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Chapter 10

Fetal Cells from Maternal Blood: Their Selection and Prospects for Use in Prenatal Diagnosis[1]

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[1]This work was supported in part by grants from the National Institutes of Health (GM-17367 and HD-13025).

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METHODS IN CELL BIOLOGY, VOL. 26

ISBN 0-12-564126-5

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I. Introduction

We are attempting to improve methods for obtaining fetal cells from samples of maternal blood early in pregnancy using a fluorescence-activated cell sorter (FACS). Our goal is to use these cells for prenatal diagnosis of chromosome abnormalities and other genetic disorders.

It would be very desirable to have a minimally invasive technique for prenatal diagnosis of genetic defects that could be applied to all pregnancies. The low but significant risks of amniocentesis restrict its use to pregnancies in which the risk of fetal defects is relatively high. Prenatal diagnosis from a maternal blood sample would not only decrease the risks in cases where amniocentesis is now recommended but would also allow testing in low-risk pregnancies, which because of their greater number actually result in the birth of the majority of infants with trisomy 21 and other chromosome abnormalities (Holmes, 1978).

In addition, such a technique might allow prenatal diagnosis results to be obtained at an earlier stage in the pregnancy than is possible with amniocentesis. This could result if fetal cells are present in maternal circulation early in pregnancy or if the cells could be cultured to yield karyotypable mitoses in a few days rather than the few weeks required for culture of amniotic fluid cells.

Our approach has been to label differentially maternal and fetal cells[3] with fluorescein-tagged antibodies to cell-surface antigens such as paternal histocompatibility (HLA) antigens. Cells with appropriate labeling are isolated from the bulk of the maternal cells using the FACS. Our verification procedure is to stain the selected cells with quinacrine mustard (QM) and examine them for the presence of Y-chromatin spots (Y-bodies), indicative of their origin from a male fetus. We hope to be able to culture the still-viable sorted cells to obtain fetal karyotype information and possibly use them to test for biochemical defects.

II. Previous Studies—Detection of Fetal Cells without Enrichment

There have been a number of reports of the detection of (male) fetal cells in maternal blood during pregnancy. The results were based on identification of XY mitoses following mitogen stimulation of cells from maternal blood (Walknowska *et al.*, 1969; de Grouchy and Trebuchet, 1971; Schindler *et al.*,

[3]In this article the term fetal cells refers to the fetal cells found in maternal blood unless otherwise specified. Abbreviations used include FACS, for fluorescence-activated cell sorter and QM for quinacrine mustard.

fetal cells were still a small minority. We are approaching this problem from several directions, including the use of monoclonal antibodies and improved staining procedures for paternal HLA, development of monoclonal antibodies to human trophoblast antigens, and use of double-staining techniques.

Besides improving the selection of fetal cells from maternal blood, this work will help to characterize such cells so that appropriate culture conditions can be chosen and information suitable for prenatal diagnosis obtained. We hope such techniques will be developed into a form suitable for prenatal diagnosis on a routine clinical basis.

In addition, FACS-based techniques are proving to be useful in investigations of fetal–maternal transfer of erythrocytes.

ACKNOWLEDGMENTS

In addition to the authors of Herzenberg *et al.* (1979) and Lipinski *et al.* (1981), work on the project as a whole has included Dr. Paul Hensleigh, Dr. Roman Malvehy, Ms. Jenny Scott, Ms. Virginia Bryan, Mrs. Jennifer Royce, Dr. Carol Greene, and Mr. Eugene Filson.

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1972) or on identification of Y-chromatin-positive interphase cells with or without mitogen stimulation (Schröder and de la Chapelle, 1972; Grosset *et al.*, 1974; Schröder *et al.*, 1974; Siebers *et al.*, 1975; Zilliacus *et al.*, 1975). The identification of rare XY mitoses by the conventional techniques used in the earlier studies (Walknowska *et al.*, 1969; de Grouchy and Trebuchet, 1971; Schindler *et al.*, 1972) is open to question (Schröder, 1975), and a later attempt to find such mitoses using quinacrine staining yielded zero XY mitoses out of 112,000 mitoses studied (Zilliacus *et al.*, 1975).

The studies on interphase cells used quinacrine or quinacrine mustard staining to reveal Y-chromatin. In each study Y-chromatin-positive cells were found primarily but not exclusively in blood samples from women who subsequently delivered boys (Schröder and de la Chapelle, 1972; Grosset *et al.*, 1974; Schröder *et al.*, 1974; Siebers *et al.*, 1975; Zilliacus *et al.*, 1975). The average observed frequencies of such cells range from less than 0.2% (Schröder and de la Chapelle, 1972; Schröder *et al.*, 1974; Zilliacus *et al.*, 1975) to almost 4% (Siebers *et al.*, 1975). The time of earliest appearance of Y-chromatin-positive cells is also different in different studies. The discrepancies among the studies and the sources of incorrect diagnoses have been discussed (Schröder, 1975; Schröder and Herzenberg, 1980), but none of the data would support the use of this technique for accurate fetal sex determination.

