetal cells can be counted, and thus the accuracy with which minute bleeds can be measured is severely limited. However, with the electronic cell sorter that we employ, over 10^5 cells/sec can be examined for fluorescence, and therefore, much greater statistical accuracy than previously attainable is possible.

In this paper we report our ability to employ our cell sorter to count accurately small numbers of Rh+ fetal erythrocytes in Rh negative maternal blood and discuss the methods and future implications of this work as a means to replace amniocentesis with simple venipuncture to obtain fetal cells for ante-natal diagnosis.

MATERIALS and METHODS

BLOOD: Two types of Rh positive-Rh negative mixtures were analyzed. Artificial (in vitro) mixtures were made by combining the necessary volumes of out-dated citrated bank blood required to achieve the desired Rh positive/Rh negative ratios. Clinical venipuncture samples (in vivo mixtures) were obtained from post-partum mothers scheduled to receive anti-Rh gamma globulin and from Rh negative primigravida mothers between their second and fourth months of pregnancy. All clinical samples were anticoagulated with EDTA. They were stored at 4C and analyzed for Rh+ cells within one week of collection.

DELEUKOCYTATION: Because fresh leukocytes stained non-specifically with our reagents, it was necessary to remove all leukocytes from the clinical samples before analysis. This was achieved by using a combination of three established means of removing white blood cells --- serial washing with buffy coat aspiration, dextran sedimentation of erythrocytes(21), and nylon wool filtration(22). By following the procedure outlined in Fig.1, white cell frequency was reduced to less than 1 white cell per 10^6 red cells, as measured by staining with acriflavine, a

fluorescent dye which binds to DNA and thus stains nucleated cells such as leukocytes. Mixtures of Rh+ cord blood and out-dated Rh negative bank blood were used to check for the possibility of selective loss of fetal erythrocytes produced by our deleukocytation method. No such losses were found.

STAINING: Because of the reported difficulties in staining Rh+ cells directly with a fluoresceinated anti-D(18), we chose to employ a sandwich technique to fluorescently label the Rh positive cells present in our mixtures. A commercial anti-D typing serum (Dade) was used as the first stain and a fluoresceinated goat anti-human IgG (Miles Laboratory F/P=3.7) as the second. The concentration of each reagent was chosen to yield the brightest possible Rh+ cells with the least amount of background staining (best S/N ratio). Anti-D, undiluted, and anti-IgG at a 1:16-1:32 dilution gave the best differential staining for our system. In addition, the fluoresceinated anti-IgG was always centrifuged at 17,000G for 15 minutes before use to remove any aggregates of fluoresceinated material.

To stain we first added 300ul of anti-D to the swirled pellet of 1.3×10^9 cells. After a 30-minute incubation at room temperature the cells were washed four times in phosphate buffered saline (PBS), pH 7.1. Three hundred ul of

the fluoresceinated anti-IgG was next added to the pellet. After this second incubation for 30 minutes at room temperature, the cells were again washed four times in PBS and finally diluted to a concentration of 10^8 cells/ml for analysis by the cell sorter.

ANALYSIS: Although our cell sorter is described in detail elsewhere(1), briefly the operation of the machine as used for these analyses is as follows: A stream of cells crosses a laser beam, which is focused so that an average of three cells is illuminated by the beam at any one time. Any fluorescent molecules attached to the cells are excited by the beam, and a fluorescent pulse is emitted. A microscope objective positioned to minimize the entrance of direct laser light focuses the emitted light through barrier filters, which block any intruding, exciting light, onto the cathode of a photomultiplier tube. Thus a signal pulse proportional to fluorescent intensity is generated at the photomultiplier output whenever a fluorescent object crosses the laser beam. The electronic pulse is suitably amplified and shaped to be stored in a pulse height analyzer. Over a period of time, then, the fluorescent-intensity distribution of the passing stream of cells can be obtained. Each accumulated pulse-height analysis spectrum is fed to a small laboratory computer,

LINC, where it is stored temporarily on tape until the data can be transferred for further processing to the larger computer, ACME, available to us.

The pulse-height analysis plotted semi-logarithmically for a typical run is shown in Fig. 2a. As can be seen, there is some degree of overlap between the Rh+ cells (signal) and background stained Rh negative cells (noise). Fluorescent cell debris and other particles also contributed to the noise.

