Leukocyte phenotype and function predicts infection risk in renal transplant recipients

Martin Blazik1, Paul Hutchinson2, Matthew D. Jose1, Kevan R. Polkinghorne1, Stephen R. Holdsworth1,2, Robert C. Atkins1 and Steven J. Chadban1

1Department of Nephrology, Monash Medical Centre and 2Department of Medicine, Monash University, Clayton, Victoria, Australia

Abstract

Background. The degree to which transplant recipients are immunosuppressed influences their risks of rejection, infection and cancer. Current measures of immune suppression are crude (clinical events) or indirect (drug exposure). We assessed a direct measure of immune status, leukocyte phenotype and function (LPF, a composite measure of five aspects of peripheral blood leukocyte phenotype and function), as a predictor of infection.

Methods. A double-blind, prospective, cohort study was conducted, to determine the burden of infection in stable renal transplant recipients with moderate-severe (Group I, n = 34) or minimal (Group II, n = 36) impairment of LPF, a composite score of: (i) CD4 count; (ii) lymphocyte proliferation in response to phytohaemagglutinin A (PHA); (iii) serum Ig concentrations; (iv) neutrophil phagocytic function; and (v) reactive oxygen species generation. Subjects completed a 6 month diary and each recorded infection was scored 1–4: 1, minor undefined infection (e.g. URTI); 2, minor, microbiologically defined infection (e.g. UTI); 3, major defined infection (requiring hospitalization); 4, opportunistic infection (e.g. Herpes zoster). Final infection score was the sum of all infective episodes. Subjects were then followed-up for 5 years for outcome measures.

Results. Groups were well matched for age, sex, diabetes, serum creatinine, rejection and trough cyclosporin concentrations. Group I (moderate to severe impairment of LPF) recorded a higher infection score, 2.4±2.8 vs 1.2±1.2 for Group II, P = 0.02, due to a higher incidence of moderate to severe infection. This relationship was confirmed by multivariate analysis (OR 1.83, CI 1.08, 3.11, P = 0.03 per unit increase in infection score). During the 5 year follow-up period they had significantly more episodes of admission to hospital, and twice as many admissions due to infections, but no difference in malignancy, graft or patient outcome.

Conclusion. LPF testing prospectively identified a cohort who incurred a higher burden of infection. Further studies are required to determine the predictive value of LPF for acute rejection, infection and cancer, and to determine whether adjustments to therapy on the basis of LPF can lead to improved outcomes.

Keywords: immunosuppression; infection; kidney transplant; leukocyte; monitoring

Introduction

The aim of immunosuppressive therapy post-transplant is to achieve freedom from rejection without toxicity. The risk of inadequate immune suppression resulting in rejection must be balanced against the risks of excess immune suppression with consequences including infection and cancer [1,2]. The therapeutic window for immunosuppressive medications is frequently narrow [3–5].

Immunosuppressive medication is currently prescribed on an empirical basis. Drug dosages are uniform for agents such as mycophenolate and steroids [3], calculated according to body weight for others or concentration controlled in the case of cyclosporin [4] and tacrolimus [5]. We are currently unable to accurately titrate dosage to the effect the drug has on an individual’s immune function.

Immune status and its response to immunosuppressive medication exhibits significant inter- and intra-subject variability [3]. Thus, empirical prescribing of immunosuppressive medication provides a range of responses across a patient population, with some
patients achieving the desired therapeutic outcome while others incur the clinical consequences of inadequate or excess immune suppression [1–3].

Direct measures of immune function have been employed in the intensive care setting [6] and have been described in transplantation [7]; however, the evidence to support their use is relatively weak [7–11]. We examined the ability of a direct test of immune status—a composite score of five aspects of leukocyte phenotype and function (LPF)—to identify renal transplant recipients with excess immune suppression and consequent increased risk of infection.

Subjects and methods

Leukocyte phenotype and function testing

LPF testing was performed on freshly collected peripheral blood of kidney transplant recipients. All recipients were >6 months post-transplant with stable graft function. Five aspects of leukocyte phenotype and function were assessed as previously described [7]. In brief, assessments were performed as follows.

