Steroid withdrawal and donor-specific hyporeactivity after cadaveric renal allotransplantation on maintenance triple therapy

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Abstract

Background. Even in low doses, long-term steroid immunosuppression is known to cause serious complications. However, the safety of steroid withdrawal has not been proven in randomized clinical trials. This study examines donor-specific hyporesponsive transplant recipients before and after steroid withdrawal, to see if reduction in immunosuppresion was associated with consistent changes in antidonor immunological reactivity.

Method. Using limiting dilution assays, the circulating precursor frequency of donor and third-party-reactive helper T lymphocytes (HTLpf) were determined in 21 consecutive cadaveric renal allograft recipients on standard triple therapy, before (pre-tx) and at different time points after transplantation (post-tx). Patients were selected for steroid withdrawal by clinical criteria (stable graft function and no or only one very mild rejection episode).

Results. Of 21 patients studied, steroids were successfully withdrawn in nine (steroid withdrawn group, SWG) for at least 187 days (mean: 380 ± 168.5), and were not withdrawn in 12 patients (steroid continued group, SCG). In the SWG seven of nine patients developed at least fivefold reduction of post-tx donor-reactive HTLpf (range 5–17), relative to pre-tx, as compared to two of twelve patients in the SCG, P = 0.01. In both groups, the third-party-reactive HTLpf levels remained largely unchanged throughout the study period. In the SWG, no significant difference of serum creatinine level was found before and at 6 months after steroid withdrawal (mean: 138 ± 24 versus 132 ± 40, P = 0.45).

Conclusion. Patients who developed donor-specific hyporeactivity as evidenced by low donor-reactive HTLpf had stable graft function and stable HTLpf levels after complete steroid withdrawal.

Key words: donor-specific hyporeactivity; limiting dilution analysis; renal transplantation; steroid withdrawal; T helper lymphocytes

Introduction

In cadaveric renal transplantation, the triple therapy consisting of cyclosporin, azathioprine, and prednisolone remains the standard protocol in many transplantation centres world-wide [1–4]. Although prolonged graft survival and acceptable patient morbidity are frequently reported with this triple drug regimen, steroid-associated complications remain a major problem. To avoid these complications, many centres have attempted to withdraw steroids after transplantation [1–11]. Some of these centres have reported encouraging results after successful steroid withdrawal [3,9] but in contrast, others have experienced a significant increase in the number of rejection episodes [2,8,10,11] and subsequent graft loss [1,7]. In one large randomized trial of steroid withdrawal, even though the acute rejection rate was low, graft function was not stable after steroid withdrawal in many patients. However, significant improvements in blood pressure, cholesterol levels, and in other parameters make steroid withdrawal an attractive goal in many patients [3].

There are no specific and sensitive in vitro tests [6,7] or clinical parameters [2,11] that are reliable in predicting the success or failure of steroid withdrawal. The usual practice is to reduce steroid dose whilst monitoring graft function. If this remains stable, steroid may be tapered to zero. Therefore, the development of immunological assays that predict graft outcome and to help to guide safe steroid withdrawal remains an important goal.

Previously we have identified the development of in vitro donor-specific hyporesponsiveness in a proportion of patients using bulk mixed lymphocyte reaction (MLR) [12]. In the present study we correlated the development of in vitro donor specific hyporesponsiveness, as detected by limiting dilution analysis (LDA) of helper T lymphocyte precursor frequencies...
(HTLpf), with the clinical outcome of 21 consecutive renal transplanted patients on standard triple therapy. We found that in a proportion of patients, the development of donor-specific hyporeactivity was associated with better graft outcome as evidenced by fewer late rejection episodes, stable renal graft function and successful steroid withdrawal for at least 6 months. This indicates that the development of in vitro donor specific hyporeactivity as detected by LDA of HTLpf may be used as an additional tool along with the available clinical criteria for identifying those patients in whom steroid withdrawal would be safe.

