

Appendixes

Diagnostic Technologies for Genetic Diseases

Diagnosing genetic diseases requires a partnership of two types of technologies: those for sampling bodily tissues and fluids, and those for analyzing such samples obtained before and after birth. Although fetal imaging is not tissue analysis in the strict sense, it will be discussed here as a technology useful both in conjunction with prenatal tissue sampling and by itself to view gross congenital malformations in utero. This section reviews the major imaging, sampling, and analysis techniques, and comments on their current and potential use as clinical tools. It applies only to prenatal and early postnatal diagnosis.

Often, the only "treatment" available for a fetus diagnosed as having genetic disease is the elective termination of pregnancy. All the stigma, emotion, and ethical controversy attached to this possible recourse can be—and has been—transferred to the diagnostic techniques themselves, and exacerbated by those techniques having been inapplicable until well into the 2nd trimester of pregnancy. With the advent of techniques minimizing risk to the fetus and allowing diagnosis within the first trimester, prenatal diagnosis may become more accepted.

Controversy also attends the use of postnatal diagnostic techniques. Genetic testing for certain disorders among high-risk populations—such as the screening for sickle cell trait among American blacks in the 1960s—have been said to stigmatize and demean individuals in those populations. Recent advances in diagnosing genetic disorders whose clinical symptoms often do not surface until adulthood—such as Huntington disease or familial hypercholesterolemia (a predisposition for arterial hardening which causes early heart attacks)—have raised further questions: Would a potential employer or insurance company have a right to this information? (see app. B). Propelled by rapidly advancing rDNA technologies, the expanding use of these diagnostic techniques will soon necessitate an answer to these ethical and political questions.

Fetal imaging

Fetal imaging involves obtaining a visual image of the fetus, either by means of special electronic techniques, or by using fiberoptic. Ultrasound and fetoscopy are the two major types of imaging. They can be used in

and of themselves, as well as being partnered with techniques for tissue sampling (see below).

ULTRASOUND

Ultrasound is commonly used in determining fetal age and defining large anatomic structures. It involves high-frequency sound waves, undetectable to the human ear, that are directed toward the uterus. A fetal image is created from the differential reflection of the sound waves bouncing off diverse fetal tissues. Various gross congenital malformations may be detected using ultrasound, including hydrocephalus (excess fluid of the brain), anencephaly (absence of all or most of the cerebral hemispheres), absent or stunted limbs, and some defects of the heart and kidney (Hobbins, Venus and Mahoney, 1981). For purposes of tissue sampling, it is generally used in conjunction with amniocentesis (see below). There is currently little evidence of risk to the fetus from the small doses of ultrasound needed for in utero visualization. However, cautioning against routine screening, the NIH Consensus Development Conference on Diagnostic Ultrasound Imaging in Pregnancy stated in its findings, "Lack of risk has been assumed because no adverse effects have been demonstrated clearly in humans. However, other evidence dictates that a hypothetical risk must be presumed with ultrasound. Likewise, the efficacy of many uses of ultrasound in improving the management and outcome of pregnancy also has been assumed rather than demonstrated, especially its value as a routine screening procedure Ultrasound examinations performed solely to satisfy the family's desire to know the fetal sex, to view the fetus, or to obtain a picture of the fetus should be discouraged. In addition, visualization of the fetus solely for educational or commercial demonstrations without medical benefit to the patient should not be performed" (Office of Medical Application of Research, 1984).

FETOSCOPY

Fetoscopy entails the insertion of a thin fiberoptic scope through the abdomen into the uterus. The procedure usually is done around the 18th week of gestation. It permits a well-defined narrow-angle view of

isolated parts of the fetus, and thus is used for fetal surgery as well as imaging. Fetoscopy, however, primarily is used to obtain fetal tissue samples (see below).

