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New Challenges in Human in Vitro Fertilization

Robert M. L. Winston and Alan H. Handyside

This review assesses some scientific and ethical problems with human in vitro fertilization. Improved selection of viable embryos, better culture conditions, and greater understanding of the uterine environment will increase success and prevent multiple pregnancy. Further advances will also improve oocyte cryopreservation, in vitro maturation of oocytes, knowledge of sperm function, and sperm microinjection. Preimplantation diagnosis will help avoid genetic diseases and increase understanding of embryonic defects and the viability of zygotes. The greatest ethical problem with all these developments seems to be delivery of these complex treatments when health-care resources are increasingly limited.

Success Rates

Human in vitro fertilization (IVF) is surprisingly unsuccessful. In the United States, the overall birth rate per IVF treatment cycle is 14%, from 16,405 oocyte retrievals (*1*). In Britain, the live birth rate from each IVF treatment cycle started is 12.5% (*2*). Success is greater when more than one embryo is transferred simultaneously. Superovulation hopefully leads to fertilization of several oocytes, and it is common to transfer several embryos to the uterus, anticipating that at least one will implant.

Pregnancy resulted from 13% (184 out of 1436) of transfers when three or fewer embryos were transferred, 25% (238 out of 944) with four, and 26% (229 out of 871) with five or six embryos (*1*). On 154 occasions, seven or more embryos were transferred. Simultaneous transfer of multiple embryos increases the incidence of multiple pregnancy and the possibility of miscarriage and prematurity. Of triplets and quadruplets born after IVF, 64.1% and 75%, respectively, required admission to intensive care, often for weeks. Multiple pregnancy also has considerable social, economic, and psychological impact on parents. Prematurity after assisted conception was associated with a perinatal mortality rate of 27.2 per 1000 (*3*), three times the United Kingdom average for births after natural conception. The increased mortality was almost entirely due to multiple pregnancy. Consequently, it was informally agreed in Britain in 1987 that there should be limits to the number of embryos transferred at any one time, with a maximum of four embryos. In 1990, the British Government established the regulatory Human Fertilisation and Embryology Authority. Since then, most transfers have been restricted to no more than three embryos. For the last 3 years, we have seldom transferred more than two embryos simultaneously (*4*), and have been able to maintain pregnancy rates of 37 to 42% per transfer with only the occasional (<1%) triplet pregnancy.

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Can We Improve the Success Rate?

The quality of both the embryo and the uterine environment affects success. Individual human embryos only have a poor chance of development to fetal stages. After natural conception, possibly as many as 60% of very early pregnancies are lost (*5*). Most losses may be due to abnormalities of the conceptus; at least one-third of postimplantation embryos may be grossly abnormal (*6*). The situation is thought to be similar after IVF. At least 22% of eggs remaining unfertilized after insemination, and 16% of diploid two- to eight-cell embryos showed chromosomal abnormalities, particularly if some blastomeres were morphologically fragmented (*7*). As many as 30% of embryos may have genetic defects (*8*). A problem with all these studies is that karyotypic analysis of embryos is difficult; moreover, such studies have largely been done on poor quality embryos left over after transfer attempts. Women who become pregnant after embryo transfer tend to have more normal additional embryos (*9*). The spare embryos of women who miscarried tended to be chromosomally abnormal, as were 50% from women who developed ectopic pregnancy. The overall proportion of normal spare embryos was 13%, similar to the live birth rate per single embryo transferred (11%; 19 infants from 171 embryos). Thus, analysis of spare embryos might predict successful pregnancy.

Chromosomal aberration is more frequent when immature oocytes are fertilized. Superovulation decreases overall quality and maturity of oocytes and increases the number of adherent follicle cells (*10*). Superovulation preceded by desensitization of the pituitary by gonadotrophin-releasing hormone (GnRH) agonists, and reduction of luteinizing hormone concentration, before egg collection may improve egg maturation, which may in turn result in fewer miscarriages. GnRH antagonists and recombinant follicle-stimulating hormone (rFSH) may possibly help reduce the incidence of defective oocytes. However, we may have reached a biological limit in quality of embryos.

