Short Communication

Stable T-cell reactivity after successful tapering of azathioprine in HLA-identical living-related kidney transplant recipients despite minor histocompatibility antigen mismatches

Jeroen H. Gerrits¹, Jacqueline van de Wetering¹, Saskia Postma¹, Jos J. M. Drabbels³, Len M. B. Vaessen¹, Jan N. M. IJzermans², Jacqueline Rischen¹, Frans H. J. Claas³, Willem Weimar¹ and Nicole M. van Besouw¹

¹Department of Internal Medicine—Transplantation, ²Department of Surgery, Erasmus MC, University Medical Center Rotterdam, Rotterdam and ³Department of Immunohaematology and Bloodtransfusion, Leiden University Medical Center, Leiden, The Netherlands

Abstract

Background. Human leukocyte antigen (HLA)-identical living-related (LR) kidney transplant recipients often receive the standard regimen of immunosuppression. We wondered whether these patients should be exposed to the side effects of these drugs any longer. Safe tapering of immunosuppression should not result in rejection and high donor-directed T-cell responses. In the present study, we investigated the effect of tapering azathioprine (AZA) on T-cell reactivity.

Methods. Fifteen HLA-identical LR kidney transplant recipients receiving a median of 150 mg/day AZA and 5–10 mg/day prednisone were tapered to a median of 50 mg/day AZA. Donor-, third-party and tetanus toxoid (TET)-reactivity were determined in interferon (IFN)-γ and interleukin (IL)-13 Elispot assays, which reflect the T-helper (Th)1 and T-helper (Th)2 response.

Results. After the tapering of AZA, none of the patients developed acute rejection and the renal function remained stable, even at 1-year follow-up. The frequency of donor-specific IFN-γ and IL-13 producing cells (pc) was low. Tapering of AZA did not influence the frequency of both IFN-γ and IL-13 pc. Also, the reactivity against third-party cells and TET remained unchanged.

Conclusions. The AZA-dose can be safely reduced in recipients of an HLA-identical LR kidney transplant without affecting kidney function and without increasing T-cell responses directed against donor or other antigens.

Keywords: azathioprine; interferon-γ; kidney transplantation; minor histocompatibility antigens; monitoring; T-cell reactivity

Introduction

Organ transplantation has become the preferred treatment for end-stage kidney function. To prevent graft rejection, transplant recipients receive immunosuppressive drugs to suppress immune responses directed to the foreign organ. Generally, lifelong administration of immunosuppressive drugs after transplantation is deemed necessary. However, chronic use of immunosuppression in transplant recipients is associated with several serious side effects, such as infections, osteoporosis, diabetes mellitus and cancer [1,2]. Consequently, discontinuation of immunosuppression is warranted, provided this is not accompanied by rejection [3–5].

In the human leukocyte antigen (HLA)-identical living-related (LR) kidney transplant setting, the major HLA molecules A, B, C, DR, DQ and HLA-DP are identical. However, mismatches may exist in minor histocompatibility antigens (mHAgs). The mHAgs are peptides derived from polymorphic intracellular proteins presented in the context of HLA molecules, which can be recognized by the immune system [6–10]. The expression of mHAgs is ubiquitous or specific on haematopoietic cells [11].

In humans, mismatches in mHAgs have been shown to induce graft-versus-host disease (GVHD) after HLA-identical bone marrow transplantation (BMT). Goulmy et al. [12] demonstrated that one or more disparities of HA-1, HA-2, HA-4 and HA-5 were associated with the development of GVHD. After BMT, male recipients who received HLA-identical graft from female donors...
are more at risk in developing GVHD than those who received a graft matched for gender [13]. This risk is due to an enhanced activity against mHAgs H-Y encoded on the Y chromosome. Studies have shown the existence of H-Y specific cytotoxic T lymphocyte (CTL) after gender-mismatched BMT [14].