CD4 count. CD4 count was assessed by flow cytometry. Briefly, 100 µl aliquots of heparinized blood was labelled with combinations of fluorochrome conjugated monoclonal antibodies. Antibodies used included CD3-Cyanine 5 (in-house reagent), CD4-Phycocerythrin (Cymbus, UK) and CD8-Alexa 488 (in-house reagent). Following incubation the red blood cells were lysed using the Beckman-Coulter Q-Prep system (Beckman-Coulter, Hialeah, FL) and run on a Mo-Flo flow cytometer (Cytomation, Ft Collins, CO). The percentage of lymphocytes positive for CD3 and CD4 was measured and from the lymphocyte count the CD4 count was calculated.

Lymphocyte proliferative response to the mitogen phytohaemagglutinin A (PHA). Lymphocyte proliferative response to PHA (Wellcome Diagnostics) was performed using a diluted whole blood assay: heparinized whole blood was diluted 1/10 in RPMI 1640 tissue culture media (Gibco). Quadruplicate wells of 100 µl aliquots were set up in U-bottomed 96-well plates with a range of doses of PHA from 0–5 µg/ml added. The cells were then incubated at 37°C and 5% CO₂ for 72 h. Over the last 4 h 0.5 µCi of ³H-Thymidine was added to each well. Cells were harvested onto glass fibre paper discs using the titertek harvester (Cambridge Technology, MA). The paper discs were left to dry overnight and then scintillation fluid was added. The incorporated ³H-Thymidine counts were measured on a beta counter (LKB-Wallac beta counter, Wallac Oy, Turku, Finland). The counts per minute obtained for each quadruplicate dose were averaged and ratioed over the starting lymphocyte count and expressed as CPM/1000 lymphocytes. The peak value was reported as the lymphocyte mitogen response value.

Neutrophil oxidative burst and phagocytic function. The simultaneous assessment of neutrophil oxidative burst and phagocytic function was done using a whole blood assay that combined two previously published techniques [12,13]. Briefly, dead Staphylococcus aureus (Pansorbin, Calbiochem) was incubated with propidium iodide (Sigma Chemicals) at 5% w/v and 50 µg/ml, respectively, for 30 min at room temperature. It was then washed twice in Hanks balanced salt solution (HBSS) and resuspended in HBSS at 5% w/v. This was then added to duplicate aliquots of undiluted heparinized whole blood at a final concentration of 0.5% w/v. These were incubated at 37°C for 20 min at which time the reactive oxygen species (ROS) fluorescent indicator dihydrorhodamine 123 (Molecular Probes, Eugene, OR) was added at a final concentration of 200 ng/ml. The sample was incubated for a further 10 min at 37°C and then a 100 µl sample from each aliquot had its red cells lysed using the Q-prep system and the neutrophil green fluorescence (dihydrorhodamine 123) and red fluorescence (propidium iodide–S.aureus) measured on the Mo-Flo flow cytometer. Neutrophils were gated by their forward and 90° light scatter characteristics and the mean intensity of both the red and green fluorescence measured for each duplicate. The averaged values were used as the ROS and phagocytosis index.

Immunoglobulin sub-type. Immunoglobulin sub-type (IgA, IgG, IgM) concentrations in serum were measured by Southern Cross Pathology (Dade Behring Dimension Clinical Chemistry System).

LPF score

For each transplant recipient, a score was given for each of the five parameters measured: if function was beneath the 10th centile of a normal population (30 healthy volunteers from the laboratory, age 20–45 years), a score of 1 was recorded vs a score of zero if above the 10th centile. The score for all parameters assessed was then averaged to give a LPF score from 0 to 1 such that a total score of 0 indicated all parameters above the 10th centile of normal LPF, whereas a total score of 1 indicated global impairment of LPF. The stability and reproducibility of LPF score over time has been demonstrated in clinically stable, healthy volunteers [7].

Subjects

All transplant recipients at Monash Medical Centre who were subjected to LPF during the 12 months prior to the study were assessed. To examine major differences in immune function, two separate cohorts were identified on the basis of LPF results and invited to participate in a prospective study: Group 1 (three or more parameters <10th centile of normal indicating poor leukocyte function, LPF score 0.5–1.0, n = 36) and Group 2 (most parameters within normal limits indicating preserved leukocyte function, LPF score 0–0.25, n = 37). The demographics of both cohorts are presented in Table 1. Transplant recipients with LPF score between 0.25 and 0.5 were not included in order to maximize the differences between the chosen groups. The LPF score across the entire cohort has been previously published [7]. As we have not found one individual parameter to be more predictive of poor immune state, a combination of parameters is used.