The goal for the future is the transfer of single embryos with a high chance of pregnancy (*11*). It is possible that the "best" embryos for transfer might be identifiable before transfer. Metabolic activity (*12*) or materials, such as cytokines, secreted into the culture media may be useful for predicting viability of embryos.

The success of embryo transfer after IVF decreases as the time after insemination increases. The reverse might be expected to be the case. Allowing IVF embryos to develop to the blastocyst stage should iden-

tify those embryos incapable of more advanced development. Single blastocysts fertilized in vivo and then flushed from the genital tract before transfer to another human recipient have approximately 50% chance of normal implantation and development (*13*). In spite of the theoretical advantages of blastocyst transfer, few IVF units transferring blastocysts after fertilization in vitro have much success. Culture conditions and media may need to be improved. To overcome presumed deficiencies in culture media, transfer of oocytes or zygotes directly to the fallopian tube has often been attempted either immediately before fertilization or just afterward. These treatments are sometimes used for women with intact oviducts. They include the transfer of oocytes with sperm (gamete intrafallopian transfer—GIFT) or newly fertilized zygotes (zygote intrafallopian transfer—ZIFT; pronuclear oocyte and sperm transfer—PROST). Such treatments are likely to become obsolete if culture conditions are improved, because transfer of embryos at more advanced stages of development, when their viability is more certain, will be preferable.

Co-culture of embryos with helper cells (such as tubal or uterine epithelium) may assist development (*14*) either by supplying growth factors or by removing waste products. Although co-culture is useful with cattle embryos, and may be valuable in improving human preimplantation development, the effect on pregnancy is less obvious. It is difficult to get enough epithelial cells for co-culture that are guaranteed "clean" of pathogenic viruses, such as human immunodeficiency virus. In culture, embryos may have difficulty hatching at the blastocyst stage when the zona pellucida is abnormally hardened. Hatching has been assisted by partial dissection of the zona or dissolution of areas of the zona with acidified Tyrode's solution (*15*). The results are promising, but have not unequivocally improved implantation rates, perhaps because the zona is not hardened in all embryos.

The effect of the uterine environment is less clear. Embryos transferred to an inadequately primed uterus are unlikely to implant. Treatment with superovulatory drugs may cause abnormal endometrial maturation, one possible cause of relatively poor success after IVF. This may account for the sometimes higher pregnancy rates achieved after treatment cycles with donor eggs which, though produced by superovulation, are transferred to a uterus exposed only to normal estrogen concentrations (*16*). Assessment of endometrial thickness, and thus progression through the proliferative phase, has been used to predict the likelihood of normal implantation after embryo transfer. A thick endometrium with an outer hyperechogenic layer (*17*) seems more likely to be receptive than a homogeneous or thin endometrium. This effect is probably not due to the high estrogen amounts associated with superovulation because even women hyperstimulated with excessive FSH and with grossly elevated blood estradiol concentrations maintain pregnancy.

The uterine environment at the time of embryo transfer is difficult to assess. Endometrial biopsy would be informative, but physicians have considered it unethical to sample the endometrium at such a critical stage of a complex treatment. Nevertheless, a minor endometrial injury should not prevent implantation. A biopsy at the time of embryo transfer could be analyzed morphometrically and biochemically for epithelial glycoprotein secretion. Proteins specifically expressed during the early luteal phase include placental protein 14. Leukemia inhibitory factor (LIF), which is a peptide growth factor, is expressed in endometrial glands on the fourth day of pregnancy in mice, and otherwise viable blastocysts fail to implant in mice lacking the LIF gene (*18*). It is likely that this cytokine and others will be implicated in implantation in other species, including man.

