Fetal cells in the blood of pregnant women: Detection and enrichment by fluorescence-activated cell sorting

(Y chromatin/HLA/prenatal diagnosis/chromosome abnormalities)

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ABSTRACT   Fetal cells, potentially usable for prenatal diagnosis, were sorted from maternal blood samples taken as early as 15 weeks of gestation. Immunogenetic and cytogenic criteria established the fetal origin of the observed cells: Y-chromatin-containing (male) cells were detected in the sorted sample if and only if the newborn proved to be male and carried cell-surface antigens detected by the fluorescent-labeled antibody used for sorting with the fluorescence-activated cell sorter.

Although the maternal–fetal barrier is generally considered to be effective in limiting cell transfer from fetus to mother, there have been several reports over the last 10 years suggesting that blood samples from pregnant women contain nucleated cells derived from the fetus. In these studies, putative male fetal cells were identified among maternal blood cells by the presence of either Y chromosome in metaphase spreads or Y chromatin after quinacrine staining of interphase nuclei. The presence of such male cells correlated reasonably well with births of male children, false negatives (births of male children but no Y-chromatin-bearing cells) being the prevalent error. The conclusions from this work, however, received only qualified acceptance (reviewed in refs. 1 and 2), in part because the data relied solely on microscopic analyses requiring recognition of one Y-chromatin-positive cell per 1000–5000 maternal lymphocytes.

In the studies presented here, we have added immunogenetic and cell-sorting techniques to the earlier methodology in order to improve the reliability of fetal cell identification in maternal blood samples. Using the fluorescence-activated cell sorter (FACS) (3, 4), we have sorted leukocytes from maternal blood and obtained samples enriched for cells that reacted with an antiserum specific for paternal antigens potentially carried by the fetus but not the mother. The enriched samples were then scored for cells containing Y chromatin, which could be derived only from a male fetus. The use of this enrichment technique facilitates detection of rare fetal cells in the maternal blood sample. More important, however, it provides an independent immunogenetic criterion for confirming the fetal origin of these cells—i.e., if Y-chromatin-containing cells were observed among those sorted from maternal blood, cells of the newborn from that pregnancy should carry the paternal antigens detected by the antiserum used for sorting. Thus, by examining FACS-enriched samples and correlating fetal cell detection with newborn reactivity with the sorting antiserum, we have definitely identified fetal cells in maternal circulation as early as the 15th week of gestation.

MATERIALS AND METHODS

For the FACS-enrichment procedure, fetal cells among maternal peripheral blood lymphocytes were stained by indirect immunofluorescence by using first a rabbit antiserum directed against paternal HLA cell-surface antigens absent in the mother followed by a fluorescein-conjugated goat anti-rabbit immunoglobulin. HLA antibodies were chosen because (i) fetus and mother frequently have different cell-surface HLA antigens due to the extensive genetic polymorphism at HLA loci, (ii) HLA antigens are expressed by the 12th week of gestation, and (iii) nearly all fetal cells would be expected to have HLA antigens.

Antisera. A rabbit antiserum against HLA-A2 antigen (kindly donated by J. Strominger, Harvard University) was used for these studies. This antiserum was made against a purified HLA-A2 antigen (5) and then repeatedly absorbed with tonsillar tissue from several HLA-A2-negative individuals until it stained only HLA-A2 (and crossreacting HLA-A28)-positive lymphocytes. Serologic studies indicate that this reagent is specific for HLA-A2; however, the specificity of the antiserum is actually irrelevant for our studies because cell separation and typing of the newborn were performed with the same reagent. The goat antiserum was absorbed with Sepharose-conjugated human immunoglobulins and tonsillar tissue.

Cell Preparation. Approximately 20 ml of blood was collected into 200 units of heparin. Whole blood was diluted with an equal volume of balanced salt solution containing 0.2% sodium azide. Peripheral blood lymphocytes were then isolated from each blood sample by Ficoll–Hypaque isopycnic centrifugation (6). The lymphocytes were washed once in balanced salt solution and then counted.

HLA Staining. The peripheral blood lymphocytes from each of the pregnant women were divided into two portions, one containing 2×10^6 cells and the other containing approximately 2×10^7 cells. Each sample was resuspended in 50 μl of a 1:40 dilution of rabbit anti-HLA-A2 and incubated for 30 min at room temperature. The samples were then washed, resuspended in 50 μl (0.9 mg/ml, F/P 1.6) of fluorescein-conjugated goat anti-rabbit immunoglobulins, incubated for 15 min at room temperature, washed again, resuspended in balanced salt solution, and finally analyzed on the FACS.

