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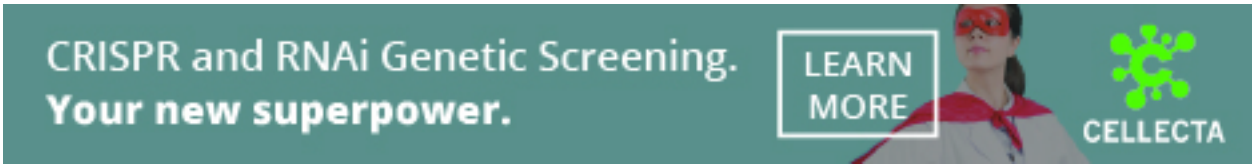

Evaluation of Bone Marrow Transplantation Efficiency by Competitive PCR on Y Sequences

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Allogeneic bone marrow transplantation (BMT) is now a common treatment for hematological diseases. Before the transplantation, the hemopoietic cells of the patient are eradicated. In spite of this pretransplant treatment, some apparently normal recipient hemopoietic cells can remain, leading to a mixed chimerism phenomenon.[1] It is very important to monitor this chimerism to follow the kinetics of recipient cell amount after BMT and possibly to prevent the relapse of a residual disease.

Early attemps to evaluate the mixed chimerism were based on immunological and cytogenetic methods. Conventional molecular detections of host cells included Southern blotting and RFLP analyses; their sensitivity was ~1–2%. More recently, PCR techniques were performed to assess the chimerism.[2] The in vitro amplification was used either to detect microsatellites[3] or Y chromosomal material.[4] The latter strategy is available in one case out of four, when there is a sex mismatch with a female donor and a male recipient.

The long arm of the human Y chromosome contains a highly repeated nucleotide sequence arranged in a head-to-tail manner.[5] The repeated area represents 800–5000 copies on the Y chromosome. These 3.56-kb *Eco*RI fragments belong to the human Y chromosome-specific repeated DNA family (DYZ1 locus) and are composed of several hundred variants of a basic pentanucleotide (TTCCA). A fragment of this sequence overlapping the *Eco*RI restriction site can be detected by PCR. This method was already used for fetal sex determination[6,7] and may also provide a way of studying the BMT chimerism.

In this paper we propose a simple, rapid, and efficient method to evaluate this chimerism after sex-mismatched allogeneic BMT. A preliminary PCR detection system is described. If Y amplified fragments are detected in recipient blood samples, we quantify the remaining Y sequences by PCR with an internal exogenous standard. This experiment is performed simultaneously on blood samples (B) and buccal epithelial cells (E) to compare hemopoietic and nonhemopoietic cells from the same patient. The B/E ratio represents the percentage of mixed chimerism. Because the analysis is repeated every 3 months after BMT, high percentages may announce a possible relapse.

MATERIALS AND METHODS

All experiments described here were performed by female manipulators to avoid male cell contaminations.

DNA Preparation

We studied four subjects: a normal male, a normal female, and two patients after sex-mismatched allogeneic BMT. The first patient (A) was analyzed 8 months after the transplantation, and the other (B) 3 yr after it. These two patients had female sibling donors and came from the Department of Haematology, Poitiers University Hospital.

Mononuclear cells from 5- to 20-ml blood samples were separated by Ficoll centrifugation. Buccal epithelial cells were obtained by mouthwash with 30 ml of 0.9% standard saline.[8] Cells from blood samples or buccal epithelium were washed with 3 ml of PBS. After centrifugation, the cell pellet was resuspended in 3 ml of cell lysis solution (10 mM Tris-HCl at pH 7.4, 50 mM NaCl, 10 mM EDTA). This solution was incubated for 1 hr at 65°C with 200 μl of 10% SDS and 200 μl of proteinase K (10 mg/ml). DNA was then precipitated with 2.5 volumes of ethanol, washed in 80% ethanol, and resuspended in sterile distilled water. DNA concentration was estimated by spectrophotometry.

