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edited by Rainer Fried

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Chapter 1

PRENATAL DIAGNOSIS OF GENETIC DISORDERS LEADING TO MENTAL RETARDATION

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I. INTRODUCTION

The prenatal detection of genetic disorders has become an area of increasing importance during the past few years. The development of reliable screening procedures for the early detection of familial disorders leading to mental retardation has stimulated the demand for an early assessment of fetal integrity in "high risk" pregnancies. The acceptance of transabdominal amniocentesis as a safe procedure and advances in tissue culture techniques as well as micromethods for biochemical

assays and chromosomal analysis have all contributed to the rapid advances in this field.

The indications, accuracy, and reliability of the procedures used in prenatal detection of chromosomal aberrations are clearly established. In contrast, the experience in management of patients at "high risk" for a familial biochemical disorder is limited and therefore the procedures should be restricted to a research center in which expertise in that specific disorder has been gained.

A number of excellent reviews have been published recently which provide guidelines as to the indications, risks, and complications of amniocentesis and related techniques used in prenatal genetic diagnosis (1-4). This chapter is designed to provide the reader with detailed methodology, as currently used in our laboratory, to perform prenatal genetic diagnosis.

II. AMNIOTIC FLUID

A. Composition

The volume of amniotic fluid at 10 weeks of gestation, averages 30 ml and gradually increases to 350 ml at 20 weeks (5-7). Amniotic fluid is composed of 98 to 99% water with 1 to 2% solids. The inorganic constituents of amniotic fluid are quite similar to extracellular fluid, and the solids are equally divided between organic solids and proteins. Excellent reviews of the composition of amniotic fluid have recently been published (8, 9).

The concentration of electrolytes in amniotic fluid prior to 20 weeks of pregnancy is essentially that found in maternal serum (10, 11). During the latter half of pregnancy, the amniotic fluid becomes increasingly hypotonic. The proteins of amniotic fluid have been investigated and although considerable variations occur, useful ranges have been established (Table 1). Conflicting reports have suggested that the proteins are of maternal origin (16, 17) while others present evidence of their fetal origin (15, 18).

TABLE 1

Protein Components of Early Amniotic Fluid (12-15)

Month of gestation	Total protein, g/liter		Albumin	Globulins			
	Mean	Range		$\alpha 1$	$\alpha 2$	β	γ
2 to 4	3.9	1.2-6.2	71.3%	10.9%	6.1%	7.4%	4.1%
5 to 6	8.1	5.6-14.3	68.0%	13.3%	7.6%	6.7%	4.4%

Other nitrogenous constituents such as urea, creatinine, and uric acid increase late in pregnancy to reach a term value approximately twice that found in maternal serum (12, 14, 19). The amino acid composition of amniotic fluid has been studied and its relevance to prenatal diagnosis of the genetic aminoacidopathies is discussed below. Many hormones have been detected in amniotic fluid (20-33). The antenatal diagnosis of the adrenogenital syndrome has been established by measuring the amniotic fluid levels of 17-ketosteroids and pregnanetriol late in pregnancy (21, 34, 35).

A number of enzymes have been detected in amniotic fluid (3). In most instances it is impossible to determine the actual source of the enzyme. Potential sources of error include maternal blood contamination and the presence of isozymes and fetal enzymes.

Amniotic fluid levels of sugars, lipids, bilirubin, protein-bound iodine, gases, and other components have also been reviewed (8, 9) and have not as yet proved useful for the in utero detection of genetic disorders. They are, however, extremely valuable for the estimation of fetal age and maturity.

B. Amniocentesis

Transabdominal amniocentesis performed between 13 and 18 weeks of pregnancy has gained widespread acceptance as being the procedure of choice to obtain amniotic fluid specimens (1-4). Transvaginal amniocentesis carries a much higher risk of complication and is not advised early in pregnancy (36). The reliability of transabdominal amniocentesis has been well established in the management of Rh disease (39-44) and lately in obtaining material for prenatal genetic diagnosis early in pregnancy (2, 45, 46).