**Subjects and methods**

**Patients**

Between November 1993 and November 1994, 21 consecutive patients (11 males and 10 females) aged between 23 and 68 years who received cadaveric renal allografts for end-stage renal failure were studied. The study was carried out in accordance with the guidelines of the local ethical committee and written informed consent was obtained in all cases.

**HLA typing**

The mean number of HLA mismatches between donor and recipients is shown in Table 1, and was carried out as described previously [12].

**Immunosuppression**

The immunosuppression therapy was based on cyclosporin (CsA), azathioprine (AZA), and prednisolone (PRED) in all these recipients. The initial dose of CsA was 5 mg/kg twice a day, adjusted according to whole blood trough level using standard radioimmunoassay (blood level 100–200 μg/l). The patients received AZA in a constant dose of 1.5 mg/kg/day as long as the white cell count did not fall below 4 × 10⁹/l. The initial dose of PRED was 10 mg twice a day in the first 3 months, which was gradually reduced by 5 mg every 3 months, so that by the end of the year the patient received 5 mg or 0.

Steroid withdrawal was attempted in patients who never experienced rejection episodes or in three cases who had only one very mild rejection episode (Banff classification [13]; borderline changes); and additionally had stable renal function (defined as stable serum creatinine level below 200 μmol/l for at least 6 months after transplantation). All patients who were selected for steroid withdrawal were maintained on triple therapy (CsA, AZA, PRED) prior to steroid withdrawal and subsequently were maintained on CsA and AZA. PRED was tapered gradually (over 3–4 months) followed by a period of 3–4 weeks during which PRED was given on alternate days. During PRED taper and 2 months thereafter, patients were observed closely on regular bases, and serum creatinine level was monitored at least once a week. The taper was halted or aborted if rejection was suspected on the basis of standard clinical criteria or if serum creatinine level rose by more than 15% in two consecutive measurements in the absence of other causes of deterioration of renal function, such as CsA nephrotoxicity or infection. If rejection was confirmed by biopsy, then the PRED was reinstated in a total daily dose of 20 mg, thereafter gradually tapered to a maintenance dose of daily 5 mg and no future attempt was made to withdraw the steroids.

According to the clinical outcome patients were divided to two groups. First group, steroid withdrawn (SW) group: those patients in whom steroid withdrawal was successful for at least 6 months; second group, steroid continued (SC) group: those patients in whom steroid withdrawal has failed because of rejection or significant rise in serum creatinine level after attempting steroid tapering, or in whom steroid withdrawal was not attempted.

**Rejection diagnosis**

Diagnosis of rejection was made based on standard clinical criteria and confirmed by biopsy in most cases. Histological assessment of biopsies was carried out and reported by three independent histopathologists. Episodes of acute rejection were treated by steroid pulses and OKT3 in resistant episodes.

**Blood samples**

Heparinized blood was collected from patients before transplantation (T0) and at appropriate different time points after transplantation. Blood samples were also taken from laboratory or hospital worker healthy volunteers at appropriate time points. Peripheral blood mononuclear cells (PBMC) were isolated from patients or healthy volunteers by standard density gradient centrifugation (Lymphoprep; Nycomed, Norway).

**Donor spleen cells**

Donor splenic mononuclear cells (DSMC) were isolated as described above after gentle disaggregation of donor splenic tissues in cold RPMI 1640 medium. DSMC were stored in 1-ml aliquots in liquid nitrogen until used.

**Flow cytometry**

The number of CD3⁺ cells in each patient or volunteer PMNC sample added per experiment was determined using flow cytometry by immunofluorescence staining. Each sample was divided into two, one incubated with 10 μl PE-labelled anti-CD3 (Becton Dickinson, CA, USA) for 45 min at 4°C