The smaller portion was used to determine the HLA-A2 type of the mother. If a substantial number (>35%) of the mother's cells were stained, she was classified HLA-A2 positive and her remaining cells were discarded. On the other hand, if the mother was HLA-A2 negative, the larger portion was sorted on the FACS. Assuming fetal HLA-A2-positive cells were

Abbreviation: FACS, fluorescence-activated cell sorter.

present, this sorting procedure would separate stained (putative fetal) cells from nonstained maternal cells.

FACS Sorting and Analysis. Representative fluorescence histograms of cells from a HLA-A2-negative mother and her HLA-A2-positive newborn are shown in Fig. 1. Only live cells in the size range of small lymphocytes were analyzed and sorted—i.e., FACS was size-gated to exclude large cells and smaller-appearing dead cells (4). Sorting fluorescent thresholds are indicated in the figure. Negative cells had fluorescent intensities below that indicated by the left arrow, and positive cells above that indicated by the right arrow. The positive fraction usually included the brightest-appearing 0.1–0.8% of cells in the appropriate size range. Sorting thresholds were chosen with the aim of excluding the maximum number of unstained maternal cells with minimal loss of stained (fetal) cells.

Populations enriched for fluorescent-stained cells were deposited directly onto microscope slides and then stained with quinacrine to allow microscopic detection of Y-chromatin-containing cells (7). Slides were read blind in the sense that, at the time of analysis, paternal HLA type, fetal HLA type and, in later cases, fetal sex were unknown.

Microscopic Aalysis. A cell was judged as acceptable for scoring if the nuclear membrane was intact and the nucleus had a uniform round or oval appearance. Polymorphonuclear leukocytes were excluded from scoring. Each cell judged acceptable morphologically was observed for Y chromatin on several planes with the fine adjustment of the microscope. Y chromatin was identified according to criteria described (7).
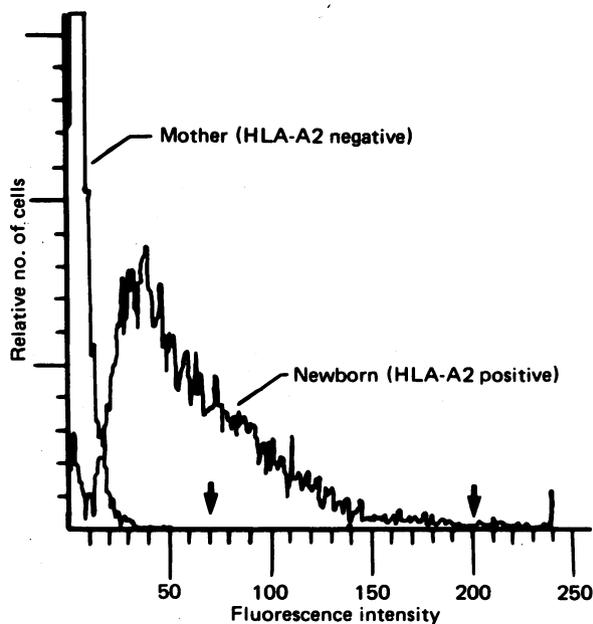

FIG. 1. FACS fluorescence intensity profiles of a HLA-A2-negative mother and her newborn HLA-A2-positive baby. Peripheral blood lymphocytes and cord blood lymphocytes were isolated by Ficoll/Hypaque isopycnic centrifugation. They were then incubated with 50 μl of a 1:40 dilution of rabbit anti-HLA-A2 serum repeatedly absorbed with HLA-A2-negative human lymphocytes, washed, and then stained with 50 μl of fluorescein-conjugated goat anti-rabbit gamma globulin (0.9 mg/ml, F/P 1.6). The maternal cells were sorted on the FACS by using scatter gates to include live lymphocytes and fluorescence gates set as discussed in the text. For the separation illustrated here, the arrows indicate the fluorescence gates used, causing deflection of the brightest 0.04% of total lymphocytes. The fluorescence pattern of cells from the newborn of this mother is shown to indicate the probable fluorescence distribution of fetal cells at the time sorting was done.