Our results are consistent with the lowest frequency estimates, because unenriched control slides usually yield no Y-chromatin-positive cells among 1000–2000 cells examined.

The tissue of origin of the fetal cells found in maternal blood during pregnancy is unknown. Those identified by quinacrine or quinacrine mustard staining usually show a morphology like lymphocytes, often with larger nuclei than the smallest lymphocytes. However, they seem to be unresponsive to phytohemagglutinin (Zilliacus *et al.*, 1975) and may in fact be precursor cells to erythrocytes or lymphocytes, and so on; or they may be of placental, perhaps trophoblast, origin.

III. Fetal Cell Project Design and Published Results

Our research program has been designed to (1) verify the presence of nucleated fetal cells in maternal blood, (2) obtain cell fractions enriched with such fetal cells in selected maternal–fetal pairs, (3) generalize the enrichment techniques to be applicable to all pregnancies, (4) improve the purity of the fetal cell-enriched fractions, and (5) find culture conditions and stimulants that will induce the fetal cells to enter mitosis so that fetal karyotypes can be analyzed. Each aspect of the work both draws on and contributes to our understanding of

TABLE III

TRANSFER OF LABELED FETAL ERYTHROCYTES TO MATERNAL CIRCULATION

Experiment no.	Time after return of labeled cells		
	0–3 hours	24 hours	3–4 days
1	0/6[a]	5/134	
2	0/75	1–4[b]/75	32/75
3	0/100	3/100	4/100
4	0/200	0/200	1/200

[a] Number of labeled cells observed/millions of maternal cells examined.

[b] Due to the low brightness of labeling in this experiment, three fluorescent cells were found whose fluorescence could not be confirmed as being the same color as that of the fluorescein label.

samples on the other. The maternal samples were run at about 30,000 cells per second (10^8 cells per hour), with the analysis circuitry threshold set to process only the few hundred most fluorescent objects per second. Those objects with fluorescence and light-scatter characteristics comparable to the labeled fetal cells were sorted into small wells filled with medium mounted on microscope coverglasses. After gentle centrifugation the sorted cells were examined with an inverted fluorescence microscope to identify erythrocytes with the uniform green fluorescence characteristic of the FITC-labeled cells.

The results of four experiments are summarized in Table III. Maternal blood samples were drawn (1) before or shortly after the return of the labeled fetal cells, (2) at about 24 hours, and (3) at 3 to 4 days. Labeled fetal cells were found in very small numbers in most of the maternal samples taken 1 to 4 days after the return of labeled cells to the fetus. Correction for cell losses in the sorting and confirmation process might double the observed frequencies.

Although the numbers of labeled fetal cells observed have been small, they indicate the magnitude of the blood transfer from the fetal to maternal circulation. Calculating back from the above data and the approximate 5000-ml maternal blood volume gives transfer rates in the range from 0 to 2 μl/day. The low but usually detectable rate of transfer implies that the placental barrier is only slightly compromised by direct blood flow.

VIII. Summary

We have demonstrated that fetal cells can be selected from maternal blood using appropriate reagents and the FACS. However, in the nucleated cell work the original techniques lacked generality and yielded sorted fractions in which the

the characteristics and possible origins of the fetal cells. This characterization is particularly necessary in finding appropriate culture conditions, but it is important for all aspects of the work.

The first two objectives have been achieved through fetal cell labeling for paternally derived HLA-A2 antigen, FACS sorting of the labeled cells, and identification of the fetal cells by examination for QM-stained Y-chromatin (Herzenberg *et al.*, 1979; Iverson *et al.*, 1981). Table I shows the data on Y-body observations in relation to typing of the informative male babies with the anti-HLA-A2 serum. The impressive correlation clearly demonstrates that Y-chromatin-positive cells of fetal origin were present in the maternal blood and that they were selectively enriched by sorting for the HLA-A2 marker. In five of the eight cases with HLA-A2-positive male infants, two or three maternal blood samples were tested beginning at 15 weeks gestation. Y-chromatin-positive cells were found in each sample. Finding such cells in all samples where they would have been expected *a posteriori* is the most convincing evidence that they are usually present as early as 15 weeks and that they continue to be present through midpregnancy. The data do not allow a firm estimate of the fraction of fetal cells in maternal blood, but the assumption that the fetal cells stain for paternal HLA like cord blood lymphocytes do and that they show a comparable frequency of visible Y-chromatin leads to estimates of 1/800 to 1/60,000 with a mode between 1/1000 and 1/5000 (Iverson *et al.*, 1981).

