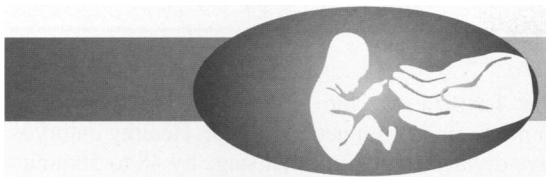

Preimplantation Diagnosis

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Preimplantation embryonic biopsy and analysis offer couples at increased risk of having offspring affected with a genetic disorder the possibility of an early prenatal diagnosis. For many couples, this approach would avoid the issue of the selective termination of affected fetuses. Substantial advances have been made in the area of preimplantation diagnosis, but the possible difficulties with this approach cannot be ignored.

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The prenatal diagnosis of genetic disease currently allows at-risk couples the option of having only unaffected offspring. To achieve this goal, they are faced with terminating a pregnancy if an affected offspring is diagnosed. Traditional prenatal diagnosis is performed by midtrimester amniocentesis at 15 to 16 weeks' gestation and provides a diagnosis from one to five weeks after the procedure. This necessitates a second-trimester termination procedure if a couple wishes to abort an affected fetus. Because of this, chorionic villus sampling was devised as an earlier prenatal diagnostic procedure (10 to 12 weeks' gestation). Although the possibility of a first-trimester termination procedure is more acceptable to some couples, to others neither approach provides a good option. The availability of preimplantation prenatal diagnosis would avoid the issue of pregnancy termination for many couples. With this approach, a biopsy specimen is taken of the early embryo after fertilization in vitro. This is then analyzed for the specific disorder the couple is at risk for, and only unaffected embryos are transferred.

Several approaches may be used to obtain a biopsy specimen of the developing embryo. The first is polar body analysis. This involves removing the first polar body, the nonfunctioning haploid product of meiosis I. The problem with this approach is that crossing over may have occurred between sister chromatids, resulting in difficulty in interpreting the analysis. Accordingly, the most common approach that has been used is the biopsy of a single blastomere removed from a four- to eight-cell embryo. This approach has the disadvantage of having only one cell available for analysis. Efforts are currently underway to culture these individual blastomeres to provide more cells for analysis. The third approach is trophectoderm biopsy of the blastocyst. This would provide more cells for analysis. Human embryos, however, are difficult to culture to the blastocyst stage, and implantation rates may be lower for blastocysts.

Many technical, ethical, and financial issues involved in this approach to prenatal diagnosis remain unanswered. Much work has already been done on animals as a prelude to human preimplantation diagnosis. In this review we will discuss the current methods being used for preimplantation biopsy, present an overview of the preclinical animal work, and discuss analyses that have been reported in humans.

Techniques for Preimplantation Diagnosis

Embryo Recovery

There are two ways in which embryos can be obtained for preimplantation diagnosis. Embryos that have been fertilized in vivo after timed intercourse or insemination can be obtained by uterine lavage.[1] This technique has the advantage of permitting spontaneous conception and therefore minimal technologic intervention for the parents, other than the extraction of the preimplantation embryo. One disadvantage of this approach is the low efficiency of embryo recovery, which imposes limitations on successful implantation. A second disadvantage arises from the possible failure to retrieve an embryo that may then implant and establish a pregnancy with an affected fetus.

The second strategy for obtaining embryos for preimplantation diagnosis is the application of techniques for in vitro fertilization. The fundamental procedures involved are the stimulation of oocyte maturation, oocyte retrieval, fertilization in vitro, and transfer of the unaffected embryos.

