Identification of expanded T-cell clones by spectratyping in nonfunctioning kidney transplants

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Background: The aim of this study was the application of complementarity-determining region-3 spectratyping analysis to determine T-cell repertoire complexity and to detect T-cell clone expansion, as a measure of immune response in nonfunctioning kidney transplants (group hemodialysis-transplant [HD-Tx]), nontransplanted dialysis patients (group hemodialysis [HD]), and normal subjects as controls (group C).

Patients and methods: Analysis of T-cell receptor (TCR) diversity by spectratyping was applied to peripheral blood samples collected from 21 subjects: eight in group HD-Tx, seven in group HD, and six in group C.

Results: Considering the extent of the skew in TCR variable region repertoires as a measure of clonal T cells, we found that the number of altered spectra showed a progressive increase from normal subjects to dialysis patients and to nonfunctioning kidney transplants, respectively. Healthy subjects had the lowest number of altered spectra, and patients with nonfunctioning kidney transplants had the highest. Differences were significant for group HD-Tx vs group C (P=0.017) and group HD vs group C (P=0.015), but not between nonfunctioning kidney-transplant recipients and dialysis patients (group HD-Tx vs group HD).

Conclusion: Although dialysis appears to be a weaker trigger for clonal expansion of T cells, our data suggest that the utilization of complementarity-determining region-3 spectratyping analysis of the TCR repertoire might be useful to monitor specific immunostimulation in patients before and after kidney transplantation.

Keywords: dialysis, nonfunctioning kidney transplant, T-cell repertoire, TCR spectratyping

Introduction

Kidney transplantation is considered the treatment of choice for patients with end-stage renal disease, since it restores patients’ quality of life and reduces morbidity and mortality rates associated with dialysis. The introduction of new immunosuppressant drugs has greatly decreased the number and impact of acute rejections, which however is still a critical complication. Unfortunately, several pathological changes due to immunological and nonimmunological components for organ loss lead to a progressive loss of graft function, and this condition does not seem to have received consistent benefits from the new pharmacological treatments. The main consequence is the necessity to retransplant patients who are often sensitized or highly sensitized by a previous transplant, with a resulting higher percentage of patients in transplant-waiting lists needing a second transplantation, longer average waiting times, and a further increase in the gap between organ demand and availability. There is much evidence suggesting how immunosuppressive therapy remains too aspecific a strategy
to improve graft survival, and additionally it is not practicable to stress systemic immunosuppression, owing to the related possible complications, mainly viral infections, malignancy, nephrotoxicity, and cardiovascular disease.10–14

Growing attention is currently focused on the development and protective mechanisms of graft tolerance, in which T-cell responses in both innate and adaptive immunity play a major role.15 Accumulating evidence suggests that innate immunity interacts with the adaptive immune system to identify antigens and eliminate them from the host.16 Both components are implicated not only in acute rejection but also in chronic allograft nephropathy, which in turn is due to the presence of preformed human leukocyte antigen (HLA) antibodies (group hemodialysis-transplant (HD-Tx)); the second group consisted of seven dialysis patients with no previous renal transplant (group hemodialysis (HD)); and the third included six healthy subjects with normal renal function, used as the control group (group C). Patients in groups HD-Tx and HD were on regular bicarbonate hemodialysis therapy three times a week at our dialysis center.

Inclusion criteria were age above 18 years, and for dialysis patients in groups HD-Tx and HD: 1) clinical stability of at least 3 months before study entry; 2) functioning arteriovenous fistula as vascular access; and 3) absence of active gastrointestinal bleeding, diabetes, amyloidosis, focal segmental glomerulosclerosis, autoimmune diseases, vasculitis, and malignancy. As shown in Table 1, the subjects included in each group were matched for general parameters (sex distribution and age). Groups HD-Tx and HD were comparable for dialysis vintage, chronic kidney-disease duration, and serum creatinine. Causes of transplant failure in the HD-Tx group were biopsy-proven chronic allograft nonimmune injury (interstitial fibrosis and tubular atrophy) in five patients, recurrent primary disease in two patients, and de novo disease in one patient.

To exclude interference due to anti-HLA antibody production in patients with nonfunctioning kidney transplants, all subjects in group HD-Tx were assayed for posttransplant HLA-specific IgG antibodies through enzyme-linked immuno- sorbent assay panel-reactive antibodies, as previously described by Costa et al.24 Therefore, patients in group HD-Tx were selected only if they tested negative for specific HLA antibodies in three consecutive controls. All patients in HD-Tx group had been tapered from immunosuppression to low-dose monotherapy steroids (5 mg/day prednisone). Dialysis patients with no previous transplant (group HD) were always negative for panel-reactive antibodies and HLA specificity.