The role of mHAgs in clinical solid organ transplantation is yet not known. Recently, Opelz [15] described an association between the presence of panel reactive antibodies (PRA) in kidney transplants from HLA-identical sibling donors and long-term graft loss. Theoretically, after HLA-identical LR kidney transplantation, mHAgs, but also other non-HLA antigens [15,16], might be responsible for the induction of acute or chronic rejection of the transplant. Therefore, HLA-identical LR kidney transplant recipients still receive immunosuppression. Considering the adverse effects of immunosuppression, we questioned whether these patients should be exposed to the severe side effects of immunosuppression.

After HLA-mismatched kidney transplantation, some studies reported a relation with increased T-cell reactivity in mixed lymphocyte culture (MLC), CTL precursor frequency (CTLpf), helper T lymphocyte precursor frequency (HTLpf) and acute rejection after tapering of immunosuppression [17–20]. However, in the HLA-identical LR kidney transplant setting, no donor-specific responses can be measured in CTLpf, HTLpf and MLC. Recently, we have shown that a sensitive method as the Elispot assay can be used to determine the reactivity against non-HLA antigens in the HLA-identical LR kidney transplant setting, no donor-specific responses can be measured in CTLpf, HTLpf and MLC. The third-party PBMC used did not share HLA antigens with donor and patient. Both before and after tapering of immunosuppression, the same third-party PBMCs were used. As control, unstimulated responder cells were used and subtracted from donor-specific responses. PBMCs from donor. As control, unstimulated responder cells were used and subtracted from donor-specific responses. PBMCs from donor.

Peripheral blood mononuclear cells sampling

PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) as described earlier [21]. Thereafter, PBMCs were stored in RPMI-1640-DM (BioWhittaker, Verviers, Belgium) containing 15% fetal calf serum (FCS) and 10% dimethyl sulfoxide (MERCK, Germany) at −140°C until use.

IFN-γ and IL-13 Elispot assay

The phytohemagglutinin (PHA) proliferation assay was performed to control the viability of the PBMCs as described before [21]. The mean counts per minute (cpm) were determined, and the stimulation index (SI) was calculated by the ratio of the cpm obtained in the presence of PHA to the cpm in the absence of PHA. Only results of viable cells (SI > 50) were analysed in the described results.

Before the first tapering of the immunosuppressive medication, we received 35 ml heparinized peripheral blood from 15 patients. Four months after the last AZA-dose reduction, we again received 35 ml blood from those patients to perform Elispot assays. The IFN-γ and IL-13 Elispot assays (U-Cytech Biosciences, Utrecht, The Netherlands) were performed as described before [21]. Briefly, in a 96-well round bottom plate (Nunc, Roskilde, Denmark), 100 μl of 2 × 10^5 patients’ PBMC in culture medium was added to 100 μl of 2 × 10^5 irradiated (40 Gy) PBMC derived from the donor. As control, unstimulated responder cells were used and subtracted from donor-specific responses. PBMCs from the patient were also stimulated with 1 μg/ml PHA (Murex Biotech), third-party cells and 30 μl/ml TET (RIVM, Bilthoven, The Netherlands). PHA was used as a positive control and showed in all experiments more than 100 spots per well. The third-party PBMC used did not share HLA antigens with donor and patient. Before and after tapering of immunosuppression, the same third-party PBMCs were used. TET stimulation was used to test the immune response to nominal antigens. After 40 h of incubation, the non-adherent cells were transferred to a 96-well plate pre-coated with anti-human IFN-γ or IL-13 monoclonal antibodies. The cells were incubated for 5 h at 37°C to allow the formation of spots. The spots were counted automatically by using a Bioreader 3000 Elispot reader (BioSys, GMBH, Karben, Germany).

Minor histocompatibility antigen typing after HLA-identical living-related kidney transplantation

Patient-PBMCs and donor-PBMCs were typed for 10 known mHAgs: H-Y, HA-1, HA-2, HA-3, HA-8, HB-1, BCL2A1 [encoded by two separate single nucleotide polymorphisms (ACC-1 and ACC-2) on a single gene].