The one case in which Y-chromatin-positive cells were observed but the baby's cells were not stained by the antiserum may have been due to an unusually high number of fetal cells in the mother's blood, allowing them to be detected without enrichment. Among the 19 cases in which sorting of fetal cells was attempted and the baby turned out to be a girl, Y-chromatin-positive cells were

TABLE I

ENRICHMENT OF Y-CHROMATIN-POSITIVE CELLS IN RELATION TO THE HLA TYPE OF MALE BABIES[a]

Y-Chromatin-positive cells found in samples sorted from maternal blood	Cord blood typing with sorting serum (no. of cases)	
	HLA-A2$^+$	HLA-A2$^-$
Yes	8	1
No	0	17

[a] The probability of obtaining such results by random association is 5.8×10^{-6} (Fisher Exact Test).

give appropriate light-scatter and fluorescence signals into a small medium-filled well mounted on a microscope coverglass. Gentle centrifugation to bring the cells to the coverglass and examination with an inverted fluorescence microscope make it possible to distinguish between properly labeled erythrocytes and artifacts, because the vast majority of artifacts are not erythrocytes at all and/or do not show the correct staining pattern on the cell.

In the initial survey, samples from Rh D^- women were drawn near term and examined for the presence of Rh D^+ red cells. They were found in all 11 patients who delivered Rh D^+ babies; the frequencies ranged from 1:4000 to 1:80,000 with an average of about 1:25,000 when the baby was ABO compatible with the mother (10 cases). The average frequency corresponds to a transfer of about 200 μl of fetal blood to the mother's circulation (taking the average maternal blood volume to be about 5 liters).

The sensitivity and accuracy of this technique will make it possible to monitor for effects of clinical procedures such as amniocentesis and to follow the decline of fetal erythrocyte frequency after delivery.

B. Rhesus Monkey Model

We have carried out several experiments to measure the amount and kinetics of the transfer of fetal erythrocytes into maternal circulation in a monkey model. The work has been carried out in collaboration with Drs. Arthur Malley and Miles J. Novy of the Oregon Regional Primate Center, Beaverton, Oregon. The surgical preparations were initiated for experimental work requiring measurement of hormone levels in maternal and fetal blood. The experiments were performed on rhesus monkeys (*Macaca mulatta*) pregnant 120–135 days (term is 167 days). Our work was started only after the animals were stabilized and we could be confident that no fetal–maternal blood contact was resulting from the surgical procedure.

After the surgery to chronically catheterize the maternal and fetal circulations, a fetal blood sample amounting to about 3% of the blood supply was drawn. The cells were labeled with fluorescein isothiocyanate (FITC) and returned to the fetus. Small samples of fetal blood were withdrawn periodically to measure the fraction of labeled cells and to confirm that it was relatively constant. We expected that fetal cells entering the maternal circulation would not be cleared rapidly because labeled fetal cells injected into unrelated adults circulated with little loss for at least 3 days. Samples of maternal blood were drawn periodically and analyzed for the presence of labeled fetal cells.

Before running maternal samples on the FACS we measured the fluorescence of the labeled cells in the fetal samples in order to select the correct brightness range for sorting from the maternal samples. Having access to two FACS machines, we avoided any risk of contamination of the maternal samples with leftover fetal cells by running the fetal samples on one machine and the maternal

found in only one sample. Later samples from the same woman did not yield Y-chromatin-positive cells. The unusual result remains unexplained and was probably due to some technical error.

The procedures used in obtaining the data just summarized have been published (Herzenberg *et al.*, 1979; Iverson *et al.*, 1981), so they will be explained only briefly here. Mononuclear cell suspensions were prepared from maternal blood samples drawn between the fifteenth and thirtieth week of pregnancy. The cells were stained with a rabbit antiserum to HLA-A2 kindly provided by Dr. J Strominger, Harvard University (Robb *et al.*, 1975), followed by a second step of goat anti-rabbit immunoglobulin. Both antisera were absorbed to obtain specific staining. If the bulk of the maternal cells were not stained by this procedure, the brightest cells having light-scatter properties like small lymphocytes were sorted with the FACS (usually the brightest 0.1 to 1% were selected). The sorted cell samples were QM-stained to reveal Y-chromatin. The reading for Y-chromatin-positive cells was carried out blindly in that neither the fetal sex nor HLA reactivity was known. When the babies were born, their sex was noted and their cord blood mononuclear cells were tested for reactivity with the selecting antiserum.

The ability of the FACS to examine several thousand cells per second and to isolate cell fractions as pure as the specificity of the fluorescent-staining reagent allows has made it possible to obtain fetal cell-enriched fractions from maternal blood. However, the low frequency of fetal cells and the great variety of cell types in maternal blood make it difficult to achieve stains that mark only the fetal cells. In the cases just quoted the Y-chromatin-positive cells averaged less than 0.4% of the total in the appropriate enriched samples. Thus even enriched fractions contained predominantly maternal cells, and the QM Y-chromatin technique was required to confirm the enrichment of fetal cells.

When sorts for fetal cells were divided into several subsamples with different fluorescence ranges, Y-chromatin-positive cells were often found in the lower fluorescence range just above unstained maternal cells and well below most positive control staining of adult lymphocytes. Thus the fetal cells seem to stain less brightly for paternal HLA than ordinary adult lymphocytes do. This may be part of the reason for the limited enrichments observed.