Embryo Cryopreservation

Preservation of embryos in liquid nitrogen, usually after slow cooling in a suitable cryoprotectant to −30° or −40°C, has largely been perfected and is widely welcomed as a major advance in IVF treatment. Vitrification, the suspension of embryos in solutions with a high concentration of cryoprotectant followed by plunging them in liquid nitrogen, has also been successful in mice, and recently humans, particularly before the blastocyst stage. However, such high concentrations of solute may be toxic. Whatever method is used, it is unclear to what extent transfer of frozen and thawed embryos improves pregnancy rate after transfer of fresh sibling embryos has already failed. This is because sibling embryos will be derived from oocytes maturing in the same, potentially deficient, endocrine environment. In any case the benefits may be less than widely believed, not least because freezing is labor-intensive and relatively costly. Cryopreservation is undoubtedly justified in certain situations, for example, for women with tumors who will be sterilized by oncologic therapy, women who suddenly fall ill during an IVF cycle, and possibly for older women for whom time is running out. Until there is absolute certainty that the cryoprotectants widely used in embryo freezing cause no long-term genetic effects, it seems preferable to regard cryopreservation as experimental.

Oocyte Donation

Donation of fresh oocytes from healthy donors is used in women with premature menopause, a relatively common cause of infertility, or ovarian agenesis or dysgenesis. Many young women request oocyte donation following radiotherapy and chemotherapy as treatments for leukemia or Hodgkin's disease. Other candidates for oocyte donation are those persistently unresponsive to superovulation and carriers of X-linked or autosomal dominant genetic defects. Success rates after oocyte donation exceed those after other methods of assisted conception, probably because oocytes are generally obtained from young women, and possibly because the uterine milieu of the recipient is not exposed to the hormonal changes induced by superovulation. Embryo transfer appears most successful in amenorrheic women stimulated with physiological amounts of added estrogen and progestogens. Donated oocytes resulted in pregnancy in 37% of transfers to the uterus after IVF (mean number of embryos per transfer, 3.6), 48% of transfers as oocytes to the fallopian tubes (*n* per transfer, 3.8), and 57% of transfers to the tubes after IVF (*n* per transfer, 3.9) (*19*). Women with idiopathic primary ovarian failure were more successful than those with iatrogenic ovarian damage. Although tubal transfer tends to be more successful, uterine transfer seems better in women with gonadal dysgenesis, perhaps because such patients may have defective tubal transport. The youth of donors is important, but older recipients are as successful as young ones, suggesting that the youth of the oocyte is more important than the youth of the uterus. It is this factor which almost certainly accounts for the poor success rates of routine IVF in older women given their own oocytes (Fig. 1).

Important ethical issues are raised by oocyte donation. First, who are suitable donors? Of five possible sources for donated oocytes, each raises difficult ethical issues (Table 1). Another issue is the age of the prospective parents (*20*). Considerable success has been reported in naturally postmenopausal women aged 50 to 59 given oocytes donated by women 15 to 20 years younger. It is difficult to believe that this is a sensible use of technology. Such parents will be of an advanced age when their children are not yet teenage, and there is concern that this cannot be in the best interest of children. A continuing caring parental relationship may not be forthcoming as parents get older and less able to supervise a vigorous, growing child. Becoming a parent is more emotionally stressful and physically demanding than many infertile couples appreciate and, although pregnancy is commenced with good

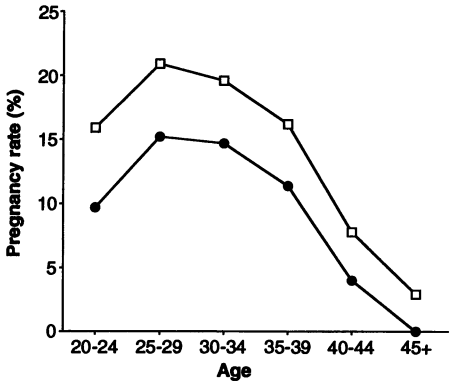

Fig. 1. IVF success rate declines with the age of the woman (*2*). Open squares, pregnancies; closed circles, live births.

intentions, the arduous nature of having a baby when one is in middle age may not be fully realized by couples considering oocyte donation. Moreover, the risks of pregnancy increase in older women and the desperation of a menopausal patient to have children should not override the very real need for most careful counseling.

