DETECTION OF MICROCHIMERISM AFTER PRETRANSPLANT BLOOD TRANSFUSIONS

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ABSTRACT

Microchimerism is the presence of a small percentage of allogeneic hematopoietic cells in an individual that can exist following blood transfusion, transplantation and pregnancy. It is associated with immunological tolerance and may be involved in the beneficial transplant outcome after pretransplant blood transfusions. Our goal was to determine the level of microchimerism in patients who received a leukocyte-containing pretransplant blood transfusion, while evaluating the conventional nested PCR technique for microchimerism detection. In one patient, donor-derived cells were detected two weeks post-transfusion. In the other ones, donor cells were absent or results were difficult to interpret due to technical limitations of the nested PCR technique. Therefore, we proposed a novel technique that combines flow cytometry-based cell sorting and confirmation of donor origin by subsequent quantitative PCR analysis. First analyses suggest that this approach is a robust and reliable approach for the detection of microchimerism. Once this assay is validated, it is possible to establish the occurrence of microchimerism after pretransplant blood transfusions and determine its role in transplantation tolerance.
INTRODUCTION

Hematopoietic chimerism can be defined as the coexistence of hematopoietic lineage derived cells with a distinct genetic background in an individual. It may be detected following cell transfer during pregnancy in the mother and in the fetus, but also after blood transfusion, stem cell transplantation, and solid organ transplantation. The existence of 1-100% donor cells in the recipient is called macrochimerism. In contrast, microchimerism refers to the state in which the percentage of circulating donor cells is low (<1%) (1). Whereas complete or macrochimerism is the purpose of hematopoietic stem cell transplantation, microchimerism is often found after solid organ transplantation through migration of passenger hematopoietic cells of donor origin from the graft into the peripheral circulation and tissues of the recipient. Many reports exist on microchimerism after transplantation of liver (2), kidney (3-5), heart (6) and lungs (7,8), even up to 30 years after the original transplantation. After blood transfusion, donor cells in the recipient’s peripheral blood are rapidly cleared within 5-7 days (9,10), although in case the recipient and blood donor share an HLA haplotype donor cell-derived DNA can be detected for several weeks (11). In immunocompromised patients due to trauma injury, long-term (>2 years) donor microchimerism has been found, even after transfusion of leukocyte-reduced blood products (12).

Detection of peripheral blood microchimerism may help to understand the immunological effects that occur after pregnancy, blood transfusion and transplantation. Fetal cell microchimerism is associated with maternal autoimmune diseases, but, in contrast, it may favor the maternal health post-partum (13). Initially, microchimerism was detected in patients who showed excellent graft function and required minimal immunosuppressive therapy (14). Although it is often questioned whether microchimerism is the cause or consequence of long-term allograft survival (15), several observational studies report now on a causal role of circulating donor cells in the induction of tolerance (16-18). Especially, microchimerism early after transplantation (<2 months) correlated with a low incidence of acute allograft rejection (19). However, it is not clear whether microchimerism indicates a tolerant state that allows reduction of immunosuppressive treatment in the individual patient.

For obvious reasons it is postulated that in order to obtain microchimerism, donor-reactive T cells in the recipient must be eliminated or suppressed. Elimination can occur when donor and recipient antigen-presenting cells (APCs) coexist in large quantities (macrochimerism), thereby deleting T cells with high affinity for donor or recipient APCs in the thymus (20). Such conditions exist after hematopoietic stem cell transplantation and require strong immunosuppressive conditioning of the recipient, associated with impaired immunocompetence. Experimental models focused on less
toxic approaches by blocking co-stimulatory signals with monoclonal antibodies (mAbs), impairing the ability of T cells to respond to alloantigens (20). Anti-CD154 antibodies that target the CD40-CD40L co-stimulatory pathway in combination with exposure to donor hematopoietic stem cells, derived from bone marrow or blood, resulted in allogeneic chimerism and tolerance (21,22). In this process, induction of CD4+ regulatory T cells may be responsible for the maintenance of tolerance (23). Microchimerism may be one of the mechanisms accounting for the beneficial effect of pretransplant blood transfusions on transplant outcome (24). However, a study in mice demonstrated that donor-recipient microchimerism, obtained by a pretransplant donor-specific transfusion under the cover of a depleting anti-CD4 antibody, is not required in itself for tolerance induction (25).

