Clin Transplant 2014: 28: 606–610 DOI: 10.1111/ctr.12359

Clinical Transplantation

Preoperative Cylex assay predicts rejection risk in patients with kidney transplant

Myslik F, House AA, Yanko D, Warren J, Caumartin Y, Rehman F, Jevnikar AM, Stitt L, Luke PP. Preoperative Cylex assay predicts rejection risk in patients with kidney transplant.

Abstract: Introduction and Objectives: The ImmuKnow assay measures cell-mediated immunity by quantifying ATP release from CD4+ T-cells in peripheral blood. Herein, we hypothesized that this assay could predict complications associated with over-/under-immunosuppression in patients with kidney transplant (KT).
Methods: Sixty-seven patients undergoing KT were recruited prospectively and had ATP levels measured preoperatively, and at specified intervals over two months. Clinicians were blinded to ATP levels. Clinical events including rejection and infection/cancer were documented with a median follow-up of 21 months. Parameters including absolute ATP levels and changes in ATP patterns (slopes, delta) were analyzed. Association between ATP parameters and clinical outcomes was compared using the likelihood-ratio test and Kaplan–Meier curves.
Results: Absolute ATP values postoperatively had poor predictive value with regard to rejection or infection/malignancy. As well, changes in ATP values were poorly associated with complications. Importantly, patients with pre-transplant ATP values <300 ng/mL had significantly less rejection episodes vs. those with ATP values >300 ng/mL ($p < 0.0001$).
Conclusions: For the first time, we have evidence that a preoperative ImmuKnow level can stratify patients with KT into low/high risk groups for rejection. Future studies used to assess the utility of this assay to design individualized immunosuppressive regimens are required.

Frank Myslik[a], Andrew A. House[a,b,c], Daniel Yanko[a,d], Jeff Warren[a,c,d], Yves Caumartin[a,c,d], Faisal Rehman[a,b,c], Anthony M. Jevnikar[a,b,c], Larry Stitt[a,e] and Patrick P. Luke[a,d,e]

[a]Western University, [b]Department of Medicine, University of Western Ontario, [c]Multiorgan Transplant Program, University of Western Ontario, [d]Department of Surgery, University of Western Ontario and [e]Department of Epidemiology and Biostatistics, University of Western Ontario, London, ON, Canada

Key words: biomarkers – immune monitoring – renal transplant

Corresponding author: Patrick Luke, London Health Sciences Center – University Campus, 339 Windermere Road, London, ON N6A 5A5, Canada.
Tel.: 519 663 3180;
fax: 519 663 3858;
e-mail: Patrick.luke@lhsc.on.ca

Conflict of interest: None.

Accepted for publication 10 March 2014

With the advent of new powerful immunosuppressive agents, rejection rates for kidney transplants (KTs) in the first year have greatly improved over the last two decades (1). However, despite this decline in incidences of acute rejection, chronic graft rejection has not seen a proportional decrease (2). One of the greatest challenges following kidney transplantation is achieving optimal immunosuppression. Consequently, a trend has moved from empiric to individualized drug therapy (3). In transplantation, our goal was to optimize immunosuppression to prevent rejection, minimize drug side effects, and avoid development of infections and malignancy (4). Current methods of monitoring rejection include measuring the plasma concentration of immunosuppressants, serum creatinine for kidney function, imaging and performing allograft biopsies, all of which are indirect ways of measuring the body's immune response (5). One FDA-approved assay that attempts to determine the true immune state of the body is the ImmuKnow® functional assay. This assay measures ATP released from CD4+ T-cells in peripheral blood, after mitogen stimulation (6). Some studies have demonstrated immune responses with ATP levels below 130 ng/mL and above 450 ng/mL are at higher risk of infection and rejection, respectively, and have suggested optimal therapeutic target ranges (7).

While the assay has been studied in solid-organ transplants, most studies have assessed Cylex levels associated with the presence of disease rather than its ability to predict development of infection or malignancy (5). Therefore, Immuknow assay® levels have yet to show any value in predicting development of immune-related complications associated with transplantation. We hypothesized that the assay could help predict complications that arise with transplantation.

Methods

Data collection

Sixty-seven patients undergoing KT were recruited prospectively with ATP levels measured preoperatively, and on day 7 (±3 d), day 21 (±7) and day 56 (±14) post-transplant. The inclusion criteria for the study indicated patients requiring kidney transplantation, >18 yr in age with the ability to provide informed consent. Patients who were lost to follow-up or voluntarily withdrew from the study were not included in the analysis of the data. Baseline individual immunosuppression regimens consisted of a triple therapy of tacrolimus, mycrophenolate mofetil (MMF), and prednisone. Initial target levels of tacrolimus were between 8–10 ng/mL for the first three months and 5–8 thereafter. Additionally, patients determined to have an intermediate risk of rejection or delayed graft function were induced with Basiliximab 20 mg IV before surgery and on postoperative day 4, and the highest risk recipients received Thymoglobulin ~6 mg/kg. Criteria for induction were based upon clinical assessment of the transplant physician/surgeon. Clinicians were blinded to the results of the Immuknow assay®.

