

Proteomic-Based Detection of Urine Proteins Associated with Acute Renal Allograft Rejection

STEFAN SCHAUB,^{*†} DAVID RUSH,^{*†} JOHN WILKINS,^{*†} IAN W. GIBSON,^{*}
TRACEY WEILER,^{*†} KEVIN SANGSTER,^{*†} LINDSAY NICOLLE,^{*}
MARTIN KARPINSKI,^{*} JOHN JEFFERY,^{*} and PETER NICKERSON^{*†}

^{*}Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; and [†]Manitoba Centre for Proteomics, Winnipeg, Manitoba, Canada.

Abstract. At present, the diagnosis of renal allograft rejection requires a renal biopsy. Clinical management of renal transplant patients would be improved by the development of non-invasive markers of rejection that can be measured frequently. This study sought to determine whether such candidate proteins can be detected in urine using mass spectrometry. Four patient groups were rigidly defined on the basis of allograft function, clinical course, and allograft biopsy result: acute clinical rejection group ($n = 18$), stable transplant group ($n = 22$), acute tubular necrosis group ($n = 5$), and recurrent (or *de novo*) glomerulopathy group ($n = 5$). Urines collected the day of the allograft biopsy were analyzed by mass spectrometry. As a normal control group, 28 urines from healthy individuals were analyzed the identical manner, as well as 5 urines from non-transplanted patients with lower urinary tract infection. Furthermore, sequential urine analysis was performed in pa-

tients in the acute clinical rejection and the stable transplant group. Three prominent peak clusters were found in 17 of 18 patients (94%) with acute rejection episodes, but only in 4 of 22 patients (18%) without clinical and histologic evidence for rejection and in 0 of 28 normal controls ($P < 0.001$). In addition, the presence or absence of these peak clusters correlated with the clinicopathologic course in most patients. Acute tubular necrosis, glomerulopathies, lower urinary tract infection, and cytomegalovirus viremia were not confounding variables. In conclusion, proteomic technology together with stringent definition of patient groups can detect urine proteins associated with acute renal allograft rejection. Identification of these proteins may prove useful as non-invasive diagnostic markers for rejection and the development of novel therapeutic agents.

Although short-term and long-term kidney allograft survival has improved over the last 15 yr (1), allograft failure is still one of the most common causes for end-stage renal disease (2). Both immunologic and non-immunologic (*e.g.*, calcineurin-inhibitor-toxicity, hypertension) factors contribute to a continuous deterioration of allograft function, which is referred to as chronic allograft nephropathy (3). Acute allograft rejection is the major immunologic risk factor for developing chronic allograft nephropathy (4,5).

At present, the diagnosis of acute rejection can only be made by renal biopsy, which is costly, inconvenient, and carries a small risk of complications (6,7). Therefore, biopsies cannot be obtained frequently (*e.g.*, weekly) to monitor the immune response to the allograft, which may be helpful, as rejection can develop in allografts before graft dysfunction occurs (*i.e.*, subclinical rejection) (8,9). Sampling error is an additional

problem, which can be diminished in part by collecting larger or multiple core biopsy samples (10,11). A non-invasive biomarker of rejection may benefit the kidney allograft recipient by allowing frequent monitoring to optimize immunosuppressive therapy. Various approaches such as mRNA measurement in urinary lymphocytes (12,13), urine flow cytometry (14), and measurement of alloreactive peripheral blood lymphocytes (15,16) have shown promising initial results, although none of these tests have yet reached wide clinical application.

As acute rejection is a complex process involving many different cell types of the donor and recipient, analysis of global changes at the gene (17–20) or protein level may provide both insights into its pathogenesis, as well as novel non-invasive biomarkers. Recent developments in mass spectrometry make it possible to rapidly profile and compare the proteome of clinical samples (21,22). In this study, we used proteomic technology and very rigid patient selection criteria, including allograft histology, allograft function, and clinical course, to detect urine proteins associated with acute renal allograft rejection.

Materials and Methods

Urine Collection

All urine samples were stored non-centrifuged at -70°C until further analysis. All patient and control urine samples were obtained

Received August 19, 2003. Accepted September 28, 2003.

Corresponding to Dr. Peter Nickerson, Immunogenetics Laboratory, Winnipeg Blood Centre, 777 William Avenue, Winnipeg, Manitoba, Canada R3E 3R4. Phone: 204-789-1125; Fax: 204-783-6780; E-mail: peter.nickerson@bloodservices.ca

1046-6673/1501-0219

Journal of the American Society of Nephrology

Copyright © 2003 by the American Society of Nephrology

DOI: 10.1097/01.ASN.0000101031.52826.BE

with informed consent and ethics approval by the University of Manitoba institutional review board.