Opposite to tolerance induction, in several studies microchimerism was identified as a risk factor for allograft rejection (26-28). It is conceivable that the nature of donor-derived cells may underlie this phenomenon, while contradictory results may also be explained by different detection techniques and a difference in control time points studied (29). Initially, the presence of the sex-determining region-y gene (SRY) was used as a marker of microchimerism to detect male cells in female recipients (2). Quantitative polymerase chain reaction (PCR) of the SRY gene is a reproducible and sensitive technique (29), but is restricted to sex-mismatched combinations only. The human leukocyte antigens (HLA) are a broader applicable target for microchimerism detection, as they are highly polymorphic (30) and thus frequently mismatched between two individuals. Classical and quantitative (real-time) PCR techniques for detection of HLA alleles are frequently used and suitable for detection of macrochimerism and microchimerism (29). The sensitivity of these conventional PCR techniques can be increased by the introduction of a preliminary PCR that first amplifies the generic HLA class I (31) or class II locus (32), followed by a second-round PCR with sequence-specific primers (nested PCR), although this may lead to false positive reactions.

In this chapter, we describe and discuss three topics: 1) the detection of microchimerism in patients who received a pretransplant leukocyte-containing blood transfusion, 2) the use of the nested PCR technique and 3) the development of a new technique that combines flow cytometry-based cell sorting and real-time PCR.

MATERIALS AND METHODS

Patients and blood samples
Seven patients were selected for microchimeric analyses after blood transfusion. While on the waiting list for simultaneous pancreas-kidney transplantation (SPKT), they received a protocolled blood transfusion (PBT) in case they did not have a history of
pregnancy or therapeutic blood transfusions. Blood donors were selected on basis of having one HLA-DR match with the patient and a negative red cell and leukocyte cross match. One unit of fresh, <24 hours stored, non-irradiated, plasma-reduced whole blood was administered. Patient blood samples were collected before and after PBT (2 weeks and >10 weeks) and from each donor a blood sample was derived from the blood product. The collection of material was approved by ethical committees of the Leiden University Medical Center (Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMCs) from patients and blood donors were isolated from heparinized blood by density gradient centrifugation with Ficoll-Amidotrizoate (Pharmacy LUMC, Leiden, the Netherlands). The cells were washed twice with phosphate-buffered saline (PBS) and frozen in RPMI 1640 supplemented with L-glutamine (RPML/gl, both from Gibco, Breda, the Netherlands), 20% fetal calf serum (FCS, Greiner, Alphen a/d Rijn, the Netherlands) and 20% dimethylsulfoxide (DMSO, Fluka, Buchs, Switzerland). The cells were frozen at -70°C and stored in liquid nitrogen until use.

DNA isolation
DNA was prepared, after thawing of cryopreserved mononuclear cells, by a salting out method (33), precipitated with ethanol, and resuspended in TE buffer (manufactured in our lab). All samples were typed for HLA class I and HLA class II using the polymerase chain reaction with sequence-specific oligonucleotide probes (PCR-SSO technique). Table 1 shows the HLA type and sex of the patients and donors.

Nested polymerase chain reaction (nested PCR)
A nested PCR, consisting of two successive rounds of PCR, was performed for the detection of HLA class I microchimerism. First-round PCR (generic amplification) was carried out in a mixture containing dH2O, Tris-EDTA based buffer (10x MG buffer; manufactured in our lab), 0.2 mM of each dNTP, 0.1 U/μl taq polymerase and 0.5 pmol/μl of the forward (5') and reverse (3') HLA locus-specific primers (HLA-A and HLA-B). If feasible, PCR reactions were carried out in a volume of 50 μl containing 0.5 μg of DNA. PCR amplification was done in a MJ Dyad discipline PCR machine (Bio-Rad, Hercules, CA, USA). After an initial denaturation step of 3min at 95°C, 32 cycles were performed: 95°C for 60s, 65°C for 60s, 72°C for 3min, and time extension at 72°C for 10min.

