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Abstract
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Reference


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Microchimerism After Liver Transplantation: Absence of Rejection Without Abrogation of Anti-Donor Cytotoxic T-Lymphocyte–Mediated Alloreactivity

Florence Bettens, Jean-Marie Tiercy, Nathalie Campanile, Emiliano Giostra, Pietro Majno, Laura Rubbia, Eddy Roosnek, Gilles Mentha, and Jean Villard

Microchimerism (MC) is defined by the persistence of <1% circulating donor cells resulting from cell migration from the graft. MC may play a role in the induction of unresponsiveness to allogeneic tissues, or may be merely the consequence of the graft’s acceptance following immunosuppression. To analyze early MC (7 patients) and late MC (12 patients) following a liver transplantation, we designed a sensitive and semiquantitative nested polymerase chain reaction (PCR) protocol based on the detection of incompatible human leukocyte antigen (HLA)-DRB1 donor alleles. MC was measured in multiple PCR samples and expressed as percent positive PCRs / time point. The detection level was 1 donor cell / 10⁵ patient cells. All patients had detectable early MC, ranging from 5 to 100% positive PCRs in the 1st 3 months after transplantation. The kinetic analysis demonstrated that MC decreased during the 1st year in 6 of 7 patients. All of the 4 patients with the lowest MC had rejection episodes, vs. none among the 3 patients with MC > 50%. However, cytotoxic T-lymphocyte reactivity (CTL) against HLA class I donor antigens could be demonstrated 1 year posttransplant in 2 patients with a high level of early MC. MC is a dynamic process, which is easily detectable <3 months after liver transplantation. In conclusion, a correlation between the level of early MC and the absence of rejection episodes was observed. However, high levels of early MC did not abrogate the persistence of an alloreactive response measured in vitro 1 year after transplantation, which suggests that MC did not lead to clonal deletion of donor-specific CTL.

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Abbreviations: MC, microchimerism; CTL, cytotoxic T lymphocytes; PCR, polymerase chain reaction; HLA, human leukocyte antigen; DNA, deoxyribonucleic acid; PBMC, peripheral blood mononuclear cells; CTLP, cytotoxic T-cell precursor.

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In organ transplantation, hematopoietic donor cells are cotransferred into the recipient and it is widely accepted that donor leukocytes may survive for prolonged periods in the recipient. Donor leukocytes can be readily detectable in the recipient’s blood during the 1st few days after transplantation by flow cytometry, but usually they decrease rapidly to an undetectable level. However, hematopoietic microchimerism (MC) has been observed with more sensitive polymerase chain reaction (PCR)-based methods after liver, kidney, lung, and heart transplantation, in peripheral blood, lymph nodes, skin, and bone marrow, for as long as years after transplantation. It has been hypothesized that this spontaneous hematopoietic MC may be essential for the development and maintenance of immunologic unresponsiveness to organ allografts, even though it is not synonymous with tolerance.

However, the correlation between the demonstration of MC after solid organ transplantation and graft outcome still remains tenuous. The presence of donor cells indicates tolerance, but it could be the consequence and not the cause of tolerance.

The controversies about the clinical consequences of MC might simply reflect the limitations of the sensitivity of the methods used to detect donor-derived material and / or differences in the time-course analysis. The detection of donor-specific deoxyribonucleic acid (DNA) sequences within recipient peripheral blood relies largely on the polymorphism of the major histocompatibility complex, usually that of the human leukocyte antigen (HLA)-DRB1 locus, since the detection of H-Y DNA obviously limits the analysis to the male-to-female transplant setting.