In order to determine the number of counts due to positively stained cells alone, it was necessary to subtract the noise from the total fluorescent-intensity distribution This was accomplished by taking advantage of the fact that, empirically, the pulse height distribution plotted semi-logarithmically for stained Rh negative cells alone (Fig. 2b) could be closely approximated with two straight lines, suggesting that there were two major components to the noise curve (labelled as dim and bright noise in Fig. 2b). Since portions of both these noise components could be identified even in the distributions for mixed populations of cells, it was possible, with the aid of computer programs and displays, to derive a noise curve from noise curve from the least-squares lines fitted to the dim and bright noise

portions of the total pulse-height analysis, much in the manner of the analysis of the decay curve for a mixture of two independently decaying radioisotopes. Subtracting the noise curve from the total then gave the pure signal curve. The precise steps are demonstrated in Fig. 3. From the signal integral, accumulation time, total cell input concentration, and cell flow rate, the ratio of positively stained cells to total cells was calculated.

RESULTS

To explore the sensitivity and accuracy of our method, we analyzed artificial mixtures of Rh positive cord blood and Rh negative out-dated bank blood. As we reduced the relative number of Rh+ cells in the mixtures, we could see that the signal peak gradually faded into the noise curve (Fig. 4) until, at a 1:1,000,000 ratio, we could no longer detect any signals above noise. Repeating this procedure with bloods blended from other individuals, we judged that, on the average, our limits of accurate detection seemed to be around one Rh+ cell in 100,000 Rh negative cells. In general, the agreement between expected and observed counts down to this 1:100,000 ratio was good. (Table I.) Practically all the values were within 20% of the expected counts.

To determine if our method worked equally well with in vivo mixtures of fetal and maternal cells, bloods from post-partum mothers scheduled to receive anti-Rh gamma globulin were analyzed for the number of Rh+ erythrocytes present. (Table II.) Our data are essentially similar to previously reported studies(10-17), with the majority of fetal to maternal ratios in our samples being <= 1:20,000.

Of interest, however, was that in none of the samples analyzed were no fetal cells found.

Because we are hopeful that our cell sorter can be used in ante-natal diagnosis, we were interested in the magnitude of transplacental leakage during the first trimester of pregnancy. Although there is literature on this time period(8-10,17), already in general, very few fetal cells are present in the maternal circulation in this early interval, making manual counting methods quite inaccurate in this range. We felt that the electronic method available to us would permit a more accurate measurement of the degree of transplacental leakage and allow us to obtain some estimate as to the number of fetal cells we could expect to isolate. We examined samples of blood from two Rh negative mothers who were in their first trimester of pregnancy. In both these cases no accumulated signal was apparent after the usual 30-minute analytic run. Thus, if any Rh+ fetal erythrocytes were present in these samples, their occurrence ratio must have been below our sensitivity level of 1 Rh+ per 10^5 Rh negative cells.

DISCUSSION

Transplacental hemorrhage, incidence and extent, has been the subject of numerous research papers. But never has a tool with the accuracy of our electronic cell sorter been applied. As in any application where random events must be tallied, statistical accuracy increases with the total number of counts recorded. With our machine, which can examine up to 100,000 cells a second, we are able in a standard run to count over 5,000 Rh positive cells in a 1:100,000 mixture of Rh+ to Rh negative cells. Others, using conventional counting techniques, such as scanning a slide for a fixed length of time or examining a certain number of high power fields, observe far fewer cells, reporting counts of zero to seven fetal cells for the same Rh positive/Rh negative ratio(12,17,18). Thus, for a 1:100,000 mixture we can state that our count of the number of fetal cells detected is accurate to within 3%. In contrast, the accuracy of a manual count of the same ratio would be 140%. Since small transplacental bleeds are the rule rather than the exception, it is possible that our system could help resolve many of the discrepancies of previous reports.

Certainly it is now possible to report accurately

fetal-to-maternal bleeds as small as 40 ul (approximately a 1:100,000 fetal-maternal cell ratio) using our method. Perhaps this increased accuracy accounts for the fact that fetal-maternal hemorrhage was discovered in all our post-partum mothers, whereas other workers could detect fetal cells in only 20 to 56% of their post-partum samplings(8-13). We agree that our total number of post-partum samples analyzed is small, but the probability, with a true expected incidence of 50%, of all nine samples being positive by chance alone is < 0.002. We are also aware that we did not examine nor control other variables such as ABO compatibility, mode of delivery, parity, time of blood collection after parturition, maternal Rh antibodies, etc. Nevertheless we suggest that the failure of other workers to detect such a high incidence of fetal-maternal leakage in the immediate post-partum period is accounted for in large part by the inaccuracy of their methods.