The amplification product was diluted 1:100 in H2O and 5 μl of the diluted product was used as input in a second round of amplification with forward and reverse sequence-specific primers for the HLA molecules of the blood donor. The reaction mixture contained dH2O, 10x MG buffer, loading buffer (10x LB; manufactured in our lab), 0.2 mM of each dNTP, and 0.1 U/μl taq polymerase and 1.6 pmol/μl of each primer. The
PCR program was as follows: denaturation for 2 min at 94°C, 10 cycles: 94°C for 10 s, 65°C for 60 s, 20 cycles: 94°C for 10 s, 61°C for 50 s, 72°C for 30 s and time extension at 72°C for 3 min.

PCR products were subsequently analyzed by agarose gel electrophoresis. PBMCs of one patient were further characterized by sequence analysis. The PCR product formed by the specific A11.2 primer combination in the second (nested) PCR was sequenced by the 3130 XL sequencer (Applied Biosystems, Foster City, CA, USA) and analyzed with SBT Engine software.

**Flow cytometry-based cell sorting**

Cell mixtures of 10% (1:10, donor:patient) and 0.1% (1:1000) were prepared from pre-transfusion PBMCs of patient 7 and donor PBMCs to mimic microchimeric situations. To prevent non-specific binding of monoclonal antibodies (mAbs) to Fc receptors, thawed patient and donor PBMCs were blocked with a non-specific and non-biotinylated antibody (FcR blocking Reagent, Miltenyi Biotec, Bergisch Gladbach, Germany). Then, cell mixtures were stained for 30 min at 4°C with an anti-HLA-B51/B35 biotin-labeled antibody (HDG8D9; manufactured in our lab), Alexa 647-conjugated streptavidin (Invitrogen, Eugene, OR, USA), anti-CD45 FITC and anti-CD14-PE from BD Biosciences Pharmingen (San Diego, CA, USA). The 10% mix was used to define the gating strategy for CD45+CD14−HLA-B35+ donor lymphocytes and CD45+CD14−HLA-B35− patient lymphocytes, whereas the 0.1% mix (1:1000) was prepared for sorting these cell subsets. Cell staining and sorting occurred with the BD FACSAria™ cell sorter (BD Biosciences, San Jose, CA, USA), equipped with a 576/26 nm filter for PE-labeled cells, a 530/30 nm filter for FITC-labeled cells and a 660/20 nm filter for Alexa 647-labeled cells. The sorted cell fractions, obtained using the yield sort settings, were used in subsequent quantitative real-time PCR analysis.

**Real-time PCR**

DNA was isolated as described before from the sorted cell fractions, the original 0.1% chimeric cell suspension and PBMCs of patient 7 and the blood donor. Amplification was carried out in a mixture containing 5 μg DNA-cells/μl, dH2O, SYBR Green mix (Bio-Rad, Hercules, CA, USA) and 4 pmol/μl HLA-A1/A11 or 0.4 pmol/μl HLA-DR7 forward and reverse primers using a real-time PCR machine (MyiQ, Bio-Rad). Primers for the human hematopoietic cell kinase (HCK) gene were used for normalization of the variable content of human DNA (internal standard) in each sample (34). The PCR program was as follows: denaturation for 5 min at 94°C, 10 cycles: 94°C for 15 s, 65°C for 45 s, 30 cycles: 94°C for 15 s, 61°C for 45 s, 72°C for 30 s, followed by 95°C for 60 s, 55°C for 60 s and 80 steps of 0.5°C/step increase of temperature from 55°C to 95°C to create the melt curve. For each reaction, the PCR cycle number that generated the first
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fluorescence signal above a threshold (the threshold cycle, Ct) was calculated with Bio-Rad iQ5 software. The delta Ct (ΔCt) was calculated as follows: Ct obtained with donor-specific primers – Ct obtained with HCK primers.

RESULTS

Study population
The characteristics of patients and blood donors are shown in Table 1. The selection of blood donors was based on one HLA-DR match with the recipient, as shown in bold, and a mismatch for the other HLA-DR allele as well as the HLA-A and HLA-B alleles.

Table 1: Patient and donor characteristics.

