nancy following IVF in an unstimulated cycle in a patient failing to respond to ovarian hyperstimulation (6).

Our results indicate that, despite the great expense and effort, ovulation induction cycles, which appear to be similar to natural cycles in the low number of oocytes retrieved, do not result in better clinical outcomes. In fact, the greater number of oocytes retrieved in Group I results in significantly lower fertilization and implantation rates. This suggests that oocyte and embryo quality are lower in stimulated cycles of poor responders than in natural cycles. A decrease in endometrial receptivity cannot be completely discounted because of the significantly reduced implantation rates, despite similar pregnancy rates for each group. The similar pregnancy rates could be explained on the basis of a Type II error (β error) due to our small sample size, especially in light of the reduced implantation rates, suggesting a decrease in endometrial receptivity for all patients using human menopausal gonadotropins. Therefore, because of suboptimal stimulation protocols, patient reluctance to proceed with ovum donation, and the advantages the natural cycle offers including acceptable pregnancy outcome, it appears to be better to proceed to natural cycles than to waste time and financial resources with controlled ovarian hyperstimulation in patients who are under 40 years of age and without male factor.

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Amplification of the RB1.20 Polymorphism in Single Spermatozoa

Submitted: July 9, 1996

Accepted: November 11, 1996

INTRODUCTION

The RB1.20 polymorphic locus identified in intron 20 of the retinoblastoma (RB1) gene (1) is a length polymorphism consisting of a variable number ($n = 14-26$) of the repeat {CTTT(T)}_n. This class of short tandem repeats (STR) is usually highly informative, thus providing valuable tool for DNA linkage studies. Brandt *et al.* (2) described a nonradioactive protocol for detecting the RB1.20 polymorphism in the genomic DNA extracted from peripheral blood lymphocytes

(PBL), for routine diagnostic use for predictive testing in familial retinoblastoma.

Recent articles have postulated a non-Mendelian transmission of defective RB1 alleles when the carrier is the male (3, 4). Familial studies do not allow accurate verification of this hypothesis because of the limited size of human pedigrees. Direct analysis of the genetic status of individual spermatozoa from retinoblastoma transmitters is an original alternative to familial survey for the analysis of meiotic segregation. For statistical considerations, such study requires the analysis of thousands of meiotic products and constitutes a very intensive work. The RB1.20 marker, with an observed heterozygosity frequency of 90%, has been chosen to study the segregation of mutant RB1.20 gene in the sperm of a male who transmitted the disease to his offspring. We have established the PCR conditions that ensure RB1.20 amplification from a single cell. We have also optimized the electrophoresis parameters to reduce drastically the length of the run (from 5 to 1 hr), as a large sample of spermatozoa has to be analyzed.

MATERIALS AND METHODS

The individual we studied was affected with a unilateral retinoblastoma and a unilateral retinoma (a regressed form of retinoblastoma). He has transmitted the disease to his two sons.

Single spermatozoa were isolated by fluorescence-activated cell sorting using a FACStar^{PLUS} cell sorter (Becton Dickinson, Oxnard, CA), in microtubes containing 5 μ l of an alkaline lysis solution (200 mM KOH, 50 mM DTT). This step allows release of the DNA molecule from the sperm head. After 10 min of incubation at 65°C, reactions were neutralized by the addition of 5 μ l 900 mM Tris-HCl, pH 8.3, 300 mM KCl, 200 mM HCl (5). The RB1.20 microsatellite was then amplified in two successive rounds of PCR, using Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, CT).

The PCR reaction mixes contained 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.01% (w/v) gelatin, a 200 μ M concentration of each dNTP, 0.6 μ M primers 310 and B103 (described in Ref. 2), and 1.25 U *Taq* DNA polymerase (Perkin Elmer Cetus), in a final volume of 50 μ l. After 4 min of denaturation at 96°C, the DNA was amplified for 40 cycles (20 sec at 94°C, 20 sec at 54°C, and 40 sec at 72°C), followed by 10 min at 72°C.