PCR Amplification

Y sequences were amplified using the Y1-1 and Y1-2 primers already described[7] (Y1-1, 5′-TCCACTTTATTCCAGGCCTGTCC-3′; Y1-2, 5′-TTGAATGGAATGGGAACGAATGG-3′). PCR reactions were performed in a total volume of 50 μl containing 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 250 μM of each dNTP (Boehringer Mannheim), 30 pmoles of each primer, 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus), and 100 ng of DNA. The reaction occurred under the following conditions in a Perkin-Elmer thermal cycler: 30 sec at 94°C and 30 sec at 60°C for 30 cycles. The PCR products were analyzed on a 2% Seakem agarose gel (FMC). When indicated, the sequencing of the amplified products was performed. Briefly, PCR fragments were purified using the Magic PCR Preps kit (Promega), subcloned in the M13 vector, and sequenced with the A.L.F. DNA sequencer (Pharmacia) ac-

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cording to the recommendations of the manufacturer.

To check the quality of the DNA and to validate a negative Y amplification, each sample was controlled by a PCR at the KM19 locus linked to the Cystic Fibrosis Transmembrane conductance Regulator gene using KMC (5′-CTGTCCAGGAAACTTTGTGT-3′) and KMD (5′-GTCTAAAAGGGTATCAGTCC-3′) primers.[9,10]

Construction of the Competitive Standard

The exogenous template for competitive amplification was constructed using two modified primers: YMA (5′-TGGAATCGAAGGGAATGTAGTG-3′) and YMB (5′-TCCAATCGATTCCTTTCCTTTC-3′). These oligonucleotides contained an artificial *Taq*I restriction site at their 5′ end, involving 1 base mismatch with the DNA template (Fig. 1). Two PCRs were performed: the first one with Y1-1 and YMA primers, and the other with YMB and Y1-2, according to the standard PCR protocol already described. Both amplified fragments (20 μl) were digested with 20 units of *Taq*I restriction enzyme (Biolabs). The cohesive fragments (5 μl of each digested solution) were ligated using 4 units of T4 DNA ligase (GIBCO BRL). The ligation product can be amplified by PCR using Y1-1 and Y1-2 primers, yielding an amplified fragment with a 30-bp deletion with respect to the native DNA sequence. The amplification product was purified through a 12% polyacrylamide gel. The 124-bp fragment was extracted from the gel and amplified as described above to obtain sufficient amounts for further experiments. The final product (YM) represented the stock solution of internal standard and was stored in aliquots at −20°C. The concentration of this solution was not measured, because our aim was to establish a ratio of two absolute quantitations. All points of the standard scale are defined as a dilution of the YM construct.

Competitive PCR

A standard scale was obtained from the YM stock solution by 10-fold dilutions from 10^{-1} to 10^{-12}. Competitive Y1-1/Y1-2 PCRs were performed on 5 μl of DNA (100 ng) and 5 μl of each standard dilution. After 30 cycles (30 sec at 94°C and 30 sec at 60°C), 10 μl of the reaction was electrophoresed either on a 2.5% NuSieve/1.5% SeaKem TAE–agarose gel or on a 3% MetaPhor TBE–agarose gel (FMC).

When indicated, DNA samples were treated with *Taq*I restriction enzyme prior to PCR. Two micrograms of genomic DNA was digested for 2 hr with 40 units of *Taq*I in 100 μl of the *Taq* polymerase buffer. After digestion, 5 μl of the reaction solution (100 ng) was directly used for PCR.

In all experiments, strict precautions were taken to avoid contamination of the samples. Negative controls (distilled water and female DNA sample) were used in all manipulations.