Patients and methods

Patients

A total of 21 subjects were enrolled in the study and assigned to three groups: the first composed of eight nonfunctioning kidney-transplant recipients under dialysis treatment, negative for the presence of preformed human leukocyte antigen (HLA) antibodies (group hemodialysis-transplant [HD-Tx]); the second group consisted of seven dialysis patients with no previous renal transplant (group hemodialysis [HD]); and the third included six healthy subjects with normal renal function, used as the control group (group C). Patients in groups HD-Tx and HD were on regular bicarbonate hemodialysis therapy three times a week at our dialysis center.
The study was carried out in conformity with the Declaration of Helsinki. Written informed consent was obtained before inclusion from all subjects, and the protocol was approved by the ethical committee of St Orsola-Malpighi University Hospital, Bologna, Italy (protocol BO-SO_NEPH2011_07).

**RNA extraction and reverse-transcription reaction**

Peripheral blood mononuclear cells were isolated from heparinized blood by Isopaque Ficoll (Lymphoprep; Nycomed, Zurich, Switzerland) gradient centrifugation, then total RNA was extracted with an RNeasy minikit (Qiagen NV, Venlo, the Netherlands) according to the manufacturer’s instructions. For each sample, 1 μg of RNA was reverse-transcribed to complementary DNA using random hexanucleotide primers and reverse transcriptase (Superscript; Thermo Fisher Scientific, Waltham, MA, USA), with a thermal profile of 10 minutes 25°C, 60 minutes 42°C, and 5 minutes 99°C.

**Polymerase chain reaction**

The TCR Vγ gene subfamilies were amplified by a constant primer labeled at the 5’ terminus with the dye FAM (6-carboxyfluorescein), coupled with other specific primers, first designed by Gorski et al.21 Briefly, 20 μL aliquots of working solution containing GeneAmp® polymerase chain-reaction (PCR; Thermo Fisher Scientific), 2 mM MgCl₂, 0.2 mM of each deoxyribonucleotide triphosphate, 0.1 mM of each primer, and 1 μL of complementary DNA were amplified with the following PCR conditions: denaturation 30 seconds 95°C, annealing 30 seconds 58°C, extension 45 seconds 72°C, 29 cycles; final extension 5 minutes 72°C. An aliquot of 5 μL of the final PCR product was analyzed by electrophoresis in a 2% agarose gel (FMC, Rockland, ME, USA) with 0.5 μg/mL of ethidium bromide in 0.5× Tris–borate–ethylenediaminetetraacetic acid buffer (Thermo Fisher Scientific), and then visualized under an ultraviolet transilluminator.

**T-cell spectratyping**

PCR product (1 μL) was denatured in 15 μL formamide and electrophoresed through POP-4® on an ABI 310 automated sequencer in the presence of Tamra 400HD ROX size standard (all Thermo Fisher Scientific). As a result of TCR β-chain rearrangements, each amplification shows a certain pattern of bands related to the complexity of the T-cell repertoire and the concentration of one or few expanded clones species within the size class. Spectratyping analysis was performed in duplicate for the Vβ families Vβ2, Vβ4, Vβ5.1, Vβ5.2, Vβ7, Vβ12, Vβ13, Vβ14, Vβ15, Vβ16, Vβ17, Vβ21, Vβ22, and Vβ24. GeneScan and Genotyper 2.1 softwares (Thermo Fisher Scientific) were used to classify each Vβ histogram peak by its PCR-size length and compute the area under curve (AUC) of each peak.

Normally, in healthy subjects, the CDR3-length distributions of each TCR Vβ family contain at least six peaks in a Gaussian distribution, suggestive of a polyclonal T-cell expansion. Any perturbation in TCR Vβ CDR3 repertoire, referred to as “skewing”, results in a markedly non-Gaussian CDR3-length distribution, suggesting a clonal or oligoclonal T-cell population. Specifically, we considered as skewed any
TCR Vβ family, defined by an AUC within a given CDR3 spectratype of greater than 40% of the sum of the total AUC.

Statistical analysis
Continuous variables are presented as mean ± standard deviation and categorical variables as number and percentage. Differences between groups were computed using Kruskal–Wallis analyses of variance followed by Mann–Whitney U tests. P-values below 0.05 were considered significant, and all statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) for Windows version 16.0.