Technologies for fetal tissue sampling

The three major fetal tissue sampling technologies in use today are: fetoscopy, amniocentesis, and chorionic villus biopsy. Fetoscopy is infrequently used because it is relatively risky and difficult to perform. Amniocentesis is relatively safe for both the fetus and mother, and is widely used today. Chorionic villus biopsy still is largely in the developmental stage in the United States, but has certain advantages that may lead to its widespread use in the near future.

FETOSCOPY

Direct tissue sampling via fetoscopy makes use of the fiberoptic scope—inserted into the uterus for fetal imaging purposes—to remove blood and skin samples. A needle or forceps, guided through the fetoscope by ultrasound, accomplishes this purpose. Various hemoglobinopathies, muscular dystrophy, and hemophilia can all be diagnosed using fetal blood samples (Hobbins, Venus and Mahoney, 1981). However, with the advent of sensitive DNA analysis techniques in the late 1970s, diagnosis of hemoglobinopathies such as sickle cell disease and thalassemia can now be obtained through the less risky procedure of amniocentesis (see below). Fetoscopy, never widely practiced, carries a 3-to-6 percent risk of fetal death over and above the natural losses from spontaneous abortion and miscarriage (Alter, et al., 1981; Rocker and Laurence, 1981), and is routinely performed at only a few medical centers.

AMNIOCENTESIS

Amniocentesis involves sampling fetal cells and other substances present in the amniotic fluid. This is accomplished via a needle inserted through the abdominal wall, through the wall of the uterus, and into the fluid-filled space that surrounds the fetus. Amniocentesis normally is done using ultrasound to direct the needle.

First used in the late 1960's amniocentesis is now routinely employed with chromosome analysis techniques to detect abnormalities such as Down's syndrome, neural tube defects (through testing of the amniotic fluid), enzyme deficiencies (as in Fabry disease and Lesch-Nyhan syndrome), and hemoglobinopathies (through testing of cultured fetal cells). Amniocentesis is widely available, has a low associated risk of fetal

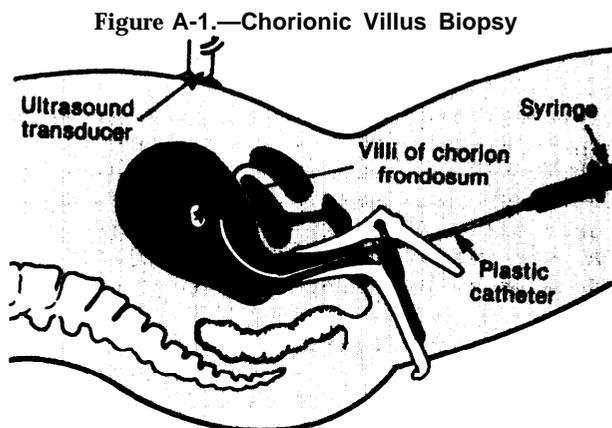
death (less than 0.5 percent), and is 99.4 percent accurate (NICHD, 1976).

Theoretically, amniocentesis could be performed early in the pregnancy since DNA analysis allows detection of the defect in any cell, regardless of tissue type or stage of fetal development. However, not enough fetal cells are available in the amniotic fluid early on, and thus, amniocentesis is usually performed no earlier than between the 16th and 19th weeks of pregnancy. Additionally, the cells from the fluid must often be cultured for 1 to 5 weeks to yield a large enough tissue sample for analysis, further delaying diagnosis. Thus, with amniocentesis the abortion of an affected fetus, if elected, must be performed well into the second trimester when the results of the analysis become available. Such a delay increases the risk of complications associated with abortion including trauma, sepsis, and hemorrhaging (Brash, 1978). Abortion that late into a pregnancy also can carry a considerably increased risk of psychological and physical stress to the parents, and is generally less acceptable to parents and the community. The further development of methods to analyze *uncultured* amniotic fluid cells continues to greatly speed diagnosis—e. g., a new technique for examining uncultured cells with an electron microscope can diagnose a glycogen storage disease 3 to 6 days after amniocentesis (Hug, et al., 1984). One of the consequences of this increasing effectiveness in neonatal and premature care is that the age at which viability is reached (see Technical Note 4) is constantly being pushed back, mandating earlier parental decisions vis a vis the course of the pregnancy.