Transabdominal amniocentesis is performed by an experienced obstetrician as an out-patient technique after thorough explanation of the risks and with signed permission of the pregnant woman and her husband. After the patient voids, a local anesthetic (1% lidocaine) is injected into the proposed puncture site. A 22-gauge, 5-in. disposable spinal needle with stylet is inserted through the abdominal wall in the midline, directed at a right angle toward the middle of the uterine cavity. After puncture, the stylet is removed and a sterile plastic syringe is used to withdraw 10 ml of amniotic fluid, after which the needle is swiftly withdrawn. Strict aseptic conditions are observed throughout the procedure (3). Other potential approaches to obtaining fetal materials is through direct biopsy of the placenta (37) and the fetus (38). These techniques have recently been performed previous to therapeutic abortion and represent highly hazardous procedures which should be done exclusively after accurate localization of the fetus and the placenta.

C. Handling of Amniotic Fluid

The amniotic fluid is placed in either a sterile siliconized glass or polystyrene tube (17 x 100 mm, 16 ml capacity) and transported at ambient temperature to the laboratory. Shipment to a distant destination is best achieved under the same conditions. The laboratory to which the fluid is to be sent should be notified before the procedure as to date, indication, test to be performed, and method of shipment. Cell viability is preserved for as long as one week at ambient temperature under sterile conditions. It is not advised to freeze the sample if cultivation of the amniotic fluid cells is planned. Shipment in culture tubes or flasks is not recommended since part or all of the cells may attach at random to the walls and thus become difficult to recover. Proper labeling of the samples is mandatory. In the laboratory, the fluid is centrifuged at 100 x g for 10 min at room temperature in a clinical centrifuge. The supernatant is removed and either processed immediately or frozen away for later reference. The cell pellet is now available for direct analysis or cultivation.

III. AMNIOTIC FLUID CELLS

A. Origin and Utilization

Amniotic fluid cells have been shown to be derived from the amnion and fetus (47-51). The origin of the fetal cells is presumably buccal mucosa, vaginal epithelium, skin, umbilical cord, and fetal urine. The number of amniotic fluid cells have been shown to increase with gestation while the percentage of viable cells decreases.

The utilization of the cellular material found in amniotic fluid initially focused upon the technique of sex chromatin analysis for the antenatal determination of sex (52-61). The presence of sex chromatin in amniotic fluid cells has been useful for the management of pregnancies in women heterozygous for X-linked recessive disorders such as hemophilia, nephrogenic diabetes insipidous, and muscular dystrophy (2, 62, 63).

The biochemical properties of uncultured amniotic fluid cells are still being investigated. Many problems such as maternal blood cell contamination (64, 65), enzyme instability (66), and insufficient number of metabolically active amniotic fluid cells have limited the usefulness of this approach to prenatal genetic diagnosis. At the present time it is mandatory to confirm the results with enzyme assay on cultivated amniotic fluid cells from the same original sample. In addition, if the uncultured amniotic fluid cells are to be used, a control enzyme must be assayed.

An increasing number of investigators demonstrate the ability to culture amniotic fluid cells. The rate of successful cultivation of amniotic fluid cells varies greatly from one laboratory to another. A number of factors probably account for the variation of these rates. In several early studies, the amniotic fluid was obtained during the third trimester of pregnancy from women with Rh isoimmunization. Cells obtained during this period are considerably more difficult to cultivate. If only those cases in which the amniotic fluid is obtained prior to twenty weeks of pregnancy are considered, the ability to cultivate these cells is over 90%.

The methods used to cultivate amniotic fluid cells differ significantly from one another; however, success rates do not appear to be dependent upon any specific factor with the possible exception of the investigator's experience and care in handling cells.