In the next sections we will discuss (1) the basic operation and capabilities of the FACS and modifications that have been made to facilitate fetal cell sorting; (2) the QM Y-chromatin technique for identification of male cells; and (3) work in progress toward improving fetal cell selection, including the use of monoclonal antibodies to HLA polymorphisms, monoclonal antibodies to trophoblast cells, and use of double-labeling techniques. Following that we will describe FACS-based investigations of fetal–maternal transfer of erythrocytes in humans and in a rhesus monkey model.

devise efficient two-reagent selection procedures to give higher purity fetal cell-enriched fractions.

Double-staining experiments are possible with our present FACS system in which both fluorescein and tetramethylrhodamine dyes can be excited by mixed laser light at 488 nm and 514.5 nm. The two fluorochromes are measured separately by a combination of optical filtering and electronic manipulation of the signals from two detectors (Loken *et al.*, 1977). However, the compromises and signal losses required for such measurements are a problem. This is especially true when monoclonal antibody reagents are used, because their monospecific binding tends to give clean but not bright staining.

To avoid such difficulties we are adding a tunable dye laser (Arndt-Jovin *et al.*, 1980) to our FACS system. It will be used together with the usual argon-ion laser to excite two dyes separately and optimally, thus allowing better measurements on doubly stained cells. With this we expect to retain the normal enrichment efficiency of the regular selective stain while using the second independent stain for defining subfractions.

VII. Fetal Red Blood Cell Transfer

A. Human Rh D-Antigen Labeling

We have been associated with Drs. Paul Hensleigh and Arnold Medearis, Department of Gynecology/Obstetrics, Stanford University, in a project assessing the frequency and amount of fetal–maternal hemorrhage during pregnancy and investigating the effect of procedures such as amniocentesis (Medearis *et al.*, 1980). The concern arises from the risk of maternal or fetal isoimmunization, particularly for Rh D, through such hemorrhage. The experimental objective is to do accurate quantitation of transferred cells when they occur at frequencies as low as 1:100,000 by differentially labeling maternal and fetal cells for erythrocyte-expressed polymorphisms and using the FACS to analyze the staining and to sort rare labeled cells (Jan and Herzenberg, 1973).

To date the work has focused on the Rh D (rhesus) antigen. The procedure is to stain erythrocytes from an Rh D^- woman with a commercial high-titer human anti-D serum followed by a fluoresceinated goat anti-human immunoglobulin second step. The sample is run on the FACS and examined for the presence of stained erythrocytes. The second-step antiserum will label B-lymphocytes and monocytes in the sample, but they can be excluded from the analysis because they give larger light-scatter signals than erythrocytes.

Under optimal staining conditions a population of only a few in 100,000 can be seen and counted as a definable stained population after several million cells have been analyzed (Jan and Herzenberg, 1973). The count of labeled erythrocytes can be verified and extended to lower frequencies by sorting the cells that

IV. Basic Flow Cytometry

Flow-cytometry systems have been described in a number of places (Hulett *et al.*, 1973; Herzenberg *et al.*, 1976; Herzenberg and Herzenberg, 1978), so we will present a brief overview, outline the capabilities and limitations of such systems, and then discuss the specific modifications we have made to facilitate selection of fetal cells.