Two microliters of the first-round products was transferred to other microtubes containing 48 μ l of

fresh PCR mix (as described above but with a final KCl concentration of 50 mM and two nested primers). The primers for this second round of PCR were selected according to the previously published sequence of the RB1 gene (6): BF 5'-CTT CAC CTT CTC TCC TCC CTA C-3' and BR 5'-GGG TAA CAG AGT GAG ACT CTA TC-3' at 0.6 μ M). All primers were synthesized on an Oligo 1000 DNA Synthesizer (Beckman, Fullerton, CA). For the nested PCR, 35 cycles consisting of 20 sec at 94°C, 20 sec at 66°C, and 40 sec at 72°C, followed by an additional 10 min at 72°C, were performed. Ten microliters of the final PCR products was mixed with 2 μ l of gel loading buffer, then loaded onto a 12% polyacrylamide gel (16 cm $\times$ 18 cm $\times$ 1 mm thick). Electrophoresis was carried out in 0.5 $\times$ TBE buffer at 10°C for 1 hr at 35 mA. The DNA fragments were then stained with ethidium bromide (2.0 μ g/ml), visualized, and photographed under UV illumination.

RESULTS

Using the method described above, we have typed a total of 354 single sperm cells from a RB1.20 heterozygous sperm donor. Among these, 288 were successfully amplified for only one allele (81.3%). Ten samples revealed the presence of two alleles, reflecting the introduction of more than one cell in these tubes. For 56 samples, we could not detect any signal on polyacrylamide gels. This might be due to the absence of spermatozoa in the microtubes, to an incomplete lysis procedure, or to a failure of the amplification reaction. No amplification was obtained for the negative controls (tubes without cells), which is consistent with the absence of contamination.

The limiting steps of the technique seem to be the isolation of a single spermatozoon per tube and DNA extraction from spermatozoa. We think that if the lysis is successful, the amplification should be as efficient as for genomic DNA samples.

PCR conditions were optimized in order to obtain a unique band for each amplified allele (Fig. 1), minimizing the presence of "shadow bands" located below the allele, which probably result from the slippage of the *Taq* DNA polymerase when amplifying short tandem repeats.

DISCUSSION

The causal mutation of the retinoblastoma gene in this family has not been identified. The RB1.20 poly-

Fig. 1. Segregation of RB1.20 alleles in sperm of an individual heterozygous for the RB1.20 polymorphism on a 12% polyacrylamide gel stained with ethidium bromide. Lanes 1 and 8: PCR products from lymphocytes DNA (1 ng) used as a control for heterozygosity. Lanes 2-7: PCR products from single spermatozoa. As expected, each of the two alleles amplified from somatic DNA was also found in the germinal cells. Lane 9: Blank control. M, molecular weight marker (1-kb ladder; GIBCO BRL).

morphism, located 56 bp downstream from exon 20 of the RB1 gene, is a highly informative microsatellite. We chose to study the familial segregation of the RB1 mutant allele in this pedigree. Amplification of the somatic DNA extracted from peripheral blood lymphocytes does not require the use of a nested PCR. Indeed, the amount of DNA initially present in the reaction mixture (1 ng) is sufficient to allow detection of the amplified product after one round of PCR.

On the other hand, we had to set up optimal conditions for reproductive PCR assays for sperm typing using the RB1.20 polymorphism. Compared to the initial description of the method established by Brandt *et al.* (2), we made two major modifications for use in sperm typing. First, we used a nesting strategy by designating nested primers which recognize sequences within the first-round products. The amplification of a single DNA molecule (3 pg) requires many PCR cycles in order to visualize the PCR products easily. However, a high number of PCR cycles leads to the emergence of nonspecific products. The nested PCR allows us to enhance both yield and specificity. Second, we improved the electrophoresis conditions to make the analysis of large series of spermatozoa conceivable.

CONCLUSIONS

The methodology described here is efficient enough to allow the amplification of small amounts of DNA

for research purposes. Using this technique, we want to analyze sperm samples from different males who transmitted a retinoblastoma, in order to estimate the origin of the segregation distortion (meiotic or post-meiotic) observed in retinoblastoma pedigrees. An application of this protocol could also be useful for the detection of mutant RB1 alleles in preimplantation diagnosis.

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