Comparing ante-partum results, we did not detect any first trimester spillage, whereas others have reported an incidence of 5-30%(8-10,17). Perhaps this difference can be accounted for by our small sample size. We accepted samples regardless of the father's Rh type; thus one would expect only one-half of our samples to come from mothers carrying

Rh+ fetuses. Furthermore, transplacental bleeds in the first trimester are usually much smaller than those at delivery(10,17), so that our method may not be sensitive enough to detect them. We analyzed ante-partum samples primarily because we were interested in assessing the possibility of using our machine to separate sufficient quantities of fetal material from reasonable volumes of maternal blood for ante-natal diagnosis. Unfortunately, such a feat is still in the future and will require improved procedures. However, present techniques could be applied immediately and quite profitably to measuring the frequency and extent of transplacental hemorrhage in numerous situations of considerable clinical importance.

Transplacental hemorrhages are of concern primarily because they are potentially immunogenic to mothers and a possible cause of anemia or death for fetuses. Naturally if any obstetrical procedure can be modified so that the extent of any leakage is reduced, then that is to the good. Clearly our electronic counting method offers an excellent way to assess the success of such modifications. Our system could also be extremely useful in conjunction with programs to prevent Rh-hemolytic disease of the newborn. If pregnant Rh negative mothers were routinely screened throughout their

pregnancy for Rh positive cells in their circulation, anti-Rh gamma globulin could be given following the first significant fetal-maternal bleed rather than following probable delivery-associated leakages as it is now. With earlier administration of anti-Rh gamma globulin, where indicated, perhaps the 2% failure rate of anti-Rh gamma globulin prophylaxis could be reduced further. With the high rate at which cells can be examined by our machine, our system ought to prove admirably suited to such mass screening programs. Finally, if we were able to make our technique even more sensitive than it is now, we might be able to establish a normal range of transplacental leakage with respect to the entire gravidic interval. Any significant spillage, then, above or below this, might serve to alert the physician to some potential danger to the fetus.

But even if, with a new staining technique, we were able to boost our sensitivity, the objection might be raised that other than the good correlation between expected and observed counts for our in vitro mixtures we have no evidence that fetal cells are in fact being detected when we process the in vivo mixtures. Thus despite the fact that no specific staining was observed during the Rh-negative

15

control runs, possible sources of false positive counts can include the following:

1)Stained white cells or non-cellular particles. If more the one white cell per 10^6 erythrocytes remains after our deleukocytation procedure, the non-specifically staining white cells can be recorded as Rh positive cell counts. However, white cells uniformly stain much brighter than Rh positive cells with our reagents, so that an unexpectedly bright signal peak can lead us to suspect white cell contamination. We have never observed such an event in our clinical runs.

The fluorescent intensity of a population of stained non-cellular objects, on the other hand, need not necessarily be brighter than Rh+ erythrocytes. Thus we have no means to distinguish a population of Rh+ erythrocytes from a population of equally bright fluorescent debris unless, using the capability of our machine to separate objects of a selected fluorescent-intensity range, we actually inspect the deflected stream. When a smear of the deflected stream from one of the in vitro mixtures run was examined, we did not observe any brightly

fluorescent objects which were not cells.

2)Faulty background noise subtraction. The decision as to which segments of the total fluorescent distribution to select to represent the two noise components is somewhat arbitrary and can affect the calculated number of fetal cells counted. In addition, our noise reconstruction certainly is only an approximation and probably not all the noise is subtracted from the total. To estimate the degree of error induced by this noise blanking system, we performed our usual computer analysis on the control curve in Fig. 4. Two hundred eighty-seven(287) counts remained as signal when we integrated the total-minus-noise difference spectrum. This leads to a 5% overestimate when we are counting numbers of fetal cells close to our sensitivity limits. But certainly, even with this overestimate, we still do much better than the 140% error with manual techniques.

3)Maternal blood group chimerism. Since we did not obtain samples of blood from the mothers in our post-partum group at a later date, when presumably all the fetal cells would have disappeared from the

maternal circulation, we cannot be sure whether we were unknowingly counting maternal cells. But the only way the Rh positive cells could be of non-fetal origin (barring erroneous transfusions) is if all our mothers were blood group chimeras. Of the twelve reported cases of blood group chimerism(23,24) none were Rh positive -- Rh negative. The likelihood that all our post-partum bleeds were from chimeric mothers seems to us very small. Certainly more plausible is the assumption that the Rh positive cells are of fetal origin and entered the maternal circulation because of a transplacental leak.