All subjects were asked to complete a written, 6 month infection diary from July 1 to December 31, 1999, including daily entries to record any symptoms of infection, medical attendance for investigation or treatment of infection.
Hospital inpatient and outpatient records for all subjects were reviewed to confirm any episodes of infection requiring medical attention. Diary completion was supervised by a single investigator who contacted subjects at least monthly. Subjects, the diary supervisor and all caring physicians were blinded to LPF results and patient management was not influenced by the results of LPF testing. All subjects were then prospectively followed to allow 5 year follow-up on patient and graft outcomes.

**Infection score**

Upon completion of the 6 month infection diary, the diaries and patient records were examined and a score (1–4) was assigned to each episode of infection by two investigators, blinded to subject grouping, according to the following criteria: minor, undefined infection (e.g. viral upper respiratory infection); minor, microbiologically defined infection (e.g. culture positive urinary tract infection); major, defined infection requiring hospitalization (e.g. pneumonia); major opportunistic infection (e.g. Herpes zoster). An overall infection score was generated for each subject as the sum of scores for every episode of infection. The infection score was the primary outcome measure of the study.

**Haematology and biochemistry**

Whole blood white cell counts (Cell Dyne 3500 automated cell counter), whole blood cyclosporin trough concentrations (TDx assay, Abbott Laboratories) and serum creatinine (Dade Behring Dimension Clinical Chemistry System) were measured at Southern Cross Pathology, Monash Medical Centre.

**Statistical analysis**

Data are presented as mean± standard deviation. Data obtained from the two groups were compared using the unpaired t-test (continuous data) or Mann–Whitney U-test (categorical data). Logistic regression was used to assess the relationship between increasing infection score and group allocation while controlling for variables known to be associated with an increased risk of infection. Factors in the multivariable model included infection score, immunosuppressant regimen (azathioprine/mycophenolate, calcineurin inhibitor, previous OKT3 use, and prednisolone), time post-transplant and gender. All analyses were conducted using Graphpad InStat (Version 3) software (Graphpad Inc., CA, USA) and Stata version 8.2 (Stata Corporation, College Station, TX, USA). Statistical significance was set at P-value <0.05 for all analyses.

**Results**

Thirty-four subjects from Group I (94%) and 36 from Group II (97%) completed the infection diary between July and December 1999 and were included in the study. The remaining subjects were excluded due to non-compliance with the diary (n=2) or relocation overseas (n=1). The demographics of each group were similar (Table 1).

Immunosuppression was cyclosporin based for 97% of subjects in Group I and 89% in Group II and cyclosporin exposure, as measured by trough concentration at the time of LPF testing, was equivalent between the groups. Additional immunosuppressants included prednisolone, azathioprine or mycophenolate (Table 2). Group I subjects received on average a higher number of immunosuppressive drugs (2.6±0.6 vs 2.2±0.5, P=0.002), with 19 subjects (56%) from Group I receiving triple therapy vs eight (22%) from Group II. The effect of double versus triple therapy on infection score was examined across the entire cohort and the average infection score for those receiving double therapy vs triple was not significantly different (infection score 1.7±1.7 vs 2.1±2.9, P=0.78).

As there were differences in the two groups with regard to the time post-transplant and number of immunosuppressive drugs prescribed, logistic regression was used to assess the relationship between the infection score and group allocation. On univariate analysis, subjects in Group I were 37% more likely to have a higher infection score compared to Group II (OR 1.37, 95% CI 1.02, 1.83, P=0.03). After adjustment for group differences in immunosuppressive regimen, gender and duration post transplantation, the association not only remained statistically significant but also increased in strength (OR 1.83, 95% CI 1.08, 3.11, P=0.03), demonstrating that infection score was higher with poor leucocyte phenotype and function.