The PCR with sequence-specific primers is currently the most powerful method used in HLA typing. However, the use of single-step PCR with sequence-specific primers typing to detect HLA alleles was found to be inapplicable for the detection of MC because of its low level of sensitivity. Sequencing has shown that nonspecific PCR products may arise from amplification of both pseudogenes or alleles with closely related sequences present in the DNA tested. A number of investigators attempting to assess the levels of MC after
solid organ transplantation have used a nested PCR approach, primarily amplifying exon 2 of HLA DRB1, followed by a 2nd PCR based on standard HLA typing procedures.\textsuperscript{4,10–13} In this study, we optimized the sensitivity of the technique by increasing the number of PCRs for each time point, thus allowing a semiquantitative estimation of MC expressed as percent positive PCRs / blood sample. We applied this technique to analyze the kinetics of MC in patients after a liver transplantation. The correlation between level of chimerism and rejection episodes was confronted with the detection of in vitro cytotoxic T-lymphocyte (CTL) alloreactivity against the donor, measured 1 year after the liver transplantation.

\textbf{Patients and Methods}

\textbf{Patients}

A total of 12 patients receiving an orthotopic liver transplantation at Geneva University Hospital were included in the analysis. All patients received a standard immunosuppressive therapy based on mycophenolate mofetil, steroids, and cyclosporine for the 1st few days, followed by tacrolimus and mycophenolate mofetil only. In 7 patients transplanted between 2001 and 2003, MC was measured at several time points during the 1st 18 months after the transplantation. Patient characteristics are detailed in Table 1. Rejection episodes were defined by the histologic analysis according to the Banff classification.\textsuperscript{14} This study was approved by our Institutional Review Board.

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>Indication for Liver Transplantation</th>
<th>HLA DRB1* rec/donor†</th>
<th>% Pos. PCRs‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hemochromatosis</td>
<td>/04,13</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>Post hep. cirrhosis</td>
<td>17, /11</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Post hep. cirrhosis</td>
<td>14,15/04,11</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>Crypto. cirrhosis</td>
<td>11,13/04,12</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Crypto. cirrhosis</td>
<td>02,14/01,11</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>Post hep. cirrhosis</td>
<td>13,15/17,04</td>
<td>15</td>
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<tr>
<td>7</td>
<td>Crypto. cirrhosis</td>
<td>11,13/01,15</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>Post hep. cirrhosis</td>
<td>11,13/04,17</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Echinococcosis</td>
<td>01,17/04,11</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Crypto. cirrhosis</td>
<td>04,17/11</td>
<td>12</td>
</tr>
<tr>
<td>11</td>
<td>Hemochromatosis</td>
<td>14,15/11</td>
<td>8</td>
</tr>
<tr>
<td>12</td>
<td>Post hep. cirrhosis</td>
<td>13/01,04</td>
<td>0</td>
</tr>
</tbody>
</table>

†DRB1* allele amplified for the chimerism analysis is shown in bold underlined type.

‡% positive PCR and numbers of positive PCR per total number of PCR done, for each patient 1 to 5 blood samples were analyzed (12 of 66 PCRs in total).

\textbf{DNA Preparation and PCR Amplification}

HLA class I typing of patients and donors was performed by serology (Biotest AB120 [Biotest AG, Frankfurt, Germany] and PelFreez 144 [Dynal Biotech, Oslo, Norway] trays) and PCR with sequence-specific primers (Genovision, Philadelphia, PA), and HLA-DR typing was performed by PCR with sequence-specific primers. DNA was extracted (FlexiGene DNA Kit, Qiagen, Hilden, Germany) in triplicate from whole blood, from mononuclear cells and granulocytes separated by Ficoll gradients, and from CD3+ peripheral blood lymphocytes isolated from blood (CD3 Dynal beads, Dynal Biotech).

