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Reduced Allele Dropout in Single-Cell Analysis for Preimplantation Genetic Diagnosis of Cystic Fibrosis*

PIERRE F. RAY,[1,2] ROBERT M.L. WINSTON,[1] and ALAN H. HANDYSIDE[1]

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Background: *For couples at risk of transmitting a known single-gene defect, preimplantation genetic diagnosis (PGD) allows the identification and transfer of only unaffected embryos following* in vitro *fertilisation (IVF), single-cell biopsy at about the eight-cell stage, and genetic analysis by PCR. This technique therefore avoids the risk of terminating an affected pregnancy diagnosed later in gestation.*

Methods and Results: *Using nested PCR, the ΔF508 mutation causing cystic fibrosis can be detected in single cells and we previously reported successful PGD in a couple in whom both partners carry the ΔF508 mutation. To date we have treated 12 couples in a total of 18 cycles. This resulted in five singleton births confirmed to be homozygous normal. Single blastomeres from disaggregated embryos which had not been transferred were analysed to confirm the original diagnosis and assess reliability in clinical practice. Amplification efficiency and accuracy were high, with blastomeres from embryos diagnosed as homozygous normal or affected. In a proportion of blastomeres from presumed carrier embryos, one of the parental alleles failed to amplify, apparently at random (allele dropout, ADO). A possible explanation is the relative inaccessibility of one of the target allele early in the PCR. To test this we have used single lymphocytes from ΔF508 carriers and investigated the effects of various denaturation temperatures in the early cycles of amplification.*

Conclusion: *Increasing the denaturation temperature reduced the rate of ADO without affecting amplification efficiency.*

KEY WORDS: single-cell analysis; cystic fibrosis; allele dropout; preimplantation genetic diagnosis.

INTRODUCTION

Current methods of prenatal diagnosis for chromosomal and single-gene defects involve sampling cells of fetal origin, for example, by amniocentesis in the second or chorion villus sampling (CVS) in the first trimester of pregnancy and use of cytogenetic biochemical, or DNA methods to detect the genetic defect. *In vitro* fertilisation (IVF) and diagnosis at preimplantation stages of embryonic development *in vitro*, or preimplantation genetic diagnosis (PGD), allow only unaffected embryos to be selected for transfer to the uterus. Any pregnancy, should, therefore, be unaffected by the disease and the possibility of a termination following diagnosis at later stages of pregnancy avoided (1). For many couples with strong ethnic or religious beliefs this is a much more acceptable solution than termination of an established pregnancy.

Cystic fibrosis (CF) is the most common, severe autosomal recessive disease in the Caucasian population, affecting about 1 in 2000 births, with a corresponding carrier frequency of about 1 in 20. The predominant mutation, accounting for 70–75% of CF mutations, is 3-base pair deletion resulting in the omission of a phenylalanine residue at position 508 in the polypeptide (ΔF508) (2).

Lesko *et al.* (3) used a nested PCR strategy to amplify the ΔF508 region, allowing efficient and accurate diagnosis of CF from single cells. Using this method, we reported the first pregnancy and birth of an unaffected child following specific PGD (4). Since then, we carried out 14 further PGD cycles for couples in whom both partners carry the ΔF508 deletion. DNA analysis was performed independently on one or two cells biopsied at the eight-cell stage on the morning of the third day postinsemination (day 3), allowing for embryo transfer later on the same day. These subsequent attemps have resulted in five homozygous unaffected singleton births and three biochemical pregnancies (detected by transient raised serum chorionic gonadotrophin levels) (5). After transferring up to two homozygous normal or carrier embryos, the reliability of the diagnosis was tested on the surplus embryos from 11 diagnosis cycles by

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[1] Human Embryology Laboratory, Institute of Obstetrics and Gynaecology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.
[2] To whom correspondence should be addressed.

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complete disaggregation and amplification from each individual blastomere. Any clinical application of single-cell analysis is critically dependent on accurate amplification. For example, with PGD it is important that both parental alleles are amplified, particularly for dominantly inherited conditions or where affected embryos are compounds heterozygotes. Although preliminary results with single-cell analysis of the ΔF508 locus indicated a high efficiency and 100% accuracy (3), this analysis of blastomeres from disaggregated embryos showed only 80% accuracy with carrier blastomeres. This appears to be due to failure of amplification of one of the parental alleles, a phenomenon we refer to as allele dropout (ADO) (6).

To investigate the phenomenon of ADO, we have used the same nested PCR protocol for the amplification of the ΔF508 locus in a series of single lymphocytes from heterozygous donors. Since ADO is likely to arise at the beginning of the amplification process (before the rise in the number of target molecules) and may be related to the accessibility of the target sequence by the Taq polymerase, we have examined the effects of different denaturation temperatures during the first few cycles of amplification with the outer primers.

