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*Original Article*

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# Laboratory assessment of immune function in renal transplant patients

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## Abstract

**Background.** Advances in immunosuppression have made renal transplantation an effective therapy for end stage renal failure; with low rejection rates and long graft survival times. However, the major adverse consequences, infection and malignancy have not diminished. To predict this risk a score of immune competence has been developed from the simultaneous laboratory assessment of multiple parameters of immune function.

**Methods.** The immune status of 152 transplant recipients (138 renal and 14 pancreas/renal) was assessed by measurement of lymphocyte subsets, mitogen-induced T-cell proliferative responses, neutrophil phagocytic capacity and reactive oxygen species (ROS) generation. A scoring system was devised based on the average number of these parameters below 10th percentile of normal.

**Results.** The most common abnormality was B-cell lymphopenia (85%) followed by reduced neutrophil ROS production (63% of patients), NK cell lymphopenia (50%), lymphocyte mitogen response (49%) and CD4 number (23%). The abnormalities were unrelated to the duration of immunosuppression (up to 15 years), and variable combinations of cyclosporine A, azathioprine, prednisolone and mycophenolate mofetil (MMF) (except for a consistent reduction in lymphocyte mitogen response in MMF treated patients). Retrospective comparison of infective episodes showed a significantly greater index of infections in patients with the worst score compared with a normal score.

**Conclusions.** The data suggests that this quantification of immune function may allow assessment of the level of host immune defence reflecting the level of drug-induced immunosuppression and thus risks of immunosuppressive complications.

**Keywords:** immunological monitoring; immunosuppression; renal move

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## Introduction

Despite advances in renal allograft survival with current immunosuppressive therapies including cyclosporine A and mycophenolate mofetil (MMF) the risks of major immunosuppressive complications such as malignancy and infection persist [1,2]. Longer graft survival involves greater exposure to immunosuppression and potential increase in these risks. A reproducible laboratory assessment of the extent of immunosuppression that predicted the risks would allow for optimal prevention and minimization of immunosuppressive therapy.

The effect of immunosuppressive drugs on transplant patients has been shown to have inhibitory effects on both the humoral [3] and cellular [4,5] arms of the immune system. It has also been shown that there are some correlations between the immune defect measured and the incidence of immunosuppressive complications [6,7]. However, it has been found difficult to quantify the level of immunosuppression on an individual basis [8]. This may be due to the fact that in most studies only one parameter of immune function was assessed.

The current study attempted to quantify the extent of immunosuppression by the simultaneous evaluation of several laboratory measures of immune function. These included assessment of neutrophil phagocytosis and reactive oxygen species (ROS) generation, enumeration of lymphocyte subsets, T-cell mitogen responses and levels of circulating immunoglobulins. From these tests a derived score of immune competence was developed. Patients with the worst score had increased incidence and severity of infection.

## Subjects and methods

### *Patient and normal groups*

A cross-sectional analysis of immune function in 138 adult renal transplant recipients and 14 combined renal and pancreas transplant recipients was performed. Inclusion criteria were: $>12$ months transplant time; serum creatinine $<250$ μmol/l (mean $\pm$ SD, $138 \pm 43$ μmol/l); no concomitant

clinical condition or therapy (other than routine immunosuppression) known to affect immune function. Normals ($n=82$) consisted of laboratory staff and others, who were under no medication and had no medical problems at the time of assessment.

Total white cell counts (WCC) were obtained from full blood evaluations performed on the Abbott Diagnostics Cell-Dyn cell counter (Abbott Park, IL) by Monash Medical Centres pathology provider Southern Cross Pathology. Immunoglobulin subclass levels, serum creatinine and blood cyclosporine trough concentration were also performed at Southern Cross Pathology using the Dade Dimension RXL autoanalyser (Dade Behring, Deerfield, IL).