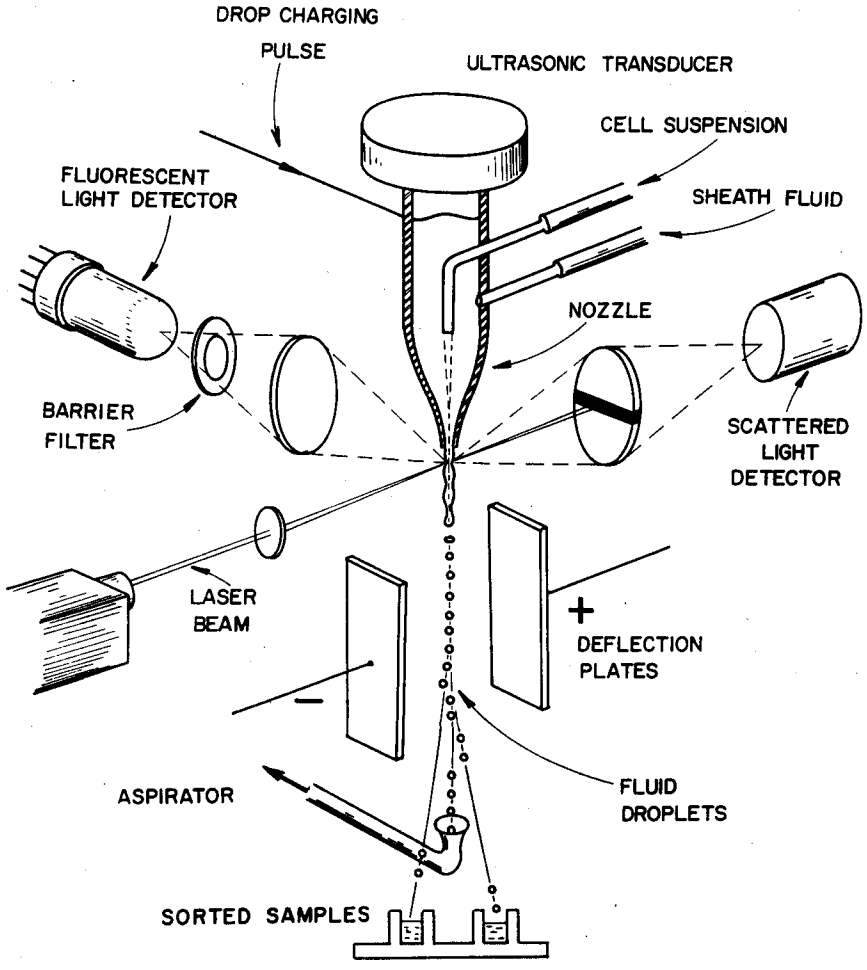

FIG. 1. Schematic illustration of the central components of a flow analysis and sorting system. The functions of the various components are described in the text.

FACS. In this application the FACS deposits single viable cells (with or without selection for fluorescent markers) in the wells of microculture plates containing "feeder" cells. This is a rapid and efficient cloning method (Parks *et al.*, 1979). This work has now yielded two trophoblast-specific monoclonal antibodies (Lipinski *et al.*, 1981).

We have begun to test several of the antibodies as immunofluorescent-staining reagents on samples of blood mononuclear cells from pregnant women. Sorting labeled cells and examining the sorted fraction for enrichment of Y-body-positive cells will show whether these antibodies selectively mark the fetal cells. Although significant results are not as yet available, these trophoblast-specific monoclonal antibodies will be useful in establishing whether fetal cells in maternal blood include cells from this tissue and should allow their routine sorting if they are there.

The other obvious possibility for obtaining fetal cell-specific antibodies is to immunize mice with human fetal blood cells and attempt to obtain monoclonal antibodies that react with fetal but not adult blood cells. Specific fetal leukocyte-differentiation antigens have not yet been described in humans, but it is worth a serious attempt with monoclonal antibody techniques. Yet another possibility for general fetal cell selection with male fetuses would be a good anti-H-Y-staining antibody. Although monoclonal antibodies to H-Y do exist (Koo and Hammerling, personal communication), so far they do not seem to be sufficiently selective of male cells for our purposes.

C. Better Fetal Cell Characterization

Increasing our knowledge of the characteristics of the fetal cells is difficult because the processing for QM Y-body analysis results in identified fetal cells that are not only dead but are even unsuitable for ordinary morphological observation. More information about their surface markers is needed for designing efficient selection procedures, and further, successful culturing to obtain karyotypes will be helped by knowledge of the exact origin of the fetal cells.

To investigate the surface antigens of the fetal cells while continuing to rely on the Y-body-identification technique, we plan to carry out FACS selection based on pairs of different fluorescent stains. One will be a proven selective stain such as antibody to paternal HLA, and the other will mark an antigen whose presence on the fetal cells we want to assess. Using different fluorescent dyes for the two stains a fetal cell-enriched fraction defined with reference to paternal HLA staining can be subdivided on the basis of staining for the second antigen. The staining pattern of the fetal cells for the second marker is then indicated by which sorted subfraction(s) yield Y-chromatin-positive cells on subsequent examination. Comparison of these results with analyses of fetal tissues such as blood and placenta stained with the same characterizing antibodies will help to identify the origin of the fetal cells. The fetal cell-characterization data will also be used to

A. Equipment and Operation

Figure 1 is a sketch of a flow analysis and sorting system. The focal point of the system is the intersection of a laser beam with a liquid jet from a small nozzle. This jet consists mostly of cell-free sheath fluid and has a diameter of 50 to 100 μm. The suspension of fluorescent-labeled cells is injected at the center of the nozzle and forms a small central core in the jet. As a cell passes through the laser beam, fluorescent molecules it carries are excited and emit fluorescent light, which is collected by a lens, filtered to block out scattered laser light, and converted to an electrical signal by a photomultiplier detector. In addition, laser light scattered by the cell in the forward direction is measured by another detector. This gives information on cell size and for some cell types allows discrimination of live and dead cells. Electronic signals proportional to the amount of light scatter and fluorescence from each cell are amplified in linear or logarithmic fashion, processed, and digitized for computer analysis or pulse height analyzer display. We have recently built high-speed logarithmic amplifiers that work over a signal ratio range of about 10,000 to 1. They are used for almost all fluorescence work because the range is enough to cover all usual fluorescence signals, making amplifier gain changes unnecessary. In addition, we have found that the logarithmic presentation makes the relevant features of immunofluorescent distributions more visible and easier to interpret than linear presentation does.

For cell sorting the nozzle is vibrated at a frequency of 20 to 40 kHz by a piezoelectric transducer. This results in breakup of the jet into extremely uniform droplets. When a cell is detected that fulfills present conditions in scatter and fluorescence, droplets containing that cell are electrically charged. The drops fall through an electric field, produced by two statically charged plates, which draws charged droplets out of the stream of uncharged ones so that they can be collected as sorted samples.