MC was analyzed by 2-stage nested PCR, based on patient / donor HLA-DRB1 incompatibilities (DRB1*01, DRB1*04, and DRB1*11). Nested PCR was set up using varying amounts of genomic DNA for the generic PCR, and varying dilutions of the 1st PCR for the allele-specific 2nd PCR. For all measurements, the 1st PCR was performed on 200 ng of genomic DNA using the generic DRBP1 / DRBP2 primers for testing DRB1 alleles.\textsuperscript{15} For each time point, 6–20 DNA aliquots were analyzed. After a 1 : 200 dilution of the 1st PCR for HLA-DRB1, the samples were subjected to a 2nd PCR step with group-specific primers. For HLA-DR the following primers were used: DRB-38 / DRB-37 + DRB47 for DRB1*04 group – specific amplification\textsuperscript{16} DRB68 / DRB54 for DRB1*11 – specific amplification,\textsuperscript{16} and DRB39 / DRB36 for DRB1*01 – specific amplification.\textsuperscript{16} For patient #6, amplification was also optimized for donor HLA-A*02 allele. Group specific PCR was performed using primers PA1N (CCACTCCTCGTCCCCAGGCTCT) and P302,\textsuperscript{16} and for nested PCR, we used primers PA1N and PA2.\textsuperscript{15,17} In all patients, the pretransplant blood sample was proven negative with the donor-specific DRB1-group specific primers. The following additional controls were included: 1 negative and 1 positive DNA sample for the donor DRB1 allele to validate the specificity of the group-specific nested PCR, an open-tube control for each PCR setup, and a no-DNA tube that was processed in parallel with patients’ blood samples from the DNA extraction procedure up to the nested PCR.

The reactions were performed in a Gene Amp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA). The amplification profiles for DRB1 were: 5 minutes at 96°C, 32 cycles of 60 seconds at 96°C / 60 seconds at 65°C / 45 seconds at 72°C, and 8 minutes at 72°C. The amplification was done in 30 µL buffer containing 50 mmol/L NH₄SO₄, 200 mmol/L Tris pH 8.8, 2 mmol/L MgCl₂, 200 µmol/L dNTP, 10 pmol of each of the primers, and 0.5 U Taq polymerase (Invitrogen, Carlsbad, CA). The nested PCR cycling conditions were: 15 minutes at 95°C, 6 cycles of 45 seconds at 96°C / 45 seconds at 70°C / 45 seconds at 72°C, plus 29 cycles of 60 seconds at 96°C / 60 seconds at 67.5°C / 60 seconds at 72°C, plus 4 cycles of 60 seconds at 96°C / 60 seconds at 58°C / 60 seconds at 72°C, and 8 minutes at 72°C. The amplification was done in 30 µL Qiagen buffer containing 200 µmol/L dNTPs, 2 mmol/L MgCl₂, 10 pmol primers,
and 1 U HotStart Taq polymerase (Qiagen). The PCRs were analyzed by agarose gel electrophoresis.

Cytotoxic T-Cell Precursor Frequency Analysis

The analysis of cytotoxic T-cell precursor (CTLp) frequencies was performed as described,18,19 with minor modifications. Briefly, peripheral blood lymphocytes from the patients (12,500 to 50,000 cells) were cultured in 96-well round-bottom plates together with 50,000 irradiated (2,500 rad) stimulator cells. The stimulator cells were peripheral blood lymphocytes from a healthy donor or lymphocytes purified from spleen (splenocytes) of the organ donor. The cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) cell culture medium with glutamine, 100 U/mL interleukin-2, and 10% pooled human serum. Limiting dilution analysis cultures were incubated for 10 days at 37°C in a humidified atmosphere containing 5% CO2. Lymphocytes to be used as target cells were set up at day 0 in RPMI 1640 cell culture medium with glutamine, 100 U/mL interleukin-2, 10% pooled human serum, and 1 μg/mL phytohemagglutinin in a 24-well plate at 1 × 10⁶ cells/well. After 10 days of culture, the limiting dilution analysis plates were tested for cytolytic activity against target cells pre-incubated with Cr51. A well was scored positive if the counts in that well exceeded the spontaneous release (mean of the wells with stimulator cells and corresponding target cells only) plus 3 standard deviations from the mean. HLA class I typing of stimulator and target cells was performed by serology and by PCR with sequence-specific primers. The target cells were selected on basis of sharing only 1 HLA-A or -B antigen with the stimulator. Positive wells were incubated for 7 additional days with interleukin-2 and phytohemagglutinin and tested against an unrelated 3rd party that shared single HLA antigen with the donor. For the CTLp analysis of patient #2, the stimulators and the target were the splenocytes from the deceased donor. Splenocytes from 2 non-HLA identical deceased donors were used as controls. Results were considered positive when the precursor frequency was 3 or more per million peripheral blood mononuclear cells (PBMC).18

