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Fetal Cells in the Maternal Circulation

Prenatal Diagnosis by Cell Sorting Using a Fluorescence-Activated Cell Sorter (FACS)

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1. Introduction

Since the development of new, accurate methods for the detection of chromosome abnormalities and sensitive techniques for diagnosis of biochemical disorders, the whole field of prenatal diagnosis has come into a completely new light. Amniocentesis is nowadays carried out as a routine procedure in most major medical centers throughout the world, and the risks of complications associated with the procedure seem to be very low (see Chapter 2).

With this low risk of complications, the physician will offer an amniocentesis to a patient if the risk of having a malformed child is high, e.g., if either parent is a carrier of a balanced translocation. Even in cases of advanced maternal age the procedure of amniocentesis involves a calculated risk, i.e., weighing the high risk of the mother having a child with a chromosomal abnormality (see Chapter 5) against the considerably lower risk of having minor complications caused by amniocentesis.

Since about 0.5% of all newborns have a chromosome abnormality and about 1% some biochemical disorder (not all of which are serious or have methods available for diagnosis in utero), in a random population the risk of mothers carrying a fetus with a malformation that could be diagnosed by amniocentesis lies close to 1%. It is not yet possible to perform amniocentesis

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on all pregnant women as a routine test. A further limitation of large-scale surveys by amniocentesis is the high cost of such studies.

Therefore, the search continues for other methods of obtaining antenatal samples of fetal tissue—methods which are cheapter, are less complicated, and reduce or eliminate known risks.

Fetal cells occur in the maternal circulation in most pregnancies. Erythrocytes of the fetus can be found in the maternal bloodstream sometimes during pregnancy but in about 50% of all mothers immediately after delivery (Schröder, 1975a).

Nucleated male cells (probably leukocytes) have been demonstrated in blood samples from pregnant women as early as the 20th week of pregnancy and in more than 70% of the cases (Schröder and de la Chapelle, 1972; Zilliacus et al., 1975). The relative proportion of these cells seems to be at least 10–20 times higher than that reported for fetal erythrocytes (Schröder, 1975a). If these fetal "leukocytes" could be isolated from a maternal blood sample, they could probably be used for prenatal diagnosis as well as amniotic fluid cells. This would mean that prenatal diagnosis could be carried out on cells isolated from a simple blood sample from a pregnant woman, and the more complicated procedure of amniocentesis would not be required.

A fluorescence-activated cell sorter (FACS) has been developed in this laboratory and is now commercially available (Herzenberg et al., 1976). With this device, different cell populations have successfully been separated from each other on the basis of differences in their fluorescence after staining with fluorescent antisera (Hulett et al., 1973; Herzenberg et al., 1976). If the fetal cells in the maternal blood could be rendered fluorescent, after staining with specific antisera, the cell sorter could probably be used to isolate fetal cells from the mother's circulation. These cells could then be cultured and used for chromosome studies or biochemical assays.

2. Fetal Cells* in the Maternal Circulation

Male cells, probably white blood cells (WBC), have been shown to occur in the circulation of most pregnant women expecting a male child (Schröder and de la Chapelle, 1972; Schröder, 1975a). The demonstration of this has involved two methods of analysis: (1) analyzing hundreds of mitotic cells from phytohemagglutinin (PHA) cultured lymphocytes of blood from pregnant women (Walknowska et al., 1969; de Grouchy and Trébuchet, 1971; Schindler et al., 1972) and (2) analyzing thousands of cultured or uncultured interphase cells from the blood of pregnant women after staining for the Y chromosome with quinacrine (Schröder and de la Chapelle, 1972; Grosset et al., 1974; Siebers et al., 1975; Zilliacus et al., 1975).

The mitotic studies were carried out using conventional methods for chro-

* When the term "fetal cells" is used in this chapter, the authors mean fetal (Y-body-positive) nucleated cells in the maternal blood, unless otherwise indicated.

mosome staining and identification (Walknowska et al., 1969; de Grouchy and Trébuchet, 1971; Schindler et al., 1972). The use of such methods for the identification of occasional XY mitoses among hundreds of XX mitoses is not an adequate method, and has been criticized (Jacobs and Smith, 1969; Schröder, 1975a). A low proportion of mitoses with tentative Y chromosomes have even been reported when large numbers of cells from newborn females were studied using conventional chromosome staining (Jacobs and Smith, 1969).

Another indication that these mitotic studies are not reliable is the fact that no such XY mitoses have been found in PHA cultures of blood from primiparous mothers when the cells were stained by quinacrine, even if the number of cells studied far exceeded that of the earlier reports (Zilliacus et al., 1975).

