Identification of Dialysis Patients with Panel-Reactive Memory T Cells before Kidney Transplantation Using an Allogeneic Cell Bank

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Donor-reactive cellular sensitization does not routinely suggest humoral sensitization and vice versa, but both predict poor kidney transplant outcome. Irrespective of donor reactivity, panel-reactive antibody (PRA) screening identifies patients who are at enhanced risk. Therefore, it was hypothesized that panel-reactive memory T cell reactivity (PRT) might be an additional risk assessment factor of dialysis patients who are on the transplant waiting list. IFN-γ–enzyme-linked immunosorbent spot memory T cell frequencies were determined in 10 healthy volunteers and 41 hemodialysis patients using for stimulation an allogeneic cell bank (ACB) from 17 healthy individuals who represented the most frequent white HLA antigens. Positive responses to ACB were analogous to PRA defined as percentage of positive assays of the ACB sets. Hemodialysis patients expressed higher PRT levels compared with healthy volunteers. Five of 10 PRT+ patients were PRA negative, and only four of 10 PRA++ patients exhibited PRT reactivity, suggesting independence of humoral and cellular sensitization. Pretransplantation PRT testing of recipients might improve individual risk assessment to make individualized therapy decisions.


The importance of sensitization for human alloimmunity has been recognized since the beginnings of transplantation medicine (1). Despite the well-established central role of T cells in allograft rejection, only assessment of humoral sensitization has been proved successful for routine use in transplant management. Terasaki and colleagues (2–4) first described antibody-mediated hyperacute rejection episodes in the setting of a positive cross-match and lower graft survival rates in kidney transplant patients with cytotoxic HLA antibodies detected before transplantation.

With the recent availability of new cellular technologies, measurement of T cell sensitization gained more attention. The IFN-γ–enzyme-linked immunosorbent spot (ELISpot) assay for determination of alloreactive memory T cells represents a highly sensitive and easily applicable technique for cellular alloimmunity (5). Using ELISpot, we and others demonstrated recently that kidney transplant recipients with high frequencies of donor-reactive memory T cells before transplantation are at risk for severe acute rejection episodes during the early posttransplantation period (6,7).

Blood transfusions, gravidities, and previous transplantations increase the risk for allosensitization, but allospecific memory does not necessarily result from alloantigen exposure. It is widely accepted that cross-reactivity is an essential feature of the human T cell repertoire and that environmentally primed T cells may cross-react with donor MHC-peptide complexes, leading to rapid T cell activation and early graft injury in individuals who have not been immunized directly by alloantigens. We could demonstrate that the presence of high frequencies of donor-reactive memory T cells is independent of established risk factors for sensitization, such as high levels of panel-reactive antibody (PRA) testing (7).

For further improvement of individual therapy decisions, early identification of T cell–sensitized individuals before transplantation might be useful. Severe graft injury could be avoided in these individuals by using more appropriate immunosuppressive regimen targeting memory T cells.

On the basis of on the ELISpot technique for the detection of alloreactive memory T cells, we developed an assay for measurement of panel-reactive memory T cell (PRT) responsiveness by using a panel of stimulator cells that represent most HLA antigens encountered in the German population (8), analogous to PRA testing. Our data suggest that high levels of T cell sensitization are more frequent in dialysis patients compared with healthy individuals but are not routinely associated with high levels of PRA, previous transplants, blood transfusions, or gravidities, supporting the concept of heterogenicity. This assay might add significant new information for the assessment of the individual risk and selection of the appropriate immunosuppressive regimen for transplant patients.
Materials and Methods

Patients and Healthy Control Individuals

Forty-one adult hemodialysis (HD) patients who had ESRD (duration range 0.5 to 29 yr) and were under renal replacement therapy were included in this study (Table 1). All patients were on the waiting list of Charité University Medicine Berlin–Campus Virchow Clinic for first or second kidney transplantation at Eurotransplant. ESRD was caused by glomerular disease (n = 9), pyelonephritis (n = 8), polycystic disease (n = 7), congenital renal dysplasia (n = 2), tubular necrosis (n = 2), hypertension (n = 1), diabetes (n = 1), and other/unknown (n = 10). One patient received a liver transplant 8 yr before and was taking tacrolimus and steroid medication. No other patient was taking immunosuppressive drugs during the performance of our study. Moreover, one patient received a liver transplant 8 yr before and was taking tacrolimus and steroid medication. No other patient was taking immunosuppressive drugs during the performance of our study. Moreover, 10 healthy adult individuals were included as control subjects. The study was performed with the approval of the Ethics Commission at the Charité University Hospital, Berlin. All patients and healthy blood donors gave informed consent.