Subjects and methods

Patients

HLA-identical LR kidney transplant recipients, treated with AZA and prednisone, were asked to participate in this study. They were at least 2 years after transplantation, had no acute rejection in the last 6 months, had stable renal function and no clinical relevant proteinuria (≥0.5 g/l). Fifteen patients agreed to participate. Characteristics of the patients are described in Table 1. At inclusion of the study, the patients received a median dose of 150 mg/day AZA (range, 100–175 mg/day; ‘high AZA’) in combination with a median dose of 10 mg/day prednisone (range, 5–10 mg/day; Prednisone). The AZA-dose was tapered twice with a 4 month interval to a median dose of 50 mg/day AZA (range, 50–100 mg/day; ‘low AZA’). The prednisone dose remained unchanged. Peripheral blood samples were analysed at inclusion and 4 months after the last mentioned AZA-dose reduction. Renal function was closely monitored.
DNA from the selected couples (Table 1) was isolated using the QIAamp DNA Mini Kit. The polymerase chain reaction (PCR-SSP) technique was used as typing method [26]. This is a PCR-based technique with the use of Sequence Specific Primers (SSP), which shortens the post-amplification processing time to a simple gel electrophoresis detection step. Each tested mHAgs is a part of a di-allelic system. This means that each locus contains two alleles. We used a specific primer combination for each allele, to test the presence of this allele (Table 2).

Statistical analysis

Numerical data were compared using the Wilcoxon signed rank test. Two sided \( P \)-values \( \leq 0.05 \) were considered significant. For statistical analysis GraphPad statistical program was used (GraphPad Software Inc., San Diego, CA, USA).

Results

Clinical results

After tapering the AZA-dose to at least 50% of the original dose, none of the HLA-identical LR renal transplant recipients developed acute rejection. Their serum creatinine levels remained unchanged before and 4 months after halving the AZA-dose (before tapering: median, 103 \( \mu \)mol/l; range, 63–128; after tapering: median, 102 \( \mu \)mol/l; range, 63–136). At 1-year follow-up, no clinical relevant increase in serum creatinine (median, 111 \( \mu \)mol/l; range, 67–138) and no proteinuria (\( \geq 0.5 \) g/l) had occurred. Additionally, haematological parameters [haemoglobin, haematocrit, mean cell volume (MCV), thrombocytes and leucocytes] remained normal during tapering of AZA (data not shown).

IFN-\( \gamma \) Elispot

Before tapering of AZA ('high AZA'), and 4 months after AZA reduction ('low AZA'), 11 out of 15 (73%) HLA-identical patients responded to donor antigens (Figure 1A). The median frequency of IFN-\( \gamma \) producing cells (pc) directed to donor antigens after reduction of AZA (median, 1 IFN-\( \gamma \) pc/2 \( \times \) 10^5 PBMC; range, 0–110; \( P = 0.99 \)) was comparable with