### *Immunosuppressive protocols*

Over the 5 years of data acquisition several combinations of standard immunosuppressive agents were employed including azathioprine 1–2 mg/kg/day, MMF 1–2 g/day (both adjusted to maintain WCC $>4500/\mu l$), cyclosporine A (maintenance dose 3–6 mg/kg/day), and prednisolone initially 20 mg/day until 3 months then tapered and withdrawn over 7 months or continued as maintenance therapy in selected patients (5–10 mg/day). Six different maintenance drug regimes were employed; prednisolone plus azathioprine, cyclosporine A plus azathioprine, cyclosporine A, azathioprine plus prednisolone, cyclosporine A plus MMF, cyclosporine plus MMF plus prednisolone and cyclosporine A plus prednisolone (for patient numbers in each group see Table 2). Rejection episodes were treated with pulse i.v. methyl prednisolone 0.5–1 g/day for 3 days and refractory rejection with OKT3 ($n=18$) for 7–10 days.

### *Lymphocyte subset analysis*

To measure lymphocyte subsets 100 µl aliquots of lithium heparin whole blood were labelled with combinations of the following fluorochrome (FITC, PE and Cy-5) conjugated antibodies: CD16, CD20, (Dako, Denmark); CD4, CD19, CD56 (Exalpha, Boston, MA); CD3, CD8, CD14, CD45 (in house preparations). The red blood cells were then lysed and the sample fixed using the Beckman-Coulter Q-prep machine (Hialeah, FL). The labelled cells were then analysed using the Cytomation Mo-Flo flow cytometer (Ft Collins, CO).

### *Neutrophil function assessment*

The simultaneous assessment of neutrophil oxidative burst and phagocytic function was performed using a whole blood assay that combined two previous published techniques [9,10]. Briefly, fixed *Staphylococcus aureus* (Calbiochem, San Diego, CA) was incubated with Propidium Iodide (PI; Sigma Chemicals, Australia) at 5% wt/vol and 50 µg/ml, respectively, for 30 min at room temperature. It was then washed twice in Hanks balanced salt solution and resuspended at 5% wt/vol. This was then added to duplicate aliquots of undiluted lithium heparin blood at a final concentration of 0.5% w/v. These were incubated at 37°C for 20 min at which time the ROS fluorescent indicator dihydrorhodamine 123 (Molecular Probes, Eugene, OR) was added at a final concentration of 200 ng/ml. The sample was incubated a further 10 min
at 37°C and then a 100 µl sample from each aliquot was Q-preped and the neutrophil green fluorescence (DHR123) and red fluorescence (PI-*S.aureus*) measured on the Mo-Flo flow cytometer. The mean intensity (MCF) of both the red and green fluorescence was measured on the neutrophils gated by their forward and 90° light scatter characteristics. The averaged values of the duplicate aliquots were then used as the ROS and phagocytosis index.

### *Lymphocyte proliferative function*

Lymphocyte proliferative response to the mitogen phytohaemagglutinin A (PHA) (Wellcome, UK) was performed using a diluted whole blood assay [11]. In this assay lithium heparin blood was diluted to a final 1/10 concentration in tissue culture media [RPMI 1640 (ICN Biomedicals, Costa Mesa, CA) supplemented with 50 U/ml penicillin (ICN Biomedicals) and 50 µg/ml streptomycin (ICN Biomedicals)]. Quadruplicate wells of 200 µl aliquots were set up in U-bottomed 96-well plates (Disposable Products, Australia)

**Table 1.** Leukocyte and lymphocyte subset numbers in the normal and transplant patient groups

| | WCC | Lymph-CC | CD3 | CD3+CD4+ | CD3+CD8+ | CD3−CD16+ | CD3−CD56+ | CD19+ |
|---|---|---|---|---|---|---|---|---|
| Normals | 6464±1752 | 2109±713 | 1564±568 | 964±344 | 526±268 | 253±161 | 234±154 | 278±133 |
| Transplant patients | 6779±2030 | 1778±704[b] | 1523±643 | 817±353[b] | 635±422[a] | 153±211[b] | 132±194[b] | 82±69[b] |

Mean±SD given. [a]$P<0.05$, [b]$P<0.01$.

