RAPID COMMUNICATION

Prenatal Diagnosis of Fetal Hemoglobin Lepore-Boston Disease on Maternal Peripheral Blood

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Molecular diagnosis of hemoglobin (Hb) Lepore-Boston in the fetus was successfully accomplished using maternal blood as a source for fetal cells in three pregnancies at risk for β-thalassemia/Hb Lepore disease. Taking advantage of the possibility of amplifying Lepore-specific DNA fragments by polymerase chain reaction and of families in which Hb Lepore was inherited by the paternal side, we demonstrated in two cases and excluded in one case the presence of this hemoglobinopathy in the fetus directly on maternal DNA. The diagnosis was concordant with that obtained by traditional approaches in all three cases. Our results unequivocally show that nucleated fetal cells are present in maternal blood during pregnancy, and demonstrate for the first time that prenatal diagnosis of a genetic disease may be feasible without invasive procedures.

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Materials and Methods

Family selection. Three families were selected for this study among those scheduled for prenatal diagnosis of thalassemia syndromes by chorionic villus sampling and DNA analysis in Milano, Italy. In all cases the mother was a carrier of β-thalassemia and the father of Hb Lepore-Boston trait. Informed consent was obtained for the study in all cases. The Lepore-Boston gene is a hybrid β-β globin gene, resulting from an unequal crossing over between misaligned β and δ genes on chromosome 11 (Fig 1). The abnormal gene is associated with a deletion of approximately 7 kilobases (kb).8,9 The Lepore gene confers a thalassemic phenotype; the coinheritance of β-thalassemia results in a severe transfusion-dependent anemia.

DNA preparation. DNA was prepared from peripheral blood by centrifugation of buffy coats. DNA from the mothers was obtained at the 8th to 10th week of gestation (before performing chorionic villus biopsy). In one case DNA was again obtained at the 24th week of gestation; moreover, in the same case, DNA was prepared from subpopulations of cells separated by the MoAb CD1 (Leu 4) as outlined below.

DNA amplification by PCR. The 7-kb deletion generating the Lepore-Boston gene is schematically illustrated in Fig 1. The sequences of the primers (a and b) used to amplify a fragment of 356 base pairs (bp) containing the 6-6 junction of the Lepore-Boston gene are reported in the same figure. These primers amplify sequences only from the rearranged Lepore chromosome, since they are more than 7 kb apart in normal DNA.

For amplification, we used Taq 1 polymerase and automated equipment (DNA Thermal Cycler; Perkin Elmer Cetus) with 30 cycles of denaturation (97°C for 5 minutes for the first, and 85°C for 30 seconds for the following cycles), annealing, and extension (53°C for 5 minutes for the first cycle, and 53°C for 1 minute for the next) as described previously.12 The same ampli-primers (a and b) were used to perform a second PCR round (30 cycles) on an aliquot (10%) of the first-round PCR product. Thirty microliters of the 100 μL volume of the amplification reaction was visualized on 1.5% agarose gels after ethidium bromide staining, and transferred on nylon membranes (Hybond C-extra; Amersham) by blotting procedure. Filters were prepared in duplicate. To further increase the specificity and sensitivity of the technique, filters were hybridized to 32P-labeled oligonucleotides internal to the amplified fragment at the boundaries of the 58-bp homology region (see below), exactly overlapping the junction of exon 2 (δ) and intron 2 (β) and c (d and c in Fig 1, respectively).

In one case an aliquot of the first-round PCR product obtained with primers a and b was reamplified using either the oligonucleotides a and d or b and c as ampli-primers (Fig 1). The amplified
DNA was then extracted by phenol-chloroform and digested with restriction enzymes *Pvu* II and *Ava* II.15

**Precautions against contamination.** Because contamination may be a major risk when performing multiple PCR cycles,16,17 several precautions were adopted.

To be sure that the reaction reagents were free of contaminants, "no DNA controls" were always included. To avoid false-positive for contamination of genomic DNAs with the Lepore-specific PCR product, DNA were prepared in Milano and PCR experiments were performed in Torino. All manipulations were performed under class II containment. DNA from a positive case (3 in Fig 2) was obtained separately by cells split in several samples. Positive results were repeated at least twice.

**Cell separation.** Leu 4 positive and negative cells were obtained by incubating Ficoll-separated cells of the mother with the CD 3-specific MoAb Leu 4 (Becton Dickinson, Mountain View, CA, cat. no. 7340), and by separating the positive cells with goat-anti-mouse immunoglobulin G (IgG)-coated immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway) as previously described.4

**RESULTS**

The breakpoint of the Lepore-Boston deletion is located between codon 87 (in exon 2 of the δ gene) and the 5' end of intron 2 of the β gene12 (Fig 1). The perfect sequence homology within a 58-bp region at the junction of exon 2 and intron 2 in normal δ and β genes18 hampers a more precise definition of the crossover site.