Success rates for in vitro fertilization cycles critically depend on the number of embryos available for transfer. The normal process of follicular maturation selects one of

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a cohort of available follicles for maturation and release at ovulation. To overcome that selection process and increase the recruitment of follicles, human menopausal gonadotropins are administered daily to the woman. The process is monitored by transvaginal ultrasonography, permitting the frequent assessment of the size and number of follicles developing. Criteria for an adequate response are determined by individual programs and are further modified for different patient circumstances. In general, stimulation is continued until the dominant follicles are larger than 16 to 17 mm and there are at least three follicles larger than 14 mm. Usually between 8 and 20 follicles are recruited. The serum estradiol level is used to verify the normalcy and maturity of the follicles visualized, anticipating that about 200 pg per ml per follicle greater than 14 will be secreted. When follicles are large enough, sufficient in number, and estrogen levels are appropriate, the final maturation of the oocyte is initiated by administering the luteinizing hormone-like hormone and human chorionic gonadotropin, and the oocyte retrieval is scheduled for 35 hours later.

Oocyte retrieval is usually accomplished using transvaginal ultrasonography to guide an aspirating needle, which is inserted once into each ovary from the vagina. Follicles are sequentially entered and the follicular contents aspirated and submitted to the laboratory for analysis. Each tube containing follicular fluid and granulosa cells is examined under a dissecting microscope to search for the oocyte. Overall efficiency of the process is about 66% to 75% recovery of oocytes per follicle aspirated. Although a small number will have two or more oocytes, most follicles (92%) will have one oocyte.[2]

After an oocyte is identified, maturity is assessed, either by observing the structure of the surrounding cumulus and corona cells or by nuclear assessment and visualizing polar body extrusion. Oocytes retrieved from different follicles within the same ovary may be at differing stages of maturation, with some mature, preovulatory, metaphase II, and others immature, with intact visible germinal vesicles. The oocytes will be inseminated at different times, as dictated by their stage of maturation. The insemination will occur six to eight hours after retrieval for mature oocytes and 24 to 36 hours after retrieval for immature oocytes that are matured in culture. Sperm for insemination is prepared by collecting an active motile concentrate. Most commonly this involves a process of washing and the recovery of motile sperm that swim up into a layer of media from a centrifuged pellet, but there are many different techniques for sperm preparation that can be used. When there is no known sperm dysfunction, a small volume of media containing 50,000 to 100,000 active sperm will be placed in the dish containing the oocyte. The inseminated oocytes are then cultured for 14 to 16 hours, after which evidence for fertilization can be detected by visualizing the second polar body or by identifying two pronuclei within the zygote. In most cases, 65% to 75% of the oocytes will be fertilized, with higher percentages seen with mature oocytes and less than 50% of immature oocytes expected to fertilize.

After fertilization, the zygotes are transferred to growth media and continued in culture. Healthy embryos will have divided to the four-cell stage by 48 to 56 hours after retrieval and will have achieved an eight- to ten-cell stage one day later. These embryos are then available for assessment by blastomere biopsy.

Blastomere Biopsy

The concepts and methods of blastomere biopsy have been recently reviewed extensively,[3,4] so we will only describe the biopsy methods used in recent reports of preimplantation diagnosis leading to human pregnancies[5,6] and as currently used in our program.

Embryos at the six- to ten-cell stage are transferred individually to the heated platform of an inverted microscope in a micromanipulation chamber that contains phosphate-buffered medium to control the pH and is sealed with paraffin oil to prevent evaporation. The embryo is anchored on a fire-polished suction pipette (outside diameter 200 μm) and oriented for biopsy (Figure 1). Using a small pipette containing acidified medium (pH 2.5), a fine stream is directed at the zona pellucida of the embryo until an opening dissolves.[7] Then a fire-polished biopsy pipette is moved into the hole produced by this zona-drilling procedure, and gentle suction is used to withdraw one blastomere.[8] If inspection at high power fails to reveal a nucleus in this blastomere, it is considered an anucleate fragment, and a second blastomere is removed for examination. After a successful biopsy, the micromanipulation chamber is moved to the dissecting microscope, where the nucleated blastomere is transferred to a microfuge tube for analysis. The presence of the blastomere in the tube is verified after transfer by inspection under the dissecting microscope. All such procedures are done inside a containment hood to minimize the risks of contamination.

