

Birth After Preimplantation Diagnosis of the Cystic Fibrosis $\Delta F508$ Mutation by Polymerase Chain Reaction in Human Embryos Resulting From Intracytoplasmic Sperm Injection With Epididymal Sperm

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Men with congenital bilateral absence of the vas deferens (CBAVD) have been regarded as presenting a mild form of cystic fibrosis (CF). In this article, we report a case of male-factor infertility, in which both partners are carriers of the $\Delta F508$ mutation and the male partner has CBAVD. Microsurgical epididymal sperm aspiration (MESA) was performed to obtain spermatozoa; intracytoplasmic sperm injection (ICSI) was carried out on the oocytes since the motility of the spermatozoa was severely impaired; and embryo biopsy and a polymerase chain reaction (PCR) were carried out for preimplantation diagnosis of the CF $\Delta F508$ mutation. Single-blastomere analysis was performed and indicated that two embryos were affected (homozygous $\Delta F508$) and three embryos were carriers. After transfer of the latter three embryos, a singleton pregnancy was established. At amniocentesis, the $\Delta F508$ carrier status of the fetus with a 46, XY karyotype was confirmed. A healthy boy was born and the presence of vasa deferentia, bilaterally, was confirmed. The CF sweat test was also normal. Successful fertilization can be obtained by combination of MESA and ICSI in patients with CBAVD. Preimplantation diagnosis of CF is indicated. Pregnancy and birth of normal children can ensue in such patients.

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CONGENITAL bilateral absence of the vas deferens (CBAVD) is a well-known cause of male infertility. More than 20 years ago, it was suggested that CBAVD might be a particular form of cystic fibrosis (CF) since obstructive azoospermia and absence of the vas deferens are the principal pathological findings for male infertility in CF. Recently, it has been demonstrated that men with

CBAVD have a higher than normal frequency of the most common CF mutation $\Delta F508$, thus confirming a possible link between CF and CBAVD.¹⁻² More recently, the detection of compound heterozygotes and homozygotes for CF mutations among men with CBAVD has led to the conclusion that CBAVD is a primarily genital form of CF in otherwise normal, healthy men with no other phenotypic manifestations of CF.³⁻⁵

Infertility in couples caused by CBAVD in the male has long been considered untreatable. However, the development of microsurgical epididymal sperm aspiration (MESA) in combination with in vitro fertilization (IVF) technology has opened up new possibilities for such couples and has led to pregnancies and the birth of healthy chil-

dren.⁶⁻⁸ The high carrier frequency for CF (about one in 25 in the white population), together with the observation that men with CBAVD may have CF, are both conducive to caution when performing MESA and IVF. Both partners should be screened for CF mutations to evaluate the couple's risk of having a child with severe CF; high-risk couples should be informed about the possibilities of early prenatal diagnosis. In such couples, preimplantation diagnosis is indicated since an IVF procedure has to be performed anyway.

In this article, we report on a couple in which both partners carry a $\Delta F508$ CF mutation and the man has CBAVD. After MESA, epididymal sperm was used for the fertilization of oocytes by intracytoplasmic sperm injection (ICSI). The ICSI technique was preferred to regular IVF, since the latter technology yields rather poor fertilization and pregnancy rates, particularly when sperm motility is significantly impaired. After preimplantation diagnosis for the $\Delta F508$ mutation, three $\Delta F508$ carrier embryos were replaced in the woman's uterus and a pregnancy ensued. A phenotypically normal, non-CF boy was born at term and the presence of both vasa deferentia was observed.

Materials and Methods

Patients.—The patients were a healthy couple, both 36 years old, with a diagnosis of male infertility caused by CBAVD. Both were screened for the presence of 12 mutations in the CF transmembrane conductance regulator (CFTR) gene⁹: $\Delta F508$, G542X, G551D,

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Embryo	Stage	No. of Blastomeres Removed	No. Amplified After PCR†	Results	Clinical Decision
1	4-cell	1	1	Homozygote Δ F508	Not used
2	7-cell	1	1	Δ F508 carrier	Transferred
3	7-cell	1	1	Δ F508 carrier	Transferred
4	5-cell	1	1	Homozygote Δ F508	Not used
5	8-cell	2	1	Δ F508 carrier	Transferred

*No amplification signals were observed in blanks.

†PCR indicates polymerase chain reaction.

R553X, W1282X, N1303K, Δ I507, 1717-1 G to A, R560T, S549N, R117H, and 621+1 G to T. Both were found to carry the Δ F508 mutation, but none of the other mutations. After genetic counseling of the couple with regard to their risk of having a CF child, they gave informed consent to perform preimplantation diagnosis for the Δ F508 mutation. This study was approved by the Institutional Ethics Committee.