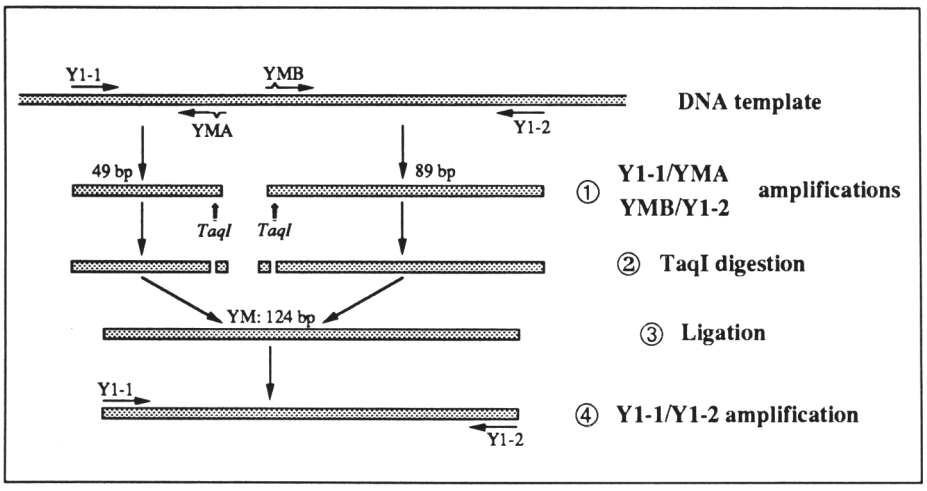

FIGURE 1 Construction of the YM standard for competitive PCR. The process is described in detail in the text. Briefly, DNA amplified with the two sets of primers (Y1-1/YMA and YMB/Y1-2) yields 49- and 89-bp long fragments, respectively. After *Taq*I restriction enzyme digestion, ligation, and amplification, the YM standard (124 bp) is obtained with a deletion of 30 bp with respect to the normal sequence.

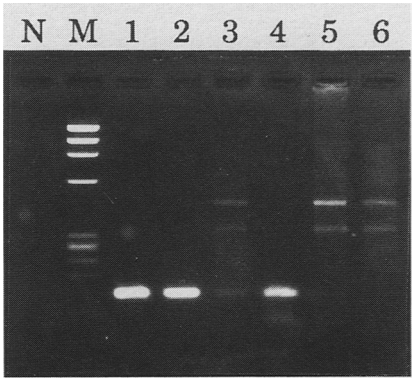

FIGURE 2 Qualitative PCR analysis of Y sequences. (N) Distilled water; (M) ϕX174/*Hae*III molecular weight marker; (lane *1*) normal male DNA; (lane *2*) epithelial mouth cell DNA from patient A; (lane *3*) blood cell DNA from patient A; (lane *4*) epithelial mouth cell DNA from patient B; (lane *5*) blood cell DNA from patient B; (lane *6*) normal female DNA.

RESULTS AND DISCUSSION

We have developed a simple and reliable strategy to evaluate the mixed chimerism after sex-mismatched (female donor, male recipient) allogeneic BMT. The first step of this protocol is based on a classic qualitative PCR on Y sequences to detect possible residual host cells in blood samples from transplanted male patients. Blood samples are preferred over bone marrow to prevent false-positive reactions owing to nonhemopoietic cells such as stromal cells. In a second step, if the detection PCR is positive, a competitive amplification is performed to quantify the remaining recipient hemopoietic cells.

Y Sequence Detection System

Two patients (A and B) and two controls (normal male and female) were studied. To test the quality of the DNAs, each sample was amplified using specific primers located in the KM19 locus. All DNA samples gave the expected 567-bp fragment (data not shown). These samples were further analyzed by Y PCR amplification (Fig. 2). The 154-bp Y-specific fragment is observed for normal male (lane 1) and epithelial mouth cells of patients A and B (lane 2 and 4, respec-

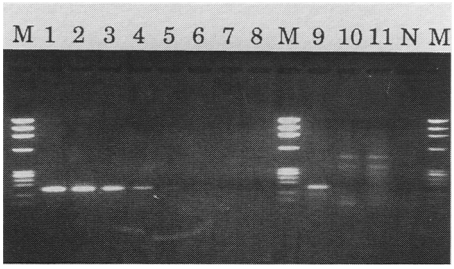

FIGURE 3 Y-chromosome amplification after 10-fold serial dilutions of DNA from a normal male. (N) Distilled water; (M) ϕχ174/*Hae*III molecular weight marker; (lanes *1–8*) 10-fold dilutions of DNA from a normal male ranging from 100 ng to 10 fg per assay; (lane *9*) mouthwash cell DNA from patient B; (lane *10*) blood cell DNA from patient B; (lane *11*) normal female DNA.

tively). A slight signal is observed in blood cell DNA of patient A (lane 3). No specific Y sequence amplification was seen with the normal female control (lane 6) or with blood DNA from patient B (lane 5). In the last three cases, two non-Y fragments, ~350 and 450 bp in length, were observed. The nucleotide sequences of the latter amplified products were analyzed. They are homologous to the human satellite III DNA found on autosomes.[11] Moreover, they are composed of a succession of pentanucleotide repeats and contain many *Taq*I restriction sites (results not shown). These non-Y fragments are obtained only when the Y sequences are absent or in a very low amount in the studied samples. However, they are easily distinguishable from the specific 154-bp fragment, and they may be a good internal control of the PCR efficiency in the absence of specific Y sequence (chimeric DNA).