CHORIONIC VILLUS BIOPSY

Chorionic villus biopsy (CVB) is a relatively new technique for the sampling of fetal tissue that can be performed as early as the 8th to 10th weeks of pregnancy. It entails taking a sample of the fronds of tissue, or villi, that root the fetal placenta to the uterus. Ultrasound is used to guide a catheter into the woman's uterus to the villi, a tiny bit of which is then suctioned off using an attached syringe (see fig. A-1). The fetal tissue is then separated from maternal tissue and subjected to biochemical or chromosomal analyses that take an average of one week to yield a diagnosis. In contrast to amniocentesis, extensive culturing of the tissue is not necessary since a sufficient quantity of DNA is obtained in the tissue sample. CVB can be performed weeks, even months, earlier than either amniocentesis or fetoscopy.

CVB was first used in China (Department of Obstetrics and Gynecology, Anshan, 1975), the U.S.S.R. (Kazy, Rozovsky and Bakarev, 1982), and Norway. It



SOURCE: Product News, "Chorionic Villus Biopsy," *Outlook*, January 1984, p. 8. Carolyn Brooks, artist.

has been considered ethically acceptable in England and used since mid-1981 for prenatal diagnosis of certain "high-risk" disorders such as hemoglobinopathies (Old, et al., 1982), and fetal sexing of pregnancies at risk of sex-linked diseases (Gosden, et al., 1982), mainly Duchenne muscular dystrophy. It is used less often there to diagnose the more common Down's syndrome. Unlike amniocentesis, however, CVB cannot be used to detect noncellular substances in the amniotic fluid, like alpha fetoprotein, whose presence indicates a high risk of neural tube defects.

According to the World Health Organization's registry, CVBs have been performed in the United States since mid-1983. By November of that year, a projected 12 percent associated fetal loss rate was reported after only 240 CVBs had been performed in the U.S. and Europe (Ward, as cited in Jackson newsletter, 1983). Because of this, several researchers (Lippman, 1984; Hecht, Hecht, Bixenman, 1984) contend CVBs are risky and should be used with caution. Current clinical data from approximately 2900 CVBs performed to date in these same countries seems to indicate that the observed fetal loss is 4.2 percent (Jackson, 1984 newsletter). This, however, includes an unquantified number of spontaneous abortions and miscarriages that are a danger to any normal pregnancy. In four similar series of ultrasound observations on ultrasound pregnancies and control groups, the observed fetal loss rate is in the neighborhood of 2 percent. Extrapolating from that, it may be reasonable to assume that the risk of fetal loss that might be associated with CVB is 2 to 3 percent. Any fetal loss rate, however, is highly dependent on the expertise of the laboratory involved, and there are laboratories with a reported 0 percent observed fetal loss (Jackson, 1984 newsletter). Although generally higher than the 0.5 percent loss rate associated with amniocentesis,

much of the discrepancy may be due to the time when the procedures are performed. There is a higher spontaneous abortion rate in the first trimester, when CVBs are done, than in the second trimester, when amniocentesis is done. Some even predict that CVB will replace amniocentesis within a few years (Product News, 1984).

Current clinical applications of chorionic villus biopsy include at least three groups in England, with four or five more testing it for clinical use in that country (Dr. Robert Williamson, Ph. D., personal communication, 2-16-84). Other countries include Italy (over 200 cases of Down's syndrome have been diagnosed by a Milan group) and France (to diagnose hemoglobinopathies; Dr. Robert Williamson, Ph. D., personal communication, 2-16-84).

