Tapering immunosuppression in recipients of living donor kidney transplants

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Abstract
We have previously suggested that the in vitro donor-specific cytotoxic T-lymphocyte precursor (CTLP) assay can guide us to identify patients in which the immunosuppressive load can be tapered. In a clinical trial we had observed that a low (<10/10⁶ PBMC) frequency of these CTLP was predictive for an uneventful rejection-free clinical course in patients that were converted from calcineurin inhibitors to mycophenolate mofetil or azathioprine. In the present prospective study in 81 stable kidney transplant recipients, already converted from calcineurin inhibitors, we measured CTLP frequencies and reduced the immunosuppressive load on a routine basis when CTLP were <10/10⁶ PBMC. Donor-specific cytotoxicity could not be measured in 50/81 patients, while their reactivity against third-party lymphocytes was not impaired. These 50 patients were tapered in their immunosuppression. Only in one patient, who had stopped all his medication, was a rejection episode diagnosed. We conclude that in patients with a low donor-specific CTLP frequency it is safe to reduce the immunosuppression.

Keywords: cytotoxic T lymphocytes; immunosuppression; kidney transplantation

Introduction
Over-immunosuppression remains a major problem after organ transplantation. Infections, cardiovascular morbidity and mortality due to the combined effects of nephrotoxicity, hypertension and dyslipidaemia, diabetes mellitus and osteonecrosis are frequent complications in transplanted patients. Especially in recipients of living donor kidneys, with an expected overall graft survival of ~15–25 years, the induction of solid non-skin carcinomas is the most frightening complication of very long-term immunosuppression. The severity and incidence of these side-effects are directly related to the immunosuppressive load and patients would therefore benefit from tapering immunosuppression [1].

In the search for a laboratory test that would predict which patients could safely be tapered in their immunosuppressive load we previously conducted a clinical trial in which the calcineurin inhibition was discontinued, followed by a 50% dose reduction of the antiproliferative agents mycophenolate mofetil (MMF) or azathioprine (Aza) [2]. On that basis we concluded that a low number of donor-specific cytotoxic T-lymphocyte precursors (CTLP) was predictive for an uneventful rejection-free clinical outcome after conversion and dose reduction [3]. We also found that tapering of immunosuppression was associated with stable, donor-specific downregulation during follow-up [4].

To ascertain whether the CTLP assay could also be prospectively used in routine clinical practice, we measured CTLP frequencies in stable kidney transplant recipients more than 2 years after transplantation and reduced the immunosuppressive load according to the results of this test.

Patients and methods

Patients
A total of 81 kidney transplant recipients was analysed. They were all transplanted more than 2 years before, had no proteinuria, had been free from acute rejection episodes in the last year and were not on calcineurin inhibitors. Inclusion criteria also demanded the availability of viable donor
lymphocytes. There were 49 recipients of a living donor kidney; patients who had received an HLA-identical organ were excluded.

**Cytotoxic T-lymphocyte precursor frequency (CTLpf)**

Limiting dilution cultures were set up in 96 well U-bottom tissue plates (Nunc.) Twenty-four replicates of grade number PBMC responders were titrated in seven-step double dilutions starting from $5 \times 10^3$ to 781 PBMC/well and stimulated with irradiated (45 Gy) donor or third-party lymphocytes ($5 \times 10^4$ cell/well) in 200 ml of culture medium. Additionally, 24 wells contained stimulator cells alone. After 3 days of culture, cells were refreshed with 100 ml of culture medium containing recombinant IL-2 with a final concentration of 20 units/ml IL-2 (proleukin; Chiron BV, Amsterdam, The Netherlands). After another 7 days of culture, the microcultures were tested for cytotoxicity. Each well was individually tested for cytolytic activity against $5 \times 10^5$ europium-diethylenetriaminepentaacetaete-labelled target cells (Fluka, Buchs, Switzerland and Sigma Chemicals, St Louis, MO). In the case of donor stimulation, T-cell blasts of donor origin were used as targets. In the case of third-party stimulation, T-cell blasts of the third-party spleen cells were used as targets. After 4 h of incubation at 37°C in a humidified atmosphere of 5% CO2, the plates were centrifuged for 5 min at 600 g and 20 ml of the supernatant was transferred to 96 well plates with a low background fluorescence (Fluorimmunoplate; Nunc, Roskilde, Denmark). Additionally, 100 ml of enhancement solution (LKB-Wallac, Turku, Finland) was added to each well. Plates were stored in the dark at room temperature until the fluorescence of the released europium was measured in a time-resolved fluorometer (Victor 1420 Multilabel Counter, LKB-Wallace). Fluorescence was expressed in counts per second. As a control for every target cell, spontaneous lysis (target cells + 1% Triton X-100) was determined.