B. FACS Capabilities and Limitations

The capabilities and limitations of flow-cytometric measurements and sorting with FACS-type instrumentation are outlined in the following points:

1. Flow-cytometric techniques can be applied to any type of cell that can be obtained in single-cell suspension. For good measurements and sorting the nozzle orifice diameter should be at lease five times the cell diameter.
2. The fluorescent label may be any dye associated with the surface or interior of the cell whose fluorescence can be excited by the laser light. The commonly used argon-ion laser has a number of available emissions from 350 nm in the near UV to 514 nm in the green. For longer wavelengths a krypton-ion laser operating at 568 nm or a tunable dye laser excited with an argon-ion laser (with emission range determined by the dye used) may be suitable.

TABLE II

RESULTS OF SORTS FROM MIXTURES OF HLA-A2$^+$ AND HLA-A2$^-$ CELLS

	Mixture HLA-A2$^+$/HLA-A2$^-$ (cells:cells)	Recovery[a]	Purity[b]	Figure of Merit[c]
Sort for bright cells	1:2900	33%	63%	600
Sort for unstained cells	4500:1	58%	24%	625

[a] Percentage of the "rare" cells run that were recovered in the sorted fraction.

[b] Percentage of sorted cells that were of the "rare" type, i.e., labeled with Hoechst 33342.

[c] Equals the product of the enrichment factor (e.g., 2900 × 0.63) and the recovery factor (e.g., 0.33).

sorting for stained fetal cells. In fact, we have now selected Y-body-positive cells from the blood of pregnant women by sorting unstained cells when the woman was HLA-A2 positive.

So far, however, the frequency of fetal cells in enriched fractions sorted with the monoclonal reagents has been similar to that found with the rabbit anti-HLA-A2. Tests using blood samples from nonpregnant persons have shown a number of stained (or unstained) cells comparable to that found with our pregnant subjects. Sorting and FACS reanalysis of such cells has demonstrated that most of them are in fact being measured correctly by the FACS. Fluorescence microscopy on such "improperly stained" cells shows some artifacts, but the staining pattern of a large fraction of the cells looks like that of properly stained cells. The sources of this limitation on staining specificity are under investigation. It is even possible that individuals express histocompatibility antigens different from their nominal HLA types on a small fraction of cells as a result of genetic variation.

B. Antitrophoblast Monoclonal Antibodies

The ideal way to select fetal cells would be with antibody to a fetal antigen not found on any adult blood mononuclear cells. We are exploring the possibility that the fetal cells in maternal blood include trophoblast cells. Monoclonal antibodies were produced by immunizing BALB/cN mice with human choriocarcinoma cells of the BeWo line and fusing their spleen cells with the mouse myeloma line NS-1 (Lipinski *et al.*, 1981). Culture fluid from each of many subcultures was tested by radioimmunoassay for antibody reacting with BeWo cells. Those that showed such activity were tested by two-step immunofluorescent staining and FACS analysis for antibody binding to human red and white blood cells and platelets. Hybridoma cells from interesting subcultures, particularly those that had antibody activity to BeWo cells but not to blood cells, were cloned with the

3. The FACS makes a quantitative measurement of the fluorescence of each cell. Using appropriate standards, this measurement may be calibrated in terms of the actual number of dye molecules per cell.

4. The sensitivity of the measurements depends on the properties of the dye and on how well its excitation efficiency is matched to the available laser wavelengths, but for the best dyes such as fluorescein, lymphocytes with only 10,000–20,000 dye molecules can be clearly distinguished from a population of unstained cells. The limit is set by the background fluorescence of the unstained cells. The number of labeled sites (such as surface-antigen molecules) required to yield adequate staining may be quite small if dye labeling is amplified by coupling the labeling reagent to fluorescent microspheres or a multiple dye molecule tail.

5. Any characteristics that allow cells to be discriminated on the basis of light scattering and/or fluorescence can be used for sorting.

6. Sorting is normally carried out at a total cell-flow rate of 2000–3000 cells per second (7–10 million cells per hour). Losses due to the appearance of cells too close together to be separated in the deflected drops increase in relative frequency as the cell-flow rate is increased.

7. The ability of FACS systems to select rare cells is limited only by the specificity of the labeling technique in distinguishing wanted from unwanted cells and by the time required to run enough cells to find the required number of the rare type.

8. The viability and functional capacities of most types of cells are not affected by passage through the FACS.

9. Sterile sorting can be carried out routinely so that sorted cell fractions are suitable for either short- or long-term tissue culture.

C. Sorter Modifications and Developments for Fetal Cell Selection

Sorts for fetal cells typically select 0.1–1% of the cells run. This presents significant problems in recovering and handling the small number of cells. Sorted cells are collected in small wells containing culture medium mounted on microscope slides. The wells are produced by attaching short lengths of 5-mm internal diameter glass tubing to the slides with wax. In sorting, the stream of undeflected droplets is intercepted and aspirated away at a point above the collecting wells (see Fig. 1).