Tissue and fluid analysis

Genetic diseases were first detected through their characteristic behavioral or physical traits. This approach, combined with family histories, still is important to the diagnosis of many genetic diseases, especially those for which the underlying biochemical and genetic defects are not known. However, behavioral and physical examination is generally not applicable to prenatal diagnosis, or on the cutting edge of diagnostic technologies, and will not be discussed here.

Many genetic diseases have surfaced in recent years whose physical and behavioral manifestations are not readily apparent, are progressive, or take several years to emerge. Many such diseases are detectable through biochemical assays for characteristic imbalances or abnormalities of certain body substances.

A small but growing number of diseases may now be diagnosed through direct analysis of the genetic material. This was once only possible for gross chromosomal abnormalities involving the absence or duplication of entire chromosomes, but recent advances in molecular genetic technology have made possible detection of minute defects within the chromosomes. Such genetic analysis can allow diagnosis of the disease before the biochemical defect is detectable, especially prenatally, and before it becomes clinically apparent, making possible early treatment and sometimes even prevention.

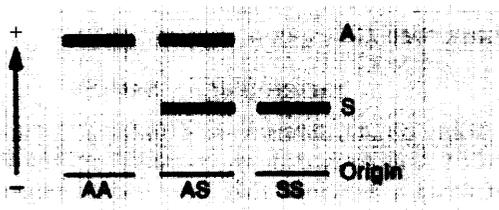
BIOCHEMICAL ASSAYS

Many genetic diseases are manifested as biochemical imbalances caused by the reduced or absent activity of certain enzymes that help manufacture a given chemical, or convert it into another useful product. Such enzyme deficiencies underlie a spectrum of dis-

orders ranging from albinism to hemolytic anemia to some immunodeficiency diseases. X-ray, urine analysis (for excretion of abnormal amounts of certain accumulating precursors) and physical or mental examinations are often used for preliminary detection. However, enzyme assays of the blood or other tissues are generally necessary to make a definitive diagnosis of such diseases. Tay-Sachs disease (TSD), for example, is a metabolic disorder primarily affecting Jews of Eastern European descent (1:3,000 US.) (Stanbury, et al., 1983) caused by the lack of an enzyme, Hexoseaminidase A, that results in the accumulation of lipids in the brain. TSD is characterized by progressive neurological degeneration including dementia, paralysis and blindness. Diagnosis routinely involves enzyme assays of cultured amniotic fluid cells prenatally and of the blood serum postnatally.

Lack of activity of an enzyme or other substance is sometimes due not to a quantitative lack of the substance, but to a structural defect that prevents it from functioning properly. Electrophoresis is a method of distinguishing such variants through the different speeds at which they migrate in an electrical field according to their total net charge—a characteristic that may vary with molecular structure. For example, electrophoresis can reveal the absence of any one of the three major classes of immunoglobulins—as well as variations within any one class—that characterize certain immune deficiency diseases. It also can detect and distinguish between both heterozygotes (i.e., sickle cell trait) and homozygotes (i.e., sickle cell disease) for the sickle cell gene (see fig. A-2). Protein electrophoresis is a relatively inexpensive and expedient technique, and is commonly used for postnatal detection of hemoglobinopathies. Because of the relatively high risk involved in obtaining fetal blood samples (see Technologies for Fetal Tissue Sampling, this appendix), electrophoresis is less commonly used for prenatal diagnosis. There are some cases in which electrophoresis is insufficient to distinguish particular genotypes, and therefore is frequently combined with a volubility test. In most cases, these two tests will suffice to identify a hemoglobin disorder.

Figure A-2



SOURCE: Bowman, J. E. and Goldwasser, E. (1975) *Sickle Cell Fundamentals*, The University of Chicago.