Using the ampli-primers a and b shown in Fig 1, a band of 356 bp is expected in Hb Lepore carriers. The results obtained on maternal DNA and on positive and negative controls are reported in Fig 2 (lanes 1 through 5).

After the first round of PCR (Fig 2A), the expected band was observed only in the positive control (lane 4); after the second PCR round (Fig 2B), multiple aspecific bands were observed; however, among them a 356-bp band was clearly distinguishable in positive control, case 1, and case 3, but not in negative control and case 2.

The oligonucleotide probes c and d, complementary to the region of δ-β junction (Fig 1), hybridize specifically to the 356-bp fragment (Fig 2, C and D, and data not shown), confirming its origin from the hybrid δ-β gene.

Hybridization experiments proved to be very sensitive. Positive autoradiographic signals were observed in cases 1 and 3 even after the first PCR round (Fig 2C), when no PCR products were appreciable on ethidium gels (Fig 2A). On the basis of these results we assumed that the fetuses in cases 1 and 3 were carriers of Hb Lepore and negative in case 2. Results of prenatal diagnoses on chorionic villi were concordant with those anticipated by these experiments.

To enrich the blood samples for fetal cells we used a CD3 MoAb (Leu 4) to eliminate maternal T lymphocytes. As shown in Fig 2, B and D (lanes 6 and 7), a 356-bp band was evident in Leu 4- fraction after the second round of PCR, and the corresponding autoradiographic signal was stronger in Leu 4- (lane 7) than in the Leu 4+ subpopulation (lane 6). These results suggest that an enrichment of fetal cells is indeed present in the T-cell-depleted fraction.

To verify further the identity of the 356-bp fragment, aliquots of the PCR product obtained with primers a and b in case 3 were reamplified using oligonucleotides a and d and b and c as ampli-primers (Fig 1). As expected, the PCR product obtained with a and d was 258 bp long (Fig 3A), and that obtained with b and c was 181 bp long (Fig 3B). This approach of using partially nested primers in the second PCR round increased both the specificity and the amplification yield, allowing to perform a restriction map of the fragments obtained. Fragments 258 and 181 were digested with *Pvu* II (which cleaves the nucleotide changes of codon 87 δ) and *Ava* II.
II (which cleaves a sequence within the IVS II of the β gene) (Fig 3). The bands obtained were those expected for the juxtaposition of δ and β sequences in the Lepore-Boston junction.

DISCUSSION

The data reported here provide evidence that nucleated fetal cells are present in the peripheral blood of the mother during pregnancy. This conclusion was achieved using the deletion associated with the Lepore-Boston gene as a fetal marker. A selective amplification of fetal DNA is obtained in this condition, since no substrate for gene amplification is present in maternal DNA. The technical device of a two-step PCR or the hybridization of the PCR product to internal oligonucleotide probes are of paramount importance in attaining the result. A dual PCR system was recently used to demonstrate the presence of Y-specific sequences in peripheral blood of mothers with male fetuses. A similar approach cannot easily identify single nucleotide substitutions in the fetus. DNA sequences surrounding the single-base change are common to both fetal and maternal DNA; therefore,
maternal sequences, largely predominant, are preferentially amplified.

The possibility of investigating the fetal genotype directly on maternal blood cells may potentially provide a noninvasive method for prenatal diagnosis of genetic diseases. Our results are indeed examples of prenatal diagnoses: the presence/absence of Lepore fragments on the DNA of the mothers was concordant with the results obtained by chorionic villi analysis. Whereas positive results are unequivocal, negative data still require careful evaluation since they could simply reflect technical failures or the absence of fetal cells in maternal circulation. Both the nature of circulating fetal cells and their number at different gestational ages remain to be evaluated further. The different degree of Lepore band intensity in positive cases (1 and 3) suggests that the proportion of fetal cells present may vary.

We must emphasize that at present only the paternal allele would be detected by this approach, and that a prerequisite for prenatal diagnosis will be that the paternal allele differ from that found in the mother. Moreover, a maternal allele inherited by the fetus would not be recognized. The full development of this strategy to the purposes of prenatal diagnosis of genetic disorders will require fetal cell separation. Attempts by means of H315 MoAb and of immunologic techniques were previously unsuccessful. The results obtained on T-lymphocyte-depleted cells (Fig 2, B and D) suggest that a certain degree of fetal cell enrichment may be realized. Immunologic maneuvers combined with selective cell culture techniques may prove useful in purifying this minor cell population. Besides providing suitable material for prenatal diagnosis, this possibility would allow the more general issue of fetal-maternal relationships to be explored.

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