Transplanted Patients

From July 1997 to March 2003, 2400 serial midstream urine samples from 212 renal transplant patients were collected. Patients were treated with a triple immunosuppressive regimen consisting of calcineurin-inhibitor (cyclosporine or tacrolimus), prednisone, and mycophenolate-mofetil or azathioprine. These 212 patients underwent a total of 693 protocol or clinically indicated core needle allograft biopsies. All biopsies were analyzed by experienced renal pathologists and scored according to the Banff 1997 classification (23) (acute Banff score: interstitial (ai 0–3), tubular (at 0–3), vascular (av 0–3), glomerular (ag 0–3); chronic Banff score: ci 0–3, ct 0–3, cv 0–3, cg 0–3). A biopsy specimen was judged adequate when ≥ 7 glomeruli and ≥ 1 vessel were present. Delayed graft function (DGF) was defined as the need for hemodialysis within the first week or a drop of serum creatinine $<50\%$ from pretransplant levels by day 5 posttransplant. On the basis of allograft function, the clinical course, and the allograft biopsy result, four rigidly defined patient groups were extracted from the whole patient population ($n = 212$) as follows.

Stable Transplant Group. Consists of 22 midstream urine samples (from 22 patients) obtained immediately before a protocol renal allograft biopsy performed within the first 12 mo posttransplant. None of these patients had experienced DGF. All had stable allograft function (*i.e.*, serum creatinine within 110% of baseline value at the time of biopsy), and none experienced a clinical or protocol biopsy-proven rejection before the date of examination. All biopsies met the criteria for adequacy, and all were required to have an acute and chronic Banff score of zero (*i.e.* ai0t0v0g0 and ci0t0v0g0).

Acute Clinical Rejection Group. Consists of 18 midstream urine samples (from 18 patients) obtained immediately before a renal allograft biopsy performed within the first 12 mo posttransplant. All experienced an elevation in creatinine $>110\%$ from baseline, and the diagnosis of acute rejection required an acute Banff score \geq ai2t2v0g0. Patients with a chronic Banff score $>$ ci1t1v0g0 were excluded to avoid chronic allograft nephropathy as a confounding variable in the analysis.

Acute Tubular Necrosis (ATN) Group. Consists of 5 midstream urine samples (from 5 patients) obtained immediately before a renal allograft biopsy performed within the first 6 d posttransplant to diagnose the cause of DGF. Antibody-mediated rejection was excluded on the basis of a negative flow-crossmatch and histologic changes on the biopsy consistent with ATN. In all biopsies, the acute Banff score was ai0t0v0g0, and significant donor pathology was excluded by requiring a chronic Banff score of \leq ci1t1v0g0.

Recurrent (or *de novo*) Glomerulopathy Group. Consists of 5 midstream urine samples (from 5 patients) obtained immediately before a renal allograft biopsy performed to diagnose the cause of proteinuria (≥ 1.5 g/d). The patients had diagnoses of membranous glomerulonephritis, focal-segmental glomerulosclerosis, or IgA-nephropathy, and all had acute Banff scores \leq ai1t1v0g0.

Non-Transplanted Control Groups

Normal Control Group. Consists of 28 midstream urine samples from 28 healthy individuals (14 women and 14 men; age, 20–50 yr).

Urinary Tract Infection (UTI) Group. Consists of 5 midstream urine samples from 5 women obtained during an episode of a lower UTI, which was defined as requiring the clinical symptoms of a UTI,

a leukocyte count in the urine sediment > 40 /high power field, and a positive bacterial culture ($>10^8$ colony-forming units).

Urine Protein Profiling with Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)

Urine samples were thawed on ice, vortexed, and centrifuged for 5 min at $10000 \times g$ to remove remaining cell particles. Five microliters of urine supernatant were applied in duplicate to normal phase chips (ProteinChip NP20; Ciphergen, Fremont, CA) and incubated for 20 min in a humidity chamber. Spots were then washed three times with $5 \mu\text{l}$ of HPLC-grade water and air-dried for 10 min. One microliter of 35% α -cyano-4-hydroxycinnamic acid (CHCA; Ciphergen) was applied to each spot and air-dried. Chips were read with a SELDI-TOF-MS instrument (ProteinChip Reader II; Ciphergen) in the positive ion mode with the following settings: laser intensity, 230; detector sensitivity, 6; detector voltage, 1700 V; 240 shots were collected per sample. Peak labeling was performed with the ProteinChip Software (Version 3.1) for peaks with a signal-to-noise ratio of ≥ 3 in the mass over charge (m/z) range from 2000 to 80000. For comparison, spectra were normalized by total ion current. Calibration was done externally with a mixture of four proteins with masses ranging from 2 to 16 kD.

Determination of Cytomegalovirus (CMV) Viremia

CMV-viremia was measured on peripheral blood buffy coat specimens using a semiquantitative PCR assay developed at the Manitoba Cadham Provincial Laboratory that is accredited by the College of American Pathologists (for details see reference 32).

Statistical Analyses

We used JMP IN software version 4.0.4 (SAS Institute Inc., Cary, NC) for statistical analyses. For categorical data, Fisher exact test or Pearson χ^2 test was used. Parametric continuous data were analyzed by Student *t* tests or one-way ANOVA. For nonparametric continuous data, Wilcoxon or Kruskal-Wallis rank sum tests were used. A *P*-value < 0.05 (two-sided test) was considered to indicate statistical significance.

