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Rapid preimplantation detection of mutant (shiverer) and normal alleles of the mouse myelin basic protein gene allowing selective implantation and birth of live young

(prenatal diagnosis/trophectoderm biopsy/polymerase chain reaction)

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ABSTRACT **As a model for the detection of human genetic disease in preimplantation embryos, we describe a method in which trophectoderm biopsy samples from viable mouse blastocysts are simultaneously analyzed for the presence of a normal or mutant allele of the myelin basic protein gene by the polymerase chain reaction. The biopsied embryos are kept in culture during analysis of biopsied material and later reintroduced to a foster mother. Prenatal diagnosis can be completed in less than 7 hr. The identity of either amplification product was proved conclusively by direct sequence analysis of amplified products. Ninety-six percent of recovered blastocysts survived biopsy, as judged by re-formation of a blastocyst cavity in culture. Fifty-nine percent of the biopsied embryos established pregnancy by day 6.5, compared to 88% of unmanipulated controls. This approach can be applied to preimplantation diagnosis of human genetic diseases by using extraembryonic cells from blastocysts obtained after *in vitro* fertilization or uterine lavage. It will make possible the elimination of a mutant allele from a family in a single generation.**

The prevention of genetic diseases is a major goal of modern medicine. To date, prevention has been achieved by genetic counseling for identified carriers wishing to have children and by antenatal diagnosis of a small number of diseases by biochemical assay (Tay–Sachs disease) or karyotyping (Down syndrome) of material obtained by amniocentesis or chorionic villus biopsy followed by elective pregnancy termination. As DNA sequence characterization of genetic defects continues, the ability to diagnose a wide variety of genetic diseases by direct analysis of DNA is becoming possible. As techniques for embryo manipulation (1) and diagnostic molecular biology (2, 3) improve, the prospect of prevention of genetic disease by preimplantation diagnosis provides an alternative to intrauterine diagnosis and the termination of pregnancy. *In vitro* fertilization has become a routine procedure in fertility clinics. Gene detection in preimplantation embryos obtained either by lavage or by *in vitro* fertilization would permit the introduction and implantation of embryos lacking the mutant allele and the eventual birth of normal children, thus preventing the transmission of mutant alleles from parents to their offspring. We present here a strategy that would permit human preimplantation embryos to be sampled harmlessly by biopsy, screened for genetic defects, and introduced into the mother.

We used as a model the mouse mutation shiverer (*shi/shi*). The shiverer mutant is characterized by tremors, hypomyelination of the central nervous system, and a shortened life-span (reviewed in ref. 4). The gene encoding myelin basic protein (MBP), a major constituent of the central nervous system myelin, has been largely deleted in shiverer mice (5). The normal MBP gene has seven exons (6, 7), which have been sequenced (6), while the mutant MBP gene has only exons I and II (5, 8). The 5′ breakpoint of the MBP gene deletion in shiverer mice has been sequenced (8). Our aim was to identify the mutant and normal MBP genes in biopsied extraembryonic material in sufficient time to allow transfer of assayed, preselected embryos to pseudopregnant recipients.

MATERIALS AND METHODS

Blastocysts were flushed from the uterine horns on the fourth day of pregnancy and incubated in M16 medium (9) at 37°C and 5% CO_2. For the biopsy, blastocysts were placed in a drop of M2 medium (10) with bovine serum albumin replaced by polyvinylpyrrolidone (1 mg/ml) and were covered with light paraffin oil. One to three or three to five extraembryonic trophectoderm cells were removed using glass needles attached to micromanipulators as described previously (11). After this operation, blastocysts were returned to M16 medium and the biopsied cells were placed in tubes in 10 μl of distilled water, frozen on dry ice, and stored at −70°C prior to analysis. The blastocysts were scored 7–12 hr later for recovery as judged by the reappearance of the blastocele cavity. Five to eight viable blastocysts were transferred to the uterine horns of pseudopregnant recipient females on day 3 of pregnancy. Each recipient had control embryos transferred to one horn and biopsied embryos transferred to the contralateral horn. The recipients were sacrificed at day 7 or days 12–15 of pregnancy to score for implantation sites or viable fetuses. In a few cases pregnancy was allowed to go to term.