However, male cells capable of mitotic division in PHA cultures have been found in maternal blood samples after delivery (Schröder et al., 1974). In these cases the identification was done after quinacrine staining.

The method we first used for identification of male cells in the maternal blood during pregnancy was simply staining of PHA-cultured lymphocytes from the mother with quinacrine and screening of thousands of interphase cells for Y bodies (Schröder and de la Chapelle, 1972). As it turned out, the method had several limitations, one of the most serious being that bright autosomal fluorescence of the mother's chromosomes could mimic a Y body in a small number of cells. Thus false "Y-positive" interphase cells could be found if the mother had brightly fluorescent chromosome regions, even if she was expecting a girl (Schröder and de la Chapelle, 1972).

To overcome this difficulty, we have lately studied the fluorescence of the mother's autosomes before looking for male cells in her blood. All mothers who had autosomes with bright fluorescence have been excluded from further studies, and only those blood samples from mothers without such fluorescent chromosomes have been studied for Y-positive cells (Schröder et al., 1974).

Also, by study of uncultured leukocytes obtained by dextran sedimentation of maternal blood, more accurate diagnoses have been obtained (Schröder et al., 1974; Zilliacus et al., 1975). Recently, we have started to use leukocytes from uncultured whole blood, after hemolyzing the red cells.

By excluding all mothers with bright autosomal fluorescence, and by using the methods described above for leukocyte preparations, we are able to find Y bodies in more than 70% of mothers expecting a boy, and false positive diagnoses are very rare (Schröder et al., 1974; Zilliacus et al., 1975).

Others have tried to use similar methods for determination of fetal sex from a maternal blood sample (Zimmerman and Schmickel, 1971; Grosset et al., 1974; Siebers et al., 1975). Zimmerman and Schmickel found Y-body-like structures in 1–5% of leukocytes from pregnant women regardless of the sex of the fetus.

Grosset et al. (1974) studied a large number of pregnant mothers with the method used by one of us, but cultured the maternal lymphocytes 6 days with PHA. The aim was to preferentially stimulate fetal lymphocytes present in the sample, and in this way obtain enrichment of fetal cells. However, fetal cells

in the maternal blood do not respond to PHA (Zilliacus et al., 1975), so their assumption was incorrect. In spite of this they were able to obtain relatively accurate diagnoses of fetal sex in a study of 86 mothers (Grosset et al., 1974). The mothers were studied between the 14th and 18th weeks of pregnancy, and the average frequency of suggested male cells in the blood of the women was around 1–2%. Since an adult has about 2×10^6 lymphocytes per milliliter of blood, and the flow volume is around 3–5 liters, the total number of lymphocytes is around 1×10^{10}. If 2% of these are of fetal origin, it would mean that 2×10^8 fetal lymphocytes circulate in the mother's blood, an amount equivalent to about 200 ml of blood. According to Grosset et al., this is already the case between the 14th and 18th weeks of pregnancy, at a stage when the fetus has very few circulating lymphocytes itself (Stites et al., 1975).

Similar figures have been suggested by Siebers et al. (1975), who studied the frequency of Y-body-positive cells in uncultured blood samples of pregnant women between the 8th and 12th weeks of pregnancy. By the 8th week of pregnancy they found about 1% of the "fetal" cells in a maternal blood sample. This is at a time when the fetus is just beginning to produce lymphocytes. Still the authors were able to predict correct fetal sex in most cases.

This is very contradictory to our own data, since we have only occasionally found fetal cells in the maternal blood before the 20th week of pregnancy, and even then at a frequency twentyfold lower than that reported by other groups. The discrepancies between the results remain obscure, but since the frequencies are so different we will mainly relate to our own data as far as frequencies and kinetics are concerned.

2.1. Frequency and Kinetics

Fetal nucleated cells, which could be leukocytes, occur in the maternal blood during pregnancy and after delivery (Schröder, 1975a). Such cells have occasionally been found as early as the 15th week of pregnancy, and can generally be demonstrated to be present around the 25th week of pregnancy (Zilliacus et al., 1975). The frequency of fetal cells in the maternal blood seems to be about 1 fetal cell per 1000 maternal cells from that stage onward, with perhaps a slight increase in the frequency immediately after delivery (Figure 1). Such a frequency would probably allow detection, and separation of these cells with the FACS, if appropriate antisera for selectively staining the fetal cells could be found. Indeed, the FACS has been used to detect Rh-positive fetal cells present in the circulation of Rh-negative mothers in frequencies as low as 1 per 500,000, which is 500 times lower than an estimate of the frequency of fetal nucleated cells (W. Jan and L. A. Herzenberg, unpublished). It is possible that fetal cells occur in the maternal circulation even before the 20th week of gestation but cannot be detected because of limitations of the methods used. The same applies to the proportion of mothers who show such cells in their blood. Male cells can be detected in blood samples of 70–80% of all mothers expecting a boy, but it cannot be excluded that such cells would be