With regard to the broader aim of this project, ante-natal diagnosis, presently our system is applicable only in cases where there is the correct Rh type difference, and, of course, only fetal red cells can be separated. We hope that in the future we can improve our techniques so that both fetal erythrocytes and fetal lymphocytes can be separated and subjected to biochemical and cytogenetic analysis. More universal fetal markers might include the blood group antigen i(25) for erythrocytes and tumor-associated fetal antigens or paternal HL-A antigens for lymphocytes. Some estimate of the utility of our system

can be appreciated in light of a recent report describing the possibility of intrauterine diagnosis of sickle cell anemia by detecting the types of globulin chains synthesized by first trimester fetuses(26). These authors estimate that 10ul of fetal blood would be sufficient to make an accurate diagnosis. Although the average magnitude of most transplacental bleeds during the first trimester is less than 100ul(10,17), ante-natal diagnosis for sickle cell disease could, under favorable conditions, be accomplished with approximately 500ml of maternal blood. At present our separation efficiency is not 100%, but yet it does not seem impossible that we might be able to obtain enough fetal erythrocytes to permit a diagnosis.

The prospect for ante-natal diagnosis using fetal lymphyocytes would be slight if these lymphocytes entered maternal blood only via frank transplacental hemorrhage. But there is some evidence to suggest that fetal lymphocytes may in fact be present in even higher numbers than fetal erythrocytes in the maternal circulation(6). If this were true then the feasibility of our system for ante-natal diagnosis using lymphocytes is even greater than for fetal erythrocytes.

In conclusion, quantitation and purification are major

goals --- and problems --- in many biological investigations. We feel that our electronic cell sorter is a potentially highly useful tool in counting and separating minor cell populations of any sort. Of particular clinical value is its potential ability to procure fetal cells for ante-natal diagnosis without the practitioner's having to perform any procedure more hazardous than simple maternal venipuncture. With this report of the use of our machine to detect and count Rh positive fetal erythrocytes in the maternal circulation during the immediate post-partum period, we present the first step in the application of our cell sorter to ante-natal diagnosis.

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DELEUKOCYTATION PROCEDURE

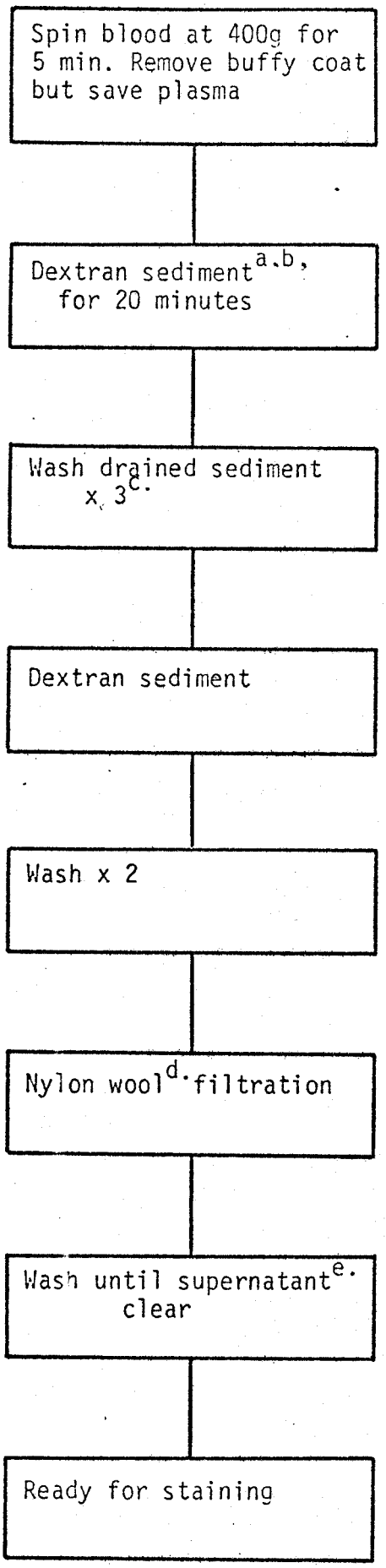


Fig. 1

Legend for Figure 1.

a. Blood and Dextran pre-heated to 37°C before mixture. Sedimentation itself done at room temperature.
b. Parenteral 6% Dextran 70 was added to the erythrocyte suspension to achieve a final Dextran concentration of 1.6%.
c. Buffy coat, if present, was always aspirated. After the final wash the cell pellet was brought up to the initial blood volume for the next step.
d. 0.1 gm of nylon wool removed from Fenwal Leuko-Pak Leukocyte Filter were used per ml whole blood. The pre-wetted nylon wool was initially packed tightly into 3 ml disposable syringe with the syringe plunger. For actual filtration the plunger was removed and the flow rate was adjusted to ~ 20 drops/min.
e. Some hemolysis was observed at times at the end of the procedure.