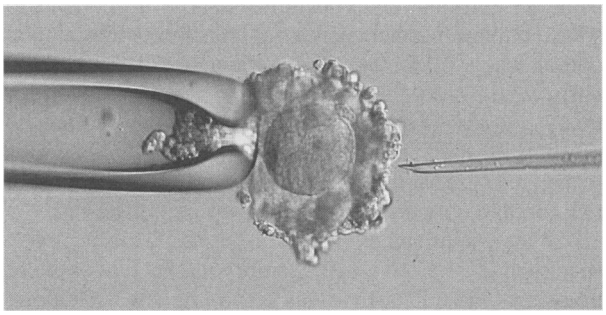

Figure 1.—The photograph shows micromanipulation of an embryo before biopsy of the blastomere.

Embryo Transfer

Embryos that survive blastomere biopsy and are determined to be unaffected can then be transferred back to the woman. This can be by either a uterine transfer, usually accomplished on the second or third postretrieval day (either four- to six-cell embryos or six- to 10-cell embryos), or a transfer into the fallopian tube. The uterine transfer is the most commonly used procedure and in-

volves the placement of a small catheter through the cervix into the uterus near the fundus. The desired number of embryos, in a small volume of about 50 μl, are layered into the uterus, with all of the embryos transferred in one procedure. For intratubal transfer, usually a laparoscopy is used to directly visualize the fimbrial end of the tube, and a transfer catheter is threaded about 3 cm into the tubal lumen. Alternatively, a catheter can be threaded transcervically into the uterus and directed into the fallopian tube for intratubal transfer. There are some advocates of intratubal transfer as a procedure offering higher probabilities of success compared with intrauterine transfer, but this is controversial. The success of implantation is then determined by the health of the embryos, and success rates are influenced predominantly by the number of embryos replaced and the age of the patient (Figure 2). Overall expectations of success for the average United States programs, without intervening manipulation of the embryos, are in the range of 18% probability of successful delivery per oocyte retrieval in women

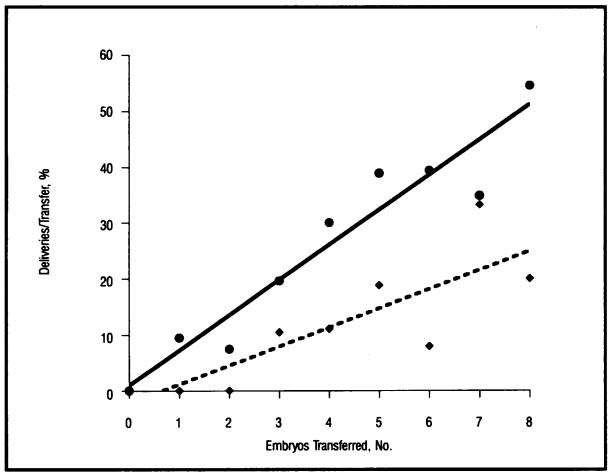

Figure 2.—The graph shows the percentage of deliveries per number of embryos transferred, University of California, San Francisco, in vitro fertilization program, 1989 to 1992. Lines are computer-derived best-fit trend lines. -◆- = women younger than 40 years, —●— = women 40 years or older

younger than 40 years. Programs in the top 10% of reporting centers, including our program at the University of California, San Francisco, have success rates in the 28% to 35% range for deliveries per oocyte retrieval in women younger than 40.

Blastomere Analysis

The polymerase chain reaction (PCR) was developed to amplify small numbers of unique gene regions from total genomic DNA using site-specific primers.[9] Beginning with the third cycle of amplification, each additional cycle logarithmically amplifies the number of unique-length target DNA sequences. Approximately 30 cycles amplify target genes about a millionfold to characterize multiple site-specific mutations from DNA obtained routinely by chorionic villus sampling or amniocentesis. About 60 cycles amplify target genes in single cells sufficiently to analyze specific mutations.[10] Because increased specificity is required for the analysis of single cells, protocols must be more robust for preimplantation diagnosis than for routine prenatal diagnosis.