ImmuKnow assay

Blood was collected in sodium heparin tubes (Becton Dickinson, Franklin Lakes, NJ, USA) to prevent coagulation and subsequently measured within a 30-h period using the Cylex™ ImmuKnow® assay (Catalog no. 4400. Cylex Inc.™, Columbia, MD, USA). CD4+ cells were selected out using methods described previously (7). Of the whole blood, 250 μL was diluted with sample diluent, and added to wells of a 96-well microtiter plate and incubated for 15–18 h with PHA in 37°C in 5% CO2. Magnetic particles coated with anti-human CD4 antibodies (Dynabeads, Dynal, Oslo, Norway) were introduced to the wells, and using a strong magnet, CD4+ T-cells were positively selected and separated. The cells were then subsequently lysed to release ATP, and with the addition of a luciferin/luciferase reagent to the ATP produce light. Using a luminometer to measure the amount of light, the concentration of ATP can then be determined. Each assay run also included a sample from a healthy volunteer as a control to validate the ATP results from the transplant recipient blood samples.

Statistical analysis

Clinicians were blinded to ATP levels, and no modification to patient care according to the ImmuKnow assay® levels was made. Clinical end points analyzed including rejection infection/cancer were documented with a median follow-up of 21 months (3–35 months). Parameters including absolute ATP levels and changes in ATP patterns (slopes, delta) were analyzed using cut points at the median or at tertiles to categorize subjects. An ATP value of 300 ng/mL approximated the median baseline value, while a range of 250–350 ng/mL approximated the middle tertile. Slopes of ATP level over time were somewhat skewed and were analyzed as both un-transformed and square-root-transformed variables. Comparison of normally distributed variables between groups was performed using one-way ANOVA. Association between ATP parameters and clinical outcomes of rejection or infection/cancer was compared using the −2log-likelihood ratio and Kaplan–Meier time-to-event curves. A significance value of $p < 0.05$ was considered statistically significant. The study was approved by the Institutional Ethics Review Board of Western University.

Results

The summary of patient demographics can be found in Table 1. Of the 67 patients, there were 20 patients that developed infections of bacterial, viral, and fungal etiology. As well, patients developed lung cancer (2), urothelial carcinoma of the bladder (3), and three skin cancers. Finally, 12 had acute cellular rejection. Postoperatively absolute ATP values were incapable in predicting episodes of infection/cancer or rejection, with no statically significant associations noted between individual levels and the clinical outcomes of interest at all time points. Likewise, relative postoperative changes in ATP levels, using whether deltas of a particular value or slope of change, were also of poor predictive value. Additionally, we found no association between falling ATP level and the use

Table 1. Summary of transplant recipient demographic factors

	n = 67
Age (mean yr)	51.6
Sex (M:F)	47:20
Etiology (DM:HTN:GN/IgA:PCKD:Other)	14:11:18:4:20
Immunotherapy (Tac, MMF, Prednisone)/other	63/67
Donor (deceased:living)	47:20
RRT (HD:PD)	50:17
Induction (Thymoglobulin:Basilliximab:None)	31:21:15
Number of previous transplants (0:1:2:3)	61:4:1:1
% PRA (0–20: >20)	64:3

DM, diabetes mellitus; HTN, hypertension; GN, glomerulonephritis; PCKD, polycystic kidney disease; RRT, renal replacement therapy; HD, hemodialysis; PD, peritoneal dialysis; PRA, panel reactive antibody; MMF, mycophenolate mofetil.

of either induction with Thymoglobulin or basiliximab (data not shown).

When comparing baseline pre-transplant ATP values, patients were divided into a high immune response group (>300 ng/mL) and a low immune response group (≤300 ng/mL). There was no discernible difference in time-to-event for infections based on this dichotomy (Fig. 1, p = NS), nor delayed time to first rejection event when compared to the high immune response group (Fig. 2, p = NS). However, the low group also had significantly less rejection episodes compared to the high group ($p < 0.0001$). Although the ATP cut-point of 300 ng/mL was associated with risk of rejection, there was no imbalance between those groups in other factors such as sex, etiology of end-stage kidney disease, sensitization risk (% PRA), or immunotherapy used (Table 2). Furthermore, consistent with a graded degree of risk, when pre-transplant ATP values were split into tertiles of <250, 250–350, and >350 ng/mL, there was a trend to having fewer rejections in the lowest tertile when compared to the remaining groups, although this failed to reach statistical significance ($p = 0.0527$).