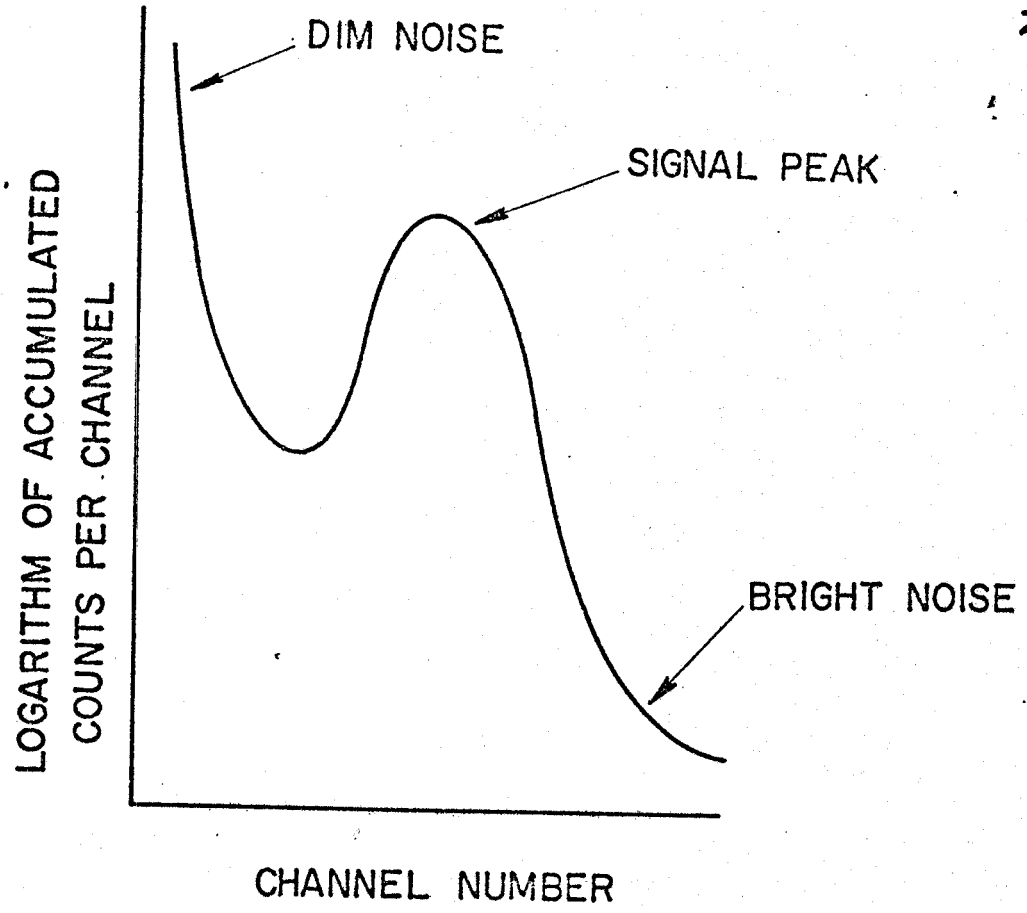

DIM NOISE
SIGNAL PEAK
BRIGHT NOISE
LOGARITHM OF ACCUMULATED COUNTS PER CHANNEL
CHANNEL NUMBER

Fig. 2a

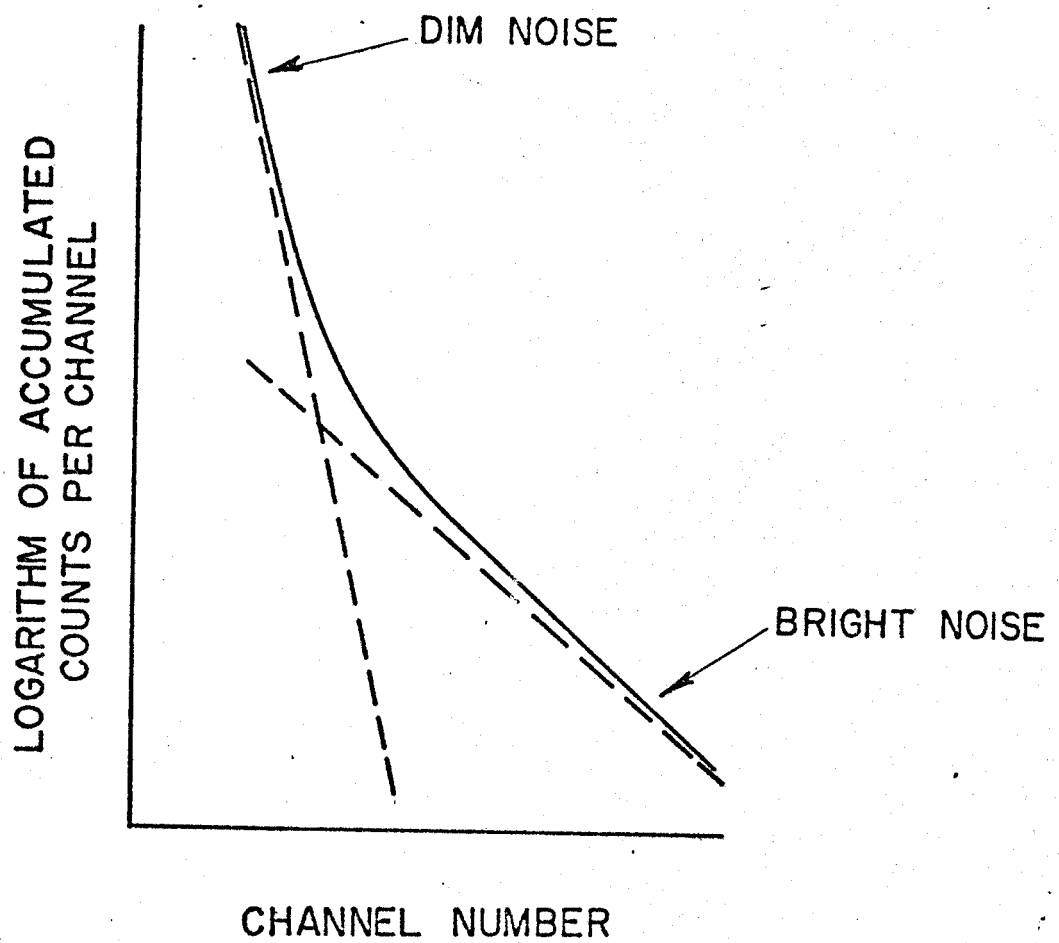
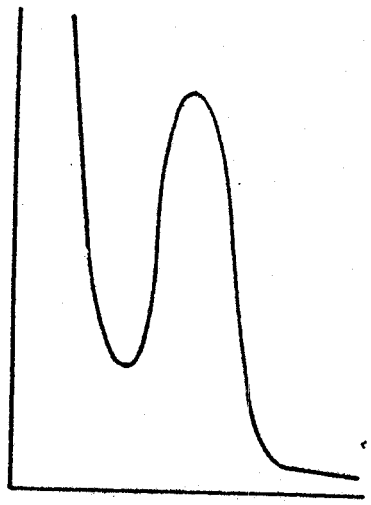

DIM NOISE
BRIGHT NOISE
LOGARITHM OF ACCUMULATED COUNTS PER CHANNEL
CHANNEL NUMBER

Fig. 2b

Legend for Figure 2a and 2b

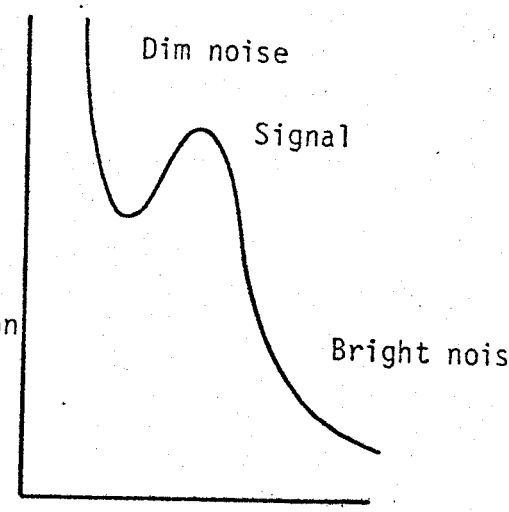