The presence of wild-type or mutant MBP alleles in the biopsy sample was detected using two sets of specific primers in the polymerase chain reaction (PCR). One set of synthetic oligonucleotide primers amplifies a 169-base-pair (bp) fragment from wild-type mouse DNA; these primers have sequences complementary to intron sequences flanking exon VI of the MBP gene (exon V in ref. 6) (5′-AGCTCTGGTCTTTCTTGCAG-3′and 5′-CCCCGTGGTAGGAATATTACATAAC-3′). As exons II–VII are deleted in the shiverer mouse, these primers do not amplify a fragment when shiverer DNA is used as a template. The second set of primers amplifies a 380-bp fragment unique to shiverer DNA. These oligonucleotide primers are complementary to sequences on either side of the shiverer "breakpoint," which is within the second intron of the MBP gene (8). They have sequences comple-

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Abbreviations: MBP, myelin basic protein; PCR, polymerase chain reaction.

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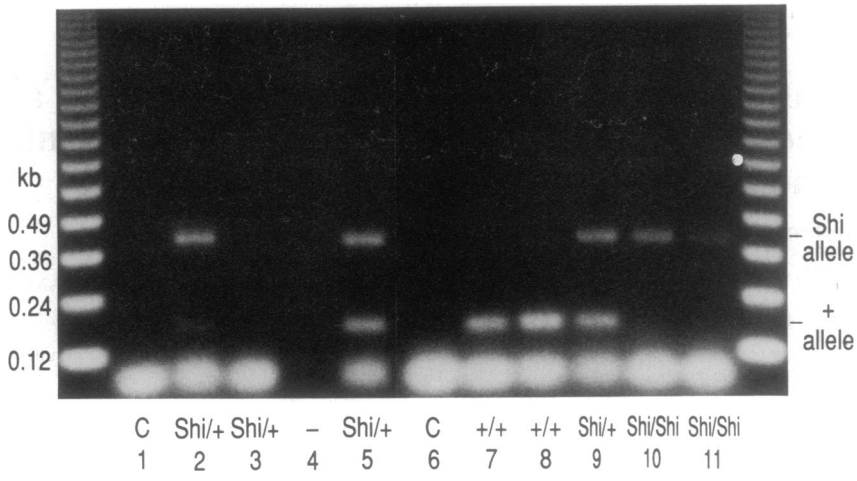

FIG. 1. Agarose gel analysis of PCR amplification of DNA in trophectoderm biopsy samples. Lanes 10 and 11, amplifications of DNA from trophectoderm biopsy material of homozygous shiverer (*shi/shi*) blastocysts. Lanes 2, 3, and 9, amplifications of DNA from heterozygous (*shi/+*) blastocysts (lane 3 false negative). Lanes 7 and 8, amplifications of DNA from homozygous wild-type (+/+) blastocysts. Lane 5, amplification of DNA from whole heterozygous (*shi/+*) blastocyst. Lanes 1 and 6, amplifications of medium control. Lane 4, empty lane.

mentary to nucleotides 201–225 (5′-CAGGGGATGGGGAG-TCAGAAGTGAG-3′) and on the opposite strand from positions 551–581 (5′-ATGTATGTGTGTGTGCTTATC-TAGTGTA-3′) (7). Both sets of primers were used in all the PCR assays.

The biopsied cells were digested by a modification of the procedure of Li *et al.* (2). Briefly, 10 μl of digestion mixture was added to each tube containing cells and distilled water, to make a final concentration of 1× PCR buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/2.5 mM $MgCl_2$/0.01% gelatin), 50 μg of proteinase K per ml, 20 mM dithiothreitol, and 1.7 μM SDS. Cells in the digestion mix were incubated at 55°C for 1 hr, then boiled for 15 min to inactivate proteinase K and denature DNA. After cooling on ice, 80 μl of amplification master mix was added to each tube to give a final composition of 1× PCR buffer; 0.5 μM for each of four primers; 2 mM for dATP, dCTP, dGTP, and dTTP; and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Cetus).