B. Cultivation of Amniotic Fluid Cells

1. Material

a. Growth medium: to 500 ml of Ham's F-10 medium is added 75 ml of fetal calf serum and 5 ml of an antibiotic-antimycotic mixture containing 10,000 units of penicillin, 10,000 μg streptomycin, and 250 μg fungizone per ml.

b. Puck's saline A: to 500 ml of sterile deionized water is added 55 ml of Puck's saline A (10 x) and 1 ml of 7.5% sodium bicarbonate.

c. Trypsin solution: a 0.25% trypsin solution is freshly prepared by diluting a 2.5% stock solution with Puck's saline A.

2. Procedure

Ten to 15 ml of amniotic fluid is gently centrifuged at 100 x g for 10 min and the cell pellet suspended in 0.1 ml to 0.2 ml of amniotic fluid, or 100% fetal calf serum. A drop of this suspension is placed in a 35 x 10 mm (8 cm^2) Petri dish (Falcon plastic). Usually three or more Petri dishes are used depending on the size of the cell pellet. A concentrated cell suspension has a better chance of attachment and therefore cultivation. A sterile glass coverslip is placed on each drop to immobilize the amniotic fluid cells, and the dishes are placed in a CO_2 incubator at 37°C for 20 min. Two to 3 ml of growth mixture are added and the dishes incubated in 5% CO_2 at 37°C. The medium is changed every other day until a number of colonies of cells are seen under the coverslips, usually 7 to 18 days. The medium is removed from the dish, and the coverslip turned cell surface up and placed in another Falcon dish. Medium is added to both the original and the new dish. Staining procedures and chromosome

analysis can be performed directly on the coverslip while the cells in the original dish are usually maintained in long term culture. When confluency is obtained in the original dish, the medium is removed, and the cells washed twice in Puck's saline A and trypsinized (0.1-0.2 ml of 0.25% trypsin until the cells detach). The cells are recovered in 0.5-1.0 ml of medium and transferred to a 60 x 15 mm (21 cm^2) Falcon Petri dish or directly to a 250-ml (75 cm^2) flask with a screw cap. A small aliquot of this cell suspension can be placed on a new coverslip and incubated 1 hr before medium is added. This coverslip can be used the next day for further chromosomal analysis. When shipment of cells in culture is planned, the cells are planted in 30-ml (25 cm^2) flasks and the flask is filled completely with medium. Shipment is best done at ambient temperature.

C. Cell Morphology

Amniotic fluid cells in culture are known to give rise to at least two morphologically distinct cell populations. One type, which is epithelial-like, proliferates in islands usually for a period limited to two to five passages. These cells adhere strongly to the culture flask and demonstrate increased resistance to trypsin and mechanical breakage. The second type, which can be maintained in culture for over 30 passages has the classical configuration of the fibroblast. Both epithelial and fibroblast-like clones proliferate with equal efficiency in short-term culture. The ratio of one cell type to the other is unpredictable and variable even in cultures derived from the same original sample. Recent work in this laboratory has indicated that morphologically distinct populations of amniotic fluid cells show biochemical differences (67). The need for establishing the normal biochemical properties for each cell type in cultivated amniotic fluid cells is desirable each time a new biochemical parameter is being studied.

D. Preparation of Cells for Enzymatic Assays

Cultivated amniotic fluid cells are rinsed with saline, collected by trypsinization and/or scraping of the flasks, washed three to four times with isotonic saline, and suspended in a small volume of the appropriate buffer. Disruption of the cells is accomplished by freeze-thawing with glass beads three times in a dry ice-acetone bath or nitrogen cavitation, a method in which the cells are exposed to a nitrogen pressure of 800 psi for 20 min followed by a rapid release of pressure until atmospheric pressure is reached. The crude homogenate is centrifuged at 200 x g for 10 min to remove unbroken cells and the supernatant used for the enzyme assays.