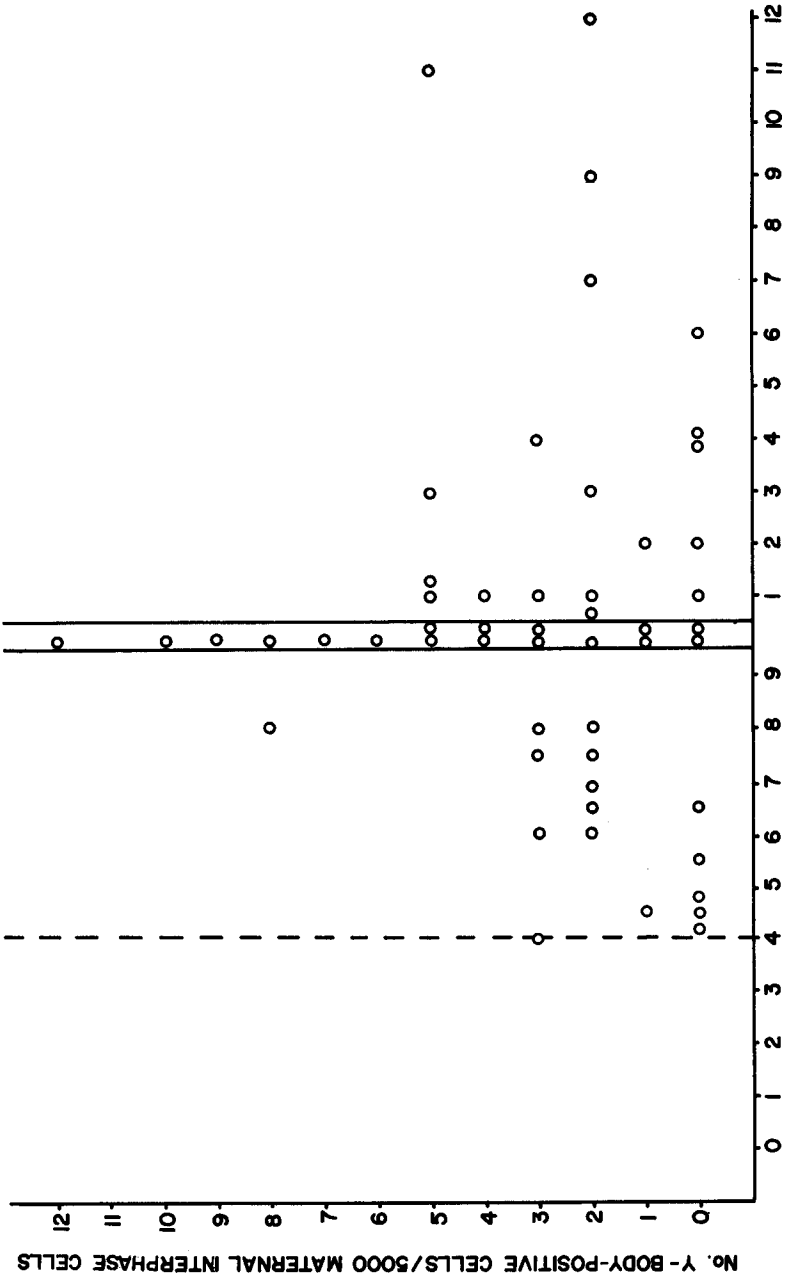

FIGURE 1. Prevalence of Y-body-containing cells in the circulation of women at various intervals of pregnancy and after delivery. "Time of delivery" means within 48 hr after delivery. The data are from papers of Schröder and de la Chapelle (1972) and Zilliacus et al. (1975). Broken line indicates time when fetal cells first occur in maternal blood.

present also in the remaining 20–30%, although in much lower numbers. Perhaps they can be found after enrichment with the FACS.