High-efficiency amplification in a thermocycler (Perkin–Elmer) was achieved using 50 cycles (92°C for 30 sec, 60°C for 45 sec, and 72°C for 90 sec) followed by 10 min of extension at 72°C. An additional aliquot of 2.5 units of *Taq* polymerase added after the 30th cycle greatly improved the signal. To avoid contamination with exogenous DNA, strict conditions, as outlined in ref. 12, were observed at all times. For analysis, 10 μl of each amplification mixture was electrophoresed in a 1.5% agarose minigel and screened for the presence of bands of expected size.

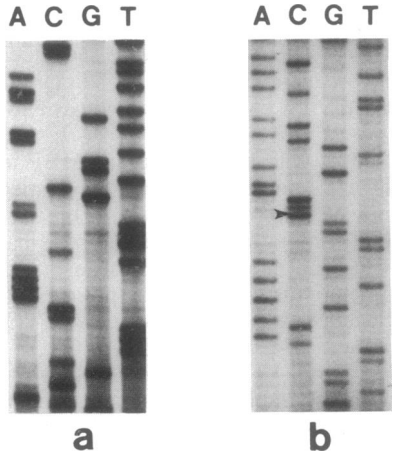

FIG. 2. DNA sequence analyses of PCR products from biopsied embryos. (*a*) Portion of exon VI (oriented with 5′ end downward) extending from position E98 (6) to 16 bases into the intron following exon VI (listed as exon V in ref. 6), sequenced from the plus strand. (*b*) Portion of shiverer MBP allele containing the breakpoint (arrowhead) (5′ end oriented downward) from nucleotide 420 to nucleotide 462 (from ref. 7), sequenced from the plus strand.

Direct sequence analysis of PCR products was performed according to Kretz *et al.* (13). Amplified DNA for use as template was obtained by electrophoresis in 1% low-melting agarose gel in Tris/borate/EDTA buffer. Bands cut from the ethidium-stained gel were heated to 96°C for 10 min and 7 μl was used immediately as template for standard dideoxy chain-termination sequencing with phage T7 DNA polymerase (Sequenase; United States Biochemical) except that after annealing at 55°C for 30 min, all reactions were performed at 37°C.

RESULTS

Detection of MBP Alleles in Trophectoderm Biopsy Samples. Amplification by PCR using the shiverer breakpoint primers leads to a 380-bp DNA product when *shi/shi* DNA is the template, but no product is observed when wild-type (+/+) DNA is the template (Fig. 1). Exon VI primers yield a 169-bp product when +/+ DNA is used as template and no product when *shi/shi* DNA serves as template (Fig. 1). This choice of primers for PCR allows for the simultaneous amplification of these regions from both alleles when present, and thus permits one to distinguish heterozygous (*shi/+*) from homozygous (*shi/shi*) embryos.

Table 1. Simultaneous assay of trophectoderm cells for mutant (*shi*) and wild-type (+) MBP alleles

Genotype of blastocyst	Number assayed	Result: Expected allele detected	Result: False positive for other allele	Result: False negative (no allele detected)
shi/shi	13	6	3	4
shi/+	7	4	NR*	3
+/+	19	15	0	4
Total	36	25	3	11
Blank†	10	—	2	—

*Not relevant.
†Medium or water.

Table 2. Summary of various steps in the procedure including embryo biopsy, culture, and transfer to foster mothers

Blastocysts	No. recovered/ no. biopsied	No. cavitated after 7–12 hr in culture	No. establishing pregnancy/ no. transferred	
			Day 6.5	Days 12–15
Biopsied	93/133	89 (96%)	22/37 (59%)	10/42 (24%)
Control	NR	NR	30/34 (88%)	15/42 (36%)

NR, not relevant.

In most trophectoderm biopsies, one or both alleles were readily detectable. Fig. 1 shows DNA fragments of the expected size that have been amplified from biopsy material by PCR and separated by electrophoresis in an agarose gel. Direct sequence analysis of the DNA fragments isolated from these bands (18 cases) confirmed the identity of these amplification products with the expected regions of the MBP gene (Fig. 2).