<table>
<thead>
<tr>
<th>Table 1. Subject demographics at enrolment</th>
</tr>
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<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>Sex (male/female)</td>
</tr>
<tr>
<td>Age (mean± SD)</td>
</tr>
<tr>
<td>Number of diabetics</td>
</tr>
<tr>
<td>Years post-transplant</td>
</tr>
<tr>
<td>Number of immunosuppressants</td>
</tr>
<tr>
<td>Acute rejection episodes</td>
</tr>
<tr>
<td>Cyclosporin trough (ng/ml)</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
</tr>
<tr>
<td>Peripheral blood WCC</td>
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</tbody>
</table>

NS, not significant.
Leukocyte phenotype and function

**Table 2.** Immunosuppressant medication

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Group I (n = 34)</th>
<th>Group II (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin monotherapy</td>
<td>1 (2.9%)</td>
<td>2 (5.4%)</td>
</tr>
<tr>
<td>Azathioprine, prednisone</td>
<td>1 (2.9%)</td>
<td>4 (10.8%)</td>
</tr>
<tr>
<td>Cyclosporin, azathioprine</td>
<td>7 (20.6%)</td>
<td>17 (48.7%)</td>
</tr>
<tr>
<td>Cyclosporin, MMF</td>
<td>2 (5.9%)</td>
<td>5 (15.3%)</td>
</tr>
<tr>
<td>Cyclosporin, prednisolone</td>
<td>4 (11.8%)</td>
<td>0</td>
</tr>
<tr>
<td>Cyclosporin, azathioprine, prednisone</td>
<td>9 (26.5%)</td>
<td>7 (18.9%)</td>
</tr>
<tr>
<td>Cyclosporin, MMF, prednisolone</td>
<td>10 (29.4%)</td>
<td>0</td>
</tr>
<tr>
<td>Tacrolimus, MMF, prednisolone</td>
<td>0</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td>OKT3 exposure</td>
<td>9 (26%)</td>
<td>6 (16%)</td>
</tr>
</tbody>
</table>

**Table 3.** Incidence of infection

<table>
<thead>
<tr>
<th>Infection</th>
<th>Group I (n = 34)</th>
<th>Group II (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number with any infection</td>
<td>23 (68.8%)</td>
<td>22 (60%)</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>16 (47.1%)</td>
<td>18 (48.6%)</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>12 (35.3%)</td>
<td>6 (16.2%)</td>
</tr>
<tr>
<td>Suppurative ENT infection</td>
<td>5 (14.7%)</td>
<td>2 (5.4%)</td>
</tr>
<tr>
<td>Gastroenteritis—bacterial</td>
<td>3 (8.8%)</td>
<td>0</td>
</tr>
<tr>
<td>Cellulitis/osteomyelitis</td>
<td>4 (11.8%)</td>
<td>3 (8.1%)</td>
</tr>
<tr>
<td>Herpes zoster/stomatitis</td>
<td>2 (5.9%)</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>3 (8.8%)</td>
<td>0</td>
</tr>
<tr>
<td>Infection score</td>
<td>2.4 ± 2.8</td>
<td>1.2 ± 1.2 (P = 0.02)</td>
</tr>
</tbody>
</table>

The majority of subjects in each group recorded at least one episode of infection (Table 3). This was largely due to an equal incidence of minor upper respiratory tract infections in both groups. In contrast, the incidence of more serious infections was greater for Group I (29 vs 11 episodes for Group I vs Group II, Table 3) and this was reflected in an average infection score that was 2-fold higher than in Group II (2.4 ± 2.8 vs 1.2 ± 1.2, P = 0.02). A major opportunistic infection only occurred in two patients within Group I within this 6 month time-period (Table 3).

Follow-up 5 years after enrolment (July 2004) demonstrated an increase in the total number of admissions to hospital for Group I recipients and a non-significant trend towards increased hospitalizations for infections (Table 4), but no difference in malignancy, graft or patient outcome.

**Discussion**

Leukocyte phenotype and function testing prospectively identified a group of renal transplant recipients who incurred a higher burden of infection. This prospective, double-blind cohort study has demonstrated the ability of a direct measure of immune status to identify patients who are excessively immunosuppressed and thereby placed at an increased risk of infection. This is the first study to demonstrate that a direct measure of immune suppression can predict clinical outcomes in renal transplant recipients.

Immunosuppressive therapy, in the context of transplantation, inflammatory conditions and malignant disease, has long been known to incur an increased risk of infective illness [1,3]. An increased incidence of both typical and opportunistic infections, as well as malignancies, is reported and the degree of risk is proportional to the intensity of immunosuppressive therapy [1,2,14,15]. However, not all immunosuppressed patients develop infection and while the dose and type of immunosuppressants administered are clearly critical in predisposing patients to infection, they are not the sole determinants. Factors including age, co-morbid conditions and biological variation in immune function are also crucial in determining susceptibility to infection [1,14]. Such factors are frequently difficult to assess in current clinical practice but may be quantifiable via direct measures of immune function, such as LPF testing.