These fetal cells are not eliminated from the mother's blood directly after delivery but have been observed for up to 1 year after delivery. Even in a few cases where the mother has been pregnant with a female child and had an earlier abortion or pregnancy with a male fetus, a few XY mitoses and Y-body-positive cells have been found in her blood samples after quinacrine staining (Zilliacus et al., 1975; Schröder, unpublished data). This suggests that indeed fetal cells persist in the maternal blood.

The question still persists, how are these fetal cells tolerated in the maternal circulation? Do they cause an immune response by the mother? Are they resistant to any maternal immune response or are they unable to induce such a response?

HL-A typing of maternal, paternal, and fetal HL-A antigens has thrown some light on these questions but is far from giving a complete answer. About 22% of all primiparous mothers produce HL-A antibodies against paternally derived fetal HL-A antigens (Tiilikainen et al., 1974). One could then argue that the cases where no fetal cells can be found in the maternal circulation are those in which a very strong HL-A incompatibility exists between fetus and mother. These cases would then be the ones where a production of HL-A antibodies is triggered, eliminating the existing fetal cells. However, this does not seem to be the case, since HL-A antibodies are produced by mothers when a strong as well as a weak HL-A incompatibility exists between mother and child (Tiilikainen et al., 1974).

HL-A typing in the parents and from the umbilical cords of the infants has shown that maternally derived fetal HL-A antigens generally can be typed without difficulty using cord blood lymphocytes; however, these cells often fail to demonstrate paternally derived HL-A antigens (Tiilikainen et al., 1974). These can generally be demonstrated on later occasions after delivery or after incubation of cord blood cells under tissue culture conditions (Tiilikainen et al., 1974). It is possible that a similar "masking" of antigens on fetal cells by maternal antibody would protect them from destruction in the maternal blood. It has been shown that the trophoblast cells, which are in immediate contact with the maternal circulation, are deficient in blood group antigens (Thiede et al., 1965; Gross, 1966). After enzyme digestion, however, transplantation antigens have been found on trophoblasts (Currie et al., 1968).

The above data clearly show that the method as such, i.e., staining for male cells by quinacrine in blood samples of pregnant mothers, is not a useful routine method for the determination of fetal sex. The following limitations exist: (1) male cells cannot be found in the circulation of all mothers pregnant with a male fetus; (2) fetal cells may persist in the maternal blood from earlier pregnancies, and lead to a wrong diagnosis; and (3) about 20–30% of all individuals have bright fluorescence on one or more autosomes, which will mimic the Y body in a few interphase cells and lead to incorrect diagnosis.

If all mothers with bright autosomal fluorescence are excluded from the sampling, the method will be considerably more accurate but still useful in

only about 70–80% of primiparous mothers. We recommend that all these facts be considered if the method is to be used for scientific purposes, and do not recommend the method at all for diagnosis of the sex of the fetus for genetic counseling. However, if a significant number of these cells could be isolated by cell sorting, it is possible that the maternal blood could be a source of fetal cells for routine prenatal diagnosis.

2.2. Characteristics of Fetal Cells in the Maternal Blood

So far we have been considering the fetal cells in the maternal circulation as nucleated cells with a Y body, probably representing leukocytes. If these cells are to be used for prenatal diagnosis after isolation, it is important that they be capable of mitotic division and suitable for diagnosis of biochemical disorders. Lymphocytes fulfill both these criteria. Can it be determined from the existing data what class of cells these actually represent?

In a leukocyte preparation of the type we have used to study the fetal cells in the maternal blood, most, and probably all, types of leukocytes are represented. After hypotonic treatment, fixation, and quinacrine staining, all these types cannot be distinguished from each other. However, four very different types of cells can be observed in such preparations. Since only the nucleus is stained by quinacrine, this means that four different types of nuclei can be distinguished. One cell type has a segmented nucleus and clearly represents a granulocyte. Another has a nucleus which is fairly large and kidney shaped. These cells probably represent monocytes. The third and fourth cell types observed in such preparations have a regular round nucleus, one being relatively small, the other relatively large. These two types probably represent lymphocytes, i.e., small and large lymphocytes.

The fetal cells in the maternal blood almost always belong to the cell types which have lymphocytelike morphology. In most cases they belong to the category with a large nucleus. Sometimes during later pregnancy and immediately after delivery fetal granulocytes can also be found in a maternal blood sample (Schröder et al., 1974; Zilliacus et al., 1975). These cells then occur in slightly lower frequencies than fetal lymphocytelike cells. When blood samples of women are studied 1 week after delivery, all fetal granulocytes have disappeared (Schröder et al., 1974). This is actually to be expected, since the mature granulocyte has a half-life of about 7 days (Wintrobe, 1967).