Allogeneic Cell Bank

An allogeneic cell bank (ACB) for stimulator cells was established from a panel of 17 healthy blood donors representing most frequent HLA antigens of the German population, analogous to PRA testing (Table 2). For calculation of the population coverage by HLA antigen selection, we used HLA-A, -B, and -DR gene frequencies determined on >13,000 German blood donors as published recently (8). Using minimal phenotype panels, a method developed for achieving maximum population coverage by HLA antigen selection, HLA-A and -B antigens were selected until >90% of the German population was covered by the ACB, meaning that >90% of the individuals in the population are expected to express at least one of the antigens in the minimal phenotype panels (9). From each of the 17 healthy blood donors,uffy coat of 500 ml of whole blood was obtained and T cell depleted using a CD3+/glycophorin A bispecific antibody (RosetteSep; StemCell Technologies Inc, Vancouver, BC, Canada). The CD3-negative fraction was stored in multiple aliquots in liquid nitrogen. All blood donors met the criteria for donor suitability required by the hemotherapy guidelines of the German Medical Association (10).

HLA Typing

HLA-A and -B antigens of patients and healthy blood donors were identified by standard lymphocytotoxicity testing and by PCR-SSP and/or PCR-SSO. HLA-DR typing was performed by PCR (11–13).

PRA Determination

All patients were on the waiting list for renal transplantation and were screened routinely for the presence of PRA every 3 mo by a complement-dependent cytotoxicity test according to the National Institutes of Health as described in detail elsewhere (14).

Responder Cells for IFN-γ ELISpot Assay

Peripheral blood samples of hemodialysis patients were T cell enriched by rosetting technologies (RosetteSep T cell Enrichment Cocktail) using tetrameric antibodies cross-linking unwanted CD16+, CD19+, CD36+, and CD56+ cells to glycophorin A on erythrocyte cell surface, which were removed along with the pellet after Ficoll preparation. Responder cells were co-incubated directly with freshly thawed stimulator cells of each of the 17 ACB donor individuals in a 96-well ELISpot plate.

ELISpot Assay

To investigate effector/memory T cell reactivity against stimulator cells of the 17 healthy blood donors, a modification of the ELISpot assay as described by Valujskikh et al. (15) was used. Briefly, 96-well plates (Millipore, Eschborn, Germany) were coated with primary IFN-γ antibody (Perbio Science, Bonn, Germany). Responder T cells and donor stimulator cells, respectively, were placed in the 96-well plates at a concentration of 1 × 10⁶/well. Wells that contained responder and stimulator cells plus medium alone, respectively, were used as negative controls. Next, the plates were incubated for 24 h at 37°C. After repeated washing, biotinylated secondary IFN-γ antibody (Perbio Science) was added for 4 h at room temperature. After an incubation with streptavidin–horseradish peroxidase for 90 min, the plates were developed using 3-amino-9-ethylcarbazol (Sigma-Aldrich, Munich, Germany). The resulting spots were counted using a computer-assisted ELISpot reader (BIO-SYS, Karben, Germany). Frequencies of IFN-γ-producing donor-reactive T cells were calculated by subtracting both the responder alone and the stimulator alone control wells from the wells that contained recipient plus stimulator cells.

Statistical Analyses

Mann-Whitney U test was used for comparison of T cell frequencies or other parameters between subgroups of patients. Spearman rank correlation was used to calculate bivariate correlations. Cross-table analysis was performed by χ² test. Data were analyzed using the statistical software SPSS (SPSS GmbH Software, München, Germany).