Figure 2a. Fluorescent-intensity distribution for stained artificial mixture of Rh+ cord blood cells and Rh- adult blood cells. (Ratio = 1:5,000). Channel number is proportional to fluorescent intensity.

Figure 2b. Fluorescent-intensity distribution for stained Rh- adult blood cells only.

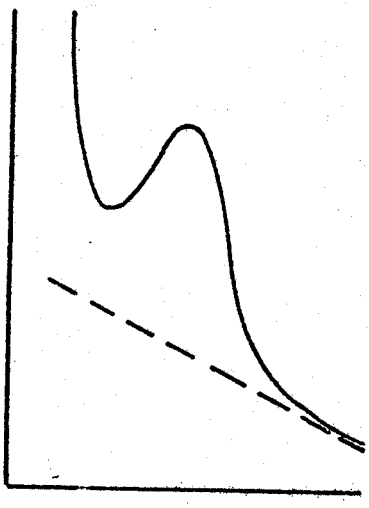


Linear input distribution

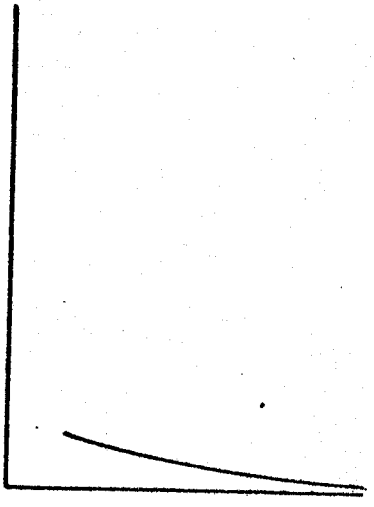
1) Take the logarithm of the linear distribution