<table>
<thead>
<tr>
<th>Pat</th>
<th>Gender</th>
<th>Relation</th>
<th>Primary disease</th>
<th>Time after Tx (years)</th>
<th>CMV</th>
<th>EBV</th>
<th>Before tapering ('high AZA')</th>
<th>After tapering ('low AZA')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>Female</td>
<td>Mother–daughter</td>
<td>Rapidly progressive</td>
<td>2.4</td>
<td>+</td>
<td>150/1.9 10</td>
<td>50/0.65 10</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>Female</td>
<td>Brother–sister</td>
<td>IgA nephropathy</td>
<td>5.0</td>
<td>+</td>
<td>150/1.6 10</td>
<td>100/1.09 10</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>Female</td>
<td>Daughter–mother</td>
<td>Focal segmental</td>
<td>10.1</td>
<td>–</td>
<td>125/1.71 5</td>
<td>50/0.67 5</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>Male</td>
<td>Brother–brother</td>
<td>Membranous</td>
<td>4.5</td>
<td>–</td>
<td>150/1.88 7.5</td>
<td>50/0.67 7.5</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>Female</td>
<td>Sister–sister</td>
<td>Medullary cystic</td>
<td>4.8</td>
<td>+</td>
<td>100/1.30 10</td>
<td>50/0.66 10</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>Female</td>
<td>Brother–sister</td>
<td>Autosomal dominant</td>
<td>4.3</td>
<td>+</td>
<td>100/1.10 7.5</td>
<td>50/0.57 7.5</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>Female</td>
<td>Brother–sister</td>
<td>Acute tubular</td>
<td>5.2</td>
<td>–</td>
<td>125/1.84 10</td>
<td>50/0.75 10</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>Female</td>
<td>Brother–sister</td>
<td>Membranous</td>
<td>6.7</td>
<td>+</td>
<td>150/1.74 5</td>
<td>50/0.56 5</td>
</tr>
<tr>
<td>9</td>
<td>Female</td>
<td>Female</td>
<td>Sister–sister</td>
<td>Hypertension</td>
<td>15.2</td>
<td>–</td>
<td>100/0.95 5</td>
<td>50/0.49 5</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>Male</td>
<td>Brother–brother</td>
<td>IgA nephropathy</td>
<td>4.9</td>
<td>+</td>
<td>175/1.58 7.5</td>
<td>75/0.65 7.5</td>
</tr>
<tr>
<td>11</td>
<td>Male</td>
<td>Female</td>
<td>Brother–sister</td>
<td>Von Hippel Lindau</td>
<td>3.4</td>
<td>+</td>
<td>150/1.74 10</td>
<td>50/0.61 10</td>
</tr>
<tr>
<td>12</td>
<td>Female</td>
<td>Male</td>
<td>Sister–brother</td>
<td>Extracapillary</td>
<td>3.0</td>
<td>–</td>
<td>150/2.17 5</td>
<td>75/0.14 5</td>
</tr>
<tr>
<td>13</td>
<td>Female</td>
<td>Female</td>
<td>Sister–brother</td>
<td>Chronic pyelonephritis</td>
<td>10.4</td>
<td>–</td>
<td>150/1.41 5</td>
<td>50/0.68 5</td>
</tr>
<tr>
<td>14</td>
<td>Male</td>
<td>Female</td>
<td>Brother–sister</td>
<td>Reflux nephropathy</td>
<td>3.7</td>
<td>+</td>
<td>150/1.58 10</td>
<td>50/0.51 10</td>
</tr>
<tr>
<td>15</td>
<td>Male</td>
<td>Female</td>
<td>Brother–sister</td>
<td>Focal segmental</td>
<td>4.7</td>
<td>+</td>
<td>175/1.70 10</td>
<td>100/0.93 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glomerulosclerosis</td>
<td></td>
<td></td>
<td>Median</td>
<td></td>
</tr>
</tbody>
</table>

Pat, patient; Don, donor; CMV, cytomegalovirus; EBV, Epstein–Barr virus; Tx, transplantation; ND, not documented; AZA, azathioprine; Pred, prednisone; mg/d, mg/day; mg/kg, mg/kg body weight.

HwA-9 and UGT2B17 (Table 2). DNA from the selected couples (Table 1) was isolated using the QIAamp DNA Mini Kit. The polymerase chain reaction (PCR-SSP) technique was used as typing method [26]. This is a PCR-based technique with the use of Sequence Specific Primers (SSP), which shortens the post-amplification processing time to a simple gel electrophoresis detection step. Each tested mHAgs is a part of a di-allelic system. This means that each locus contains two alleles. We used a specific primer combination for each allele, to test the presence of this allele (Table 2).
before dose reduction (median, 2 IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC; range, 0–88).

Before reduction of AZA, 13 out of 15 (87%) patients responded to HLA-mismatched third-party PBMC. After tapering to low AZA, 15 out of 15 (100%) patients responded to third-party antigens (Figure 1B). The median frequency of IFN-\(\gamma\) pc directed to third-party antigens after tapering of AZA (median, 12 IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC; range, 2–324; \(P=0.95\)) was comparable with before tapering (median, 14 IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC; range, 0–305). The response to HLA-mismatched third-party antigens was significantly higher than the response to donor antigens \((P<0.01)\).