To assess the sensitivity of our qualitative PCR protocol, DNA from a normal male was diluted in distilled water in proportions ranging from 100 ng to 10 fg per 50 μl assay (Fig. 3). We detect up to the fifth dilution (lane 6), which corresponds to ~1 pg. Our PCR sensitivity is almost similar to those of other reports[2,4] and is better than the conventional Southern blot analysis. Therefore, patient B (lane 10), who appeared negative, presented <1 pg of male hemopoietic cell DNA per 100 ng of total DNA. On the other hand, for patient A (positive in Y detection), the remaining hemopoietic recipient cells may be apprehended by quantitative PCR.

Quantitative PCR on Y Sequences

Over the last several years, PCR has become a powerful tool for quantitation. Several methods are commonly used: Labeled nucleotide incorporation, hybridization with a specific probe, and competitive PCR. The latter strategy uses an exogenous template as an internal standard and seems to be the best way to practice a real quantitation of nucleic acid template by in vitro amplification.[12–14]

To standardize our competitive PCR on Y chromosomes, 100 ng of DNA from a normal male was coamplified with successive 10-fold dilutions of the YM standard using Y1-1 and Y1-2 primers (Fig. 4A). The equivalent signal intensity between the standard and the control male DNA corresponds to the 10^{-3} YM dilution. When the same experiment is realized with DNA from a normal female (Fig. 4B), the YM 124-bp fragment is observed up to the 10^{-8} dilution. The parasitic fragments appeared toward the 10^{-7} dilution.

We have then tested DNA from blood or from mouthwash of patient A (Fig. 5). The equivalence points correspond to the 10^{-4} YM dilution for epithelial mouth DNA (Fig. 5A, lane 4) and to the 10^{-7} YM dilution for blood (Fig. 5B, lane 7). This result indicates that the blood cell DNA of patient A contains ~10^3-fold less Y chromosome sequences as compared with his epithelial cell DNA. In the

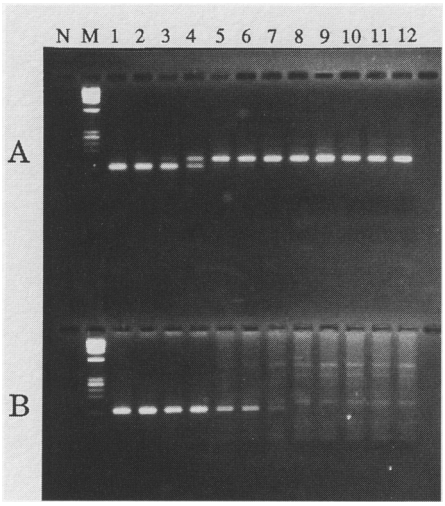

FIGURE 4 Competitive PCR standardization with normal male or normal female DNAs. One hundred nanograms of male (*A*) and female (*B*) DNA was studied. (N) Distilled water; (M) ϕχ174/*Hae*III molecular weight marker; (lanes *1–12*) 10-fold serial dilutions of the YM standard (from 10^{-1} to 10^{-12}).

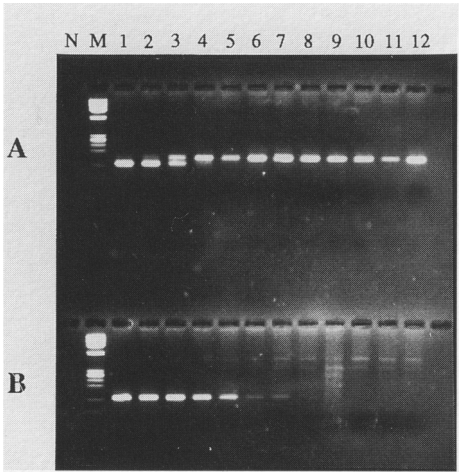

FIGURE 5 Competitive PCR analysis of patient A DNA. One hundred nanograms of DNA from mouthwash (*A*) and blood (*B*) cells was analyzed in the presence of 10-fold serial dilutions of the YM standard from 10^{-1} to 10^{-12} (lanes *1–12*). (N) Distilled water; (M) ϕχ174/*Hae*III molecular weight marker.

previous experiment, the equivalence point for normal blood DNA was found at the 10^{-3} YM dilution. This difference of one dilution may originate from the repeat number polymorphism at the DYZ1 locus. Our positive control with buccal epithelium of each patient eliminates the dependence of the assay on the number of Y repeats.