Results

MC Analysis: Sensitivity and Semiquantitative Measurement

MC analysis was performed by a nested PCR approach on the HLA-DRB1 locus (Fig. 1A). To determine the detection level of the method, serial dilutions of a DR4-positive DNA sample were performed in 200 ng non-DR4 DNA. As shown in Figure 1B, the detection level of the nested PCR is 2 pg of DRB1*04-positive DNA in 200 ng DRB1*03 / DRB1*11 DNA. Assuming the DNA content is 6.6 pg / cell, this represents a detection level of 1 DR4-positive cell in 1 × 10⁵ recipient cells. Nested PCR performed on 200 ng of genomic DNA as starting material would theoretically not allow the discrimination of >1 donor cell in 3 × 10⁶ recipient cells.

To increase the sensitivity of the method, multiple 200-ng samples from 1 to 3 parallel DNA extracts of a given patient’s blood sample were tested. To determine the reliability of the method, 4 dilutions of 0, 2, 20, and 100 pg of donor DNA in 200 ng of recipient DNA were tested by performing 21 individual PCRs for each dilution. The results showed a 100% efficiency rate (21 / 21 positive PCRs) in detecting 30 cells (20 pg) in 3 × 10⁴ cells, a 90% efficiency rate (19 / 21) in detecting 3 cells (20 pg) in 3 × 10⁴ cells, and a 24% efficiency rate (5 / 21) in detecting 2 pg (0.3 cell) of donor DNA in 3 × 10⁴ cells (data not shown). Therefore by testing multiple 200-ng DNA samples / time point, a semiquantitative determination of MC could be achieved for a range between 1 / 10⁴ to 1 / 10⁶ cells.

For the clinical study, 6-20 DNA samples were therefore tested for each patient’s blood sample, and MC results were calculated as a percentage of positive
PCRs / time point. An example of such a semiquantitative determination is illustrated in Figure 2 for patient #3. The patient was typed as DR15 / 14 and was transplanted with a DR4 / 11 liver. Several patient DNA samples were amplified by the nested PCR approach for DRB1*11 before and after the transplantation. The results showed the detection of all DNA samples after the generic DRB1 PCR amplification before and after transplantation (day 9 and 5 months) (Fig. 2A). The DRB1*11 donor-specific PCR was negative for all samples before the transplantation (0 / 6), and all samples (6 / 6, 100%) were positive at day 9 (lanes 10-15), and 3 of 6 (50%) samples were positive at 5 months (lanes 18-22).

**Time Course of MC After Liver Transplantation and Clinical Rejection**

For each of the 3 periods considered in this study (<3 months, 3 to 12 months, and >12 months), 1 to 6 time points were analyzed per patient and 2–20 PCRs were performed on each blood samples (Tables 1 and 2). The MC is expressed as the total number of positive PCRs divided by the total number of PCRs performed on DNA samples extracted from blood samples drawn during the time period considered. MC analyses were performed by nested PCR on the HLA DRB1 locus in 12 patients 1 year after an orthotopic liver transplantation (Table 1). The results showed that MC could be detected in 58% (7 / 12) of the patients more than 1 year after the transplantation. The level of MC as determined by the number of positive PCR / time point was low (<20%) for all patients except 2 (patients #1 and 3; see Table 1). For the 1st 7 patients (patients #1-7), a kinetic analysis of the MC was performed during the 1st year following the liver transplantation. In all patients, the presence of donor DNA could be detected during the 1st 3 months, although at highly variable levels (Table 2). MC decreased progressively during the 1st year with the exception of patient #1, but could still be detected in 6 of 7 patients at 12 months. No correlation was found between the persistence of high-level MC in patient #1 and any special event documented in the patient’s follow-up data.