Table 1 summarizes the results of assay of 36 trophectoderm biopsy samples. In homozygous shiverer embryos the shiverer allele was detected 6 out of 10 times. In 3 of these 6 cases the homozygous shiverer DNA was contaminated with normal cells, leading to a false positive for MBP exon VI. In 4 other shiverer homozygote samples no amplification was obtained. In 4 of 7 heterozygous samples (*shi*/+) the normal and mutant alleles were detected; in 3 cases no amplification was obtained or the PCR reaction failed. In 15 of 19 wild-type biopsy samples the correct allele was identified and in 4 cases no amplification was obtained. Thus, in 25 out of 36 trophectoderm biopsy samples the expected MBP allele was detected; in the remaining 11 samples no allele was detected (i.e., false negative results were obtained). Three false positives were obtained using trophectoderm biopsy samples and 2 false positives were obtained using medium or water.

The analyses were completed and the genotypes of the blastocysts were identified in 7 hr from the time of biopsy (1 hr for digestion, 6 hr for amplification and separation of products by agarose gel electrophoresis).

Biopsy and Transplantation of Blastocysts. The viability of the biopsied embryos was assessed by monitoring the maintenance or re-formation of a blastocele cavity and their subsequent ability to establish pregnancy (implantation sites), form fetuses (Table 2), or produce live young (Fig. 3). The viability after biopsy (as judged by recovery of blastocele) (96%) was improved over a previous study (60%) (11). The implantation rate of biopsied embryos was 59% compared with 88% for control embryos. As judged by development of fetuses, the overall success of transfer of both biopsied (24%) and control unbiopsied (36%) embryos was lower than in a previous study (11) (36% and 55%, respectively). This was almost certainly due to the inexperience of the person performing the transplants.

DISCUSSION

Over the next decade the number of genetic diseases that are characterized at the DNA level will greatly increase (14). One important application of such knowledge would be the early detection of harmful mutations in the fetus or the preimplantation embryo.

We have used the shiverer mutant mouse as a model system for the preimplantation diagnosis of a genetic disease. Living embryos were biopsied and the DNA was assayed for the presence of shiverer mutant (*shi*/*shi*) and wild-type MBP genes by PCR. This study demonstrates the practical application of a rapid PCR gene-detection assay for screening preimplantation embryos and transfer of selected normal embryos to pseudopregnant foster mothers.

At present there are two methods to sample DNA from preimplantation embryos: (*i*) removal of a blastomere at the two- to eight-cell stage (15, 16) and (*ii*) removal of cells from the extraembryonic trophectoderm layer of the blastocyst, so-called trophectoderm biopsy (11). Both biopsy sources have been used for preimplantation diagnosis of embryos either by biochemical assays to measure enzyme activity (11) or by PCR for the detection of a particular mutation (16). We used trophectoderm biopsy for several reasons: (*i*) sufficient material is obtained from trophectoderm biopsy to repeat the assay if necessary; (*ii*) the viability of the embryos following biopsy is good, and the implantation rate is only somewhat less than that of unmanipulated embryos; and (*iii*) the portion of the blastocyst that is sampled, the trophectoderm, is destined to become placenta and is thus distinct from the embryo proper. When the use of such techniques on human embryos is considered, biopsy of trophectoderm may be ethically more acceptable than removal of one of four or eight cells destined to form part of the embryo proper. We believe that these factors outweigh the disadvantage of trophectoderm biopsy, which is that it is technically more difficult than using blastomeres and it is subject to an increased likelihood of sample loss.

FIG. 3. Shiverer mouse (at left) born after trophectoderm biopsy and PCR analysis at blastocyst stage. Embryo was maintained in culture for 10 hr prior to transfer to foster mother (at right). Mice were photographed with slow shutter speed to reveal shiverer phenotype.

In this study we screened embryos for the presence of the wild-type or mutant MBP gene. Two sets of oligonucleotide primers were used in every PCR reaction. One set amplified a fragment of DNA that is present only in the wild-type gene, while the other pair amplified a DNA fragment unique to the mutated gene. It is important to identify both the normal and the mutated allele. The dangers of not doing so are best illustrated by a recent study in which PCR was used to screen preimplantation embryos for β-thalassemia (16). One set of oligonucleotide primers was used to amplify part of the wild-type hemoglobin gene. Failure to amplify this fragment was interpreted as absence of the wild-type gene and, by inference, the presence of the mutant allele. With this strategy it would not be possible to distinguish biopsy material with mutant alleles from a PCR assay that failed for technical reasons or the loss of the sample material. More importantly, heterozygote embryos carrying the mutation could not be distinguished from wild-type embryos. Finally, if the sample was contaminated with normal cells, as readily happens, a mutant embryo would be falsely diagnosed as normal.