Results

Patient characteristics are depicted in Table 1. The 41 HD patients were selected to achieve a great diversity regarding presensitization status among the study population (Table 1). Ten of 41 patients showed previous PRA testing of ≥80%.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients</td>
<td>41</td>
</tr>
<tr>
<td>Age (yr ± SD, range)</td>
<td>47 ± 14.2 (18 to 71)</td>
</tr>
<tr>
<td>PRA recent 0 to 10/10 to 49/50 to 79/≥80% (n)</td>
<td>32/6/1/2</td>
</tr>
<tr>
<td>PRA historic 0 to 10/10 to 49/50 to 79/≥80% (n)</td>
<td>25/5/1/10</td>
</tr>
<tr>
<td>Time of ESRD (yr ± SD, range)</td>
<td>9.3 ± 7.6 (0.5 to 29)</td>
</tr>
<tr>
<td>No. of patients with previous kidney transplants (range)</td>
<td>18 (1 to 3)</td>
</tr>
<tr>
<td>No. of patients with blood transfusions (range)</td>
<td>22 (1 to 95)</td>
</tr>
<tr>
<td>No. of women (n = 17/41) with gravidities (range)</td>
<td>6 (1 to 6)</td>
</tr>
</tbody>
</table>

*PRA, panel-reactive antibody.
whereas two of them had recent PRA ≥80%. Eighteen of 41 patients had received at least one previous kidney transplant (range 1 to 3). Twenty-two of 41 patients received blood transfusions in the past (range 1 to 69), and six of 17 female patients had gravidities (range 1 to 6).

**PRT**

ELISpot frequencies of IFN-γ-producing alloreactive memory T cells of 10 healthy volunteers and 41 HD patients were determined using a panel of stimulator cells from 17 healthy blood donors of our ACB. On the basis of previous studies correlating heightened pretransplantation ELISpot donor-reactive memory T cells with early acute rejection, we arbitrarily defined positive assays as ≥50 spots/100,000 T cells and calculated PRT levels as percentage of positive responses to the 17 ACB blood donors (7).

Figure 1 summarizes the results of our experiments. We observed a wide range of responses against the allogeneic stimulators. Approximately half of the HD patients showed no signs of T cell sensitization, whereas 19 of 41 patients exhibited positive responses (PRT > 0%) against at least one of the ACB donors, approximately 15% even a very strong panel reactivity (PRT > 25%). The median PRT level among the 19 PRT+ patients was 17.7% (mean 20.4%). The peak PRT level was 58.8% in a patient who had a positive ELISpot response to 10 of 17 ACB stimulators.

Patients with an ELISpot reactivity of >100 spots/100,000 T cells against at least one ACB donor were considered as highly T cell sensitized against that particular donor. High T cell sensitization against at least one ACB donor was associated with broad HLA T cell priming, as individuals with any response >100 spots/100,000 T cells to ACB stimulators exhibited highly significant enhancement of PRT levels compared with patients with <100 spots/100,000 T cells against all ACB stimulators ($P < 0.001$). ELISpot frequencies of IFN-γ-producing T cells to ACB stimulators showed a slight but statistically significant correlation (Figure 2) with the cumulative mismatch of HLA A, B, and DR antigens ($r = 0.139$, $P < 0.001$, $n = 697$).

**HD Patients Have Significantly Higher PRT Compared with Healthy Adult Control Subjects**

First, we investigated whether the 41 patients with ESRD (HD) would show higher levels of T cell sensitization compared with healthy adult control subjects. Because of limited material, PRT assays in the healthy control subjects could be performed only in 12 of the 17 ACB stimulator individuals. However, HD patients showed significantly elevated median ($P = 0.002$) values of alloreactive memory T cell responses as well as higher PRT levels ($P = 0.033$) to ACB stimulator individuals compared with control subjects (Figures 4 and 5). In comparison with median values in healthy control subjects, 19 of 41 HD patients displayed higher PRT levels (controls 0%) and 40 of 41 HD patients displayed higher median ELISpot responses (controls 1.75 spots/100,000 T cells) to the 17 ACB stimulators.

**High T Cell Sensitization Is Not Routinely Associated with High PRA or History of Allosensitization**

One major aim of our study was to analyze the relation of PRT to established humoral PRA testing. It is interesting that in general, historic but not recent PRA slightly correlated with median ($r = 0.402$, $P = 0.009$) ELISpot responses of the 41 HD patients to the ACB stimulators, whereas neither recent or historic PRA correlated with PRT levels or peak ELISpot responses to ACB stimulators. Furthermore, comparison of PRT and PRA subgroups according to their level of sensitization (e.g., ≥0, ≥20%) did also not reveal statistical significance in $\chi^2$ cross-table analysis.