<table>
<thead>
<tr>
<th>HLA type patient*</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA type blood donor*</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-DR</th>
<th>Sex</th>
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<td>1, 3</td>
<td>13, 62</td>
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<td>35, 60</td>
<td>4, 7</td>
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*a HLA type: split level. M: male; F: female.

Microchimerism after blood transfusion
Amplification of post-transfusion DNA of patient 7 showed positive bands on agarose gel when donor-specific primers were used. In the other 6 patients, we found no positive bands. Results of the nested PCR of P7 and one other patient (P5) are shown in Figure 1A and 1B respectively. For each HLA class I allele, two different combinations of forward and reverse primers were compared (Figure 1D).

In the left part of Figure 1A a clear dilution is shown in lane 1 to 7 of positive DNA (see Figure 1C for order) for each HLA-A specific primer. However, primer combination A1.1 depicted the negative control DNA as a positive sample, which makes interpretation of the results obtained with this primer combination difficult. Chimeric donor cells were detected, in duplicate, in the 2 wk post-transfusion samples, while absent in the pre-transfusion test samples when the HLA-A1.2, -A11.1 and -A11.2 primer combinations were used. No cross reactive response of these primers was observed with HLA alleles corresponding to the patient (data not shown). The positive bands are of similar intensity as the positive DNA control in lane 5, which means that chimeric DNA was detected at a level of 0.005% (equivalent to a dilution of DNA of
Sequence analysis of the 2 wk post-transfusion PCR product acquired by using primer combination A11.2, confirmed donor origin. The HLA-B35 specific primers failed, while neither positive DNA nor chimeric cells after transfusion could be detected. When using the HLA-B60 primers, false-positive reactions (prior to transfusion and positive water controls) were found. Thus, chimeric cells after blood transfusion were detected in P7 with the primers specific for the HLA-A alleles, but could not be confirmed with the HLA-B specific primers. More than 10 weeks after transfusion, chimeric cells had disappeared below detection level as none of the primers was able to amplify a specific product.
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<table>
<thead>
<tr>
<th>Standard dilution</th>
<th>Test samples</th>
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</tr>
<tr>
<td>1:100</td>
<td>pre-transfusion</td>
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<td>H₂O</td>
</tr>
<tr>
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<td>2 wk post-transfusion</td>
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<td>2 wk post-transfusion</td>
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<tr>
<td>1:100000</td>
<td>H₂O</td>
</tr>
<tr>
<td>1:1000000</td>
<td>&gt;10 wk post-transfusion</td>
</tr>
<tr>
<td>negative</td>
<td>&gt;10 wk post-transfusion</td>
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<tr>
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Chapter 6

<table>
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<td>tcgggtcctgagggagagga</td>
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<tr>
<td>HLA-B</td>
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<td>atagggtgc(gc)ggggtgaggggc</td>
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Figure 1: Detection of microchimerism by nested PCR using HLA class I specific primers. Figure 1A and B represent Polaroid pictures of agarose gel of P7 and P5 respectively. Each lane represents a different sample. Sample 1 to 7 are dilutions of DNA of the blood donor (positive control) started with 70,000 positive cells (1:1); sample 8 only contains negative DNA; sample 9 is the DNA size marker; sample 10 to 17 are patient PBMC of different time periods and water controls (C). Figure D shows the sequence of the HLA-A and HLA-B primers for first round PCR and of each sequence-specific primer for second round PCR. In second round PCR, two different primer combinations are used for each specific HLA molecule.

In Figure 1B, representing P5, a clear response was shown in the 2 wk post-transfusion samples when using primer combination HLA-A1.1. However, due to positivity of one pre-transfusion sample, results obtained with this primer combination are not reliable. A slight response was detected after amplification with the HLA-A3.1 primers, but this could not be confirmed with the duplicate sample or with any of the other primer combinations.

Nested PCR technique

Frequently, technical problems occurred while using the nested PCR technique. Some primer combinations amplificated specific as well as non-specific DNA in a similar quantity, as shown by primer combination B60.2 (Figure 1A) and A1.2 (Figure 1B). In contrast, other primers did not bind at all (B35.2, Figure 1A).
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An internal control is the use of pre-transfusion DNA. Only patients who were not exposed to alloantigens via pregnancy or therapeutic transfusions were included into the pretransplant blood transfusion protocol, which makes the chance of detecting microchimeric cells in the pre-transfusion samples very low. Therefore, positive pre-transfusion samples (as obtained by A1.1 primers, Figure 1B) were regarded as false-positive, as well as the positive water controls (B60.1, lane 12, Figure 1A). It is clear that nested PCR is a very sensitive technique prone to contamination leading to false positive results. A more robust and reliable approach for the detection of microchimerism is necessary for routinely monitoring of patients.