The correlation between MC and the absence of rejection leading to long-term graft survival remains a matter for debate. We addressed this issue by taking advantage of the semi-quantitative approach to determine MC in a time-dependent manner. The time-course analysis carried out in 7 patients showed that none of the 3 patients that exhibited the highest levels of MC (55-100%) during the 1st months experienced rejection episodes during the 1st year (Table 2). In contrast, all 4 patients with low-level MC (<25%) had rejection episodes at 3 to 12 months according to the Banff classification.

To determine the cell origin of the DNA amplified

### Table 2. Microchimerism and Graft Rejection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Chimerism &lt;3 months*</th>
<th>Chimerism 3 to 12 months†</th>
<th>Rejection‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81% (13/16)</td>
<td>79% (26/33)</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>55% (11/20)</td>
<td>26% (8/31)</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>100% (10/10)</td>
<td>60% (15/28)</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>23% (6/26)</td>
<td>16% (10/60)</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>20% (2/10)</td>
<td>12% (4/34)</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>8% (3/36)§</td>
<td>0% (0/30)§</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>5% (1/20)</td>
<td>5% (2/37)</td>
<td>+</td>
</tr>
</tbody>
</table>

*% positive PCRs (numbers of positive PCR per total number of PCR done), for each patient 1 to 5 blood samples were analyzed (10 to 36 PCRs in total).
†% positive PCRs (numbers of positive PCR per total number of PCR done), for each patient 2 to 6 blood samples were analyzed (28 to 60 PCRs in total).
‡Presence of at least 1 rejection episode during the 1st year according to the Banff classification.
§Donor-specific PCRs were performed with DR4- (6) and A2-specific (30) primers.
||Donor-specific PCRs were performed with DR4- (9) and A2-specific (21) primers.
with the nested PCR technique, cell fractionation was performed at several time points in 3 patients. Blood samples from patients #1, 2, and 3 were separated into lymphocytes or monocytes and granulocytes fractions by Ficoll gradients; CD3+ lymphocytes were sorted from whole blood by anti-CD3 beads. The results show that donor-derived DNA was found in the mononuclear and CD3+ cells fraction but not in the granulocyte fraction (data not shown).

Absence of Rejection and In Vitro CTL Alloreactivity

Based on the correlation between low-level MC and a higher incidence of rejection episodes, we hypothesized that higher levels of passenger leukocytes might lead to donor-specific tolerance. Therefore, we investigated whether 2 of the patients, with high levels of early MC, still had alloreactive T cells against incompatible HLA class I antigens present on the donor graft more than 1 year after transplantation. The absence or persistence of alloreactive CTL against HLA class I mismatched antigens was determined using CTLp frequency analyses.

In a 1st experiment, CTLp frequency was determined on patient #3, whose MC decreased progressively during the 1st year from 100%, to 60%, and to 38% at 12 months. PBMC from patient #3 were used as responding cells, and splenocytes from the donor that had been frozen on the day of transplantation were used as stimulators. Cells from unrelated splenocytes were used as a 3rd party. The results demonstrated that the patient still had CTL reacting against donor splenocytes as strong as against a 3rd party with totally different mismatched antigens (Fig. 3A). A 2nd experiment was performed with PBMC of patient #2. The MC level of this patient decreased progressively during the 1st year and was negative at the time of the CTLp assay, i.e., 12 months after the transplantation. In this case, we compared the CTL reactivity against a mismatched major histocompatibility complex—antigen shared by the donor or not. For this purpose, PBMC of the patient were cultured for 10 days under limiting dilution conditions with irradiated 3rd party stimulator cells (Fig. 3B; Panel A: B*3501,58). Subsequently, we tested 96 T-cell lines derived from wells displaying a substantial cytotoxic activity against panel B (1st CTLp). These cell lines were tested (CTLp restimulation) against panel B, panel C (which shared a single class I antigen expressed by panel B and the donor [B*3501, underlined]), and panel D (which shared another single class I antigen expressed by panel B and not by the donor [A11, underlined]). Autologous control was negative (neg).