Figure 3 summarizes the distribution of PRT+ and PRA+ patients of our population. Next, we analyzed whether high cellular (PRT) and humoral (PRA) allosensitization might be closely related or occur independent of each other. Table 3 shows PRA levels and other parameters of the 10 highly T cell-sensitized HD patients of our study cohort (>100 spots/100,000 T cells against at least one ACB stimulator). Most important, five of 10 highly T cell-sensitized individuals had both negative recent and historic PRA and no history of classic allosensitization, clearly indicating that high cellular alloreactivity does not routinely imply humoral allosensitization. The other five of 10 patients with >100 spots/100,000 T cells against at least one healthy blood donor all had significant historic PRA of >40% and a history of allosensitization by transplants, transfusions, or gravidities. It is interesting that the patient with the highest ACB response in our study cohort of 529 spots/100,000 T cells was the liver transplant patient.
Complementary, only four of 10 patients with high levels of historic PRA testing (>80%) had >100 spots/100,000 T cells against at least one healthy blood donor in PRT testing, demonstrating again the independence of high PRA and high PRT responsiveness (Table 3).

Independent Association of PRA and PRT with the Number of Previous Transplants, Blood Transfusions, and Duration of ESRD

Next, we investigated whether PRT testing would correlate with well-established risk factors for allosensitization. Both
recent and historic PRA correlated significantly with number of previous kidney transplants \((n = 41; r = 0.529, P < 0.001/r = 0.690, P < 0.001),\) blood transfusions \((n = 40; r = 0.553, P < 0.001/r = 0.759, P < 0.001),\) duration of ESRD \((n = 41; r = 0.478, P = 0.002/r = 0.792, P < 0.001),\) cumulative duration of dialysis \((n = 41; r = 0.376, P = 0.015/r = 0.784, P < 0.001),\) and cumulative duration of previous transplantations \((n = 41; r = 0.463, P = 0.002/r = 0.634, P < 0.001).\) Both recent and historic PRA testing did not correlate with number of previous gravidities and patient age.

Regarding the parameters median ELISpot response against the ACB stimulators as well as PRT level, a significant correlation was found between median \((r = 0.391, P = 0.012)\) ELISpot responses and numbers of previous transplants. Moreover, me-
Median ELISpot responses were significantly higher in HD patients with previous transplantations compared with HD patients without previous transplantations (Figure 5). Furthermore, both HD patients with and without previous transplantations showed significantly heightened median \( P = 0.006 \) ELISpot alloreactive T cells compared with control subjects, whereas patients with former transplants also exhibited heightened PRT levels compared with control subjects \( P = 0.010 \); Figure 5).

Median ELISpot responses \( r = 0.363, P = 0.020 \) as well as PRT level \( r = 0.333, P = 0.034 \) correlated with duration of dialysis. Synthetic dialyzer membranes were used in most of the HD patients in our study. We found no difference in alloreactive T cell frequencies between HD patients on polysulfone high-flux versus polyamide low-flux membranes. No ELISpot parameter correlated with age, numbers of transfusions, or gravidities.

Other Demographic Parameters

It is interesting that female HD patients had significantly higher PRT levels \( P = 0.003 \) and median \( P = 0.003 \) ELISpot responses compared with male HD patients (Figure 6), whereas they showed no statistically significant difference regarding recent or historic PRA. In contrast, nullipara and multipara groups could not be distinguished by PRT frequencies. We found no difference in any of the PRT parameters regarding patients who were younger or older than 55 yr. Moreover, there was no difference between cytomegalovirus IgG-positive and cytomegalovirus IgG-negative individuals.

**Figure 6.** Female HD patients have significantly higher levels of PRT compared with male HD patients, irrespective of previous gravidities.
Discussion

Several pretransplantation tests are used routinely to improve short- and long-term allograft survival, such as HLA typing, ABO matching, humoral cross-match, and PRA screening. Assessment of donor-specific and panel-reactive humoral sensitization helps to prevent hyperacute rejection and to identify patients who are at enhanced rejection risk and require higher immunosuppression, respectively.

According to the principle of PRA screening, we established a test system for measuring cellular sensitization of patients who are on the transplant waiting list, called PRT assay. As previous studies have shown that highly elevated pretransplantation donor-reactive IFN-γ–ELISpot frequencies were associated with severe early acute rejection episodes, we focused on the direct pathway of allorecognition, which was suggested to dominate the early phase after transplantation, whereas the indirect pathway of alloreactivity has been associated with later forms of allosponses, chronic allograft nephropathy, and antibody production.