When analyzing the subset of patients who underwent induction with Thymoglobulin, there was significantly more rejection events in the first 90 d following transplant for the high immune response group compared to the low immune response group (Table 3). There was no statistical difference between cancer types encountered in the induction group (p = NS).

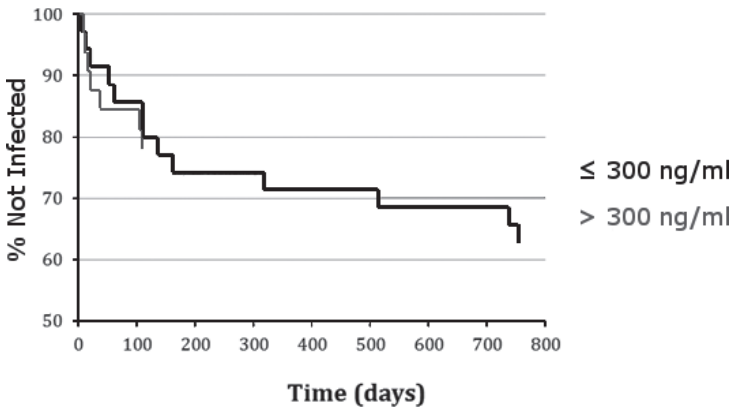
Discussion

Using a prospective group of KT recipients, we have shown for first time that preoperative ATP values are valuable at predicting rejection episodes (6, 8–11). Those with lower values showed had a reduced risk of developing rejection compared to those with higher values. However, postoperative ATP values had poor predictive value for complications. Interestingly, these results support that of Heeger's group (12), who showed that another assay-measuring immunoreactivity, namely the ELISPOT assay for interferon-gamma, can predict rejection in patients with KT when preoperative levels were assessed (6).

In our study, assay results were divided into two groups around the ATP value of 300 ng/mL. Furthermore, results were also analyzed according to tertiles, and within the lowest tertile, there were fewer rejection rates vs. other groups. This coincidently corresponds to the Cylex™ guidelines that classifies weak immune response as <224 ng/mL, moderate 224–524 ng/mL, and strong >525 ng/mL (5).

Clinicians were blinded to ATP values when selecting induction for patients who were felt to be at the highest risk of rejection. Importantly, there was no significant difference in preoperative ATP values for patients with induction who were in the clinically high immune risk group compared to the low group. However, when looking at patients in both low and high immune reactivity groups induced using Thymoglobulin, there were significantly more rejection events in the first 90 d for the high group compared to the low group. This corroborates our primary finding that lower preoperative ATP levels correlated with a decreased risk of developing rejection, independent of clinical risk assessment.

Limitations of this study include the small sample size of patients with KT analyzed. Further-



Pre-operative Immuknow	≤300	>300
Days until infection	6,12,26,51, 61, 110,110,135,162,318,513737,754	9,12,14, 20,37 ,105,109

Fig. 1. Kaplan–Meier survival curve showing no difference in infection rates over time between groups according to preoperative ImmuKnow® levels (p = NS).

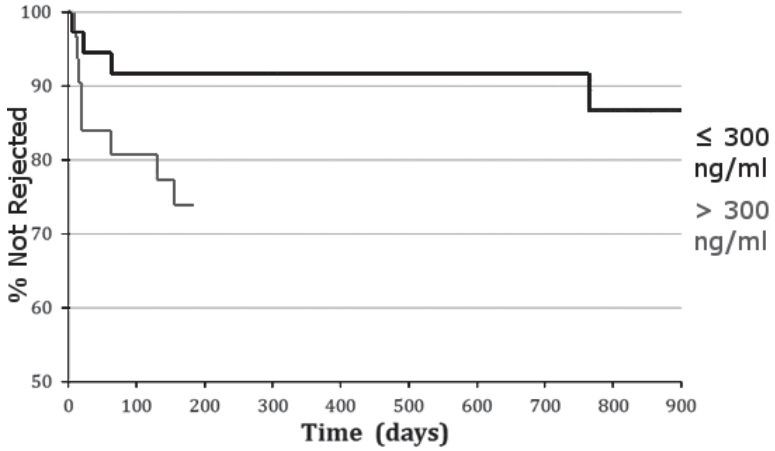


Fig. 2. Kaplan–Meier survival curve showing significant difference in rejection rates between patients with ImmuKnow® levels ≤300 and >300 ($p < 0.05$).