The primary outcome measure of this study was the significantly increased number and severity of infections in a group of transplant recipients displaying a poor LPF score. The mean infection score for Group I was double that of Group II, indicating a significantly higher burden of infection. Although the incidence of minor infections, typically viral upper respiratory tract infections, was equal between the two groups, severe infections were more prevalent for Group I, consistent with their inferior leukocyte parameters and consistent with previous observations that more intense immune suppression results in an increased incidence of severe infections [1,15]. These prospective data confirm our previous observations that subjects with abnormal LPF appear to incur an excess of infection [7].

Several groups have reported alterations in numbers and/or function of peripheral blood leukocytes obtained from patients acquiring infection following organ transplantation. Depressed lymphocyte (CD3+, CD4+ and B-cells) and monocyte/macrophage counts have been reported to precede infective episodes following bone marrow [8] and solid organ [9] transplantation and diminished polymorph migratory capacity (ex vivo) has been found to identify patients at an increased risk of sepsis [10]. Conversely, elevated serum concentrations of interleukin-10 (IL-10) and
soluble intracellular adhesion molecule-1 (sICAM-1) and an elevation in the CD4:CD8 ratio have been reported to precede rejection episodes [7,9]. Rejection has also been associated with elevated intracellular IL-2 expression by T-cells in one study [7], but not in a second study that found reduced IL-2 and IL-10 mRNA expression by T-cells from patients experiencing rejection [11]. Thus, the existing studies have provided inconsistent results and have also been weakened by a general lack of information on possible confounders (immunosuppressant medication, diabetic status, age), univariate analysis and retrospective trial design. The present study provides the best evidence to date that a composite measurement of leukocyte phenotype and function can identify patients at increased risk of infection whilst receiving maintenance immunosuppression after renal transplantation.

Several limitations in the present study should be considered. At enrolment, Group I subjects were at an earlier stage post-transplant and were more frequently receiving triple rather than double immunosuppressive therapy. However, this difference was not present throughout the follow-up period with longer follow-up time. In addition we have previously reported that LPF scores are not significantly affected by duration post-transplant or by the number of immunosuppressive medications taken at the time of testing [7]. The pattern and frequency of infection is known to vary with duration post-transplant [1,14]; however, as the current study examined subjects during the maintenance phase, the fact that Group I subjects were initially a mean of 3.2 years post-transplant vs 6.0 years for Group II is unlikely to have significantly affected the outcome. Similarly, as the infection score was not different between those on double vs triple therapy when considering the entire cohort, the fact that more subjects in Group I than in Group II were receiving triple therapy is unlikely to have confounded the results. To confirm this, multivariate analysis demonstrated that even accounting for these differences, infection score was strongly related to poor LPF scores.

The work presented provides preliminary evidence that direct measures of LPF may have a role in optimizing clinical outcomes in transplantation. In the current study, the measures of LPF were predictive of infective episodes. Whether alterations in immunosuppressive drug therapy in response to measures of LPF can reduce infections remains to be demonstrated.

The focus of the present study was infection; however, cancer and rejection are equally important consequences of excess and insufficient immune suppression, respectively. The predictive value of LPF testing for these additional outcomes is of interest but will require subsequent studies. Ultimately, a clinical role for LPF testing will only be justified if changes in drug therapy, made as a result of the test, are shown to produce improved outcomes. A randomized, controlled trial will be required.

In summary, the present study has shown that LPF, as a direct measure of immune status, was able to identify a cohort of renal transplant recipients who subsequently incurred a high burden of infection. Direct measures of immune status, such as LPF, may provide a means to improve tailoring of immunosuppressive therapy and thereby improve outcomes for transplant recipients.

Conflict of interest statement. None declared.

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8. Storek J, Espino G, Dawson MA, Storer B, Flowers ME, Maloney DG. Low B-cell and monocyte counts on day 80 are associated with high infection rates between days 100 and 365 after allogeneic marrow transplantation. Blood 2000; 96: 3290–3293

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