Before tapering AZA-dose, we found that 13 out of 15 (87%) patients responded to TET. The median frequency of IFN-\(\gamma\) pc directed to TET antigens before tapering AZA was 7 IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC (range, 0–1200). After tapering to low AZA, 11 of the 15 patients (73%) responded to TET, and tapering of AZA did not influence the number of IFN-\(\gamma\) pc (median, 3 IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC; range, 0–1200; \(P=0.38\)).

Some of the HLA-identical patients had high responses against donor PBMC in the IFN-\(\gamma\) Elispot assay (\(>45\) IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC; high responders). We analysed whether other parameters could clarify the differences between high responders and nonresponders (0 IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC). Haematological parameters, transaminases and renal function (proteinuria and creatinine clearance) were comparable between high responders and nonresponders.

After third-party stimulation, we observed in four patients (patient 3, 4, 7 and 15) high frequencies of IFN-\(\gamma\) pc before and/or after tapering of AZA \((>45\) IFN-\(\gamma\) pc/2 \(\times\) 10^5 PBMC; Figure 1B). The response against donor antigens in two of the four patients (patient four and seven) remained low and stable after tapering of AZA (Table 3). This could indicate that before and after tapering the AZA dose, the Th1 cells in those patients are still able to recognize foreign antigens other than those of the donor antigens of the kidney. It was remarkable that one of those four patients (patient 3) showed high third-party responses and donor-specific responses after tapering of AZA (Table 3). In this patient, renal function was stable during tapering of immunosuppression. Two patients (patient 6 and 15) demonstrated a decrease in third-party antigens. The donor-specific response also decreased significantly in one patient (patient 6), and slightly in another patient (patient 15). Both patients showed no abnormalities in renal function, liver function or haematological parameters.

**IL-13 Elispot**

As shown in Figure 2A, before reduction of AZA a detectable frequency of IL-13pc against donor cells was observed in 8 out of 15 (53%) patients.
Stable T-cell reactivity after successful tapering of azathioprine

After tapering to low AZA, 10 out of 14 (71%) patients responded to third-party antigens. Reduction of AZA also did not increase the number of IL-13 pc (median, 4 IL-13 pc/2 x 10^5 PBMC; range, 0–20; \( P = 0.18 \)). The response to third-party antigens was significantly higher than the donor-specific response (\( P = 0.01 \)).

Before and after reduction of AZA, 10 out of 14 (71%) patients responded to TET. No difference in the frequency of IL-13 pc to TET was found before (median, 2 IL-13 pc/2 x 10^5 PBMC; range, 0–187) and after (median, 2 IL-13 pc/2 x 10^5 PBMC; range, 0–127; \( P = 0.46 \)) tapering of AZA.

Similar to the IFN-\( \gamma \) Elispot result, patient 3 demonstrated high frequencies of IL-13 pc against donor antigens before (30 IL-13 pc/2 x 10^5 PBMC) and after (53 IL-13 pc/2 x 10^5 PBMC) tapering of AZA. Whether these high frequencies of IL-13 pc were caused by mismatches in mHAgs remains unknown, because no mismatches for known mHAgs were found with the correct HLA restriction element between donor and recipient (Table 3).

**Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) after HLA-identical LR kidney transplantation**

Table 1 shows CMV and EBV serology of donor and recipient. Before transplantation, eight patients were CMV-positive and seven were CMV-negative. From the CMV-negative patients, five out of seven received a CMV-positive graft. Those five patients (CMV-/+\) were not different in their cytokine profile of IFN-\( \gamma \) and IL-13 from the other 10 patients (CMV+/+, CMV+/−, CMV−/−).