Results
All the subjects participating in the study presented skewed spectra, including those in the control group. The technique showed high specificity and repeatability. The number of altered spectra revealed no correlation with sex, age, or dialysis vintage. Qualitative evaluation of skewed spectra revealed high variability in the three groups, although the number of patients was too small to provide statistically significant correlations of specific altered Vβ-chain CDR3 regions with clinical outcomes. Figure 1 shows the complete spectra patterns for patients in each group who presented an intermediate number of alterations.

To determine the number of skewed spectra for each patient in the three groups, we observed electropherograms of TCR Vβ amplicons and expressed the “area under the peak” as the quantity of labeled Vβ-fragment PCR product of a defined length measured by the detector. From the evaluation of the amount of altered spectra in each group, we applied Kruskal–Wallis analyses of variance, followed by Mann–Whitney U tests to compare the AUC for each peak of all Vβ families across the three groups. Table S1 shows the AUC of each peak for all Vβ families analyzed for each subject in the three groups: HD-Tx (n=8) vs HD (n=7) vs C (n=6).

As Table 2 shows, there was a progressive increase in the number of skewed spectra from healthy subjects to dialysis patients and to nonfunctioning kidney transplants, respectively. When each Vβ family was considered separately, no significant differences were found among the three groups, but the differences were significant for the sum of total skewed spectra (P<0.001).

Healthy subjects had the fewest altered spectra and patients with a nonfunctioning kidney transplant the highest. Applying post hoc Mann–Whitney nonparametric tests, we found that differences were significant for group HD-Tx vs group C (P=0.017) and group HD vs group C (P=0.015), but not for nonfunctioning kidney-transplant recipients vs dialysis patients (group HD-Tx vs group HD).

Discussion
The possibility of identifying oligoclonal T-cell expansions of TCR Vβ families in kidney transplants can represent a fascinating research field in kidney-transplant tolerance. The analysis of TCR diversity through spectratyping in our patients suggested that renal transplant recipients who experienced graft failure were characterized by more persistent expanded T-cell clones, with a potential specific role in the acceptance of transplanted solid organs.27,28 Previous evidence has indicated that the extent of skewing of TCR repertoires and levels of clonal T-cell expansion are significantly correlated with renal transplant outcomes. It has been reported that a decrease in the number of donor-reactive T-cell clones is more directly associated with tolerance induction, whereas allograft rejection can trigger clonal expansion of T cells.30,31

In our study, we selected a limited number of renal transplant recipients with a nonfunctioning graft negative for donor-specific antibodies to avoid the introduction of a further confounding variable. The eight-patient HD-Tx group had lost graft function, due to nonimmune injury (interstitial fibrosis/tubular atrophy) previously diagnosed by renal biopsy in five, recurrent primary disease in two, and de novo disease in one. The group of renal transplant recipients with a nonfunctioning graft showed the maximum number of skewed spectra, although the difference from dialysis patients did not reach statistical significance. It is feasible that previous cell-mediated acute and/or chronic reactions might have elicited persistent specific HLA-sensitization responsiveness, leading to allograft intolerance. On the other hand, the limited number of patients prevented us from applying other statistical analyses or to draw any firm conclusion about the real influence of dialysis on T-cell-clone expansion. The higher extent of skewing in patients with a failed graft seems to indicate a link between altered Vβ-chain CDR3 regions and immunological response against HLAs.

In spite of the small sample size and differences in the patient population, these findings show a certain consistency with a recent report by Morris et al, who described a drop in donor-reactive T-cell clones in tolerant combined kidney and bone marrow-transplant recipients. The same reductions were not observed in intolerant patients or standard renal transplant patients treated with standard immunosuppressive regimens. T-cell-repertoire turnover due to lymphocyte-depleting conditioning only partially accounted for the observed reductions in tolerant patients, supporting the hypothesis that one key mechanism of allograft-tolerance induction may be T-cell clonal deletion.31

The actual difficulty is related to the apparent variability of the spectra-alteration pattern detected in each group.
Spectratyping in renal transplants and not characterizing the particular status of the subject (posttransplant, dialysis or healthy), in line with previous data by Barth et al, suggesting that T-cell Vβ-repertoire is restricted and individual in both acute and ongoing chronic allograft rejection before getting to transplant failure.26 Thus far, these preliminary data might suggest a possible follow-up approach for renal transplant recipients, considering the changes over time in T-cell CDR3 regions as a reflection of a specific immune status after kidney transplant.