2) Fit a least squares line onto a suitable segment of the bright noise component



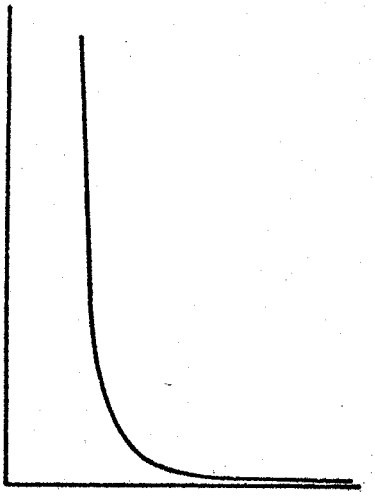
3) Take the antilogarithm of this bright noise line



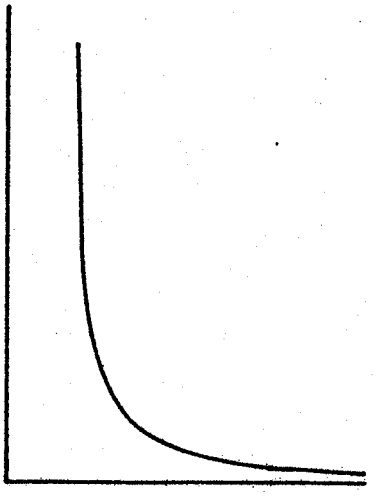
4) Subtract this line from the initial linear distribution

5) Take the logarithm of this difference spectrum

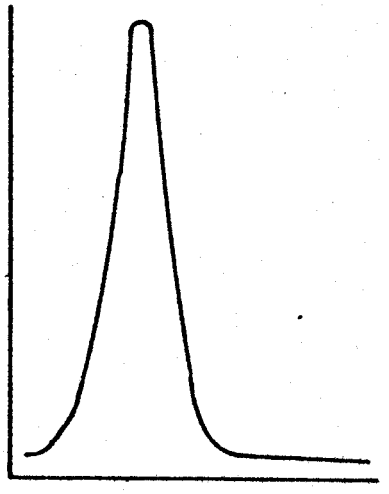
6) Fit a least squares line onto a suitable segment of the dim noise component



7) Take the antilogarithm of this dim noise line



8) Add the two derived noise components



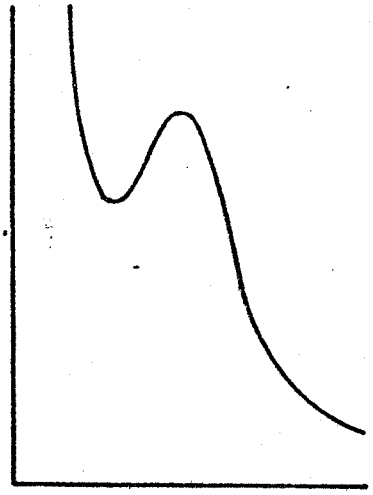
9) Subtract this curve from the initial distribution

10) Integrate under the derived

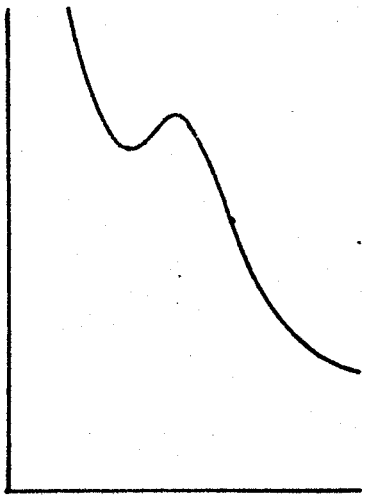
Legend for Figure 3.

Method of subtracting background noise from total fluorescent-intensity distribution.
An artifical mixture of Rh+ cells and Rh- cells (ratio = 1:10,000) is used as an example.