The PCR mixtures routinely include the four nucleotides deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxyadenosine triphosphate, and deoxythymidine triphosphate; a buffer that maintains a physiologic pH and optimizes polymerase enzyme efficiency; and a heat-resistant DNA polymerase derived from bacteria growing in hot springs, such as *Thermus aquaticus* (Cetus). This polymerase must withstand heating numerous times to 95°C to denature the DNA during each cycle (described later). Because all enzymes in bacteria growing in hot springs are heat resistant, this was a logical source from which to purify a heat-resistant polymerase. The PCR also requires cycling tubes at different temperatures for multiple cycles at controlled time periods. Automated thermocyclers accomplish this task easier, faster, and even more reliably than technicians with multiple temperature heating blocks and a stopwatch did in the original experiments.

As each human cell carries 6 billion base pairs of DNA packaged in 46 chromosomes, specifically amplifying a single locus requires synthesizing primers that recognize only one locus along with a partner that recognizes another unique locus within 2,000 base pairs. Amplifying 150 base pairs of unique target DNA among these 6 billion base pairs with an efficiency of between 55% and 70% per cycle and not detecting other sequences that give extraneous fragments is the equivalent of consistently identifying and amplifying a 1-oz needle in a 160-ton haystack. The minimum primer length expected to recognize unique sequences is at least 17 base pairs ($4^{16} = 4 \times 10^9$). In practice, primers 20 to 30 base pairs long are synthesized for routine amplifications. Because primers cannot be allowed to recognize repetitive sequences, each other, or sequences within themselves, computer programs (such as Primer, version 0.5, Whitehead Institute for Biomedical Research, 1991) have been developed to choose the optimal primer sites in sequenced DNA.

The polymerase chain reaction begins with double-stranded DNA that is denatured at 90°C to 95°C for two to five minutes to provide single-stranded target DNA (Figure 3). Although the most reliable means to generate amplifiable DNA from prenatal amniocyte or chorionic villus cell DNA is to purify the DNA before beginning (R.V.L., unpublished data, March 1992), the most reliable way to amplify DNA from a single cell is to place the cell in hypotonic solution and proceed directly to PCR, which begins with denaturing the DNA.

The temperature is lowered until the high concentration of primers can specifically anneal to identical DNA sequences. Annealing to homologous sequences is minimized because mismatches have fewer hydrogen bonds between base pairs that do not hold the primer to the target as tenaciously and may also destabilize the double-stranded molecule by suboptimal tertiary structures.

The elongation step then begins at the double-stranded stable primer or target DNA locus and synthesizes a longer complementary DNA fragment from the annealed primer. The time is chosen so that the double strand can be extended past the second primer site but not so long as to generate secondary artifacts during the many PCR cycles. This completes the first cycle with the number of target sequences doubled and the amount of total DNA practically unchanged.

The second cycle (Figure 4) again denatures, anneals, and elongates unique target DNA sequences. This step, however, now generates two unique-length DNA sequences defined by the positions of both primers. The third cycle repeats the denaturing, annealing, and elongating steps but, most important, increases the number of unique sequences from two to eight. From this step onward, the number of unique-length synthesized fragments increases logarithmically. In this way it is possible to generate more unique-length DNA than the total amount of DNA originally in the beginning tube. This unique-length DNA can be sized by acrylamide gel electrophoresis, digested by restriction enzymes to detect DNA base-pair mutations or polymorphisms,[11] or analyzed by dot blot or reverse dot blot analysis to determine target gene content.[12]