In competitive PCR, a standard and a target sequence compete for the same primers and, therefore, for amplification. In our case, other sequences, such as the human satellite III, might behave as endogenous competitors. To eliminate this possible artifact and to achieve a classic competitive reaction, we have digested DNA samples with *Taq*I restriction enzyme before PCR. This enzyme was used because the 154-bp Y fragment does not contain the restriction site, whereas autosomal products present many *Taq*I restriction sites. Under these conditions, only Y sequences are amplified, thus allowing an accurate competitive PCR (Fig. 6). The previous experiment was repeated with DNA treated with the *Taq*I restriction enzyme; the equivalence point corresponds to a 3.3×10^{-4} dilution in the epithelial cells (lane 4) and 10^{-7} in the blood cells (lane 8). These results show that *Taq*I digestion eliminates the non-Y sequence amplification and allows a real competitive PCR. Altogether, one can estimate

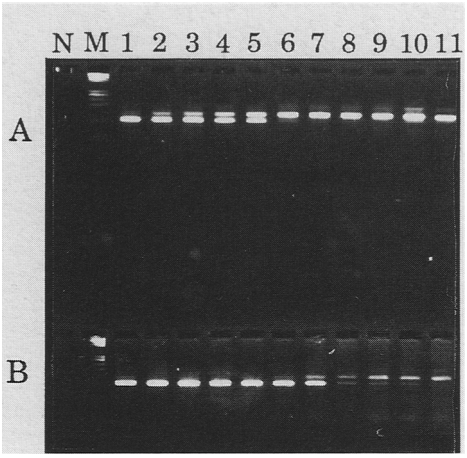

FIGURE 6 Competitive PCR analysis of patient A DNA after *Taq*I digestion. One hundred nanograms of DNA from mouthwash (*A*) and blood (*B*) cells was analyzed in the presence of the following dilutions of the YM standard: 10^{-2} (lane *1*); 10^{-3} (lane *2*); 6.6×10^{-4} (lane *3*); 3.3×10^{-4} (lane *4*), 10^{-4} to 10^{-7} (lanes *5–8*), 5×10^{-8} (lane *9*), 10^{-8} and 10^{-9} (lanes *10,11*). (N) Distilled water; (M) $\phi\chi174$/*Hae*III molecular weight marker.

that in patient A, 1 blood cell out of 3300 is from male origin.

The strategy we propose for the monitoring of sex-mismatched BMT is very efficient. The extra signals obtained with female DNA do not really interfere with the competitors. Moreover, the results obtained after a *Taq*I restriction enzyme digestion are even more reliable. Our purpose is not an absolute quantitation of the remaining host cells in blood but the establishment of a ratio between the remaining Y cells in blood and in buccal epithelium. This percentage is equivalent to a pretransplant/posttransplant blood host DNA ratio. Owing to the Y repetition polymorphism, it is not possible to compare the amount of residual Y clones in different patients. Thus, our quantitation assay using DNA from blood and DNA from epithelial cells in the same patient is perfectly adapted to our purpose.

The methodology described here might also prove useful in sex determination or forensic medicine. In our laboratory it is used for the molecular monitoring of patients after sex-mismatched BMT. The PCR analysis is performed before the transplantation and every 3 months after it. When the qualitative PCR remains negative for a patient, this is of excellent prognosis for him and allows a favorable prediction for the clinical outcome. On the other hand, when we detect Y recipient DNA in blood after BMT, this result does not mean necessarily a recurrence of the disease, because these Y residual cells may be without tumorigenic potential. However, in this case, competitive PCR facilitates the monitoring of the kinetic behavior of the residual clone during the post-BMT period. Systematic investigation of larger patient series will substantiate the value of our Y competitive system for the management of BMT when a male patient is transplanted with a female donor.

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