Figure 3. Persistence of alloreactivity against mismatched HLA antigens of the donor and 3rd party. (A) Alloreactivity of the PBMC of patient #3 against splenocytes of the donor and 3rd party (control spleen) performed 1 year after the liver transplantation. A panel control (A2,24 B44,18) was positive against splenocytes from the donor and 3rd party (not shown). The autologous controls were negative with the patient and the panel control (not shown). Results were considered positive (+) when the precursor frequency was 5 or more per million PBMC. (B) (Left) Alloreactivity of the PBMC of patient #2 1 year after liver transplantation against 3rd party (panels A and B). A panel control (A2,26 B44,56) was positive against both panels A and B (not shown). The autologous controls with the patient cells and the panel control were negative (not shown). Results were considered positive (+) when the precursor frequency was 3 or more per million PBMC. (Right). T cell lines were derived from responding cells (patient #2) isolated from wells displaying a substantial cytotoxic activity against panel B (1st CTLp). These cell lines were tested (CTLp restimulation) against panel B, panel C (which shared a single class I antigen expressed by panel B and the donor [B*3501, underlined]), and panel D (which shared another single class I antigen expressed by panel B and not by the donor [A11, underlined]). Autologous control was negative (neg).
the results showed that the CTL reactivity against B*3501 and A11 was comparable: 8 of 17 T cell lines recognized B*3501, 9 of 17 T cell lines recognized A11, while 2 of 17 T cell lines recognized both HLA molecules. We therefore conclude that alloreactive CTL against donor antigens persisted in the recipient at 1 year posttransplant, and that a high level of early MC did not lead to clonal deletion of donor-specific CTL.

Discussion

Hematopoietic chimerism denotes the presence of donor hematopoietic cells in a recipient (more than 1% of donor cells for macrochimerism, less than 1% for MC). Therefore, the presence of donor-specific HLA DR allele in the recipients' peripheral blood samples, as detected by the nested PCR technique, corresponds to passenger leukocytes released from the graft, which are, at least transiently, viable in the circulation. We report in this study the detection of donor cells in all patients analyzed early, i.e., within the 1st 3 months after liver transplantation. As compared to previous protocols used for the detection of MC, we have introduced a semiquantitative analysis by performing multiple PCR / time points, taking into account the fact that 1 cell in 10^5 detectable cells with high efficiency. Limited DNA amounts tested per time point in previous studies could account for fluctuations with time for individual patients or for the inconclusive results with respect to correlations with rejection episodes. The nested PCR technique based on DR1, DR4, DR11-incompatible donor / recipient pairs analyzed in this study could be adapted to any DR-mismatched combination, since the primers used for the allele- or group-specific PCR are the same as those used for routine HLA-DR typing. The follow-up demonstrated that MC decreases with time in most cases but can also fluctuate as part of a dynamic process. The semiquantitative method developed in this study allowed us to show a correlation between the level of chimerism and the episodes of acute rejection. The potential role or significance of MC is still a matter for debate. MC could merely be a reflection of effective immunosuppression; moreover, donor leukocytes could mediate tolerance by functioning as immature antigen presenting cells, or they could also act as surrogate targets of rejection, protecting the graft.9

Hisanaga et al., using a similar sensitive methodology (1 in 10^5 detectable cells) showed that 75% of heart and 72% of liver allograft recipients were microchimericas at some time in the posttransplant period. MC did not correlate with rejection and had no predictive value for the individual patient. Devlin et al. subjected 18 microchimeric liver allograft recipients with stable function (>5 years) to a staged program of immunosuppressive drug withdrawal. The comparison between complete or partial drug withdrawal showed no statistical difference between the group of patients who were chimeric and those who were not. Schlitt et al. reported an extraordinary case of extensive donor-type MC associated with graft rejection 8 years after liver transplantation. The study of Ciancio et al., in which 63 deceased donor renal allograft recipients were treated with donor-specific bone marrow infusions and compared to 219 untreated controls, produced significant data in favor of an association between MC and donor-specific hyporesponsiveness. Actuarial graft survival at 5 years was superior in bone marrow--infused patients. Acute and chronic rejections were significantly reduced in patients given bone marrow infusions. The degree of MC increased in the group of patients given bone marrow and among those who showed no rejection episodes. A recent published meta-analysis was not able to confirm the existence of a significant association between MC and donor-specific hyporesponsiveness after solid organ grafting.