To ensure that the trajectories of the deflected drops remain constant it is necessary to monitor the deflections during the sorting. However, observation of the deflected drops is difficult when only a few cells are being sorted per second, so we devised a variable-delay strobe-light system triggered by the drop charging

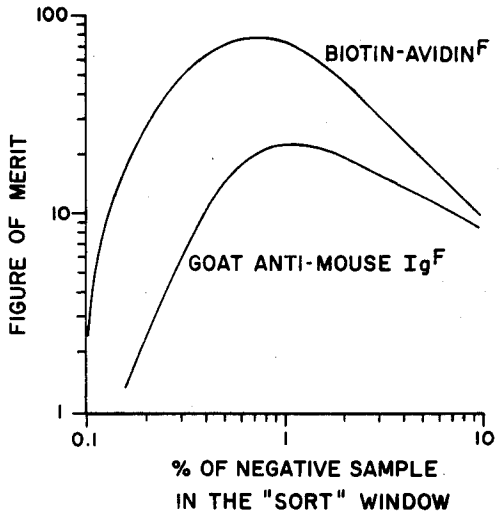

FIG. 2. Comparison of the quality of two-step stains for fetal cell enrichment. Adult blood mononuclear cell populations positive and negative for HLA-A2 were stained by different two-step procedures, using the monoclonal anti-HLA-A2 first step. Each cell sample was analyzed on the FACS to yield a computerized data set of cell frequencies versus cell fluorescence. For various simulated sorting ranges in fluorescence, the fraction of the HLA-A2$^-$ cell population (fNeg) and the fraction of the HLA-A2$^+$ population (fPos) that would have been sorted were calculated. The Figure of Merit for the expected quality of a sorting of rare HLA-A2$^+$ cells from a mixture of the two is as follows:

$$\text{Figure of Merit} = (\text{Estimated enrichment factor for rare cells}) \times (\text{Fraction of the rare cells in the sort window}) = (f\text{Pos})^2/f\text{Neg}$$

The figure plots the Figure of Merit as a function of fNeg.

cells with an appropriate HLA difference, and the mixture was stained with the appropriate anti-HLA reagent at the optimal concentration indicated by the previous testing. Mixtures in which the "rare" cells should be stained and those in which the "rare" cells should be unstained and the majority stained were both tested. Cells with the appropriate fluorescence for "rare" cells were sorted with the FACS and collected into small wells mounted on microscope coverglasses. An inverted fluorescence microscope with appropriate excitation and emission filters was used to count Hoechst 33342-labeled cells and other cells in each well. In the course of such experiments each prelabeled cell retains most of its Hoechst 33342, and no other cell absorbs enough of what is released to cause any confusion. Data taken with biotin–avidin staining are shown in Table II.

The biotin–avidin system gave better results than other two-step stains. Such tests also indicated that sorting for unstained fetal cells should be as good as

pulse. The delay is set so that the flash occurs when the deflected drops are about to enter the collection vessel. The drops of each deflection event can be seen and sorting conditions adjusted to ensure that each deflected cell is collected.

The assurance of collecting each deflected cell has also been increased by shortening the path from the FACS nozzle to the collector and by grounding the collection vessel to prevent static charge buildup, which can cause the charged drops to be repelled out of the well.

V. Quinacrine Y-Chromatin—Background and Techniques

Either quinacrine or quinacrine mustard can be used to reveal Y-chromatin in interphase cells. The results are similar, but QM is probably better because it is used at a lower concentration for staining, it binds more strongly (even covalently) to DNA, and its fluorescence is more resistant to fading under illumination (Caspersson and Zech, 1970). They are intercalating dyes that bind relatively uniformly to DNA with little preference for particular local nucleotide base compositions (Latt *et al.*, 1974). However, the fluorescence quantum efficiency shows a strong maximum in an A-T rich environment (Latt *et al.*, 1974). The distal half of the long arm of the human Y-chromosome contains A-T-rich heterochromatin, and that area shows bright fluorescence when stained with QM. The Y-chromosome fluorescence is the brightest area in metaphase chromosome spreads from most males. Other chromosomes may show relatively bright areas particularly at the centromere (Caspersson *et al.*, 1972).

A QM bright spot (Y-body), identifiable with the relatively condensed Y-chromatin, is visible in interphase nuclei of many male cells (Pearson *et al.*, 1970; Mukjerjee *et al.*, 1972). The fraction of cells exhibiting a Y-body varies from one cell type to another and from person to person. Fibroblast cells have been reported to show a Y-body frequency of 71–81% (Mukjerjee *et al.*, 1972), whereas blood lymphocytes are commonly in the 35–66% range (Schröder and de la Chapelle, 1972). In lymphocytes the Y-body appears as a small bright spot often near the margin of the nucleus. The treatment used for QM staining makes it difficult to distinguish different cell types, but in our experience the Y-body-positive cells found in maternal blood generally have round nuclei and look more or less like small or medium lymphocytes.