Fetal cells in the maternal blood have never been observed to have a monocytelike morphology. Always when we refer to the frequency of fetal cells in maternal blood, we mean the number of Y-body-positive cells per number of round lymphocytelike cells in the sample, if we have not specifically stated that we have also studied granulocytes. This means that our average frequencies of about 1 fetal cell per 1000 maternal cells actually means 1 fetal lymphocytelike cell per 1000 maternal lymphocytelike cells. Since only about 20% of the cells in our leukocyte preparations belong to this category, the actual frequency of fetal cells in our samples is much lower than 1 per 1000.

From these data, it would be tempting to speculate that the fetal cells in

the maternal blood are mostly lymphocytes and that granulocytes also occasionally cross the placenta but generally in connection with the delivery. The fact that the fetal cells often have a large nucleus could be explained by suggesting that they actually are primitive lymphocytes, i.e., lymphoblasts, which are larger than mature lymphocytes. On the other hand, erythrocyte, granulocyte, monocyte, and lymphocyte precursors all are large cells with a round nucleus and cannot be distinguished from each other after quinacrine staining. This means that if we go by morphological criteria alone and consider that a high proportion of fetal blood cells are immature, any of these cells could actually be candidates for the fetal cells in the maternal blood.

Another question worth pursuing is, are these round mononucleated fetal cells blood cells at all?

It is known that large numbers of trophoblastic cells are continuously sloughed into the maternal circulation throughout pregnancy (Iklé, 1961). Most of these cells are probably broken down enzymatically in the mother's blood, while the remaining cells are passed to the mother's lungs where they are gradually eliminated (Iklé, 1961). It is evident that the fetal cells in the maternal blood are not trophoblasts, since trophoblastic cells are large, often polyploid, with multiple nuclei (Salvaggio et al., 1960). Such cells could easily be differentiated from blood cells. However, some other placental cells might accompany the trophoblastic cells into the maternal bloodstream and be morphologically impossible to distinguish from blood cells after quinacrine staining. Cells of different morphology occur in the amniotic fluid (Milunsky, 1973). Besides large polygonal cells (probably epithelial), many small round cells also occur which are of fetal origin. These cells are not contaminating maternal or fetal leukocytes, since they are found even when no erythrocytes are present in the sample. They do not stain like leukocytes with the May-Grüwald-Giemsa technique (Schröder, unpublished data). However, if these cells are fixed and stained with quinacrine as the leukocyte preparations from maternal blood samples, they cannot be morphologically distinguished from lymphocytes (Schröder, unpublished data). This indicates that in the fetus itself, in the amniotic membranes, or in the placenta there are nonlymphoid fetal cells which could easily be mistaken for lymphocytes by morphological criteria alone.

However, the following facts speak against the theory that the fetal cells in the maternal circulation would be cells other than blood cells. It is very unusual for cells that are not blood cells to stay in the circulation. Such cells are generally sticky and are probably eliminated rapidly from the bloodstream, as are trophoblastic cells (Iklé, 1961; Beer and Billingham, 1971). Since it is also known that fetal erythrocytes cross the placenta in almost 50% of all pregnancies, and since the same applies to fetal granulocytes, it seems reasonable to assume that fetal lymphocytes would be capable of the same, or even a preferential passage, since lymphocytes are capable of active movement and have a long life span. Even if the fetal cells in the maternal blood were not lymphocytes but rather, for example, placental cells, the cell sorter could

probably still be used for their separation and the cells cultured on monolayer without mitogens instead of in a suspension with mitogens.

If these cells are lymphocytes, one would expect that they should respond to mitogen stimulation and undergo mitotic division in lymphocyte cultures of maternal blood, as the mother's own cells do. However, this does not seem to be the case. So far, an unequivocal XY mitosis has not been found in a PHA culture of lymphocytes from a primiparous mother (Zilliacus et al., 1975). An unequivocal XY mitosis is, according to our criteria, one that has been identified by quinacrine staining (Schröder, 1975a). Only in a few cases have such cells been found in blood cultures of pregnant women, and this has always been after delivery of a boy (Schröder et al., 1974). Since bleeding through the placenta is known to occur in connection with the delivery, the occasional PHA-responsive male cells probably enter the maternal circulation at this time. The question still persists as to why these fetal cells do not respond to PHA in a maternal blood sample. Lymphocytes obtained from peripheral blood of 13- to 14-week-old fetuses will respond to PHA (Stites et al., 1975). However, only human T lymphocytes respond to PHA by mitotic division, while B cells, which comprise 20–30% of all peripheral lymphocytes, are unresponsive (Greaves et al., 1974). If the fetal cells in the maternal blood were mainly B lymphocytes, this could explain their unresponsiveness to PHA.