The level and the kinetics of MC were not evaluated in most of the studies that analyzed the presence of passenger leukocytes after solid organ transplantation. In animal models, evidence supports the idea that passenger leukocytes are determinant to induce tolerance in solid organ transplantation. Ko et al. demonstrated that early MC is a key event for the induction phase of heart allograft tolerance, irrespective of any long-term persistence of donor leukocytes. Elimination of the donor cells 18 days after transplantation did allow long-term graft acceptance whereas eliminating the cells within the 1st 24 hours following the transplantation did prevent long-term graft survival. However, in this article, it should be noticed that donor cells were not totally depleted at either time point. Indeed, long-term positive PCR MC was detected in spleen and liver.

Potential mechanisms of graft tolerance include induction of anergy by transferred donor T cells, presence of regulatory cells or peripheral clonal deletion by priming for activation-induced cell death of T cells. If the level of chimerism is high (more than 1%) and prolonged (mixed chimerism), the mechanism of tolerance is likely to be due to central clonal deletion.

In our study, the early chimerism analysis performed within the 1st 3 months suggests that, in at least 2
patients, donor cells represented over 1% of the PBMCs. High level chimerism in the 1st few days after the transplantation corroborates the findings of a previous study where 0.2% to 20% of donor cells were found in the peripheral blood of recipients during the 1st week after a liver transplantation. A number of factors may influence the peak level of early chimerism, including the total number of cells released from the organ transplanted, the rate of clearance of the alloantigenic cells, and their migration to peripheral lymphoid tissue. Among them, the size of the liver and its high content in hematopoietic cells (resident or circulating) explain why liver transplantation is associated with a significant number of donor hematopoietic cells in the early posttransplantation period.

As suggested by Starzl and Zinkernagel and other researchers, macrochimerism or MC is a necessary condition for, but is not synonymous with, the long-term persistence of the tolerant state. In our study, high level early MC was correlated with an absence of rejection episodes. However, the detection of CTL against donor cells in 2 patients with high level chimerism early after the transplantation indicated that, in liver transplantation, the absence of rejection episodes is not synonymous with antigen-specific CTL deletion. CTL has already been demonstrated after organ transplantation including in the liver. In stem cell transplantation and in kidney transplantation, the presence of CTL against donor cells is known to be associated with decreased overall survival or graft survival. Several arguments may explain the persistence or the reemergence of an alloimmune response observed in our patients. A tolerant state induced by mechanisms such as peripheral anergy, regulation, and / or peripheral deletion can be broken by several environmental factors, such as inflammation or viral infection. Alternatively, the absence of donor hematopoietic stem cell engraftment, especially in the thymus, allowed the emergence of nontolerant T cells. In addition, it is well known that calcineurin inhibitors such as cyclosporine or FK 506 block priming for activation-induced cell death of T cells by interleukin-2 and play a role in tolerance inhibition.

The role of donor passenger leukocytes is certainly complex, as these cells play different roles at different posttransplantation stages. Animal models and human studies, including our data, support the hypothesis that a large number of donor leukocytes present at the time of the transplantation helps induce a state of tolerance. However, the persistence of MC does not prevent the alloreactivity against donor cells.

In liver transplantation, the higher dose of passenger leukocytes provides a prime window of opportunity for tolerance induction and might be 1 reason for the permissiveness with respect to HLA compatibility. These elements stimulated the development of a novel therapeutic approach based on a sequential infusion of hematopoietic stem cells combined with solid organ transplantation.

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