RESULTS

Blood samples taken from pregnant HLA-A2-negative women were examined for the presence of fetal cells by FACS sorting for HLA-A2-positive (fetal) cells and then visual scoring of the sorted population for Y-chromatin-containing cells. The group of mothers included in this study were all carrying male fetuses. Samples were usually obtained between 21 and 27 weeks of gestation from women who had undergone amniocentesis for detection of genetically abnormal fetuses at 15 weeks and who were thus known to be carrying male fetuses. Two samples are included that were taken from women at 15 weeks of gestation who later were shown to be carrying male fetuses.

Data are presented in Table 1 for those pregnancies ($n = 12$) in which mothers delivered male infants whose cord blood or peripheral blood cells could be obtained for testing with the anti-HLA-A2 (sorting) antiserum. FACS-enriched samples from maternal blood in five of these pregnancies contained Y-chromatin-positive cells. (Frequencies of Y-chromatin-containing cells in the enriched samples are: 2/331, 3/484, 4/610, 3/1017, and 7/1065.) In each of these cases, the newborn baby's lymphocytes showed clear staining with the anti-HLA-A2 antiserum.

No Y-chromatin-containing cells (0/400–0/1078) were found in FACS-enriched samples in seven pregnancies. These mothers all delivered infants whose lymphocytes did not stain with the anti-HLA-A2 antiserum.

The perfect correlation demonstrated in these data between detection of fetal (male) cells and newborn reactivity with the sorting antiserum demonstrates the dependence of enrichment on the fetal cell-surface phenotype. Thus, the immunogenetic testing confirms the fetal origin of the sorted Y-chromatin-containing cells. The probability (P) that the observed correlation occurred by chance is <0.0013 (Fisher exact test). The Y-chromatin morphology of the sorted cells also supports fetal origin (Fig. 2). In each case, the size of the Y chromatin observed was the same as the Y chromatin observed in the baby's cord blood and consistent with the size of the Y chromosome in metaphase spreads of the fetal cells obtained by amniocentesis. Thus, although the number of Y-chromatin-containing cells observed in each of the FACS-sorted, positive samples was small, the aggregate data clearly demonstrate fetal origin of these cells.

DISCUSSION

How and when the fetal cells enter maternal circulation is still an open question. The serologic and Y-chromatin morphologic similarities observed between the FACS-enriched cells and the newborn's cells suggest that entry occurs during the first or second trimester—i.e., sometime between conception and maternal blood sampling (15–27 weeks). These data, however, do not absolutely exclude the possibility that the fetal cells detected originated in an earlier pregnancy, perhaps entering maternal circulation during parturition or during an abortion. The strong correlation reported previously between the pres-

Table 1. Fetal cell detection is correlated with fetal cell reactivity with sorting antiserum

| | HLA-A2 phenotype of fetus† | |
Fetal cell detection*	Positive	Negative
Yes	5	0
No	0	7