It has been shown that human B cells can be separated from human T cells by a variety of procedures (Wigzell et al., 1972; Eisen et al., 1972; Schlossman and Hudson, 1973). A very simple and efficient method is to incubate lymphocyte suspensions in nylon wool columns, since most of the B lymphocytes adhere to the columns, whereas almost all the effluent cells are T lymphocytes (Eisen et al., 1972; Julius et al., 1973; Greaves and Brown, 1974).

If blood from pregnant women who have fetal cells in their blood is fractionated by Ficoll-Hypaque centrifugation (Thorsby and Bratlie, 1970), the lymphocytes can be separated from the erythrocytes and from other leukocytes. The interphase layer will contain mostly lymphocytes and some monocytes, while the pellet will contain the remaining leukocytes and all the red cells. If the lymphocyte layer now is screened for fetal cells, most of them can be found in this layer (Schröder, unpublished data). However, if the red cells are hemolyzed from the pellet and the remaining cells studied, at least in some cases a few fetal cells can be found in this lymphocyte-depleted population also. No definitive conclusions can be drawn from this, since a few lymphocytes are known to pass the Ficoll-Hypaque gradient and can be demonstrated in the pellet.

However, if the lymphocyte suspensions (from a pregnant woman's blood) which contain fetal cells are passed through nylon wool columns, almost all fetal cells will adhere to the nylon wool together with the maternal B lymphocytes (Schröder, 1975b). This suggests that the fetal cells in the maternal blood are B cells. However, immature cells, even some T cells, seem to adhere to nylon wool more readily than mature T lymphocytes (R. Stout, personal

communication). If we assume that these cells really are B lymphocytes, it does not rule out the possibility of using them for prenatal diagnosis. Such "fetal B lymphocytes" isolated by the FACS might be stimulated to mitosis by B-cell mitogens, such as pokeweed mitogen or dextran sulfate.

The following can be said concerning the characteristics of fetal cells in the maternal circulation:

1. Male cells occur in the blood of most women pregnant with a male fetus.
2. These cells have a lymphocytelike morphology, except after delivery when some fetal granulocytes also can be found.
3. These cells do not respond to PHA by mitotic division, except in occasional cases after delivery.
4. Most of these cells can be found among the lymphocytes after Ficoll-Hypaque centrifugation.
5. These cells adhere to nylon wool columns, as do B lymphocytes.

Many of these characteristics actually fit those of B lymphocytes, but they could well be immature blood cells from the fetus, or some other cells, perhaps even of placental origin.

3. Prenatal Diagnosis by Cell Sorting

By means of the FACS (mentioned earlier) different cell populations can successfully be separated from each other, either by differences in cellular fluorescence, by differences in light-scattering characteristics (an indicator of cell size, type of cell, etc.), or by different combinations of these two variables (Hulett et al., 1973; Herzenberg et al., 1976). In this instrument, individual cells are observed in suspension in the central stream of a very small coaxial liquid jet as they pass through a laser beam. The jet is later broken into uniform droplets, and those droplets containing the desired cells are electrically charged and then deflected by an electric field (Herzenberg et al., 1976). Thousands of cells can be processed per second. In this way enrichment of a selected cell population by factors of more than 1000 can be achieved, with viabilities of more than 90%. Cell populations present in fractions as small as 1 in 5×10^5 have been identified by this device (Herzenberg et al., 1976).

A simplified diagram of the cell sorter is shown in Figure 2. Cells are forced through a micronozzle under pressure in a liquid suspension into the center of a cell-free fluid and then out through an effluent nozzle 50 μm in diameter. In this way a coaxial flow is created, which keeps the cells near the axis of the effluent jet. The nozzle is vibrated axially at 40,000 Hz, breaking the jet into 40,000 uniform droplets per second. Immediately below the nozzle, before formation of droplets, the stream is illuminated by an argon ion laser operating at a wavelength selected to excite fluorescence in cells tagged with the appropriate fluorescent material. Some of the fluorescent light, filtered to remove the exciting wavelength, is focused onto a photomultiplier tube which