**Table 2.** Immune function score for different drug regimens used

| Immune function score | Pred+Aza ($n=15$) | CsA+Aza ($n=64$) | CsA+Aza+Pred ($n=24$) | CsA+MMF ($n=19$) | CsA+MMF+Pred ($n=25$) | CsA+Pred ($n=5$) |
|---|---|---|---|---|---|---|
| 0.00 | 2 | 14 | 4 | 0 | 0 | 0 |
| 0.25–0.49 | 7 | 32 | 12 | 4 | 10 | 1 |
| 0.50–0.74 | 6 | 17 | 8 | 13 | 11 | 4 |
| 0.75–1.0 | 0 | 1 | 0 | 2 | 4 | 0 |
| Average±SD | 0.32±0.16 | 0.27±0.19 | 0.29±0.17 | 0.46±0.14 | 0.45±0.20 | 0.47±0.07 |

with a range of nine doses of PHA from 0 to 5 μg/ml final concentrations. The cells were then incubated at 37°C and 5% $CO_2$ for 72 h.

Proliferation was measured by addition of 0.5 μCi of $^3$H-thymidine (Amersham International, UK) per well over the last 4 h of culture. The cells were harvested onto glass fibre disks (Cambridge Technology, MA) using a PHD multi-well cell harvester (Cambridge Technology). The disks were left to dry over night before the addition of scintillation fluid (Optiscint Hisafe, Wallac, Finland). Incorporated $^3$H-thymidine was assessed using a LKB Wallac beta-counter (Wallac). The counts per minute obtained for each quadruplicate dose was averaged and ratios over the starting lymphocyte count and expressed as CPM/1000 lymphocytes. The peak value was reported as the lymphocyte mitogen response value.

### *Defining immune function score and infection score*

Results of the analysis of CD4 counts, phagocytic capacity and ROS generation by neutrophils, and T-cell mitogen induced proliferative responses were used to assign an immune competence score. Individual patients were assigned one point for each parameter below the 10th percentile of normal and the average was the immune competence score. The variability of these measurements was assessed by comparing results of two separate tests performed for 17 normal donors over a period ranging from 2 to 12 months. No significant differences were found between the results obtained at these different time points. The average difference among measurements made on the same donor between time points was $<10\%$.

The burden of infection for each subject was quantified by applying a score to each recorded infection episode: 2, infection leading to admission; 1, infection requiring antibiotics without admission and 0.5, infection requiring neither hospital admission or antibiotics. The final score for each patient was the sum of each episode over the life of the transplant.

### *Statistics*

Student's *t*-test was used to determine significant differences between groups. Significance was defined as $P<0.05$. Linear correlation coefficients and their significance was determined using Graph Pad Prism software.

## Results

### *Leukocyte phenotypes*

Total WCC of the transplant patients were not different from normal, but total lymphocyte numbers were significantly lower than normal (Table 1). B cells (CD19 positive) were significantly lower than normal, with 85% of patients having numbers below the normal 10th percentile. This was not due to downregulation of CD19 as another pan B-cell marker CD20 gave a similar result [patients $4\pm3\%$ (mean $\pm$ SD), $80\pm71/\mu l$; normals $13\pm5\%$, $290\pm132/\mu l$]. A random sample of immunoglobulin levels (22 patients) found normal levels of IgG (patients $11.0\pm3.8$ g/l compared with normal $11.5\pm2.3$), IgM ($1.7\pm0.9$ compared with $1.4\pm0.5$) and IgA ($2.8\pm1.8$ compared with $2.4\pm0.8$).

The number of CD3+CD4+ positive lymphocytes (T-helper cells) in transplant patients was significantly less than normal. The range of the patient results suggested a bi-model distribution (Figure 1), with 23% of transplant patients below the 10th percentile of controls. CD3+CD8+ lymphocyte numbers were significantly elevated in transplant recipients compared with normal, while NK cells were significantly lower in the patient group when assessed as either CD3−CD16+ or CD3−CD56+ with, respectively, 50 and 49% of patients below the normal 10th percentile.