Use of the Y-body technique for identification of male cells is limited by the fact that not all male cells show a Y-body and by the appearance of fluorescent spots in cells for reasons other than the presence of Y-chromatin. In particular, a small fraction of interphase cells from people who have bright fluorescent spots on one or more autosomes may show a spot bright enough to be taken for a Y-body. Staining artifacts also occur that may occasionally be difficult to distin-

about 28% of pregnancies.[4] For HLA-B7 with a gene frequency of about 8% the sortable fraction is 14%. A battery of perhaps 5 or 10 of such reagents would make it possible to select for fetal cells in most (but not all) pregnancies.

We have compared several two-step staining procedures with these reagents, including the use of fluorescein-conjugated whole goat anti-mouse-IgG1 and F(ab')2 of this as second steps. We have also used biotin-conjugated forms of the monoclonal anti-HLAs with fluorescein-conjugated avidin as a second step (Bayer and Wilchek, 1978). For our purposes it is not staining brightness or staining specificity per se that is important, but rather the ability to discriminate a few (fetal) cells of one type from a large heterogeneous population of another. To test the staining reagents, each was titrated for staining of cells of the appropriate type and of cells that should be negative. Fluorescence histograms for each sample were collected on the FACS and analyzed by positive–negative pairs with the same reagents and concentrations.

To optimize the titrations and compare one reagent to another, we used computer analysis of the histograms to ask, "If a few of this kind of cell had been mixed with a large number of that kind of cell and the mixture sorted for cells in a particular fluorescence range, what fraction of the few cells would we expect to recover, and what enrichment of these cells would we expect in the sorted fraction compared to the original mixture?" A single optimizing measure or Figure of Merit was obtained by weighting the factors of fractional recovery and enrichment equally. Sample results are shown in Fig. 2 for a biotin–avidin stain and a goat anti-mouse immunoglobulin second-step stain. The optimum in each case occurs with the "sort" fraction, including a little less than 1% of the nondesired cells, but the Figure of Merit is four times higher for the biotin–avidin stain. The set of such analyses showed that the biotin–avidin system was superior to the anti-mouse Ig second steps tested, even though its staining brightness was not as great as with some of the second-step antisera. In these tests the anti-HLA-A2 performed better than the anti-HLA-B7 because background staining levels were comparable, whereas the anti-HLA-A2 staining brightness was more than twice that of the anti-HLA-B7. This may reflect a real difference in average antigen density or may be due to differences in the monoclonal antibody affinities.

The reagent analysis was extended by sorting from actual mixtures of adult blood mononuclear cells at ratios of one to several thousand. In order to minimize postsorting variables, the "rare" cells were prelabeled with the UV-excited DNA-labeling dye Hoechst 33342 (Arndt-Jovin and Jovin, 1977). This is a viable cell dye and is totally invisible under the 488-nm laser illumination used to excite fluorescein in the FACS. These cells were then mixed with unlabeled

[4]Assuming random assortment, the relationship between gene frequency (f) and total sortable cases is: fraction sortable $= 2f(1 - f)^2$.

guish from Y-bodies. Often examination of such a cell by white light will expose the artifact because Y-bodies are essentially invisible without fluorescence (Holbrook and Tishler, 1980).

The other major limitation on the technique is that the reading is subjective. Obtaining reliable results depends on the training and consistency of the reader as well as on careful experimental design to include adequate controls and to assure that the reading is "blind."

VI. Broadening Fetal Cell-Selection Capability and Reagent Evaluation

In order to improve the enrichment of fetal cells and devise fetal cell-selection techniques applicable to most or all pregnancies, we are exploring several types of labeling reagents and different selection strategies. The reagents include monoclonal antibodies to HLA types and to trophoblast antigens. Selection strategies have been expanded to include selection with two independent fluorescent labels.

A. Monoclonal Antibodies for HLA Polymorphisms

There were three problems with the rabbit anti-HLA-A2 serum used in our earlier enrichment of fetal cells: (1) it was available in limited quantity; (2) even after extensive absorptions to make it specific, most sorted cells were of maternal origin; and (3) as an HLA-type reagent it was usable only when the mother and fetus differed in that particular HLA type. Monoclonal antibodies to HLA-A2 and HLA-B7 (named PA2.1 and BB7.1, respectively) were obtained as mouse ascites fluids from Dr. Peter Parham (Brodsky *et al.*, 1979). Both are of the IgG1 isotype and are available in large quantities. They require only minimal processing, such as ammonium sulfate precipitation and ion-exchange chromatography, to give almost pure antibody. By binding assays on cells of known HLA type and inhibition of binding by purified HLA antigens, the two monoclonal antibodies show the same pattern of reactivities as the HLA-A2 and HLA-B7 types defined by conventional serology.

Our previous work was limited to HLA-A2-incompatible pregnancies in which the mother was HLA-A2 negative, partly because of the need to conserve the antiserum. With the monoclonals this limitation is removed, and it is reasonable to sort for unlabeled fetal cells in a sample of maternal mononuclear cells that stain with the antibody. This doubles the fraction of mother–fetus pairs that are appropriate for fetal cell selection. With an HLA-A2 gene frequency of about 25%, fetal cell selection based on an HLA-A2 difference should be possible in