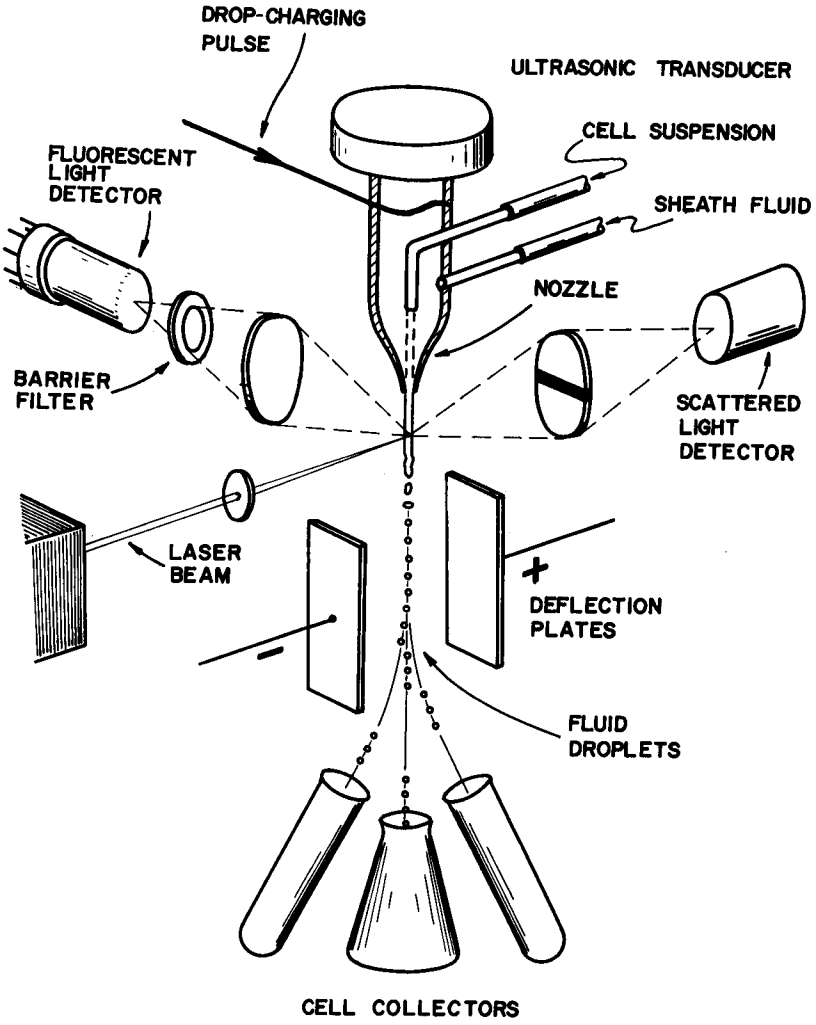

FIGURE 2. Simplified block diagram of the fluorescence-activated cell sorter (FACS).

generates an electrical signal proportional to the number of fluorescent molecules on each cell. A second signal, related to the volume of the cell, is generated by detecting the light scattered forward out of the illuminating beam by the passing cell.

Signals produced in the scatter and fluorescent channels are processed, delayed, and combined as required to produce electrical pulses which are used to charge the liquid stream at the time the droplet containing the desired cells is forming. Further downstream the droplets pass through an electric field between two charged plates. Droplets that carry a charge are deflected, while uncharged droplets continue their original course. To ensure that the droplet carrying a desired cell is charged, and thus deflected, the charging pulse lasts

for the period of three droplets, centered at the time when the cells are expected to enter a droplet. In this way the cells with the right fluorescence or scatter properties will be deflected and collected separately from the uncharged cells. A more detailed description of the cell sorter and its operation can be found elsewhere (Herzenberg et al., 1976).

A variety of experiments have been carried out with this device. T and B lymphocytes have successfully been separated from each other, as have numerous other functionally different cell populations (Kreth and Herzenberg, 1974). Could this sophisticated machine with its high electronic and optical resolution power be used to separate fetal cells from a maternal blood sample? For this purpose the fetal cells in a blood sample from a pregnant woman would have to be rendered fluorescent without making the mother's cells fluorescent. Methods have been suggested for accomplishing this (Hullet et al., 1973). At least as a theoretical model, staining of fetal cells in a maternal blood sample could be achieved by use of a fluoresceinated antiserum directed against paternal, but not maternal, HL-A antisera. In this way, if the fetus inherited one or two paternal HL-A antigens not present in the mother, such a reagent should stain only the fetal cells and not the maternal ones. Such experiments are under way in this laboratory in collaboration with Dr. G. M. Iverson and Dr. H. M. Cann. The assay for HL-A staining used involves binding of a hapten (e.g., arsanilic acid) to the specific HL-A antiserum (Cammisuli and Wofsy, 1976). If this antiserum is used as the first step of the staining procedure, it will bind to all the cells which carry the HL-A antigen against which the antiserum is directed. A fluoresceinated anti-arsanilic acid will now bind to these cells and make them fluorescent. Using artificial mixtures of cells from individuals with known differing HL-A antigens, we have been able to obtain up to a 200-fold enrichment of male cells present in a ratio of 1 male cell per 1000 female cells. The enrichment has been evaluated blindly after quinacrine staining for the Y chromosome. Some preliminary data exist already on the use of the method for isolation of fetal cells from pregnant mothers. However, strong monospecific HL-A antisera are quite difficult to obtain, which limits the method.