Cryopreservation of Oocytes

Considerable energy has been spent in finding effective methods to cryopreserve human oocytes. Many of the ethical difficulties (Table 1) surrounding oocyte donation would be overcome were cryopreservation possible, because surplus eggs donated by superovulated infertile IVF patients could be held until treatment was completed by a confirmed pregnancy. Following this, spare, frozen oocytes could be thawed for fertilization by the sperm of the partner of a suitable recipient with ovarian failure. Such a strategy would also avoid problems posed by possible infection by human viruses.

Although human oocytes have been successfully frozen and thawed—indeed, a pregnancy has been reported (*21*)—there are serious concerns regarding the safety of human oocyte freezing. The main problem is that the mature oocyte is arrested at the metaphase II stage of meiosis, when chromosomes are arranged on the temperature-sensitive microtubular spindle. Disruption of this structure may cause loss of chromosomes. Some studies, mostly in mice, have suggested that nondisjunction does not occur after cryopreservation, but others (*22*) found a threefold increase in aneuploidy. It is possible that the particular cryoprotectant used is crucial; currently dimethyl sulfoxide seems the most promising. However, caution is required because the effect on chromosome segregation may vary between species, as the cytoskeletal architecture varies between mouse and man. Mitotic division may also be affected by aneuploidy and this could generate abnormal blastomeres. Polyploidy occurs frequently after oocyte cryopreservation (*23*). This is likely to be digynic polyploidy, with retention of the second polar body because of disruption of the meiotic spindle. Frozen oocytes may be also more susceptible to polyspermic fertilization. Fertilization is frequently impaired after oocyte cryopreservation. The mechanisms that inhibit fertilization may include changes in the zona pellucida, possibly zona hardening, and changes in sperm receptors on the surface of the zona. The problems associated with oocyte freezing seem solvable and it is probable that cautious attempts to fertilize and transfer embryos after storage of frozen oocytes will soon result in clinical success.

Table 1. Implications of donated eggs.

Source	Main advantage	Main disadvantage
IVF patients	Oocytes readily available without extra risk to the donor	Recipient may get pregnant at the expense of donating patient whose treatment fails
Relations or close friends	Often highly motivated to help infertile couple; therefore may be readily available	Child may become involved in serious family conflicts
Gynecologic patients	Oocytes can be collected without extra surgical procedure	Usually in age group when there is extra genetic risk of oocyte abnormality
Altruistic members of public	Anonymity easy to protect	Complex and difficult recruitment unless (possibly unethical) financial incentive

In Vitro Maturation of Oocytes

An exciting clinical advance is in vitro maturation of immature human oocytes. This would solve many problems associated with oocyte donation and could be used for women whose ovaries respond very poorly to superovulatory drugs, or for women whose ovaries are largely destroyed by endometriosis, inflammation, or neoplasia. One square millimeter of ovarian cortex taken from a young woman contains hundreds of primordial follicles—possibly more eggs than would be ovulated during an entire reproductive lifetime. This rich source could be exploited if there were means to mature such eggs in vitro. Four possible strategies by which immature oocytes might be matured in vitro and subsequently fertilized have been proposed (*24*).