MESA and Sperm Preparation.—The details of the MESA procedure have been described previously.⁷⁻⁸ The semen characteristics were assessed by standard procedures for sperm density, motility, and morphology. The spermatozoa were then washed once in Earle's medium containing 3% bovine serum albumin (BSA) and centrifuged at 1800g for 5 minutes. The supernatant was discarded and the sperm suspension was put on top of a two-layer Percoll gradient (95% to 47.5%). After centrifugation, the 95% Percoll fraction was removed and mixed with 3 volumes of Earle's medium to remove the Percoll particles. This sperm suspension was used first for the injection procedure and the remainder was cryopreserved.

Oocytes and ICSI Procedure.—Twelve oocytes were retrieved after ovarian stimulation by an association of gonadotropin-releasing hormone analogs and human menopausal gonadotropins.⁹ The cumulus and corona cells were removed as previously described.¹⁰⁻¹¹ Intracytoplasmic sperm injection was carried out on all 12 oocytes that had extruded the first polar body.

The details of the ICSI procedure have been previously described.¹⁰⁻¹² After injection, the injected oocytes were transferred into 25- μ L droplets of B2 medium under lightweight paraffin oil (29436 SH, British Drug House, Dorset, England) and incubated at 37°C in an atmosphere with 5% O₂, 5% CO₂, and 90% N₂. About 16 hours after ICSI, the injected oocytes were examined carefully under the inverted microscope, in order to observe whether they were intact and fertilized. Normal fertilization was assumed to have occurred when two clearly distinct pronuclei were present.

After another 24 hours of in vitro culture, the fertilized oocytes were examined for their cleavage. Four- to eight-cell embryos were then used for embryo biopsy and preimplantation diagnosis by PCR.

Embryo Biopsy and PCR Assay for the Region of the CF Δ F508 Mutation.—One or two blastomeres were removed from each embryo by micromanipulation and checked for the presence of a nucleus.¹³⁻¹⁴ After removal of the blastomeres, single blastomeres were put into PCR tubes containing 20 μ L of distilled water. Each of the blastomeres had its own blank, which was taken from the washing droplet of the blastomere. Polymerase chain reaction tubes containing blastomeres or blanks were stored at -20°C until the PCR assay. Two consecutive PCRs with nested primers were performed. The details of these PCRs and the analyses of the resulting products have been reported before.¹⁴⁻¹⁶

Results

After MESA, the sperm concentration was 14.3×10^6 /mL, the total motility was 2%, and the morphology of the spermatozoa was normal. Twelve oocytes were retrieved. All were injected by the ICSI technique and 11 oocytes (92%) remained intact after injection. Five of the 11 oocytes (45%) showed normal fertilization 16 hours after ICSI and these cleaved to the 4- to 8-cell stage another 40 hours later. From each embryo one or two blastomeres were removed by micromanipulation. All five embryos remained intact after biopsy. Single-blastomere DNA was analyzed by PCR (Table). After PCR, the results indicated that two embryos were affected (homozygous for the Δ F508 mutation) and three embryos were carriers of the Δ F508 mutation. In one blastomere, no DNA amplification was obtained by PCR. None of the six blanks showed any signal of false-positive amplification. The three carrier embryos were morphologically sound and were transferred into the uterus of the female patient on the third day after ICSI. A pregnancy test was done 14 days after embryo

transfer. The human chorionic gonadotropin in the blood increased to 236 IU/L and to 519 IU/L on day 16 (human chorionic gonadotropin in blood is <10 IU/L in nonpregnant women). On day 30, one embryo and a yolk sac were observed and a normal heartbeat was present. At 16 weeks and 3 days of gestation, an amniocentesis was carried out and the result showed (1) a normal 46, XY karyotype and (2) the heterozygous carrier status for the Δ F508 mutation. In April 1994, a Δ F508 carrier boy was born at term and weighed 2700 g. At 3.5 months a sweat test was performed with normal results (sodium, 18 mmol/L; chloride, 30 mmol/L). Physical examination revealed the presence of vasa deferentia, bilaterally.

Comment

To our knowledge, this is the first report on the combined use of MESA, ICSI, and preimplantation diagnosis for the CF Δ F508 mutation in a couple with male infertility caused by CBAVD. For fertilization of the oocytes with epididymal sperm, the ICSI technique was used instead of standard IVF. Both fertilization and pregnancy rates are greatly reduced in regular IVF using epididymal sperm with severely impaired motility from CBAVD men. The sperm motility of our patient was severely impaired (2%). In our experience, such couples have never achieved fertilization or pregnancy through MESA and conventional IVF. A low number of motile sperm is often an exclusion criterion for couples having regular IVF. On the other hand, high fertilization and pregnancy rates are observed in such couples after ICSI.¹⁷ For this reason, ICSI, which seems to be independent of sperm characteristics, was used for fertilization of this patient's oocytes.