DIRECT ANALYSIS OF DNA

Cytogenetics: Visualization of Chromosomes.—Cytogenetics is the examination of chromosomes under a microscope in order to detect gross changes in chromosomal structure. One of the first clinical applications of this technique was the detection of Down syndrome (Lejeune, 1959), in which the cell carries an entire extra chromosome 21. Although many reports on other numerical chromosomal aberrations followed, researchers were often unable to identify which chromosome was involved. Characterization of banding patterns on particular chromosomes in the late 1960s and early 1970s allowed the identification of specific chromosome pairs as well as parts of each chromosome (Hirschhorn, 1981). An array of chromosomal deletions, duplications and translocations, and the corresponding syndromes, have since been identified (Borgoankar, 1980). Down syndrome and other major numerical and structural chromosomal defects afflict about 1 in 160 live-born infants (Hook and Hamerton, 1977). Such chromosomal defects—including abnormalities of the sex chromosomes such as Klinefelter syndrome in which the male possesses an extra X chromosome, causing sterility and feminization—are now routinely diagnosed both before and after birth using cytogenetics.

Genetic Markers.—The rapid progress in recombinant DNA techniques since the mid-1970s has made possible detailed analysis of particular genes on the chromosomes, and the characterization of minute genetic defects that are not detectable through examination of gross chromosomal structure. In recent years, the defective genes underlying various other heritable diseases have been identified—including Lesch-Nyhan syndrome (Jolly, et al., 1982, Brennand, et al., 1982) familial hypercholesterolemia (Bishop, 1983), and phenylketonuria (Woo, et al., 1983). Within the past 2 years, genetic “markers” (though not the actual genetic defect) have been discovered for two important genetic disorders: Duchenne muscular dystrophy (Murray, et al., 1982) and Huntington disease (Gusella, et al., 1983).

The discovery of identifiable markers for genetic defects opens up the possibility of using them to aid in diagnosis. Such markers might be useful as prenatal tests, for postnatal identification of risk, or perhaps even for genetic screening. For example, phenylketonuria (PKU), an inherited enzyme deficiency that, if untreated, can cause severe mental retardation, could not be detected before birth until recently (although biochemical tests were available for postnatal screening). The recent discovery of a genetic “probe” or

short stretch of DNA specific to the defective gene, has made possible not only prenatal detection, but also carrier identification (Woo, et al., 1983). This means that parents at risk of having a child affected by PKU can be identified. This opens up the technological prospect of parental carrier screening to supplement or replace routine newborn screening.

Direct analysis of DNA is being used now in the diagnosis of several other diseases as well, including some disorders of hemoglobin and one form of dwarfism (Antonarakis, et al., 1982). Direct binding of DNA probes to patient DNA could theoretically be developed for any disease caused by a single gene.

Genetic markers can be of several types. Some depend on the presence or absence of specific biochemical activities. Others depend on the differences in how DNA is cut by enzymes specific to certain nucleotide sequences in combination with DNA probes—specific detectable stretches of DNA constructed in the laboratory that bind directly to either the gene causing the disease or a piece of DNA so close to the disease-causing gene that it can be used as an indicator for the defective gene. The techniques are briefly described below.

Restriction Enzymes.—Restriction enzymes are proteins that are found in bacteria that cut DNA at specific sequences. Human DNA is composed of roughly 3 billion pairs of nucleotides (see Technical Note 1); restriction enzymes look for stretches of 4 to 12 nucleotides that are arranged in a particular order, and cut the DNA at a site either in the middle of the sequence or near to it. When the DNA from human cells is so treated, DNA fragments of many lengths are generated. The DATA from any one individual will have a specific pattern because the sequence recognized by a particular enzyme will occur in characteristic places in that person's DNA.

People generally have very similar patterns of DNA fragmentation when their DNA is treated with restriction enzymes, and so most enzymes have not yet been shown to be useful for diagnosis. Some enzymes, however, generate differences that correlate with disease. The DNA coding for sickle cell disease, for example, is cut by an enzyme that does not cut the normal gene. Therefore, when this enzyme is used on DNA from a patient, the one DNA fragment found in normal individuals is cut into two smaller pieces. These pieces can be seen using standard laboratory methods, and the technique has been used to detect both sickle cell disease and sickle cell trait (Orkin, et al., 1982).