Biopsied ovarian tissue may be cultured in vitro but has a high oxygen consumption rate, and the low ratio of surface area to volume makes adequate oxygenation in culture difficult. Immature ovaries are another possible source; they are more easily perfused. Fetal ovaries could also potentially be used as a source of oogonia. The use of such fetal tissue from abortuses raises all kinds of ethical problems and is bound to be fraught with debate. Depleted adult ovaries could also be repopulated by grafted fetal oogonia. Another strategy is to use thin cortical slices of ovary perfused by hyperbaric oxygen. Meiotic maturation has been observed in such explants. Isolated follicle culture seems more promising. Follicles are relatively avascular and therefore the nutrient and oxygen gradients that occur with larger pieces of tissue are not such a problem. Isolated follicles can be dissected from the ovary together with some adhering stroma-theca tissue; the follicles remain arrested in the absence of adenosine 3′, 5′-monophosphate and resume maturation after exposure to luteinizing hormone. Following culture in microdroplets of suitable media, approximately 60% of mouse follicles will reach the size of Graafian follicles (*25*). At present it is uncertain whether oocytes matured under these conditions could give fertile zygotes. Oocytes may also be matured in granulosa cell complexes. Many preantral follicles can be recovered after disaggregation in protease or collagenase. Although normal follicular morphology is lost in culture, resumption of oocyte meiosis, fertilization, and embryonic development have been observed (*26*).

The acme of oocyte culture technology would be the growth of isolated, naked, primordial oocytes to full maturity. Various attempts to achieve this have failed, even with conditioned media. Oocytes need to synthesize macromolecules and have a high energy requirement. Also macromolecules are transported into the maturing oocyte from surrounding cells. Separating them from the granulosa cell layer may obviate all chance of normal development.

Sperm Function and Sperm Microinjection

Poor function of spermatozoa accounts for one-quarter of all human infertility; IVF is one of the only effective treatments. Failure of fertilization is a frequent outcome of routine IVF treatment for male infertility. IVF generates useful research information

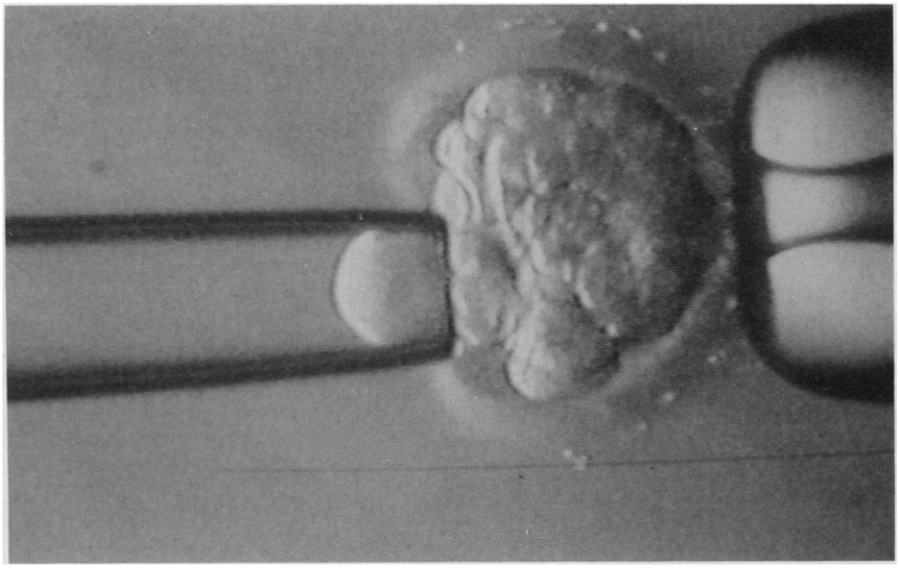

Fig. 2. Cleavage stage biopsy of a 12-cell polyspermic human embryo 3 days after fertilization. The embryo is immobilized on a flame-polished holding pipette and a sampling pipette is pushed through a hole drilled in the zona pellucida to aspirate a single cell. Photograph from a video recording (×60). The diameter of the human embryo at this stage of development is about 140 micrometers.

about the various stages of fertilization, under varying physiological and pharmacological conditions. Computer analysis of sperm movements, studies of oocyte-sperm interaction, and analysis of reactive oxygen species and creatinine phosphokinase produced in cases of impaired male fertility (*27*) all seem potentially useful.