Eleven patients were EBV-positive and four were EBV-negative before transplantation. From the EBV-negative patients, two out of four received an EBV-positive graft, one received an EBV-negative graft, and from one the EBV serology was not documented. The donor-specific cytokine profiles were comparable between those two ‘EBV-negative patient EBV-positive donor’ (EBV−/+\) combinations and 11 EBV-positive patients.

**Minor histocompatibility antigens typing after HLA-identical LR kidney transplantation**

Patient PBMCs and donor PBMCs were typed for 10 known mHAgs that could cause a T-cell response (Table 2). We analysed whether the IFN-\( \gamma \) and IL-13 Elispot results could be explained by mismatches in known mHAgs expressed with the correct restriction element.

In 11 out of 14 patient–donor combinations, we found mismatches for mHAgs (Table 3). The correct HLA-restriction element was present in only four patients. In two of these four patients, positive Elispot responses were found. In six out of the seven patients without the correct HLA-restriction element, also positive Elispot results were found. Three patient–donor combinations were identical for the 10
determined mHAgs. Those patients had no significant response in the IFN-\(\gamma\) and IL-13 Elispots.

### Discussion

In the present study, we show that the immunosuppressive medication of HLA-identical LR kidney transplant recipients, who were more than 2 years after transplantation, can be safely reduced. Four months after the last AZA reduction to at least 50% of the original dose, no acute rejection or decrease in renal function had occurred. Also, at 1-year follow-up, the kidney function remained stable. Tapering of AZA did not increase the frequency of Th1-cells (IFN-\(\gamma\)) as well as the frequency of Th2-cells (IL-13).

The Elispot assay becomes an attractive method to determine donor-specific responses in kidney transplant recipients and may be used to monitor patients. A study of Gebauer et al. [27] determined the frequency of IFN-\(\gamma\) pc in 11 stable HLA-mismatched renal allograft recipients, who were more than 18 months after transplantation, and found low frequencies of donor-specific IFN-\(\gamma\) pc (1-45 IFN-\(\gamma\) pc/3 \(\times\) \(10^5\) PBMC). Hricik et al. [28] also found low frequencies of alloreactive IFN-\(\gamma\) pc (<10 IFN-\(\gamma\) pc/3 \(\times\) \(10^5\) PBMC) in renal allograft recipients with low serum creatinine levels within 6 months after kidney transplantation. Also, after bone marrow transplantation with HLA-identical sibling donors, detectable IFN-\(\gamma\) pc were found in 20% of the donor cells stimulated with irradiated recipients cells [29].

During the current study, comparable frequencies of IFN-\(\gamma\) pc were found in the HLA-identical setting (high AZA: median, 2 IFN-\(\gamma\) pc/2 \(\times\) \(10^5\) PBMC; range, 0–88; low AZA: median, 1 IFN-\(\gamma\) pc/2 \(\times\) \(10^5\) PBMC; range, 0–110). It seems that those frequencies of IFN-\(\gamma\) pc are common in an immunological quiescence period after transplantation and are independent of HLA-matching.

The number of IFN-\(\gamma\) pc directed to HLA-mismatched third-party cells and TET did not change after tapering of AZA, while those reactivities were significantly higher than the reactivity to donor antigens. This suggests that the immune response to nominal antigens, allo-antigens as well as to donor antigens is not affected by the reduction of AZA. Therefore, our HLA-identical LR kidney transplant recipients are both clinically (stable kidney function, haematological parameters and transaminases) and immunologically stable. These results are in line with our experience in patients who received a kidney transplant with one or more HLA-mismatches [30]. In this study also no increase in T-cell reactivity was found.