In most cases, the differences between the normal and the abnormal gene will not be so easily identified: the disease-causing mutation will not occur where restriction enzymes are known to cut. In this case, one can sometimes identify people who might carry the

abnormal gene by identifying differences in a piece of DNA close to the gene causing the disease. This technique depends on using restriction enzymes indirectly, rather than directly, and is correspondingly less precise.

People show characteristic variations in how their DNA is cut by certain restriction enzymes, just as they have specific blood groups. These variations usually are not significant in and of themselves. The place along the DNA that is responsible for the variations can be located. The utility of such variations comes when, by chance, a particular pattern is caused by differences close to a disease-causing gene. When this occurs, it is often possible to track the abnormal gene by following the restriction fragment pattern. This technique of establishing "guilt by association" is called linkage analysis (denoting a physical genetic linking between a trait of interest and an identifiable marker) and has permitted tracing of the Huntington disease gene, (Gusella, et al., 1983) the Duchenne muscular dystrophy gene, and genes underlying several hemoglobin disorders (Boehm, et al., 1983). Because the technique does not require any special knowledge about which gene causes a disease, or the biochemical lesion responsible, restriction fragment analysis may be used to diagnose diseases whose molecular mechanisms are not yet known, such as cystic fibrosis. A major disadvantage of the technique is that the restriction enzyme pattern usually varies from family to family, and many members of each family must be tested before genetic detection within any one family is practical.

The technique is analogous to searching for passengers of a downed plane. In thousands of miles of mountainous territory, the wreckage of an aircraft is found by tracing its radio distress signal. This does not give much information about the condition of the crew or the circumstances of the crash, but it does permit restriction of the search to a smaller area, and increases the probability of finding the passengers. The crash site itself may provide some clues about the cause of the mishap and where to look for survivors. In this analogy, the radio signal is like a linked genetic marker, while the defective gene is like the crash site.

DNA Probes.—Gene probes are short stretches of DNA that bind to a specific DNA sequence. Through cloning, many identical copies of a probe can be made. Probes are usually made out of DNA that has been specially labelled with either a radioactive or chemical tag that allows the probe to be used to detect specific DNA sequences, employing standard laboratory methods.

Probes are often used in combination with restriction enzymes. First the DNA is chopped into manageable sizes by restriction enzymes, and then a probe

is bound to the DNA. In the instance of the sickle cell gene mentioned above, for example, the probe corresponds and binds to an abnormal variation of the hemoglobin gene that causes the disease, allowing its detection.

Probes come in different sizes. Most sequences used as probes are fairly long, composed of many copies of a single ordered sequence of hundreds or thousands of nucleotides. These are usually made using bacterial clones of a gene or DNA fragment. Some short probes, called oligonucleotide probes ("oligo-" means few), can be chemically manufactured in the laboratory. These small highly specific probes can, under carefully controlled conditions, detect the difference between genes that differ only in a single nucleotide in their sequences. This property has been used to detect sickle

cell disease (Wallace, et al., 1981; Orkin, 1982; Conner, et al., 1983), and some thalassemias. (Orkin, et al., 1983; Pirastu, et al., 1983) An oligonucleotide probe has also been developed for another genetic disease, alpha-1-antitrypsin deficiency (an inherited deficiency of a blood protein that can lead to lung and liver disease), making prenatal diagnosis possible (Kidd, et al., 1984).

The power of the new diagnostic techniques can be imagined by noting that they can detect differences of a single letter in a book composed of three billion letters. If each gene is a paragraph, then only a few paragraphs in a long monograph have been investigated using the new techniques; more of the text will be tested over the next few decades, and the meaning of the book may thus slowly become clearer.