One revolutionary approach has been micromanipulation of the egg to enhance fertilization. Improved fertilization has been observed after the zona pellucida was disrupted by drilling holes (*28*) or by partial dissection (*29*). Sperm have also been injected directly into the perivitelline space. Of 307 patients who had all previously failed fertilization after attempted IVF, almost 50% achieved fertilization with microinjected sperm (*30*). The pregnancy rate was 23% per embryo transfer. Several sperm were injected under the zona to increase the chance of fertilization. All eggs that subsequently appeared to be unfertilized or polyspermic were discarded. Single sperm have also been injected directly into the ooplasm (*31*). Although there are obvious problems with this, such as potential damage to the chromosome spindle, the method holds considerable promise. All microsurgical methods of enhancing fertilization presumably threaten genetic risks, because abnormal sperm may be inadvertently injected.

Preimplantation Diagnosis

The incidence of genetic diseases resulting from identifiable genetic defects is about 1 to 3%. Chromosomal abnormalities predominate over single gene defects, of which close to 5000 are known. IVF with preimplantation genetic diagnosis (PD) allows for identification of unaffected embryos before transfer to the uterus. The hope is that the fetus should be unaffected by the disease and termination of a pregnancy avoided.

There are two basic approaches to the detection of genetic defects before implantation. One is the removal of the first polar body from the unfertilized oocyte, but this procedure is only informative for maternal genes. The other involves biopsy of one or more cells from a developing embryo at the cleavage (Fig. 2) or blastocyst stage. To minimize the reduction in embryonic mass involved in removing a single cell, embryos have been biopsied as late as possible—at the eight-cell stage—leaving only 8 to 12 hours for genetic analysis before transferring selected embryos on the same day. This does not appear to affect preimplantation development (*32*).

The time and numbers of cells available for genetic analysis place restrictions on the methods used. For chromosomal abnormalities, fluorescent detection of in situ hybridization (FISH) with chromosome-specific DNA probes is sensitive and rapid and can be used for analysis of interphase nuclei (*33*). Multicolored fluorescence can be used for the simultaneous detection of several probes, and mixtures of short DNA probes for a particular chromosomal region can be used for "chromosomal painting" and detection of translocations and other structural abnormalities. For single-gene defects, the polymerase chain reaction (PCR) enables amplification of short fragments of DNA over a millionfold within a few hours, making it possible to detect even single base changes in the DNA of single cells.

FISH has been applied to analysis of both human oocytes and preimplantation embryos with chromosome-specific DNA probes. Use of several chromosome-specific probes simultaneously raises the possibility of screening the embryos of older women for common trisomies (*34*). Indeed, simultaneous detection of the X and Y chromosomes is already possible (*35*). The most frequent autosomal trisomies found prenatally involve chromosomes 21, 18, or 13; these trisomies are more common in the pregnancies of older women. Each of these trisomies is compatible with development to term, but the development may be abnormal and both fetus and newborn have higher rates of death than do those with the normal diploid chromosome complement.

FISH for PD of aneuploidy of chromosome 18 has been evaluated with a probe specific for the centromeric region of that chromosome (*36*). Although most interphase nuclei had the expected diploid number of two signals, others had fewer or more signals. This may arise through technical artifacts. Analysis of several nuclei would be needed, therefore, when only a single probe is used. It is interesting that a number of tetraploid nuclei with four hybridization signals were also present, possibly representing precursors of polyploid trophectoderm cells (*37*).

About 300 X-linked recessive conditions have been described, the most frequent being Duchenne muscular dystrophy (DMD). Prenatal diagnosis of males affected by DMD is possible by multiplex amplification of frequently deleted exons. Some of the mutations are not well characterized, however, and in those families identifying sex is all that can be done. The sex can be determined in embryos at the cleavage stage by amplification by PCR of Y-specific repeat DNA sequences from single cells. Healthy births, including two sets of twins, have followed this approach in couples at risk of X-linked conditions (*38*). A singleton pregnancy has been established following transfer of a female embryo identified by coamplification of X and Y alphoid DNA sequences in a couple at risk for hemophilia A (*39*).