**Statistical analysis**

The mean counts per second with their standard deviation (SD) of the wells in which only stimulator cells were present were considered as background. Experimental wells were scored positive if the counts in that well exceeded the mean +3 SD of the wells in which only stimulator cells were present. For each cell concentration, the number of negative wells was determined and used to calculate the frequency with a computer program designed by Strijbosch et al. [5]. The CTLpf (expressed as the number of CTL per $10^6$ cells) and the 95% confidence interval were calculated by the Jackknife procedure for maximal-likelihood [5]. The calculated frequencies were accepted when the goodness-of-fit did not exceed 12.

**Immunosuppression**

Patients eligible for a 50% dose reduction on the basis or a low CTLpf started with MMF 1 g b.i.d. or Aza 2 mg/kg in combination with 10 mg prednisone. First, a dose reduction to MMF 0.75 g b.i.d. or Aza 1.5 mg/kg was prescribed, followed by a further reduction to MMF 0.5 g b.i.d. or Aza 1 mg/kg 4 months later. Steroids where kept at 10 mg prednisone.

**Results**

In 50/81 patients no donor-specific CTLpf could be measured. In contrast, in all but three patients we were able to detect a CTLpf frequency against third-party lymphocytes. There was no difference in the proportion of patients with undetectable CTLpf between recipients of a living vs a post-mortal donor kidney: 31/49 vs 19/32 patients (Figure 1). No correlation was found in the CTLpf and the time after transplantation in this cohort of patients transplanted more than 2 years ago.

After obtaining informed consent we reduced the immunosuppressive load in all 50 patients with CTLpf < $10/10^6$ PBMC according to the scheme mentioned in the Patients and methods.

There were 30 patients on MMF and 20 on Aza. Median follow-up after transplantation was 50 months (range 20–186 months). In the median observation period of 12 months after the first dose reduction, only one patient was diagnosed with acute rejection. This patient had discontinued all his medication for several weeks. This rejection episode proved to be reversible after reintroduction of the immunosuppression and 3 × 1 g methylprednisolone IV.

Proteinuria developed in another patient; a kidney biopsy showed recurrence of glomerulonephritis.

**Discussion**

In the majority of stable kidney transplant recipients more than 2 years after transplantation, we were not able to detect donor-specific CTLp in their peripheral blood. This suggests donor-specific downregulation as
the CTLp against the third-party lymphocytes proved to be unimpaired. This observation confirms our earlier reported data from before [4].

In the present prospective study we also confirmed our previous results that it is possible to taper the immunosuppression in patients with low CTLpf. Apparently, this test is not only valuable in patients on calcineurine inhibitors [3] but also in patients without cyclosporine or tacrolimus, as the present study has been performed in patients on MMF or Aza. It appears that most patients, even when they had already been converted from calcineurin-based immunosuppressive schemes, are still over-immunosuppressed with all the potential risks for accruing lethal side-effects. A negative CTLp test can identify patients in whom the immunosuppressive load can safely be reduced, but we still do not know whether a positive CTLp test in the context of MMF or Aza is associated with clinical problems after dose reduction. It is conceivable that it is possible even in these patients with high CTLpf to reduce the dose MMF or Aza by 50%. We are now in the process of analysing exactly that. Furthermore, more ‘bold’ withdrawal of immunosuppression could also be envisaged, especially in those patients who still have undetectable CTLps after dose reduction. Discontinuation of steroids is an obvious option [6]. Prescribing an immunosuppressive agent every other day or even twice a week is another possibility [7]. Particularly, recipients of living donor kidney transplants with their expected long-term overall graft survival could benefit from drastic reduction of the potentially dangerous immunosuppressive medication. We will continue our efforts to do so, guided by donor-specific cytotoxicity tests.

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Conflict of interest statement. None declared.

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