Pre-operative Immuknow	≤300	>300
Days to rejection	5,22,64,764	8,12,15,20,20,62,129,155

Table 2. Comparison of demographics of patients with low (<300 ng/mL) and high (>300 ng/mL) preoperative ImmuKnow® levels

	≤300 ng/mL	>300 ng/mL	p-Value
n	36	31	
Age	51.9	51.2	0.83
Male:Female	23:13	24:7	0.23
Etiology (DM:HTN:GN/IgA:PCKD:Other)	3:3:6:3:16	6:8:12:0:14	0.11
Immunotherapy (Tac, MMF, Pred)/other	34/36	29/31	0.98
Induction (Basiliximab: Thymo:None)	7:18:10	14:13:5	0.10

DM, diabetes mellitus; HTN, hypertension; GN, glomerulonephritis; PCKD, polycystic kidney disease; Tac, tacrolimus; MMF, mycophenolate mofetil; Pred, prednisone; Thymo, Thymoglobulin.

Table 3. 90 d rejection events in patients induced using Thymoglobulin stratified by preoperative ImmuKnow levels

	≤300 ng/mL	>300 ng/mL	p-Value
n	18	13	
90 d rejection events	0/18	4/13	0.023

Assessment of significance was performed by Fisher's exact test.

more, we cannot completely rule out the ability of the Immuknow assay to predict episodes of rejection and infection postoperatively. There have been multiple studies across multiple organ transplant groups that have shown that no single time point postoperatively can accurately predict complications, but rather it is the overall trend that is a stronger predictor of complications (13–15). Because there were few events in our analysis and only three ImmuKnow® levels were taken postoperatively, this limited our ability to predict complications.

Using the ImmuKnow® assay as a one-time test during evaluation for transplant may provide useful information in determining the need for induction therapy, and potentially the development of personalized protocols for immunotherapy and rejection monitoring. For example, during initial assessment, patients can be stratified into low/intermediate or high risk independent of assessment of % PRA, HLA, and patient demographics. In patients with high preoperative Immuknow ATP levels, closer follow-up monitoring for rejection should be considered. Patients with lower preoperative levels may require less stringent follow-up or reduced immunotherapy long term. Further studies need to be performed to determine the usefulness of the assay in designing individualized regimens of immunotherapy.

References

1. Patel J, Kobashigawa JA. Minimization of immunosuppression: transplant immunology. Transpl Immunol 2008: 20: 48.
2. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D. Improved graft survival after renal transplantation in the United States, 1988 to 1996. N Engl J Med 2000: 342: 605.
3. Cadillo-Chavez R, de Echegaray S, Santiago-Delpin EA et al. Assessing the risk of infection and rejection in Hispanic renal transplant recipients by means of an adenosine triphosphate release assay. Transplant Proc 2006: 38: 918.
4. Gautam A, Fischer SA, Yango AF, Gohh RY, Morrissey PE, Monaco AP. Cell mediated immunity (CMI) and post transplant viral infections–role of a functional immune assay to titrate immunosuppression. Int Immunopharmacol 2006: 6: 2023.

5. Kowalski RJ, Post DR, Mannon RB et al. Assessing relative risks of infection and rejection: a meta-analysis using an immune function assay. Transplantation 2006: 82: 663.
6. Heidt S, San Segundo D, Shankar S et al. Peripheral blood sampling for the detection of allograft rejection: biomarker identification and validation. Transplantation 2011: 92: 1.
7. Kowalski R, Post D, Schneider MC et al. Immune cell function testing: an adjunct to therapeutic drug monitoring in transplant patient management. Clin Transplant 2003: 17: 77.
8. Serban G, Whittaker V, Fan J et al. Significance of immune cell function monitoring in renal transplantation after Thymoglobulin induction therapy. Hum Immunol 2009: 70: 882.
9. Gupta S, Mitchell JD, Markham DW et al. Utility of the Cylex assay in cardiac transplant recipients. J Heart Lung Transplant 2008: 27: 817.
10. Huskey J, Gralla J, Wiseman AC. Single time point immune function assay (ImmuKnow) testing does not aid in the prediction of future opportunistic infections or acute rejection. Clin J Am Soc Nephrol 2011: 6: 423.
11. Torio A, Fernandez EJ, Montes-Ares O, Guerra RM, Perez MA, Checa MD. Lack of association of immune cell function test with rejection in kidney transplantation. Transplant Proc 2011: 43: 2168.
12. Heeger PS, Greenspan NS, Kuhlenschmidt S et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. J Immunol 1999: 163: 2267.
13. Gralla J, Huskey J, Wiseman AC. Trends in immune function assay (ImmuKnow; Cylex) results in the first year post-transplant and relationship to BK virus infection. Nephrol Dial Transplant 2012: 27: 2565.
14. Husain S, Raza K, Pilewski JM et al. Experience with immune monitoring in lung transplant recipients: correlation of low immune function with infection. Transplantation 2009: 87: 1852.
15. Kobashigawa JA, Kiyosaki KK, Patel JK et al. Benefit of immune monitoring in heart transplant patients using ATP production in activated lymphocytes. J Heart Lung Transplant 2010: 29: 504.