In situ hybridization with X- and Y-specific DNA probes can be used to identify the sex of each embryo and is less susceptible than PCR to misinterpretations caused by contamination. The strength of in situ hybridization lies in simultaneous detection of both probes, with the use of different colored fluorescence probes. This avoids potential errors due, for example, to hybrid-

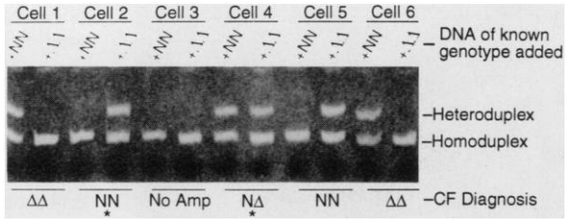

Fig. 3. Preimplantation diagnosis of the predominant ΔF508 deletion causing cystic fibrosis by polyacrylamide gel electrophoresis of PCR products from single cells biopsied from cleavage stage embryos. Additional slow-migrating heteroduplex bands were present when DNA amplified from cells of known genotype was mixed with the unknown amplification product. Asterisks denote analysis of cells from embryos that were later transferred; implantation of the second embryo produced an unaffected girl. NN, homozygous normal; NΔ, heterozygous carrier; ΔΔ, homozygous for deletion (*40*).

ization failure of one or other probe. With dual FISH, only if two X signals or one X and one Y signal are detected is the sex confidently predicted to be female or male, respectively. Several pregnancies have been established after dual FISH, and one normal female born.

Specific diagnosis is being attempted for prevalent single gene defects, especially those caused by a predominant or a limited number of mutations. PCR is used to amplify a fragment of DNA containing the defective sequence. Amplification from single cells has been achieved by developing sensitive, nested-primer strategies in which a second, shorter fragment is reamplified. Several groups have now succeeded in amplifying, from a variety of single cells, by PCR the region of the cystic fibrosis transmembrane conductance regulator gene that encompasses the ΔF508 deletion indicative of cystic fibrosis. The deletion is then detected by heteroduplex formation, that is, the formation of double-stranded DNA from a normal single strand and a deleted single strand, followed by gel electrophoresis to analyze the hybrid (Fig. 3). Migration of the heteroduplex is retarded relative to a normal double-stranded fragment, and the genotype of the cell can be deduced from the presence or absence of the heteroduplex bands in comparison with various mixtures with amplified fragments from homozygous normal or affected cells. PD of cystic fibrosis has in that way been attempted in three couples where both parents carry the ΔF508 deletion (*40*). Carrier and affected embryos have been identified, and unaffected or carrier embryos transferred. One woman became pregnant, delivering a healthy baby free of both deleted alleles.

Appropriation of Resources

The most significant problem concerning these advances in IVF-related technologies is the allocation of hard-pressed medical resources. In Britain, during the last 6 years, fewer than 5,300 babies were born following IVF, but there may be as many as 600,000 infertile couples (*41*). In 1990 in the United States, a total of 25,744 stimulation cycles were reported to the IVF–Embryo Transfer Registry. From these, there were 5,150 pregnancies and just under 4,000 live births. Assuming that approximately 10% of couples suffer infertility involuntarily, an estimate based on a number of different clinical studies, and allowing for the possibility that at least 50% of the population of childbearing age will at some time desire a pregnancy, upwards of 2 million women in the United States may at some time be candidates for IVF treatment. Considering that IVF currently has a success rate of only 10 to 15%, only a tiny proportion of the North American population in need currently has access to assisted reproductive treatments. Clearly, the situation is far worse in the developing world, where infertility often causes huge social and psychological problems. Although there are fewer couples known to be at risk of genetic disease, the use of IVF for PD will place an additional burden on resources. All these treatments remain in the realm of privileged therapy only available to relatively few couples, who are mostly in a sufficiently sound economic position to afford it.

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42. We are grateful to many colleagues who have discussed these issues with us, in particular K. Hardy, J. Conaghan, K. Dawson, and R. A. Margara. We particularly express gratitude to the Associazone Donatella Flick which has generously supported much of our work on the preimplantation diagnosis of genetic defects described in this paper.