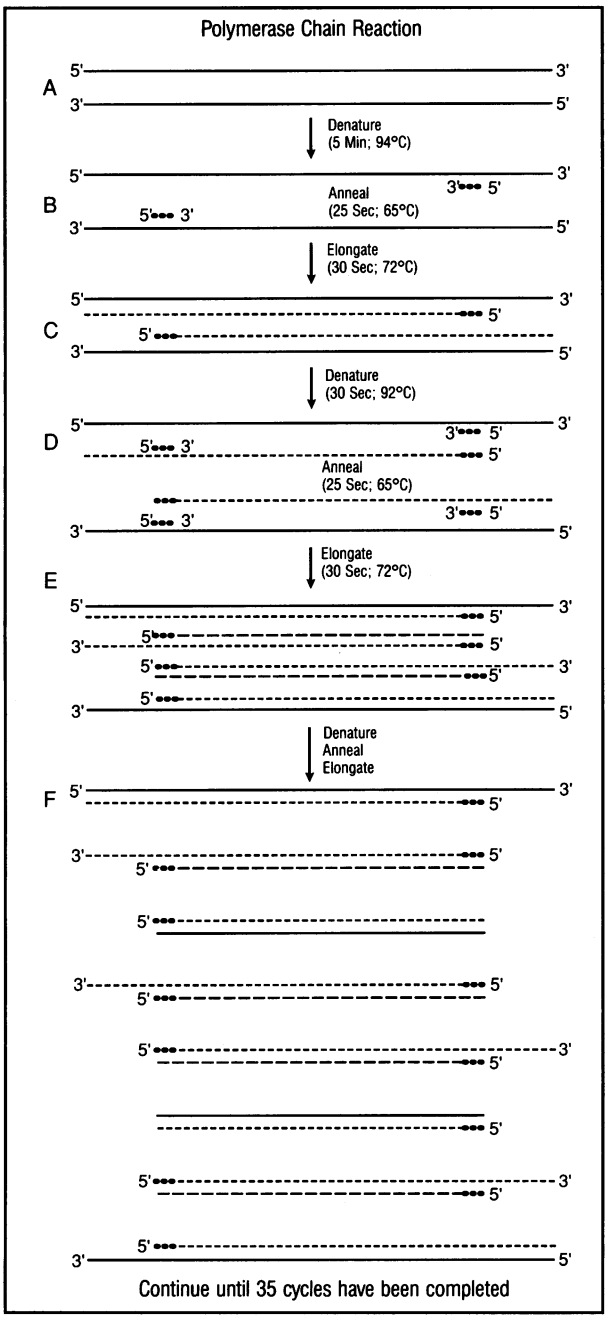

Figure 3.—A polymerase chain reaction (PCR) scheme is shown. Three complete PCR cycles amplify template sequence to 8 unique-length segments defined by primer location. Each cycle includes a denaturing, an annealing, and an elongation step.

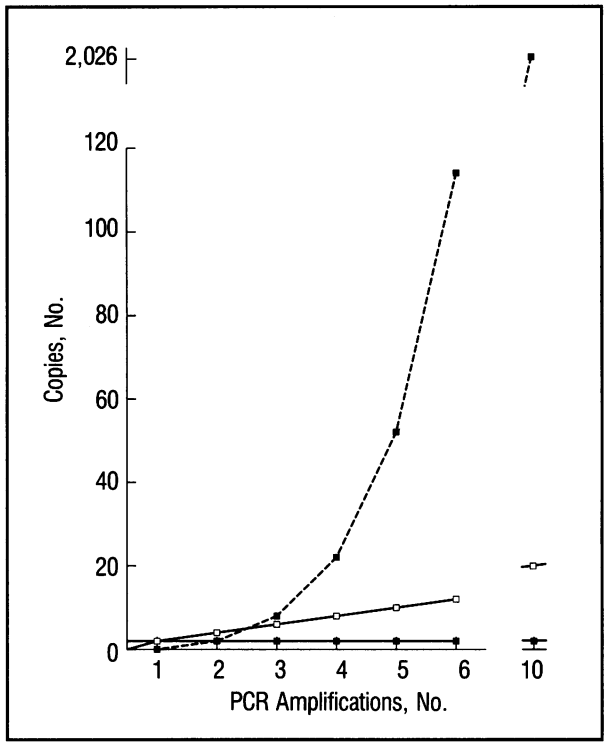

Figure 4.—The graph shows polymerase chain reaction (PCR)-amplified copies. The number of original DNA templates remains constant at 2 per haplotype, the number of longer products with a primer on one end increases linearly by 2 each cycle, and the number of unique-length sequences increases logarithmically beginning with the third amplification cycle.

This approach, or variations on it, can be used to determine the identity of alleles present in a single cell obtained by blastomere biopsy, thus leading to preimplantation diagnosis of the individual embryo.