### *Mitogen-stimulated lymphocyte proliferation*

Transplanted patients demonstrated a significant reduction in PHA stimulated lymphocyte proliferation [patients $1855\pm1510$ (mean $\pm$ SD) c.p.m./1000 lymphs, normals $2766\pm917$, $P<0.001$]. Half of the transplanted patients (49%) had lymphocyte proliferation below the 10th percentile of normals (Figure 2).

### *Neutrophil function*

Neutrophil phagocytic function in transplanted patients was similar to normal. Review of the distribution showed only 3% of transplanted patients were below the 10th percentile of normal. The *S.aureus*-induced neutrophil oxidative burst measured in the transplant patients was significantly less than in normals [ROS index: patients $140\pm81$ (mean $\pm$ SD), normals $235\pm102$, $P<0.001$] with 63% of patients below the 10th percentile of the normal range (Figure 3).

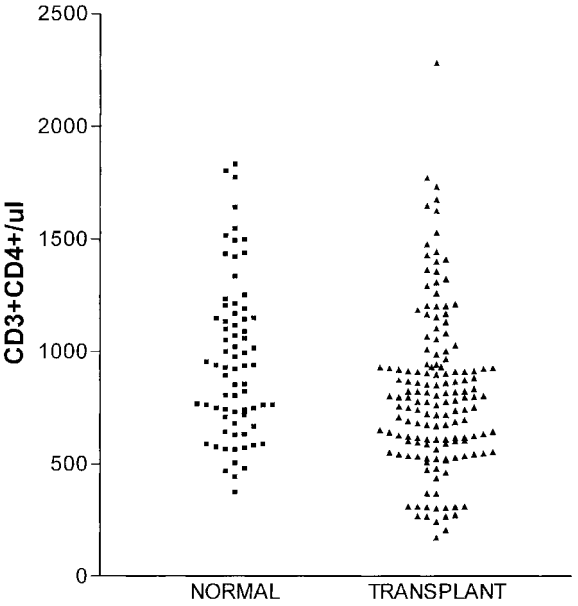


**Fig. 1.** Distribution of CD4 T-cell numbers in normal and transplant groups.

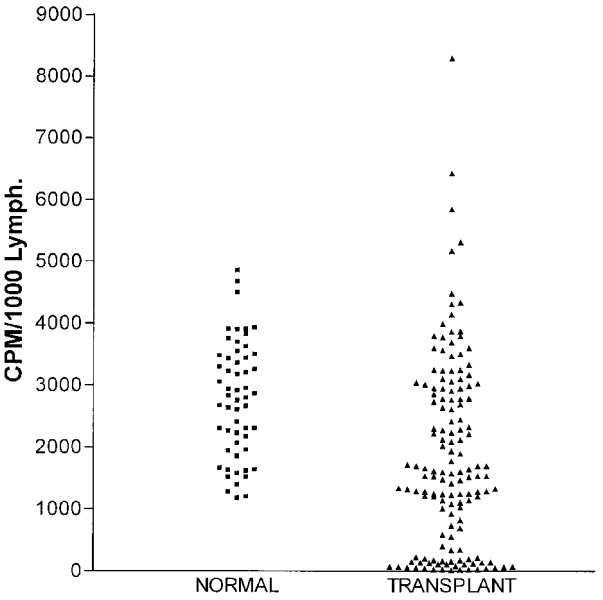


**Fig. 2.** Distribution of lymphocyte mitogen responses in normal and transplant groups.

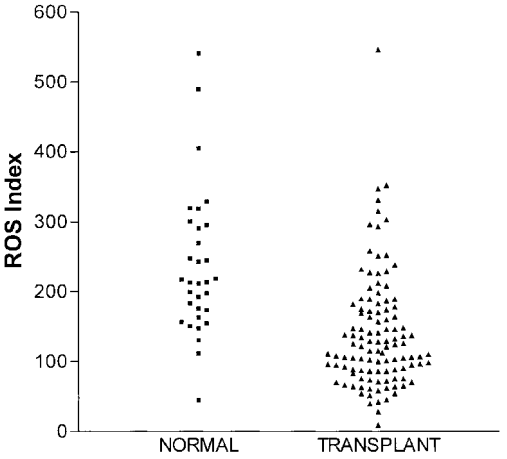


**Fig. 3.** Distribution of neutrophil ROS index in normal and transplant groups.

### *Immune function score*

The immune function score was determined using the four parameters CD4 count, lymphocyte mitogen response and neutrophil phagocytosis and oxidative burst level (as per materials and methods). Only 13% of the transplant patients had all four parameters measured above the 10th percentile of normal (Table 2), whereas 5% had three or more parameters below the 10th normal percentile.

### *Effect of duration of therapy and renal function*

There was no significant change in immune function score with longer exposure to immunosuppressive therapies: graft time 1–2 years $0.38 \pm 0.20$ (mean $\pm$ SD), 2–5 years $0.37 \pm 0.20$, 5–10 years $0.33 \pm 0.19$ and $>10$ years $0.29 \pm 0.18$. No significant correlations were found between serum creatinine levels and the four immune function parameters measured.