Flow cytometry-based cell sorting

HLA specific monoclonal antibodies (mAbs) can be used to distinguish between cells of two genetically different individuals using flowcytometry (35). To validate sensitivity of using HLA specific antibodies, we mixed pre-transfusion PBMCs of patient 7 (P7) and the blood donor (Table 1) in different quantities in order to mimic a microchimeric situation. Subsequently, cells were labeled with an anti-HLA-B35 mAb specific for donor but not patient cells. The 10% mix was used to define gates for the CD45⁺CD14⁺ HLA-B35⁺ donor cells (pos) as well as the CD45⁺CD14⁻HLA-B35⁻ patient cells (neg) (Figure 2A). The 0.1% mix was used for cell sorting using the defined gating strategy. More than 3x10⁶ events were acquired during cell sorting, resulting in a yield of 2522 cells in the positive fraction (pos) and 706,266 cells in the negative fraction (neg). The positive and negative fractions were used for further quantitative PCR analysis.

Figure 2: Gating strategy and cell sorting. Dotplots of CD45 and HLA-B35 in the 10% (A) and 0.1% (B) donor-patient mix. The gates for CD45⁺CD14⁺HLA-B35⁺ donor cells (pos) and CD45⁺CD14⁻HLA-B35⁻ patient cells (neg) are set in the 10% mix (A) and applied to the 0.1% mix (B). The neg and pos fraction of the 0.1% mix (B) are sorted and further analyzed in quantitative PCR.
As shown in Figure 2B, the analysis of so many cells, which is necessary to sort sufficient cells for PCR analyses, hampers a reliable discrimination between patient and donor cells. Additional staining of the unwanted (patient) cells with another HLA specific mAb (as exclusion marker) can overcome this problem. This is shown in a separate experiment, where we prepared a mixture of HLA-A2+ chimeric cells and HLA-A3+ bulk cells (1:100) and stained them with HLA specific mAbs (Figure 3). The chimeric cell fraction (HLA-A2-A3) can easily be discriminated and is more pure compared to the positive fraction of Figure 2B.

Figure 3: Gating strategy using two distinct HLA specific mAbs. A 1% mixture of chimeric cells (HLA-A2, -A3, -B62, -B17, -DR17, -DR15) and bulk cells (HLA-A3, -A11, -B7, -B35, -DR4, -DR13) was prepared and stained with an anti-HLA-A2 Alexa 647 conjugated mAb, an anti-HLA-A11/A3/A24 biotin labeled antibody (BR011F6; both manufactured in our lab), and PE conjugated streptavidin. Chimeric cells are defined as CD45+CD14-HLA-A2+A3- and bulk cells as CD45+CD14-HLA-A2-A3+.

Real-time PCR
After sorting the HLA-B35+ donor cells, a real-time PCR was performed to confirm donor origin on the DNA level in a quantitative way with primers specific for other donor-specific HLA molecules (HLA-A1/11, HLA-DR7). The sorted fractions (pos and neg), the artificial 0.1% chimeric cell suspension as well as the original patient and blood donor PBMCs were analyzed in real-time PCR. Results of this quantitative assay are shown in Figure 4A and depicted as Ct values, which refer to the PCR cycle numbers that are necessary to generate a fluorescent signal above a certain threshold. The lower the Ct value, the fewer PCR cycles are needed to generate a specific product, showing that more specific DNA was present in the starting sample. Since each starting
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sample contained a variable amount of DNA, this had to be normalized by using primers for the house-keeping gene human hematopoietic cell kinase (HCK). Figure 4A shows that the positive fraction (pos) after sort contained the lowest amount of DNA (Ct HCK=19.71) and the blood donor PBMCs the highest amount of DNA (Ct HCK=14.76). To correct for DNA input, the Ct value for the HCK gene was subtracted from the Ct values obtained after amplification with the donor-specific HLA-A1/A11 or HLA-DR7 primers (ΔCt). The ΔCt values are used for comparisons.