We also determined the Th2-response by using an IL-13 Elispot assay. IL-13 is an immunoregulatory cytokine secreted by activated Th2-cells [24,31]. The importance of Th2-responses has been shown in several studies in acute as well as in chronic rejection [23,32,33]. In our study, we found that before and after tapering of AZA, 53 and 27% respectively, of the patients’ PBMC made IL-13 after stimulation with donor cells. Those frequencies of IL-13 pc were comparable before and after reduction of AZA. This suggests that the donor-specific Th2-response, as well as the Th1-response, was not affected by reduction of AZA. The number of IL-13 pc after stimulation with third-party PBMC and TET remained also in the same range before and after reduction of AZA, indicating that tapering does not affect either Th1- or Th2-reactivity towards foreign HLA and nominal antigens. Alternatively, the AZA in the given dose has no immunosuppressive effect on Th1- and Th2-response. In a pilot study, we found comparable frequencies of

### Table 3. Known mismatched minor histocompatibility antigens (mHAgs) and donor-specific IFN-\(\gamma\) and IL-13 Elispot results

<table>
<thead>
<tr>
<th>Patient Donor</th>
<th>Mismatched mHAgs in the HLA-identical donor</th>
<th>Restriction element present</th>
<th>IFN-(\gamma) Elispot(^{b})</th>
<th>IL-13 Elispot(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>high AZA</td>
<td>low AZA</td>
</tr>
<tr>
<td>1 Female</td>
<td>Female HA-1R, HA-2Y, HA-8R(^{c})</td>
<td>A2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2 Male</td>
<td>Male No known differences</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 Female</td>
<td>Female HA-2M, BLC2A1Y</td>
<td>88</td>
<td>110</td>
<td>30</td>
</tr>
<tr>
<td>4 Male</td>
<td>Male No known differences</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5 Female</td>
<td>Female HA-1H, HA-3M, HB-1Y</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 Male</td>
<td>Female HA-3T, HwA-9G</td>
<td>12</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>7 Male</td>
<td>Male HB-1Y</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>8 Male</td>
<td>Female ND(^{a})</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>9 Female</td>
<td>Female HB-1Y</td>
<td>5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>10 Male</td>
<td>Male No known differences</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11 Male</td>
<td>Male HA-3M, HA-8R, HwA-9R(^{a}), A1, A3</td>
<td>12</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>12 Female</td>
<td>Male HwA-9G</td>
<td>12</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>13 Female</td>
<td>Female BCL2A1Y, BCL2A1D</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>14 Male</td>
<td>Male HB-1H</td>
<td>54</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>15 Male</td>
<td>Female HwA-9G</td>
<td>63</td>
<td>59</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\)Not determined. \(^{b}\)Results are presented as the number of donor-specific IFN-\(\gamma\) and IL-13 producing cells/2 \(\times\) \(10^5\) PBMC. \(^{c}\)Mismatched mHAgs with the correct HLA-restriction element are presented as underlined. Elispot results: negative = 0 IFN-\(\gamma\) or IL-13 pc; positive \(\geq\) 1 IFN-\(\gamma\) or IL-13 pc.
granzyrne B (GrB) pc before and after tapering of AZA in PBMC from HLA-identical LR kidney transplant recipients, suggesting that the functional activity of CTL were also unaffected [34]. Unfortunately, not enough cells were available to test the frequency of GrB pc in this patient cohort.

In the present study, we observed that some of the HLA-identical patients demonstrated detectable (>10 IFN-γ pc/2 x 10^5 PBMC) frequencies of IFN-γ pc against donor antigens, whereas other patients had undetectable frequencies (0 IFN-γ pc/2 x 10^5 PBMC). No relation was found between IFN-γ frequencies and proteinuria, serum creatinine, CMV or EBV serology. Recently, Opelz [15] showed an association of PRA reactivity with long-term graft loss in kidney transplant from HLA-identical sibling donors. Those lymphocytotoxic antibodies could reflect high B-cell responsiveness and alternatively could be directed to mHAgs expressed on donor cells, but also to other non-HLA antigens [16]. Autologous responses are unlikely, because no difference was found between donor-responses corrected for unstimulated patients’ PBMC and donor-responses corrected for the autologous control. Accordingly, in our study the donor-specific Elispot responses could be directed to mismatches in mHAgs, but also to other non-HLA antigens.