Animal Models of Preimplantation Diagnosis

The effect of removing a portion of the early embryo on subsequent development is a major issue that had to be considered before doing preimplantation diagnosis on human embryos. Although the mouse, *Mus musculus*, is the predominant species that has been studied as a model for preimplantation embryo diagnosis, evidence from studies

of it and several domestic species indicates that there will be little, if any, lasting effect from such ablations. First, the cells of the cleavage-stage mammalian embryo (or blastomeres) appear to be developmentally equivalent and totipotent until just before the blastocyst stage.[13,14] Consequently, removing one or two blastomeres does not alter the developmental program of the early embryo, as it regulates to form a normal but smaller blastocyst. Single blastomeres of the two-cell mouse embryo (defined as one-half blastomeres) can form an intact blastocyst that develops normally to term after transfer to a foster mother with no lasting effects on an individual's size or rate of growth.[15] Although isolated one-quarter and one-eighth blastomeres of mouse embryos do not generally develop into complete blastocysts, embryos of some domestic mammals have an even greater regulatory capacity, as indicated by the development of identical siblings after the transfer of isolated one-quarter or one-eighth blastomeres to the uteri of foster mothers.[13,16] The difference between the mouse and domestic species may be the stage at which they form a blastocyst, which occurs at the 32-cell stage in mice, but at the 64-cell or later in the other species.[17] Because human embryos form a blastocyst at about the 64-cell stage,[18] they may be expected to be more similar in their regulative capacity to the domestic species than to mice. In any case, removing one or two one-eighth blastomeres from an embryo is far less a developmental challenge than forming a complete embryo from a single isolated blastomere.

Human embryos from which biopsies have been taken using the procedures described here were capable of developing to the blastocyst stage at least as frequently as unmanipulated embryos and had the same proportion of inner cell mass (destined to form the embryo proper) as trophectoderm cells (destined to form the placenta); when correction was made for the reduction in mass resulting from biopsy, they had the same number of cells at the blastocyst stage as normal embryos.[19] Therefore, biopsy procedures used in preimplantation diagnosis do not seem to substantially perturb normal embryonic development, as evidenced by successful development to term when biopsied embryos were returned to the mother by embryo transfer.[5,6]

Embryos of laboratory mammals have also been used to develop the general variables of preimplantation diagnosis. Blastomere biopsy of mouse embryos at the eight-cell stage was less detrimental to subsequent development than biopsy at earlier or later stages.[20,21] Mouse embryos biopsied at the eight-cell stage have also been cryopreserved and thawed before transfer to foster mothers, with no notable differences in the frequency of implantation or full-term development as compared with sham- and intact-control embryos.[22] Rabbit, mouse, and marmoset embryos have also been biopsied at the blastocyst stage.[23-26] Similar preclinical studies have been done with human blastocysts,[27,28] but this approach has not yet been used clinically because of the modest frequencies of human embryo development to the blastocyst stage,[18] and it is not considered further here.

Mouse embryos have been studied most extensively as a model for preimplantation diagnosis using PCR. This approach was used to diagnose homozygous embryos for the β-thalassemia,[29] the shiverer mutation in the myelin basic protein gene,[30,31] ornithine transcarbamylase deficiency or sparse fur mutation,[32] a human β-globin transgene,[33] and for sex identification on the basis of Y-specific sequences.[34-36] These studies have established PCR as an efficient and sensitive method for detecting alleles in single cells that, combined with blastomere biopsy, can be used to distinguish between normal and mutant embryos with a high degree of confidence (90% to 100%). Despite these high efficiencies of correct allele identification, continued caution is advised in transferring this approach to human embryos because of the compounding effect of errors in PCR efficiency, blastomere transfer, and inadvertent DNA contamination.[37]

In situ hybridization or other cytogenetic analyses have also been used to analyze the biopsied blastomeres of mouse embryos. In these studies 70% to 90% of embryos were successfully diagnosed with X chromosome-specific probes[38,39] or by karyotyping.[40,41] Because of the lower rates of success than with PCR and the limitation to large genetic differences that can be distinguished with these approaches, cytogenetics has not yet been used clinically for human embryos. Nonetheless, cytogenetic methods, particularly in situ hybridization, remain promising for diagnosing numerical chromosomal abnormalities in biopsied blastomeres because this cannot be done efficiently by PCR.