<table>
<thead>
<tr>
<th>No of patients</th>
<th>HLA-A ± SD</th>
<th>HLA-B ± SD</th>
<th>HLA-DR ± SD</th>
<th>HLA-DQ ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone withdrawn (n=9)</td>
<td>0.78 ± 0.44</td>
<td>0.78 ± 0.67</td>
<td>0.22 ± 0.44</td>
<td>0.33 ± 0.5</td>
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<tr>
<td>Prednisolone continued (n=12)</td>
<td>1.17 ± 0.58</td>
<td>0.67 ± 0.65</td>
<td>0.33 ± 0.65</td>
<td>0.58 ± 0.51</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD number of mismatches.
and the other with isotype-matched control antibodies (Becton Dickinson). After two washes in cold PBS, cells were fixed with 1% paraformaldehyde and stored in the dark at 4 °C until analysis. Immunofluorescence was measured on Epics Profile-II Coulter Counter flow cytometer. Markers were set based on background staining with the isotype-matched control antibodies. Data were stored into list-mode files until further analysed using Flowmate software (Dako Ltd, UK).

Culture medium

The culture medium was RPMI 1640 (Gibco; Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (Sigma; Dorset, UK), 25 mM of hepes buffer, 2 mM L-glutamine, 100 µg/ml penicillin and streptomycin and 5 × 10−5 M 2-mercaptoethanol (Sigma) and was used throughout the study.

Limiting dilution analysis of HTLpf

The LDA is based on a bioassay for IL-2 detection using the IL-2 dependent CTLL-2 as described previously in detail [14,15]. Briefly, serial dilutions of responder PMNC were cultured in 96-well microtitre plate (Nunc, Denmark) in 24 replicates together with 20 000 appropriate mitomycin C inactivated stimulator mononuclear cells, either donor spleen- or third party (a mixture of spleen cells from 10 donors) in a total volume of 200 µl. After 72 h of incubation, 100 µl of supernatant was transferred from each well to 96 flat-bottom microtitre plate (Nunc) for detection of IL-2 using the IL-2 dependent CTLL-2 cells. Proliferation of CTLL-2 was assessed by [3H]-thymidine incorporation over 8 h. Individual microcultures were considered positive for IL-2 secretion in wells that exhibited proliferation greater than the mean plus 3 times the SD of 24 control wells containing supernatant of stimulator cells only.

Statistical analysis

The HTLpf in each assay was calculated based on the Poisson distribution method, and was corrected for number of CD3+ cells. The number of the frequency, 95% confidence intervals (CI), and the probability for single-hit kinetics were estimated by maximum likelihood analysis using the Jackknife modification of the χ² minimization as described by Taswell [16] using computer software [17]. Assays were considered to conform to single-hit kinetics when the goodness-of-fit was <12.5 and P value >0.05 as calculated by χ² analysis. Assays for which P value was <0.05 were excluded. Individual HTLpf were considered to be statistically different when the 95% CI did not overlap. As appropriate, Mann–Whitney U test, Fisher’s exact test, and paired or unpaired Student t test were used to compare between the groups. Statistical significance was defined as P value <0.05. A patient was considered as hyporesponsive to his donor antigen if the donor-reactive HTLpf reduced at least fivefold (as compared to pre-tx) at least two time points post-tx provided that the post-tx third-party-reactive HTLpf has not reduced by more than twofold.

Reproducibility of the assays

Sensitivity and reproducibility of the assay was established by measuring HTLpf in different healthy volunteers from hospital or laboratory staff at different appropriate time points. We repeatedly found that Con A stimulated mitomycin C inactivated stimulator mononuclear cells did not produce IL-2. The chosen mitomycin C dose did not greatly reduce allostimulatory capability of the stimulator cells (unpublished results). As a control, CTLL-2 cells were also incubated with increasing concentrations of rIL-2. The culture period of 72 h was found to be optimal for measuring the IL-2 production by a single cell type and the assays conformed to single-hit kinetics, indicating that IL-2 production was dependent on a single limiting cell type. In general the HTLpf of the healthy volunteers were found to be similar with overlapping 95% CI and < twofold variation during the monitoring period (results not shown).