Animal studies have shown that mHAgs are involved in the development of allograft arteriosclerosis, GVHD and skin graft rejection [35–38]. In humans, the influence of mHAgs on transplant outcome has mostly been seen after BMT [12,39]. Therefore, in our HLA-identical LR kidney transplant setting, mHAg disparities between patient and donor may theoretically trigger T cell-mediated rejection after tapering of immunosuppression. In our study, 14 patients were typed for 10 known mHAgs, which could give a T-cell response. Four of those patients had mismatches for mHAgs and the correct HLA restriction molecule. Only PBMC from two of the four patients had a T-cell response after donor-stimulation. We also found that PBMC from six of the seven patients with mismatches for known mHAgs, but not with the correct restriction element, had a donor-specific T-cell response in the IFN-γ and/or IL-13 Elispot. A possibility for the response in those patient–donor combinations with mHAgs mismatches, but not the correct HLA-restriction, is that other HLA molecules can present the specific mismatched mHAgs. Therefore, not only mHAgs could be unknown, but also their HLA-restriction molecule. In theory, all genetic polymorphic proteins presented in MHC could give peptides recognized as mHAgs, but not all mHAgs have the potential for inducing strong T-cell responses. The hierarchy of mHAgs could clarify the variety of the donor-response. When a high donor-response was found, immunodominant mHAgs could be present [39,40]. Recently, Mori et al. [41] found that there is a hierarchy of immunodominance among mHAgs in mice, which is dependent on mHAgs density on host cells and the repertoire of donor T-cells capable of responding to mHAgs. However, this phenomenon is unknown in humans. Mori et al. [41] suggested that GVHD is a consequence of activation of multiple T-cell populations against multiple mHAgs. In contrast, we found low numbers of IFN-γ and IL-13 pc towards multiple donor mHAgs, and those were insufficient to induce rejection of the graft. This could indicate the presence of low affinity T-cells recognizing donor mHAgs or that these mHAgs were not immunodominant. Therefore, these patients could be tolerant for the
donor mHAs. The last possibility could be that these patients are still over-immunosuppressed. After tapering of AZA, the patients received a median of 1.1 mg/kg AZA and 5–10 mg prednisone, and this may still be sufficient to prevent cytokine production of lymphocytes after donor stimulation, and therefore rejection.

Some mHAs as HA-1, HA-2 and BCL2A1 are only expressed on haematopoietic cells, while HA-3, HA-8 and H-Y are ubiquitously expressed [14]. Therefore, theoretically, T-cell responses could be found directed to those mHAs only expressed on haematopoietic cells, while epithelial cells were not affected. Unfortunately, epithelial cells from the graft were not available in our laboratory.

In conclusion, this study shows that in HLA-identical LR kidney transplant recipients the AZA-dose can be safely reduced without the occurrence of acute rejection. Tapering of AZA showed no effect on kidney function, even at 1-year follow-up. No increase in T-cell reactivity directed to donor antigens is found. Therefore, we think that these patients are still over-immunosuppressed, and we suggest to reduce the immunosuppressive medication even further.

Acknowledgement. This study was supported by grant C02.2002 from the Dutch Kidney Foundation.

Conflict of interest statement. None declared.

References

20. van Besouw NM, van der Mast BJ, de Kuiper P et al. Donor-specific T-cell reactivity identifies kidney transplant patients in whom immunosuppressive therapy can be safely reduced. Transplantation 2000; 70: 136–143
21. van Besouw NM, Vaessen LMB, Zuijderwijk JM et al. The frequency of interferon-g producing cells reflects alloreactivity against minor histocompatibility antigens. Transplantation 2003; 75: 1400–1404


Jordan WJ, Brookes PA, Szydlo RM et al. IL-13 production by donor T cells is prognostic of acute graft-versus-host disease following unrelated donor stem cell transplantation. *Blood* 2004; 103: 717–724


Mori S, El-Baki H, Mullen CA. Analysis of immunodominance among minor histocompatibility antigens in allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2003; 31: 865–875

Received for publication: 24.3.06
Accepted in revised form: 19.10.06