Of 12 retrieved oocytes, one (8%) was damaged by the injection procedure. This number corresponds well to the number of damaged oocytes found in a large series of ICSI procedures conducted in our center.^{11,12} In contrast, the fertilization rate (five embryos from 11 oocytes [45%]) was lower than that obtained in this series (71.2%), although it was close to our standard IVF rate of 55% and much higher than the 7% fertilization rate in IVF with epididymal sperm from men with CBAVD.

About 55 hours after the ICSI procedure, the five embryos developed to the 4- to 8-cell stage and one or two blastomeres were removed from each embryo for PCR analysis. We have previously described a reliable and sensitive PCR method for the analysis of the region containing the CF Δ F508 mutation in blastomeres.¹⁴⁻¹⁶ This same pro-

cedure was used successfully for the diagnosis of the five embryos, as a result of which two homozygous $\Delta F508$ and three heterozygous carriers of the $\Delta F508$ mutation were found. No noncarriers of the $\Delta F508$ were found, contrary to what would be expected from the autosomal recessive pattern of inheritance of CF. Of course, the number of embryos analyzed is low and this observation will probably be due to chance.

The three carrier embryos were replaced in the uterus less than 10 hours after the start of the preimplantation diagnosis procedure and a singleton pregnancy resulted, confirmed by human chorionic gonadotropin values and ultrasound. Amniocentesis, carried out at 16 weeks and 3 days of gestation, confirmed the $\Delta F508$ carrier status of the fetus and indicated a normal 46, XY karyotype. A healthy boy was born at term. At 3.5 months of age, a normal sweat test was obtained, and the presence of both vasa deferentia was confirmed.

The injection of a single spermatozoon may also have an advantage for the PCR procedure. Since in ICSI the oocytes are not brought into contact with several thousands of motile spermatozoa, the possibility of contamination by DNA of sperm origin is impossible.

One important question remains with regard to the phenotype of the $\Delta F508$ carriers as related to CF. The increased frequency of CF mutations in men with CBAVD, together with the finding of several compound heterozygotes among them, has led to the suggestion that these patients represent a special form of CF primarily characterized by a genital phenotype. In our male patient who has no phenotypic symptoms of CF, only a $\Delta F508$ mutation was found, but he still

might be a carrier of a second rare mutation in the CFTR gene. Consequently, some of the three $\Delta F508$ carrier embryos replaced in the uterus might be compound heterozygotes for CF with a $\Delta F508$ mutation from their mother and a rare CF allele from their father. Before the preimplantation diagnosis, the patients were seen in two genetic counseling sessions. They were told that we would preferentially replace non- $\Delta F508$ carriers, but if this were not possible, as was the case here, $\Delta F508$ carriers could be replaced, with the concomitant risk of male offspring with CBAVD or children of either sex with more severe CF. The patients wished to take this risk because the man was completely free of symptoms of the pulmonary and pancreatic phenotype of CF and because of their 10 years of infertility. Indeed, the risk of having children with more severe CF cannot be excluded. For instance, four males with CBAVD have been described with a genotype $\Delta F508/R117H$, which is a well-known CF genotype. These men were described as being free of symptoms of CF. However, in a recent study by the Cystic Fibrosis Genotype/Phenotype Consortium,¹⁸ CF patients with this genotype were compared with age-matched $\Delta F508$ homozygous patients and no significant statistical difference was found between these two groups with regard to the severity and course of pulmonary disease. On the other hand, $\Delta F508/R117H$ patients were pancreatic-sufficient, were older at diagnosis, and had lower sweat chloride concentrations than the $\Delta F508$ homozygotes. Apart from these, all other parameters studied showed a wide variability in each group (mild vs severe CF phenotype) and were not statistically

different. A detailed study of CF patients with the $\Delta F508/R117H$ genotype and men with CBAVD with the same genotype will be necessary to clarify the extent of clinical overlap in these two groups of patients. However, the clinical variability in CF patients with this genotype suggests that in some cases a rare CF mutation from a CBAVD man in combination with a $\Delta F508$ mutation from the female partner may lead to a more severe CF phenotype in the progeny. In a recent effort, we were not able to find a second mutation in the father by screening most of the CFTR gene. We also looked for informative intragenic and extragenic polymorphisms to discriminate between the alleles of the parents and to correlate these with the alleles found in the two affected (homozygotes for the $\Delta F508$ mutation) embryos, which were frozen after preimplantation diagnosis. These attempts were also unsuccessful in finding genotype differences in the parents. Since CBAVD was not present in the baby boy, it seems most likely that he has inherited the $\Delta F508$ mutation from his father and a normal CF gene from his mother. However, further genotype studies will be required to confirm this.

In summary, successful fertilization can ensue from a combination of MESA and ICSI in patients with CBAVD even if the quality of spermatozoa is severely impaired. Preimplantation diagnosis of CF is indicated and pregnancy and birth of normal children can result in such patients.

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