The gene-detection procedure provided genotype information within 7 hr of biopsy. We were able to detect the correct MBP alleles in 25 out of 36 cases, with 3 false positives (incorrect allele also detected) arising from the contamination of *shi/shi* embryos with wild-type cells or amplified material. There were 11 false negatives (no allele detected). The false negatives were due to either the loss of biopsy material during transfer of cells from the operating drop to the assay tube or the failure of the amplification reaction for technical reasons. We favor the former explanation because, using the same conditions and oligonucleotides, we were able to detect both alleles in a single blastomere (data not shown). The important point is that in every case where PCR products were obtained, the presence of a mutant allele was correctly established.

In the case of the three false positives both the wild-type and mutant alleles were detected in biopsies from homozygous shiverer embryos, due to contamination of the biopsy material with normal mouse genomic DNA, even when the strictest precautions were observed (12). Consequently three of the homozygous shiverer embryos (*shi/shi*) were misdiagnosed as carrier embryos (*shi/+*). In practice when dealing with human genetic disease, embryos with either the homozygous mutant genotype or the heterozygous carrier genotype would not be transplanted. In no case was a shiverer allele detected in wild-type embryo biopsy material (0/19). Thus only embryos unequivocally lacking the mutant allele would be transplanted by our procedure, ensuring the elimination of a given mutant gene from a family in a single generation and the birth of only genetically normal offspring.

We tested the specificity of our assay directly by sequencing amplified DNA products after separation in agarose gels (Fig. 2). We have seen that in the 18 cases tested the fragment amplified was identical to the region of DNA (exon VI or breakpoint) flanked by the primers. This latter observation also demonstrates the power of PCR amplification, which has made possible the sequencing of DNA originally amplified from as few as one or two cells.

A number of embryos (30 out of a total of 133) were lost during transfer from the drop of medium in which they were biopsied to the culture medium. The number of embryos transferred safely would be improved with practice and by scheduling the biopsy of fewer embryos at a time. If smaller sample numbers were processed at a time, the *in vitro* period following the biopsy would also be reduced, and the transfer could be undertaken within 10 hr of biopsy. The percentage of embryos that survived biopsy was high (96%). This may be ascribed to the fact that fewer cells need be removed for the PCR assay as compared to the enzyme assay. Fifty-nine percent of the viable biopsied embryos implanted as compared to 88% for the unmanipulated control embryos. These data were not recorded in the previous study (11). Twenty-four percent of biopsied blastocysts as compared to 36% of unbiopsied blastocysts were found to have established pregnancy in foster mothers sacrificed at days 12–15. The pregnancy rates of both biopsied and control embryos were lower than those reported previously (36% and 55%, respectively). Because of the inexperience of the investigator performing the uterine transfers it is likely that these rates would greatly improve with further practice. The biopsy and culture of blastocysts seem to have had less effect (as judged by differences in pregnancy rates) on pregnancy rate in this study than in the previous one (11). This may be due to the smaller biopsy size (PCR assay needs fewer cells) or to the reduced duration in culture.

This strategy may be applied to the screening of human embryos obtained by *in vitro* fertilization or lavage to identify those appropriate for transfer to the mother. This study illustrates a number of factors that are important to consider. (*i*) The method of biopsy is important for the survival of the embryo as well as the success of the assay. Trophectoderm biopsy is the method of choice from both standpoints, as it provides enough material to repeat the assays if necessary and the survival of the biopsied embryo is good. (*ii*) The strictest conditions must be observed to avoid contamination of the sample. Methods for avoiding contamination have been outlined (12). (*iii*) DNA primers should be chosen to detect mutant and normal alleles directly. Both sets of primers should be able to function together in the same biopsy sample. (*iv*) Sequencing of the amplified products should be done initially to confirm that the correct fragment has been amplified. (*v*) Application of other methods of gene detection, such as the oligonucleotide ligation assay (3), will allow detection of any characterized gene mutation including single base-pair substitutions. Rapid advances are expected in this important application of clinical genetics and embryology. As the number of genetic diseases that are characterized at the DNA level increases, this embryo screening procedure could be of great use.

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