Results

Steroid withdrawal

Of the total 21 patients studied, PRED was withdrawn completely in nine patients for average of 380 ± 165 days (range 187–684) and PRED was continued in 12 patients. Among the 9/21 patients in whom PRED was withdrawn, PRED was reinstated in two patients at 210 and 187 days respectively after PRED withdrawal. The reasons for restarting PRED in these two patients were mild acute rejection episode confirmed by biopsy in one patient; the other patient had a significant rise in serum creatinine level but no acute rejection episode was confirmed. In this patient the PRED was restarted at a total dose of 2.5 mg/day. In both these patients the serum creatinine level returned to the level before withdrawal in 4 weeks time after restarting the PRED.

PRED was not withdrawn in 12/21 patients because of previous significant acute rejection episode in eight patients or unsuccessful complete withdrawal of PRED in four patients because of significant rise of serum creatinine level after PRED tapering to below 5 mg/day. PRED requirement was not correlated with HLA compatibility (Table 1).

Rejection episodes

A total of four rejection episodes occurred in four of nine patients in the SW group, three before steroid withdrawal (see material and method) and one at 207 days after PRED withdrawal. In the SC group a total of 16 rejection episodes occurred in nine of 12 patients, 12 within 6 months and four episodes after 6 months post-tx. No grafts were lost from chronic rejection in the SW or SC group after a mean follow up of 854 ± 102 days (range 714–1083).

Renal function

In the nine patients in whom PRED was discontinued, the mean serum creatinine was 138 ± 24 before and 132 ± 40 after PRED withdrawal (P=0.45). The mean serum creatinine at 18 months was significantly lower in the SW group than in the SC group, 132 ± 40 and 173 ± 39 respectively, (P=0.04). Table 2 summarizes the results in both groups.
Table 2. Comparisons of patient demographics and clinical outcome in steroid withdrawn (SW) and steroid continued (SC) group

<table>
<thead>
<tr>
<th></th>
<th>SW (n=9)</th>
<th>SC (n=12)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Age in years (mean±SD)</td>
<td>50±14</td>
<td>47.8±12.3</td>
<td>0.85</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>4/5</td>
<td>7/5</td>
<td>0.85</td>
</tr>
<tr>
<td>Number of rejection episodes before SW or with comparable period in SC group</td>
<td>3</td>
<td>12</td>
<td>0.07</td>
</tr>
<tr>
<td>Number of rejection episodes after SW or with comparable period in SC group</td>
<td>1</td>
<td>4</td>
<td>0.4</td>
</tr>
<tr>
<td>Mean number of total rejection episodes (mean±SD)</td>
<td>0.44±0.52</td>
<td>1.33±1.43</td>
<td>0.06</td>
</tr>
<tr>
<td>Serum creatinine at 18 months (µmol/l) (mean±SD)</td>
<td>132±40</td>
<td>173±39</td>
<td>0.04</td>
</tr>
<tr>
<td>Follow-up in days (mean±SD)</td>
<td>876±127</td>
<td>838±81</td>
<td>0.64</td>
</tr>
<tr>
<td>Changes* of donor-reactive HTLPf (mean±SD)</td>
<td>9.5±6</td>
<td>2.8±2.5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*In multiples relative to pretransplant level determined at least 6 months after steroid withdrawal or at 12 months in the steroid continued group.

Kinetics of HTLPf

The post-tx values of donor-reactive HTLPf ranged between 156–486 (/million) and were without a predictive value for the clinical outcome. The post-tx values of donor-reactive HTLPf indicated that there were two different reactivities towards donors. In seven of nine patients in the SW group (Figure 1), the donor-reactive HTLPf determined at least 6 months after steroid withdrawal were reduced at least fivefold (range 5–17) compared to pre-tx level, while third party-reactive HTLPf frequencies remained mostly unchanged (<twofold) through the same period. In one (1/7) patient in whom complete steroid withdrawal was successful had a persistent high post-tx donor-reactive HTLPf. In two (2/9) patients in whom steroid withdrawal was not successful, one had a high post-tx donor-reactive HTLPf, and in the other patient the post-tx donor-reactive HTLPf decreased to a significant level. The mean (95% CI of mean) of the third-party-reactive HTLPf in these nine patients at different time points are as follows: at pre-tx 1/2760 (2044–3475), before steroid withdrawal 1/3655 (2745–4563) and after steroid withdrawal 1/3586 (2395–4776).