Figure 4A shows that the pre-transfusion sample failed to generate a Ct value while using the donor-specific primers, which confirms its suitability as a negative control. Blood donor PBMCs serve as a positive control, which is confirmed by a low ΔCt value (ΔCt A1/A11: 0.06, ΔCt DR7: 1.88). Higher ΔCt values were found when less donor cells were present, such as in the 0.1% chimeric cell suspension (ΔCt A1/A11: 9.42, ΔCt DR7: 10.70). As expected, ΔCt values decreased when using the positive fraction after sort containing donor cells (ΔCt A1/A11: -0.57, ΔCt DR7: 0.70) and increased when using the negative fraction after sort containing only patient cells (ΔCt A1/A11: 9.49, ΔCt DR7: 13.53).

The enrichment of donor cells by flowcytometry-based cell sorting is shown graphically in Figure 4B and C. If the ΔCt value of the 0.1% chimeric pre-sort sample is considered as zero, the ΔCt value of the positive fraction after sort is about ten times lower (note the reverse y-axis) and thus contains more donor-specific DNA. Similarly, the negative fractions contain less donor-specific DNA, as the ΔCt values, especially obtained with the HLA-DR7 specific primers, are higher as compared to the 0.1% chimeric cell suspension.

Also the 2 wk post-transfusion sample of P7 was taken in the flowcytometry-based cell sorting and quantitative PCR analysis. As expected, the ΔCt value of the positive fraction was lower compared with the ΔCt value of the negative fraction when using the HLA-A1/A11 specific primers, however this could not be confirmed with the HLA-DR7 specific primers (data not shown).

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<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
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<td>Blood donor PBMCs</td>
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<td>14.82</td>
<td>0.06</td>
<td>16.64</td>
<td>1.88</td>
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<tr>
<td>0.1% chimera pre-sort</td>
<td>15.60</td>
<td>25.02</td>
<td>9.42</td>
<td>26.30</td>
<td>10.70</td>
<td></td>
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<tr>
<td>Pos after sort</td>
<td>19.71</td>
<td>19.14</td>
<td>-0.57</td>
<td>20.41</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Neg after sort</td>
<td>14.81</td>
<td>24.30</td>
<td>9.49</td>
<td>28.34</td>
<td>13.53</td>
<td></td>
</tr>
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Chapter 6

**DISCUSSION**

Although it is not clear whether microchimerism is the cause or consequence of transplant tolerance, it is widely accepted that a certain degree of tolerance must exist in order for donor cells to persist in the recipient’s circulation (15). One of the goals of this study was to investigate the occurrence of microchimerism in recipients of a leukocyte-containing blood transfusion. In one out of 7 patients we detected leukocytes of donor origin in the peripheral blood two weeks after transfusion. In this patient, microchimerism was evaluated by the nested PCR technique that is now used for microchimerism detection and a new technique that combines flow cytometry-based cell sorting and direct PCR.

The degree to which donor cells survive after allogeneic blood transfusion depends on several factors. Microchimerism is thought to develop more often after transfusion of traumatic patients with a suppressed immune system (36) and less often after...
transfusion of HLA-mismatched (11) and irradiated or stored blood products (37). Patients in this study received a fresh, non-irradiated, one HLA-DR shared blood transfusion, while on dialysis awaiting simultaneous pancreas-kidney transplantation. In one out of seven patients, microchimeric cells of donor origin were detected two weeks after transfusion by nested PCR (sensitivity: 0.005%) and this was confirmed by sequence analysis. None of the patients showed microchimeric donor cells 16 weeks after transfusion. Due to the small study population, no conclusions can be drawn yet about the prevalence of microchimerism after pretransplant leukocyte-containing blood transfusions.