In studies utilizing cellular fractions, the cells are suspended in 0.25 M sucrose and disrupted by one of the above methods, and the homogenate is fractionated by differential centrifugation (68) in a Model L-Beckman preparative ultracentrifuge. The procedure is as follows: the homogenate is centrifuged at 600 x g for 10 min to obtain the nuclear pellet. The supernatant is spun at 4200 x g for 8 min to obtain the mitochondrial pellet. This supernatant is then centrifuged at 30,000 x g for 8 min for the lysosomal pellet. The microsomal pellet is obtained by centrifuging the supernatant at 60,000 x g for 30 min. The final supernatant obtained is called the high-speed supernatant.

IV. DETECTION OF CHROMOSOMAL ABNORMALITIES

A. Clinical Commentary

Sex determination and chromosome analysis of amniotic fluid cells before and after cultivation provides a reliable method to manage pregnancies at risk for cytogenetic aberrations. The indications for study in our laboratory include chromosomal translocation carriers, maternal age over 40 years, previous trisomic Down's syndrome, and carriers of X-linked recessive disorders. In very unusual carrier states, in which the abnormal chromosomes cannot be identified with certainty or for which the relation to mental retardation has not been securely established, karyotyping the amniotic fluid cells will not be helpful at the present time.

B. Staining for Sex Chromatin

Fresh amniotic fluid is spun in a clinical centrifuge at 100 x g for 10 min. The supernatant is discarded and part of the cell button is resuspended in 2 ml of chilled methanol:acetic acid fixative (3:1, v/v) and incubated at 4°C for 30 min. The suspension is centrifuged at 200 x g for 10 min and the supernatant discarded. The cell pellet is resuspended in 0.25 ml of fresh fixative. One drop of this final solution is mixed with one drop of 1% cresyl violet (69) or 2% aceto-orcein (61) on a glass microscope slide. The preparation is covered with a coverslip and examined under a light microscope for the presence of sex chromatin.

C. Fluorescent Staining Technique for the Y Chromosome (70)

1. Reagents

a. Buffer: Dissolve 2.72 g of sodium acetate trihydrous in water, adjust to pH 5.5 with glacial acetic acid and make up to 100 ml.

b. Quinacrine: Dissolve 500 mg of quinacrine dihydrochloride (Sterling-Winthrop) in 100 ml of water.

c. Fixative: 25% methanol in water.

2. Procedure

A cell pellet is prepared by centrifugation of fresh amniotic fluid at 100 x g for 10 min. The supernatant is discarded and smears are prepared on a number of glass slides. The smears containing 100 to 200 cells are successively:

(a) Fixed in methanol for 15 min.
(b) Stained with quinacrine for 5 min.
(c) Washed in distilled water for 3 min.
(d) Rinsed in buffer.
(e) Mounted in 1:1 glycerol and buffer.
(f) Viewed immediately.

The Y chromosome can be detected as a fluorescent body in interphase nuclei. Between 2 and 10% of the cells may show a fluorescent body if the fetus is a male.

D. Technique of Chromosome Analysis

1. Giemsa Reagents

(a) Giemsa stain; mix 10 g of Giemsa powder in 666 ml of glycerin and 666 ml of methanol.

(b) Sodium phosphate buffer; dissolve 93 mg of $NaH_2PO_4 \cdot H_2O$ and 428 mg of Na_2HPO_4 anhydrous in 300 ml of water.

(c) To 19 ml of sodium phosphate buffer add 1 ml of Giemsa stain and filter twice.

2. Procedure for Amniotic Fluid Fibroblasts

Eighteen hours after a cell-layered coverslip is inverted into a new Petri dish or after subculture onto a new coverslip, 0.1 ml of diacetylmethyl colchicine (Colcemid) (0.01 mg/ml) is added per milliliter of medium. Four to six hours later the medium is removed, the coverslip rinsed gently with hypotonic solution (5 parts distilled water to 1 part fetal calf serum) and then incubated in this hypotonic solution at 37°C for 30 min. The coverslip is gently rinsed in fixative for 20 min. The coverslip is then rinsed in 50% acetic acid and placed cell side up on a slide. A few more drops of 50% acetic acid are added on the preparation which is gently passed back and forth about 5 in. above a flame to promote