### *Effects of different immunosuppressive drug contribution*

Similar immune functional scores were seen in patients treated by each immunosuppressive protocol except for those having a regimen including MMF (Table 2). This difference could be attributed to an effect on a single parameter; with 41 out of 42 patients taking this drug having a lymphocyte mitogen response below the 10th normal percentile (Table 3).

Eighteen of the 152 patients had received OKT3 treatment for acute rejection. There was no significant difference between these patients and the rest for any of the measured immune function parameters. No significant correlations were found with blood CsA levels and the measured immune function parameters.

### *Retrospective comparison of infection and immune functional score*

A retrospective comparison of infection incidence and severity was made between 16 patients with the lowest score (0) and 15 with the highest scores ($\geqslant 0.75$; six with score of 1, and nine chosen randomly from patients with score of 0.75). Patients with the lowest immune score were similar in age (low score $46 \pm 12$ *vs* high score $42 \pm 8$), sex (M/F 8/8 *vs* 8/7), duration of transplants ($36 \pm 9$ *vs* $29 \pm 9$ months) and number of immunosuppressive drugs (mean $2.2 \pm 0.3$ *vs* $2.6 \pm 0.5$) to transplant patients with the higher immune function score. The patients with the higher

**Table 3.** Immune function parameters and drug regimens used

| | CD4 | ROS index | Phagocytosis index | PHA proliferation |
|---|---|---|---|---|
| Pred + Aza ($n=15$) | $1031 \pm 578$ (4/15) | $173 \pm 63$ (10/14) | $196 \pm 70$ (2/14) | $2223 \pm 739$ (3/15) |
| CsA + Aza ($n=64$) | $710 \pm 224$ (15/64) | $178 \pm 126$ (37/62) | $213 \pm 65$ (1/62) | $2707 \pm 1364$ (16/60) |
| CsA + Aza + Pred ($n=24$) | $902 \pm 338$ (4/24) | $167 \pm 86$ (14/24) | $209 \pm 71$ (1/24) | $2050 \pm 1021$ (9/23) |
| CsA + MMF ($n=19$) | $967 \pm 379$ (4/19) | $160 \pm 113$ (11/16) | $194 \pm 51$ (0/16) | $208 \pm 277$ (18/18) |
| CsA + MMF + Pred ($n=25$) | $792 \pm 277$ (6/25) | $160 \pm 69$ (14/24) | $192 \pm 55$ (0/24) | $383 \pm 755$ (23/24) |
| CsA + Pred ($n=5$) | $683 \pm 419$ (2/5) | $78 \pm 24$ (5/5) | $181 \pm 64$ (0/5) | $2829 \pm 2107$ (2/4) |

Mean $\pm$ SD given. Numbers in brackets refer to how many patient values were below the 10th percentile of normal for that parameter.