Our definition of a positive PRT assay's being >50 spots/100,000 T cells is yet arbitrary and should be reevaluated in future studies that analyze the clinical relevance of the PRT assay for transplant outcome.

The alloreactive IFN-γ–ELISpot frequencies measured in this study are slightly lower compared with previously published pretransplantation donor-reactive IFN-γ–ELISpot frequencies in kidney transplant recipients, which were generally in the range of <10 to 1000 spots/300,000 peripheral blood mononuclear cells (6,7). This can be explained by different responder and stimulator cell preparation. We now use a standardized number of 100,000 enriched T cells/well (95% purity) instead of 300,000 peripheral blood mononuclear cells/well. Furthermore, previous studies used unseparated donor cells (containing also donor T cells) as stimulators, which have been shown to be capable of IFN-γ production despite irradiation or treatment with mitomycin C. Another difference to North American studies might be that they included black patients who are more sensitized (Heeger et al., Cleveland Clinic Foundation; personal communication, April 2005).

Whereas only few healthy donors expressed alloreactive T memory responses, HD patients showed enhanced cellular alloreactivity. It is widely known that uremic toxins, dialytic devices, and water contamination in chronic HD exhibit complex immunologic effects such as chronic inflammation, complement activation, and upregulation of adhesion molecules on leukocytes. Dialysis patients may also experience chronic infections (16). Moreover, a predominance of Th1 CD4+ T cells has been reported in dialysis patients, probably related to an overproduction of monocyte-derived IL-12 (17). Thus, repeated antigenic stimulation and bystander activation by chronic inflammation might explain heightened frequencies of cross-reactive alloreactive T cells in HD patients without a history of allosensitization. We found, however, no differences between patients using distinct dialysis membranes.

In patients with a history of allosensitization, we found further enhanced T cell alloreactivity. Most important, we could demonstrate that approximately 50% of highly PRT+ patients exhibit negative recent and historic PRA and have no history of allosensitization, supporting the concept of heterogeneity and cross-reactivity for the generation of alloreactive memory T cells. Hence, PRT testing allows the identification of PRA negative but clearly memory T cell–alloreactive patients irrespective of known risk factors for allosensitization, thereby providing important new informations for the assessment of the pretransplantation immune status in transplantation candidates. Further clinical studies that analyze the relevance of cellular allosensitization in PRT screening for posttransplantation outcome are now warranted. In accordance with our results, a significant number of transplant patients experience early acute rejection and poor 1-yr graft function despite negative testing for humoral sensitization in PRA and final cross-match testing (7). These early acute rejections might be related to preformed alloreactive memory T cells, as we and others recently showed that heightened frequencies of pretransplantation donor-reactive memory T cells predict acute rejection in kidney transplant recipients irrespective of humoral reactivity (6,7).

High levels of humoral sensitization (PRA+ +) increase the probability of positive pre/posttransplantation cross-match associated with poor outcome, but also in the absence of donor-reactive alloantibodies, PRA++ patients have a poorer prognosis compared with PRA recipients, suggesting that broad humoral sensitization might be a marker of “high immune reactivity.” Similarly, high levels of pretransplantation PRT levels may result in higher probability of donor-reactive memory T cells associated with poorer outcome (6,7). However, it also might be possible that broad T cell sensitization simply indicates general cellular hyperreactivity. Further studies have to address this issue.

Conclusions

We have established an easily applicable technique for assessing pretransplantation T cell allosensitization status to a broad panel of allogeneic stimulators in patients who are on the waiting list for transplantation. Future studies that analyze the predictive value of PRT testing for posttransplantation outcome are required. Considering the effects of preformed donor-reactive T cells on rejection frequencies in transplant patients irrespective of PRA and cross-match testing, it is expected that PRT testing will provide useful additional information for identifying transplant candidates who are at high immunologic risk to improve individualized immunotherapy.

Pretransplantation ELISpot analyses by our HLA-typed ACB identified PRA-negative but T cell–presensitized HD patients before transplantation. We suggest introducing PRT testing and memory T cell cross-match for an early identification of presensitized transplant patients.

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