Delayed T-cell recovery and restricted TCR diversity after both hematopoietic stem cell transplantation and solid-organ transplantation appear to increase the risk of opportunistic infections. Therefore, monitoring T-cell repertoire over time may provide valuable insights into the immune status of transplant recipients. Figure 1 shows the complete spectra patterns for patients 2, 7, and 12, who were in the intermediate number of skewed spectra groups (HD-Tx, HD, and C, respectively).

**Figure 1** Complete spectra patterns for patients 2, 7, and 12.

**Notes:** Patients 2, 7, and 12 were those with the intermediate number of skewed spectra in group HD-Tx, group HD, and group C, respectively.

**Abbreviations:** C, control; HD-Tx, hemodialysis-transplant; HD, hemodialysis.

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infections and cancer, with a significant impact on transplant-associated morbidity and mortality.\textsuperscript{32} van Heijst et al investigated clinical parameters potentially able to affect T-cell-repertoire features in nonfunctioning kidney-transplant recipients vs dialysis patients vs healthy subjects, without examining the outcomes specifically. Although the limited sample size prevents us from drawing firm conclusions about a possible correlation between a given pattern of spectra and a specific clinical condition, the main preliminary finding emerging from our study is the elevated number of skewed spectra in nonfunctioning kidney-transplant recipients under dialysis treatment. Perhaps dialysis treatment alone represents a weaker trigger for clonal expansion of T cells, even if the difference between nonfunctioning kidney-transplant recipients vs dialysis patients did not meet statistical significance, maybe owing to the small number of subjects.

### Conclusion

Our data indicate that early identification of T-cell clonality renal transplantation might predict a state of immunoca- 

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### Disclosure

The authors report no conflicts of interest in this work.

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### Table 2

|     | HD-Tx (n=8) | HD (n=7) | C (n=6) | P     |
|-----|-------------|----------|---------|-------|
| V1  | 3/8 (4.928) | 0/7 (9.316) | 0/6 (6.861) | NS    |
| V4  | 3/8 (7.182) | 2/7 (10.367) | 0/6 (11.598) | NS    |
| V5.1| 2/8 (4.940) | 3/7 (4.885) | 0/6 (5.429) | NS    |
| V5.2| 3/8 (3.493) | 3/7 (1.465) | 0/6 (2.594) | NS    |
| T2  | 4/8 (8.501) | 2/7 (9.850) | 0/6 (17.732) | NS    |
| T3  | 2/8 (4.284) | 2/7 (2.260) | 0/6 (11.509) | NS    |
| T9  | 1/8 (2.096) | 0/7 (3.145) | 0/6 (7.19) | NS    |
| T13 | 1/8 (12.303) | 2/7 (12.693) | 0/6 (23.654) | NS    |
| T14 | 2/8 (1.935) | 1/7 (25.177) | 0/6 (1.876) | NS    |
| T15 | 2/8 (5.846) | 3/7 (7.575) | 0/6 (2.802) | NS    |
| T16 | 3/8 (3.094) | 2/7 (7.457) | 0/6 (6.777) | NS    |
| T17 | 3/8 (1.586) | 1/7 (3.963) | 0/6 (6.169) | NS    |
| T19 | 1/8 (11.208) | 1/7 (25.098) | 0/6 (15.592) | NS    |
| T20 | 1/8 (4.354) | 3/7 (2.018) | 0/6 (13.481) | NS    |
| T21 | 3/8 (10.385) | 1/7 (15.294) | 0/6 (27.751) | NS    |
| T22 | 1/8 (1.614) | 3/7 (1.410) | 0/6 (2.520) | NS    |
| Total | 35/128 (27.3%) | 29/112 (25.9%) | 4/96 (4.2%) | <0.001 |

**Notes:** Although no differences across the groups emerged when each V\textbeta family was considered separately (Kruskal-Wallis analysis of variance), significant differences were found in the total number of skewed spectra (\textchi^2 test). Individual comparisons through post hoc Mann–Whitney nonparametric tests revealed that healthy subjects had the fewest altered spectra (group HD-Tx vs group C, \textchi^2 test, \textchi^2 = 23 3/8 [NS]; group HD vs group C, \textchi^2 = 21 1/8 [NS]; group HD-Tx vs group C, \textchi^2 = 19 1/8 [NS]; group HD vs group C, \textchi^2 = 17 3/8 [NS]; group HD-Tx vs group C, \textchi^2 = 15 2/8 [NS]; group HD vs group C, \textchi^2 = 14 2/8 [NS]; group HD-Tx vs group C, \textchi^2 = 13 1/8 [NS]; group HD vs group C, \textchi^2 = 5.3 3/8 [NS]; group HD-Tx vs group C, \textchi^2 = 2.5 3/8 [NS]).
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