Alternative ways of staining fetal but not maternal cells would be of importance. If an antiserum could be raised that was specific for fetal cells but not reactive with adult cells, an ideal situation would exist. Attempts to raise such antisera in rabbits against various human fetal tissues are under way (A. de la Chapelle, personal communication).

Placental cells contain alkaline phosphatases that differ from those in other tissues (Sussman et al., 1968). If the fetal cells in the maternal blood are of placental origin, an antiserum against placental alkaline phosphatases could be used for their detection and isolation.

The cell sorter has also been used for the detection of Rh-positive fetal cells in the circulation of Rh-negative mothers (Jan and Herzenberg, 1973). Staining of Rh-positive cells was carried out as a two-step process. First Rh-positive antibody was added to the erythrocyte suspension, and then the cells were treated with fluorescent goat anti-human γ-globulin. The fluorescent goat

anti-human γ-globulin will bind only to cells with Rh-positive antibody (Rh-positive cells) on their surface, rendering them fluorescent. This staining method is very sensitive, and it turned out that the cell sorter easily could detect Rh-positive cells among Rh-negative cells at concentrations of 10^{-5} to 10^{-6} both in artificial mixtures and in samples of blood from pregnant women (Jan and Herzenberg, 1973).

We have reasonable hopes that fluorescence-activated cell sorting can be used to isolate fetal cells from blood samples of pregnant women. Even if the fetal cells are not T cells, or lymphocytes at all, we believe that methods for their culture can be found and mitotic divisions obtained. So far, we have methods to detect and sort only in pregnancies where we have suitable anti-paternal HL-A antisera, but even if fetal cells can be isolated from maternal blood in only a few model cases at this time, we feel confident that the method can be a useful tool in prenatal diagnosis in the future.

Since the presence of fetal cells in the maternal blood has so far been demonstrated only by chromosome studies, it is of great importance to demonstrate their presence by an independent method. A method such as we have described for prenatal diagnosis from maternal blood samples would make chromosome abnormalities such as trisomies, monosomies, translocations, and biochemical disorders detectable with no physical risk beyond the negligible ones of maternal venipuncture.

The costs would be far below those incurred by the family and society under present circumstances. This would naturally allow large-scale surveys to lower the costs, without any risk for the mother.

4. Addendum

During the past year, Drs. G. M. Iverson, D. W. Bianchi, and H. M. Cann and ourselves have successfully isolated fetal cells from maternal blood samples in a number of pregnancies (Herzenberg et al., 1979). Since the isolation is carried out using the fluorescence-activated cell sorter (FACS), fetal cells among maternal peripheral leukocytes were stained by indirect immunofluorescence using first a rabbit antiserum directed against paternal HLA antigens absent in the mother's cells, followed by a fluorescein-conjugated goat anti-rabbit immunoglobulin. Our best reagent for this purpose is a rabbit anti-HLA-A2; therefore, separations were done in 12 HLA-negative women carrying a male fetus fathered by an HLA-A2-positive male. The fetal sex was ascertained through amniocentesis, since all 12 women underwent amniocentesis for detection of genetically abnormal fetuses. In 5 of the cases male (Y-chromatin-positive) cells could be found in frequencies of 0.3–1% of the enriched population. All these mothers subsequently delivered HLA-A2-positive boys. In 3 of these pregnancies maternal blood was obtained before amniocentesis, ruling out the (small) probability that all fetal cells found passed as a result of amniocentesis. In the 7 cases where no enrichment of Y-chromatin-positive cells were seen, the male children were HLA-A2-negative. These studies were

carried out completely blindly, the observer never being cognizant of the source of the population of cells on a given slide (e.g., control or separation).

Fetal cells in the maternal blood do not seem to respond to T or B cell mitogens, or in mixed leukocyte culture (Schröder et al., 1977). So far we have not been able to stimulate the fetal cells in the enriched populations to division, but such studies are under way.

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