To be able to detect a low number of donor cells in the recipient’s peripheral circulation, a technique has to fulfill strict criteria in order to obtain reliable results. The nested PCR technique is characterized by its high sensitivity (31) and is commonly used in detection of microchimerism (8,32,35). However, a drawback of the technique is the appearance of unexpected amplification products (32). We also found positive bands on the gel in the water and negative control samples and even in a few pre-transfusion samples, which are the most adequate controls. However, individuals without previous pregnancies, transplants of transplantations are not necessarily negative for microchimerism, due to two-way fetal-maternal cell transfer. An underlying problem of the extra bands could be contamination of the samples. Amplification of contaminating molecules in two successive rounds of PCR can easily be misinterpreted to indicate the presence of donor alleles. Currently, we are evaluating the possibility of using ultraviolet light for the degradation of contaminating aerosols. Working in an air cabinet may be an additional option to reduce the risk of contamination. False-positive results can also arise after misannealing of the primers. In many cases, a single mismatch at the 3’ residue was not sufficient to prevent mispriming events. Cross reactivity of the primers with other HLA molecules is another possibility. In any case, the results can be strengthened with an additional nested PCR on other markers than HLA, such as the Y-chromosome (if possible) or mitochondrial DNA. Besides the high probability of contamination and mispriming events, the nested PCR is a complicated and time-consuming technique and not quantitative. Therefore, we developed an alternative and more robust approach to detect microchimerism: a flow cytometry-based method to select the microchimeric cells with HLA-specific monoclonal antibodies (mAbs) and confirmation of donor/patient origin with quantitative real-time PCR.

Labeling of mAbs with magnetic beads or fluorescent markers makes it possible to sort individual cells with advanced techniques (MACS: magnetic-activated cell separation and FACS: fluorescent-activated cell sorting). While a high percentage of cells is lost during MACS separation (35), we used the FACS-based method to select microchimeric cells for further analysis. Flow cytometry has already been described as a sensitive and
valuable technique in the detection of macro- or mixed chimerism, however highly specific and good binding antibodies are needed for the detection of a low number (<1%) of specific cells (38,39). While using the biotinylated anti-HLA-B35 mAb that was manufactured in our own lab, we were able to detect the donor populations in the 10% as well as in the 0.1% mixture.

A real-time PCR technique confirmed the donor origin of the sorted positive fraction from the 0.1% mixture. Irrespective of the used primers, DNA product corresponding to the donor was detected after less rounds of amplification in case of the positive fraction sample as compared to the 0.1% mixture. This direct PCR technique was also able to detect 1 donor cell in 1000 patient cells (sensitivity: 0.1%), as the ΔCt values of the 0.1% mixture were lower than the sorted negative fraction and at least detectable in contrary to the pre-transfusion sample.

This chapter proves the principle of the combined approach to detect microchimerism. Flow cytometry-based sorting of cells followed by confirmation of donor origin in real-time PCR was possible for microchimeric cells at a level of 0.1%. At lower concentrations of donor cells, more cells have to be counted in order to sort enough cells for reliable PCR analyses. We showed that the non-specific binding that occurred with increasing numbers of counted cells can be circumvented using an additional HLA specific mAb that targets the patient cells. In ongoing experiments this set-up is applied to establish the detection limit of the combined approach.

The advantage of the combined approach is the detection of microchimerism on cell level as well as DNA level. This way, the PCR primers can be chosen in such a way that they target other donor HLA molecules than the mAbs for flowcytometry, which strengthens the outcome of microchimerism detection. Our results showed that donor cell sorting facilitates the real-time PCR technique and results in an enriched donor cell population that can also be used in further (functional) assays.

In conclusion, microchimerism after a leukocyte-containing blood transfusion was established after two weeks in one patient using the nested PCR technique. In the other six patients, donor cells were absent or not detected due to technical limitations. The nested PCR is a sensitive technique, but contamination remains a major problem. Our proposed approach, combining flow cytometry-based cell sorting and confirmation of donor origin by subsequent quantitative PCR analysis, may be a valuable and reliable tool for microchimerism detection. As our laboratory has developed more than 100 human monoclonal antibodies directed against different HLA antigens, it is possible to apply this method in almost all allogeneic combinations. Once this assay is validated, it is possible to establish the prevalence of microchimerism after pretransplant blood transfusions in a reliable way and determine its role in transplantation tolerance.
Development of microchimerism after pretransplant blood transfusions

References

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