MATERIALS AND METHODS

The primer sequence and nested PCR protocol used to amplify the ΔF508 locus was as described previously (4), with the following modifications: the primary PCR was increased to 22 cycles, and the denaturation temperature (D_t) of each cycle altered to 90, 93, or 96°C (only for the first 10 cycles, after which it was lowerd to 94°C to avoid excessive denaturation of the Taq polymerase). The amplification product was electrophoresed on a 10% polyacrylamide gel and detected by ethidium bromide staining under UV light. The heteroduplex band was clearly present when both alleles had amplified and the determination of the missing allele in case of ADO was done by adding previously amplified normal or affected DNA and analysing the pattern of heteroduplex formation as in (4). Blood was obtained from heterozygous ΔF508 carriers and white cells were separated by centrifugation over Ficoll Paque (Pharmacia). Lymphocytes were diluted into microdrops of PBS (Gibco BRL) containing 10 mg/ml of crystallised bovine serum albumin (BSA; ICN, Immuno Biologicals). Following several washes single lymphocytes were isolated manually, using a mouth-controlled fine glass pipette, under a dissecting microscope and transferred into 10 μl of double-distilled water (ddH_2O). Samples were stored at −20°C until analysed. Before cycling the cells were denatured by heating to 95°C for 5 min. Thermal cycling was performed with a Perkin Elmer/Cetus DNA thermal cycler. This research was performed under licence from the Human Fertilisation and Embryology Authority, UK, and approved by the Research Ethics Committee of the RPMS.

RESULTS

A total of 308 blastomeres from 83 embryos of different ΔF508 genotypes, not transferred following PGD, was amplified to validate the accuracy and efficiency of amplification on blastomeres of different genotype. Overall amplification rate was 85% (93% for nucleated blastomeres). Diagnostic accuracy for normal or affected homozygous blastomeres was 95 and 97%, respectively. With presumed carrier embryos, however, only 80% amplified both parental alleles and ADO of normal or affected alleles occurred with equal frequency (9).

Here we tested the amplification efficiency and ADO rate of single carrier lymphocytes. A total of 190 single lymphocytes was analysed. Amplification efficiency was high (≥92%) at the three initial denaturation temperatures tested (Table I; Fig. 1). The rate of ADO was strongly correlated with the denaturation temperature. At 90°C, the frequency of ADO was 70%. Raising the denaturing temperature to 96°C reduced the incidence to 13% (Table I, Fig. 1). Of the 67 cases of ADO, 36 (54%) and 31 (46%) amplified the normal or affected allele, respectively, showing the random nature of ADO. Preliminary results with lymphocytes lysed in a lysis buffer as described in Ref. 7 showed that ADO was reduced overall but followed a similar pattern; at the highest denaturation temperature tested, 96°C, ADO was reduced to 5%.

Table I. Amplification Rate and Frequency of ADO with the CF Primers at Different Denaturation Temperatures (D_t), Using a Water Lysis Protocol

D_t	n^a	Amp (%)	ADO(%)
90°C	50	92	70
93°C	62	97	44
96°C	78	94	13

[a] Number of cells analysed.

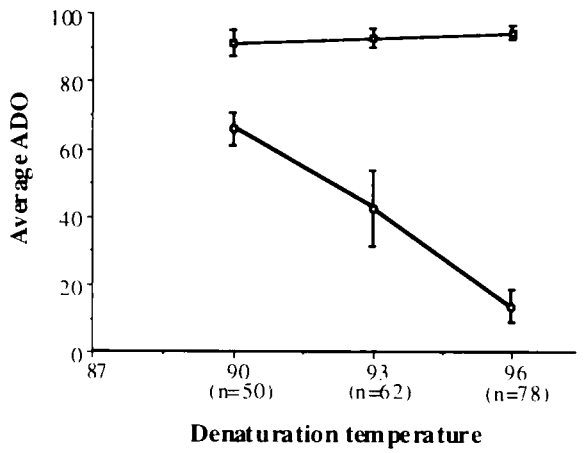

Fig. 1. Amplification rate–■–and frequency of ADO–●–of single lymphocytes lysed in ddH_2O at different denaturation temperatures. *n* = number of single lymphocytes analysed. Error bars are standard error of the mean.

DISCUSSION

For an autosomal recessive disease, such as CF, a serious misdiagnosis occurs only when a homozygous affected embryo is diagnosed as normal or carrier. Among the blastomeres analysed previously (9), disaggregated from presumed homozygous affected embryos not transferred following PGD, 4 of 366 (1 homozygous normal and 3 heterozygote carriers) fall into this category, giving an estimated risk for an embryo of unknown genotype of about 1%, when relying on diagnosis from only one cell. More striking, however, was the relatively high incidence of random ADO in blastomeres from presumed carrier embryos (20%). In these cases, the adverse consequences would be relatively minor: carrier embryos diagnosed as homozygous affected would not be considered for transfer, whereas those diagnosed as normal could be preferentially selected over correctly diagnosed carrier embryos of better morphology.

ADO could be caused simply by single- or double-strand breaks in the target DNA sequence during preparation and lysis of single cells. Alternatively, accessibility of single-stranded target DNA for both alleles in the first cycles of PCR may depend on efficient initial denaturation, which in turn is critically dependent on temperature and influenced, among other factors, by GC-rich regions. Preliminary work on single lymphocytes with primers amplifying part of the β-globin cluster showed a similar relationship between D_t and ADO rate, thus suggesting that ADO is not primer specific. For amplification of the ΔF508 fragment, the results with single carrier lymphocytes have demonstrated that the denaturation temperature in the initial cycles can dramatically affect the incidence of ADO independently of amplification efficiency. This therefore indicates that ADO is not due primarily to DNA breakage of one of the alleles; otherwise a parallel increase in total amplification failure would be expected. We suggest that the optimal temperature is higher (96°C) than that previously used. In combination with a two-step lysis protocol originally described for amplification from single sperm (7), ADO was reduced to 5% but not eliminated. This may still be too high for PGD of dominant conditions and further work is needed to ascertain whether these remaining cases are caused by degradation of one of the target sequences, partial DNA denaturation, or chromosomal mosaicism.

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