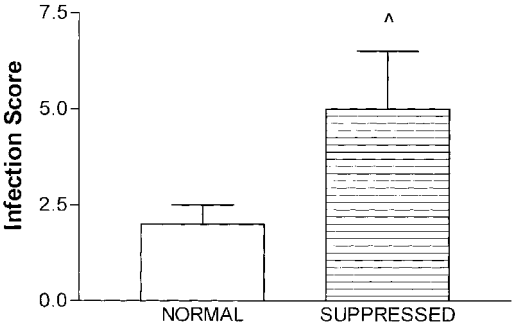


**Fig. 4.** Infection score for transplant patients with low and normal immune function. $^P < 0.05$.

immune function score had a significantly higher infection score: low score $2.0 \pm 0.5$ *vs* high score $5.0 \pm 1.5$ (Figure 4).

## Discussion

Immunosuppressive drugs have a variety of individual toxicities and side effects [12], but all share the adverse consequences of impairment of normal immunity. The major harmful consequences of immunosuppression are impairment of host immune defence against infection and the development of malignancy. Unfortunately accumulated data over many years confirms the strong association between immunosuppressive drugs, infection [1] and malignancy [2]. It was hypothesized that stratification of the population by quantification of multiple parameters of immune function would predict the rate of infectious complications.

Analysis of total leukocyte counts showed no overall differences between normal controls and renal transplant recipients. This was not unexpected as all treatment protocols included drug reduction to prevent leukopenia. The transplant group showed mild neutrophilia (related to steroid administration) and a significant lymphopenia. This was predominantly due to the reduction in circulating B-cell numbers. Measurement of circulating B cells is widely assessed by immunolabelling with anti CD19 antibodies [13], and our finding was confirmed by measurement of another pan B-cell marker CD20 [14]. The B-cell lymphopenia was profound in most patients. Eighty five per cent of all patients had B-cell numbers below the 10th percentile of the normal group. Other studies involving lymphocyte subset analysis have suggested a similar result [6,13]. A subgroup of patients had immunoglobulin levels assessed. All subclasses were in the normal range demonstrating a clear distinction between circulating B-cell numbers and overall immunoglobulin production. Other lymphocyte abnormalities included increased circulating CD8 T-cell numbers and decreased NK cell numbers, which, although statistically significant, were small. Similar findings have also been reported previously [13]. Overall there was a modest but significant decrease in circulating CD4 numbers in the patient group. The distribution indicated that a substantial proportion were below the 10th percentile of the normal range. The association of reduced CD4 numbers and immune dysfunction has been widely studied [15,16]. A study of CD4 counts in long-term renal transplant patients found a similar result to ours with 17% of patients having a CD4 count below 300/μl [6]. The patients with the low CD4 counts were also found to have a higher incidence of both skin cancers and infectious complications.

The current study demonstrated that lymphocyte mitogenic response to phytohaemagglutinin A was significantly lower in the renal transplant group than controls. This is consistent with previous studies [5]. It was also found that all patients on drug treatment regimes that contained MMF had consistently lower than normal mitogenic responses. Previous studies on the effect of MMF treatment have produced different results. One group found that isolated peripheral blood mononuclear cells from renal transplant patients on MMF therapy had no inhibition of T-cell proliferation following stimulation with recall antigen (tetanus toxoid) and in mixed lymphocyte cultures [17]. Others have found that there was an $\sim 80\%$ suppression of the mixed leukocyte culture proliferative response in patients receiving MMF compared with those on AZA as measured by carboxyfluorescein diacetate succinimidyl ester enumeration of cell division, although no effect of MMF on patient PHA response was observed [8]. The inconsistencies in the reported and current effects of MMF treatment on *in vitro* lymphocyte function may relate to the conditions of the assay used. The current studies used a diluted whole blood assay, which contains autologous plasma. This technique would contain trace amounts of circulating mycophenolic acid (the bioactive byproduct of MMF), which is known to inhibit proliferative responses of human T and B lymphocytes to nanomolar amounts [18]. Removal of the plasma of patients treated with MMF restores peripheral blood mononuclear cell mitogen responses to normal [18].