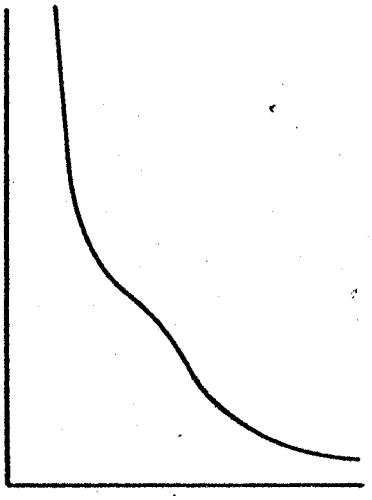
Abscissa represents fluorescent intensity.
Ordinate represents linear or logarithmic accumulated counts per fluorescent-intensity channel.



1:10,000
(Rh+/Rh- ratio)



1:50,000



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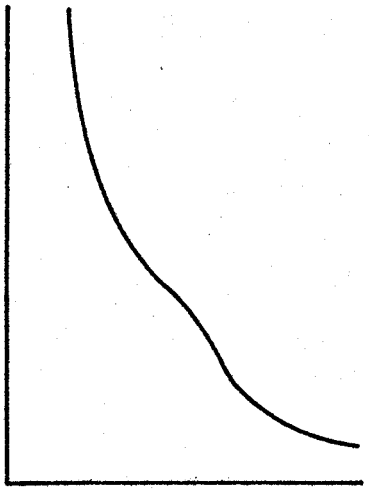

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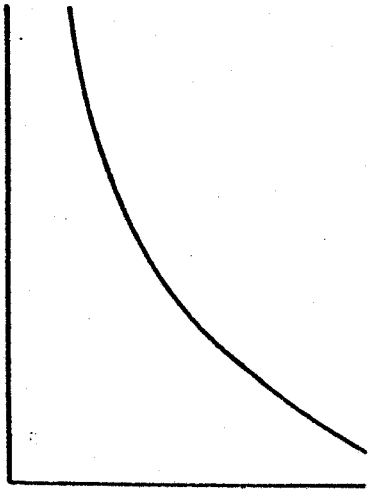

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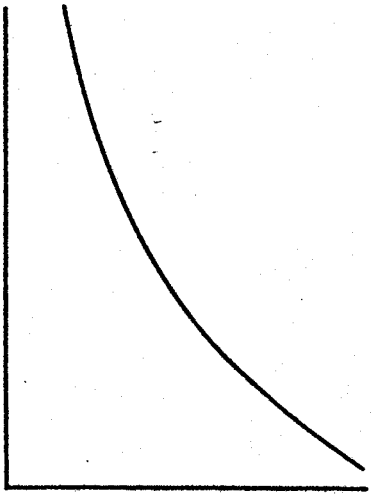

Rh neg alone

Legend for Figure 4.

Fluorescent-intensity distribution for stained in vitro mixtures of Rh+ and Rh- erythrocytes. Abscissa represents fluorescent intensity;ordinate represents logarithm of accumulated counts per fluorescent intensity channel.

Table I. Number of Counts Detected vs. Expected in *In Vitro* Mixtures of Rh+ and Rh- Erythrocytes

Rh+/Rh- Ratio	Expected counts x 10^{-3}	Observed Counts x 10^{-3}	Fetal Blood Spillage
1:10,000	43.0	44.4[a]	0.36 ml[b]
	43.0	42.1	
	101	107	
1:50,000	8.45	9.62	0.07 ml
	14.9	14.6	
	15.8	19.4	
	21.5	19.9	
1:100,000	5.3	5.6	0.04 ml
	7.4	6.0	
	7.9	6.3	
	9.9	8.9	
1:500,000	1.5	1.2	
	1.6	None[c]	
Rh Neg. only	0	0.3	

a. Each entry is a new sample and not a repeated run on the sorter.

b. Fetal blood spillage is computed assuming 4700 ml maternal blood volume, 4×10^6 maternal red cells/mm^3 and 5.2×10^6 total red cells/mm^3.

c. Beginning at this ratio positively stained cells could no longer uniformly be distinguished over the background noise.

Table II. Rh Positive to Rh Negative (Fetal to Maternal) Erythrocyte Ratios in Samples of Post-Partum Maternal Blood

Mother	Rh+/Rh- Ratio	Equivalent transplacental leakage*
SC	1:41,100	90 ul
LG	1:43,600	80 "
DL	1:12,100	300 "
BW	1:14,600	250 "
BJ	1:105,000	30 "
TO	1:5,940	600 "
"	1:5,310	680 "
"	1:4,910	740 "
EL	1:75,200	50 "
JP	1:163,000	20 "
JM	1:11,900	300 "

* Transplacental leakage computed assuming 4700 ml maternal blood volume, 4×10^6 maternal red cells/mm^3 and 5.2×10^6 fetal red cells/mm^3.