In 10 (10/12) patients in the SC group (Figure 2), both donor and third-party-reactive HTLPf remained little changed (<threefold) at 12 months post-tx as compared to pre-tx. One of eight patients in whom no attempt was made to withdraw steroid showed a significant decrease in post-tx donor-reactive HTLPf. In four patients in this group in whom steroid withdrawal was not successful, two had a high post-tx donor-reactive HTLPf in all the determinations. In the other two patients, one had an initial 1.5-fold reduction in post-tx donor-reactive HTLPf but then increased during the last determination, the last patient showed a significant decrease in post-tx donor-reactive HTLPf.

Fig. 1. Significant reduction of post-transplant donor-reactive HTLPf in seven (7/9) cadaveric renal allograft recipients in whom steroid withdrawal succeeded for at least 6 months. All seven patients were free from the steroid until final evaluation (minimum of 10 months after withdrawal). In two patients steroid was reinstated after 6 months of withdrawal (see text).
Fig. 2. Post-transplant donor-reactive HTLpf remained little changed in 10 (10/12) cadaveric renal allograft recipients on continued steroid at different time points as indicated. Two patients showed a low post-transplant HTLpf, in one patient no attempt was made to withdraw steroid, and in the other one complete steroid withdrawal was not successful (see text).

in all the determinations. The mean (95% CI of mean) of the third-party-reactive HTLpf in these 12 patients at different time points are as follow: at pre-tx 1/2394 (1951–2837), 6 months post-tx 1/3272 (2568–3975), and at 12 months post-tx 1/3519 (2627–4410).

Discussion

Several previous studies have reported the advantages of steroid withdrawal after solid organ allotransplantation. These advantages include lower incidence of opportunistic infection [10], lower incidence of cataracts [9,10], lower serum lipids [3,5,10], less hypertension [3,5,9,10], good control of diabetes [10], and improved growth rate in young recipients [18]. These advantages should be weighed against the risk of chronic rejection and subsequent graft loss as a result of steroid withdrawal [1,7]. Based on the standard clinical criteria, the decision to withdraw steroid cannot be undertaken lightly, and it would be advantageous if in vitro immunological assays could predict the safety of steroid withdrawal.

Our focus has been on studying IL-2 production and proliferation, given the important role played by T helper lymphocytes in the initiation and the development of organ allotraft rejection [19,20], and the fact that the majority of these T helper cells are proliferative cells reactive with allogeneic MHC class II antigens. Previously we quantified the alloreactive response by measuring the proliferation of T cells in MLR and we identified donor-specific hyporeactivity in 11/19 studied [12]. In the present study we quantified the donor-reactive HTLpf in a longitudinal study using LDA, known to be a powerful and sensitive technique [21] and we correlated these results with the success or failure of steroid withdrawal.

Our study provides evidence that the development of in vitro donor-specific hyporeactivity in certain patients as detected by LDA of HTLpf correlates with successful steroid withdrawal. In the SW group there were fewer rejection episodes (3 before and one after steroid withdrawal) compared with the SC group where a total of 16 rejection episodes (12 within 6 months post-tx and four after 6 months) occurred in nine patients. Whilst suggestive of better graft tolerance the differences are not significant ($P=0.06$, Table 2). In a study by Flechner et al. [22] rejection episodes occurred in four of 21 recipients of HLA identical grafts and in two of 12 recipients of haplo-identical grafts after steroid withdrawal. Flechner et al. selected candidates for steroid withdrawal based on low anti-donor reactivity as detected by MLR. A continuation [9] of this study confirmed the excellent tolerance to steroid withdrawal in the donor-specific hyporesponsive patients after 5 years of follow-up. In another study by Reinsmoen and Matas [23], the development of in vitro donor specific hyporeactivity was found to be associated with a lower mean serum creatinine level at 6, 12, and 24 months following transplantation and fewer rejection episodes in 26% of renal allotraft recipients studied, as compared to patients who had persistent reactivity towards donors 2 years post-transplantation. However, no attempt was made to
withdraw steroids in these patients, although they suggest that these hyporesponsive patients may benefit from steroid withdrawal.