Various parameters of neutrophil function in transplant patients have been examined previously. The phagocytosis and killing of the pathogen *Kelbsiella pneumoniae* by isolated polymorphonuclear leukocytes from renal transplant patients has been found to be significantly lower than normals [4]. *In vitro* studies of the effect of CsA on neutrophil function have shown that it can decrease both phagocytosis [19] and ROS production [20]. An *ex vivo* study on renal transplant patients found that ROS production after zymosan stimulation was negatively correlated with CsA blood levels but was not significantly different from the normal controls [21]. Our results are consistent with the reports of decreased neutrophil function in renal transplant patients, and demonstrated that significant reduction ($<$10th percentile of normal) of ROS production occurs in 63% of renal transplant recipients.

The patients studied here were treated with a range of combinations of immunosuppressive drugs. The

extent and distribution of immune abnormalities was similar regardless of drug treatment (Table 3) except for the previously discussed association of MMF treatment and decreased mitogen response. The duration of therapy also appeared to have no direct effect on the immune function score.

Several other laboratory assessments of immune functional status have been devised and applied to measure potential abnormalities in renal transplant recipients. These include flow cytometric analysis of cytokine production by T cells following polyclonal stimulation with phorbol myristate acetate and ionomycin [22,23]. Using this method it was shown the proportion of CD4 T cells staining for IL-2 was significantly lower in the transplant recipients, and that this proportion negatively correlated with blood CsA levels [23]. Another postulated method is quantification of the proliferative response of patient's lymphocytes to third party lymphocyte stimulator cells [24]. However, this method lacked the resolution to distinguish individual patient differences on a day-to-day basis [8]. A study of a panel of ploymorphonuclear leukocyte functions and products found that the combination of increased peripheral blood elastase levels and decreased chemotactic function was a postive indicator of sepsis in solid organ transplant recipients [7]. The advantage of our method of immune function quantification is that it assesses simultaneously both cognate and non-cognate parameters on the same sample of blood.

In the current study four parameters were used to devise a score of functional immune competence arbitrarily based on the number of test results below the 10th percentile of controls. Although abnormal numbers of circulatory NK cells and B cells were commonly observed neither of these parameters were included in determining the score. In the case of B cells, 85% of all patients were below the 10th percentile of normal. Thus, this test would not allow humoral immunity to be stratified within the transplant population. Furthermore, measurement of immunoglobulin levels did not show differences between normals and transplant recipients. Thus, no parameter of humoral immunity is included in the scoring system employed. More sophisticated measures of humoral immunity such as responsiveness to vaccinations has been shown to be abnormal in transplant recipients [3]. As these tests are unlikely to find practical routine application they were not included. Practicability and reproducibility was a major factor in decisions on the tests to be included in the scoring system. Neutrophil function, mitogen responsiveness and CD4 count were easily performed on small volume whole blood assays. Although NK numbers would allow stratification of patients, lack of clinical data on the association between circulating NK cell numbers and infection or malignancy rates predicated against their inclusion. However, longer-term studies may indicate that NK cell reduction is a risk factor and these assessments may strengthen the validity of immune functional indices.

Infection is the most frequent adverse occurrence in renal transplant recipients available to test the relevance of the devised immune competence score and was therefore studied on a retrospective review of 31 patients. Patients with the worst immune functional score were compared with an equal number from the group with the best score. Both groups were similar in age, duration and adequacy of graft function, number of rejection episodes and exposure to immune suppressive drugs. Both frequency and severity of infection was significantly higher in patients with poor immune function.

These observations suggest that a laboratory profile of immune functional status may be relevant to predicting risks of immune suppression related complications. A number of future developments are indicated: to compare the relative predictive powers of the individual parameters; to define the positive and negative predictive values of various levels of abnormality; and to assess the capacity of the score to predict malignancy. In summary, routine immunosuppressive protocols induce a variety of quantitative abnormalities of immune function. Simultaneous analysis of a spectrum of functions allows for semi-quantitative evaluation of the level of immune function that correlates with the frequency of infective complications.

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