* Cells containing Y chromatin were found in maternal blood. Each of these mothers was carrying a male fetus.
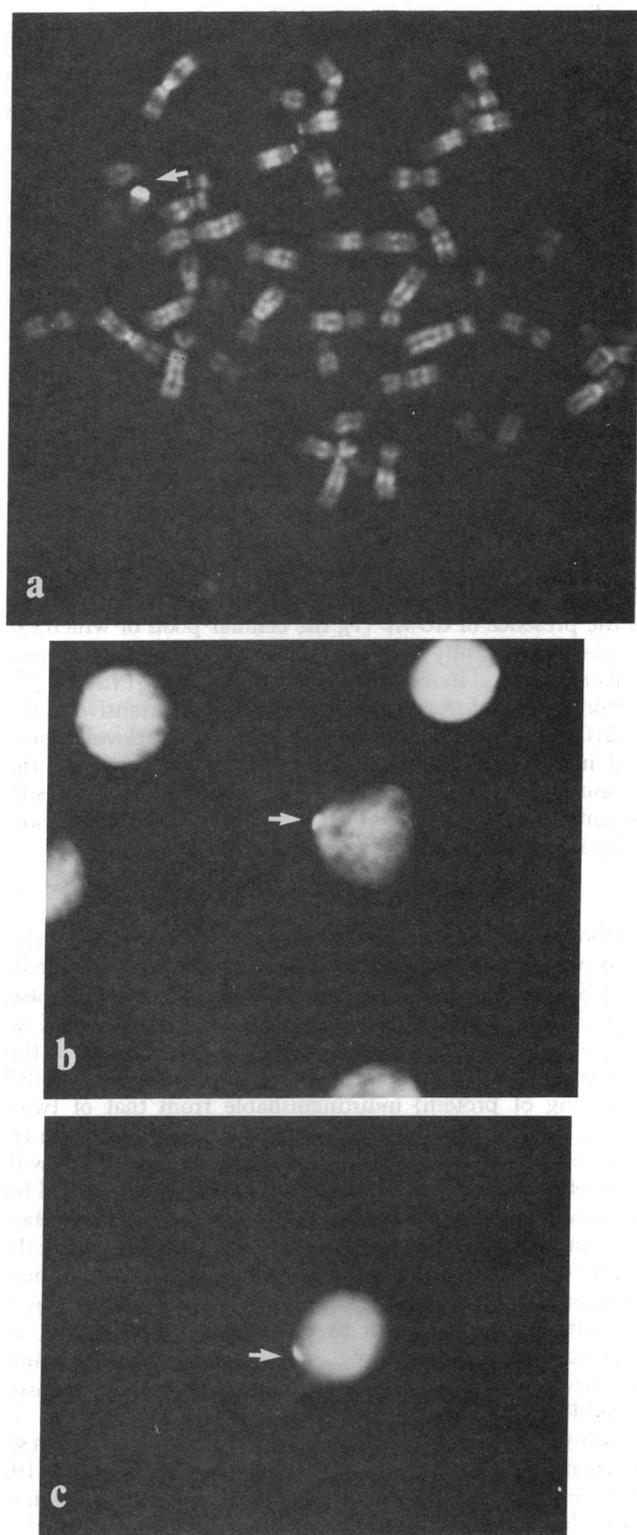† Cells of newborn react with anti-HLA-A2 (sorting) antiserum.

FIG. 2. Quinacrine-stained cells obtained from a single fetus. (*a*) Mitotic cell cultured from amniotic fluid. Arrow indicates Y chromosome. (*b*) Interphase cell from cord blood. Arrow indicates Y chromatin. (*c*) Fetal interphase cell isolated by FACS from maternal blood. Arrow indicates Y chromatin.

ence of Y-chromatin-containing cells and the carrying of a male fetus, however, makes this improbable (8). If Y-chromatin-containing cells originate in earlier pregnancies, such cells

should have been detected in some mothers carrying female fetuses.

Regular and frequent entry of fetal cells into maternal blood is supported by our findings. The sorted samples from five of five women carrying HLA-A2 male fetuses whose cells we could expect to obtain by the FACS-enrichment procedure showed Y-chromatin-positive cells. This number of cases is too small to allow an accurate prediction of success on a larger scale. It does, however, suggest that with the appropriate staining reagents FACS sorting would reveal fetal cells in many mothers as early as 15 weeks of gestation since two of the positive samples were obtained at this gestational time (all negative samples reported were obtained at greater than 21 weeks of gestation).

In addition to its basic significance, the demonstration here that fetal cells enter maternal circulation and can be isolated by FACS-enrichment procedures could be of enormous practical importance. If the frequency of successful isolations of fetal cells is high at 15 weeks and if the isolated cells can be induced to divide, then FACS sorting with an extended series of HLA or other cell-surface reagents could provide a universal non-invasive screening technique for prenatal diagnosis of genetic abnormalities. It could replace (or be used in conjunction with) midtrimester amniocentesis techniques currently used to monitor pregnancies with a relatively high empirical risk of chromosomal abnormalities in the fetus—e.g., pregnancies in women over 35 years old.

Furthermore, because the FACS procedure requires sampling of maternal blood rather than amniotic fluid, it could make widespread screening of pregnancies in younger women feasible. Chromosomal abnormalities detected by the FACS procedure would, of course, be verified by examination of cultured amniotic fluid cells. Widespread screening is desirable because the relatively large number of pregnancies in women below 35 years old means that they bear the majority of children with chromosomal abnormalities despite the relatively low risk of such abnormalities in pregnancies in this age group (9). Thus, if the remaining obstacles can be overcome, FACS sorting of fetal cells from maternal blood could enable early recognition of large numbers of abnormal fetuses that currently go to term before diagnosis.

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