In contrast to these previous studies [9,22,23], Ingulli et al. [7] found that MLR failed to predict the graft outcome, as hyporesponsive status to donor antigens was seen in patients who experienced rejection episodes after steroid withdrawal, and also in patients in whom steroids were never withdrawn. Goulmy et al. [6] also reported that a partial or complete steroid withdrawal was successful in both responsive and hyporesponsive patients to donor antigens as detected by cell-mediated lympholysis. It is not clear why these studies are contradictory. One might speculate that this may be due to the small number of patients (n = 13) studied by Ingulli et al. [7], and another possible factor is that the increase in the immunosuppression during the study period might have affected the in vitro results of these patients or the fact that Goulmy et al. [6] studied cell-mediated lympholysis rather than helper T-cell function.

Previous studies by others [1,7,11] have found that the degree of HLA compatibility did not appear to predict the success of steroid withdrawal. In our study, the total number of HLA mismatches was lower in the SW group than in the SC group, but this was not statistically significant. This was also true for the total number of HLA-DR mismatches (lower in SW group) between donors and recipients (P = 0.66, Table 1). In an attempt to determine the effect of MHC class II antigens mismatches on the alloreactive HTLPf, the data were further analysed on the basis of donor-recipient pairs with and without HLA class II mismatches separately (regardless of steroid withdrawn or not). Fifty per cent (8/16) of patients who were HLA-DR matched to their donor organs showed a significant reduction in post-tx donor-reactive HTLPf. While only one (20%) of five HLA-DR mismatched renal allograft recipients showed a significant reduction of post-tx donor-reactive HTLPf. Furthermore, 50% (8/16) of patients who were HLA-DR matched to their donor organs had a total of 10 early rejection episodes (<3 months post-tx) and two late episodes. While four (80%) of five HLA-DR mismatched renal allograft recipients had a total of six early rejection episodes and two late episodes. Although the influence of HLA-DR on post-tx donor-reactive HTLPf (P = 0.51) and rejection episodes (P = 0.51) did not reach statistical significance, it may be more apparent when studying larger numbers of patients.

In summary, this study showed that with time a proportion of renal transplanted patients on standard triple therapy developed a state of donor-specific hyporeactivity as evidenced by the specific reduction of donor-reactive HTLPf. This state of donor-specific hyporeactivity was associated with a lower incidence of late rejection episodes, and surprisingly stable renal graft function after complete successful steroid withdrawal. Based on these findings, we intend to carry out a prospective study involving large series of patients to establish whether the clinical selection for safe reduction in immunosuppression correlates with in vitro immunological tests.

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4. Orosz C, Adams P, Ferguson R. Frequency of human alloreactive, interleukin-2-producing T cells in humans. In summary, this study showed that with time a proportion of renal transplant patients on standard triple therapy developed a state of donor-specific hyporeactivity as evidenced by the specific reduction of donor-reactive HTLPf. This state of donor-specific hyporeactivity was associated with a lower incidence of late rejection episodes, and surprisingly stable renal graft function after complete successful steroid withdrawal. Based on these findings, we intend to carry out a prospective study involving large series of patients to establish whether the clinical selection for safe reduction in immunosuppression correlates with in vitro immunological tests.

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5. Kahan B, Kerman R, Van Buuren C, Flechner S, Golden D, Lewis R. Clinical outcome in 36 patients after at least one and two late episodes. Although the influence of donor-drug mismatch on post-tx donor-reactive HTLPf. Furthermore, 50% (8/16) of patients who were HLA-DR matched to their donor organs had a total of 10 early rejection episodes (<3 months post-tx) and two late episodes. While four (80%) of five HLA-DR mismatched renal allograft recipients had a total of six early rejection episodes and two late episodes. Although the influence of HLA-DR on post-tx donor-reactive HTLPf (P = 0.51) and rejection episodes (P = 0.51) did not reach statistical significance, it may be more apparent when studying larger numbers of patients.

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