In sum, studies to date of animals have provided a sound preclinical basis for the continued application of preimplantation diagnosis to human embryos by PCR and will continue to serve for the development and validation of protocols for other single-gene defects by PCR and chromosomally detectable defects by cytogenetic methods.

Experience With Human Embryos

Much of the experimental analysis of human embryos has been by the use of PCR. Some initial investigations suggested the use of miniaturized enzyme systems for analyzing individual blastomeres. This approach appears not to be useful in that much of the enzyme activity in blastomeres from four- and eight-cell embryos appears to be of maternal origin. Braude and co-workers measured hypoxanthine phosphoribosyltransferase activity in individual blastomeres removed from four- to eight-cell human embryos.[42] The addition of α-amanitin to block the transcription of messenger RNA produced no change in activity. This suggests that activity at this stage is maternally inherited.

The most common analysis done on human embryos has been the determination of gender for use in the prenatal diagnosis of X-linked disorders. Handyside and associates initially reported preimplantation diagnosis from six- to eight-cell embryos in two couples at risk for X-linked adrenoleukodystrophy.[5] At the time of the report, the pregnancies were in midgestation. Recently the same

group reported on eight couples at risk for X-linked disorders.[19] Five pregnancies were achieved, two with twins and three with singleton pregnancies. Six of seven fetuses were confirmed to be female on follow-up chorionic villus sampling.

The analysis of several single-gene disorders has also been reported. The successful analysis of single human blastomeres, oocytes, or sperm has been reported for cystic fibrosis,[6,43-45] Duchenne-type muscular dystrophy,[43] and sickle cell anemia.[46,47] Handyside and colleagues reported the birth of an unaffected girl following preimplantation diagnosis for cystic fibrosis.[6]

Discussion

The successful birth of infants who have undergone preimplantation embryonic biopsy has opened a new approach to prenatal diagnosis. Many problems remain to be solved, however, before this approach can be more widely applied. The biggest problem involves the accuracy of the technique, and several issues are involved. The first is contamination of the specimen. Because PCR is being used to amplify a single cell in most cases, any extraneous DNA contamination could potentially also be amplified. Various strategies have been devised to minimize this risk, but it continues to exist. Another serious problem that is inherent to the PCR procedure is inefficient amplification. Various studies have reported amplification efficiencies using PCR with single cells of 45% to 80%.[45-48] Another amplification problem is that in as many as 29% of cases, a single allele only may amplify.[46] Several strategies to deal with these problems using coamplification have been devised.[48,49] None of these approaches completely eliminate the problem.

Another concern is the safety of the procedure to the developing embryo. As noted, "normal" infants have been born following the procedure. Too few infants have been born to know if there will be any long-term effects from this procedure, but the studies in animals argue for a minimal or insubstantial effect of biopsy on mammalian embryos in general.

This approach to prenatal diagnosis is a complicated and expensive one. The efficiency of in vitro fertilization is low, although the current statistics relate to infertile couples. The hope is that a population with normal fertility undergoing preimplantation diagnosis would have higher pregnancy rates.

Ethical issues also exist. Whereas the ethical status of the early embryo has been widely discussed, the emergence of new technologies, such as preimplantation diagnosis, raises further issues. Many people are uncomfortable with this type of early manipulation of the embryo and think that it may open the door to germ-line genetic therapy or other manipulations. Robertson feels that the selection of embryos on genetic grounds is ethically acceptable because it obviates the birth of offspring with severe genetic disorders without the need to undergo prenatal diagnosis and abortion.[50]

Clearly preimplantation diagnosis will remain an experimental procedure until these problems are solved. In addition, because of the current need for in vitro fertilization with its attendant high cost, the procedure will probably be used only by couples at high risk for having an offspring affected with a genetic disorder.

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