Results

Patient Characteristics

The acute clinical rejection group had more HLA-mismatches and a higher mean serum creatinine level at the time of the renal allograft biopsy compared with the stable transplant group. Otherwise, there were no significant differences between these groups (Table 1).

Characterization of Urine Protein Profiles Associated with Individual Patient Groups

In the m/z range from 5000 to 12000, we observed two distinct urine protein patterns when comparing the normal control group or stable transplant group to the acute clinical rejection group. One urine protein profile (rejection pattern) had prominent peak clusters in three regions corresponding to m/z values of 5270 to 5550 (region I; 5 peaks), 7050 to 7360 (region II; 3 peaks), and 10530 to 11100 (region III; 5 peaks) that always occurred together, whereas the other urine protein profile (normal pattern) had no peak clusters in these m/z regions (Figure 1). All 28 urine samples (100%) from the normal control group, 18 of 22 urine samples (82%) from the

Table 1. Patient characteristics

Variable	Stable Transplant (n = 22)	Acute Clinical Rejection (n = 18)	ATN (n = 5)	Recurrent or <i>de novo</i> Glomerulopathy (n = 5)
Female gender, n (%)	12 (55)	6 (33)	2	2
Age, mean ± SD	45 ± 13	43 ± 10	40 ± 18	47 ± 9
Caucasian race, n (%)	14 (64)	15 (83)	3	5
Nephropathy				
diabetic, n (%)	6 (27)	3 (17)	1	0
glomerulonephritis, n (%)	6 (27)	6 (33)	3	4
others, n (%)	10 (46)	9 (50)	1	1
First transplant, n (%)	21 (95)	16 (89)	5	4
Cadaveric donor, n (%)	15 (68)	10 (56)	3	5
HLA-mismatches, median (range)	3 (1–5)	4 (2–5) ^a	3 (2–4)	3 (3–5)
Panel-reactive antibodies (PRA)				
peak PRA >10%, n (%)	2 (9)	0	0	1
current PRA >10%, n (%)	1 (5)	0	0	1
Cytomegalovirus serology				
recipient neg./donor pos., n (%)	3 (14)	3 (17)	1	1
recipient neg./donor neg., n (%)	7 (32)	4 (22)	0	2
recipient pos./donor pos., n (%)	4 (18)	9 (50)	3	1
recipient pos./donor neg., n (%)	8 (36)	2 (11)	1	1
Allograft biopsy				
week posttransplant, median (range)	8 (3–51)	8 (1–18)	day 5 or 6 ^d	253 (7–442)
rejection type (Banff 1997)				
IA (moderate tubulitis), n (%)		7 (39)		
IB (severe tubulitis), n (%)		8 (44)		
IIA (moderate arteritis), n (%)		3 (17)		
Creatinine at biopsy [μmol/L], mean ± SD	91 ± 26	180 ± 59 ^b	942 ± 80 ^e	122 ± 29
% above baseline, median (range)		25 (11–76)		
Proteinuria at biopsy [g/L], median (range)	0.07 ^c (0.03–0.17)	0.09 (0.03–0.28)		3.20 (0.58–6.00)

^a $P = 0.003$ versus stable transplant group.

^b $P < 0.001$ versus stable transplant group.

^c $P = 0.14$ versus acute clinical rejection group. $P < 0.001$ versus recurrent or *de novo* glomerulopathy group.

^d Not included for statistical analysis.

^e Not included for statistical comparison (3 of 5 patients were on hemodialysis).

stable transplant group, and 1 of 18 urine samples (6%) from the acute clinical rejection group showed the normal pattern. The rejection pattern was detected significantly more often in the acute clinical rejection group (17 of 18; 94%) than in the stable transplant group (4 of 22; 18%) ($P < 0.0001$) (Figure 2). The ATN, the recurrent (or *de novo*) glomerulopathy, and the UTI groups had urine protein profiles that were different from both the normal and the rejection pattern (Figure 1 and Figure 2).

Influence of CMV-Viremia on Urine Protein Profile Pattern

Twenty-seven of 40 patients (68%) in the stable transplant and acute clinical rejection groups were tested for the presence of CMV viremia at the time of renal allograft biopsy. Five patients tested positive; however, none had or developed CMV disease subsequently. CMV viremia was found in 2 of 21

patients (10%) with the rejection pattern and in 3 of 19 patients (16%) with the normal pattern ($P = 0.83$) (Table 2). We could not detect any additional peaks in the urine protein profiles from patients who had CMV-viremia.

Sequential Urine Protein Profile Analysis

To further determine the specificity of the normal and rejection pattern, we examined serial urine protein profiles in the stable transplant and acute clinical rejection groups and correlated them with the clinicopathologic course of the renal allograft. In particular we were interested in four specific outcomes: (1) the stable course persisted; (2) the stable transplant patient subsequently had an acute clinical rejection; (3) acute clinical rejection resolved to a stable course; (4) acute clinical rejection recurred.

In the stable transplant group, we had sufficient urine and histology samples for sequential analysis to evaluate 12 of the

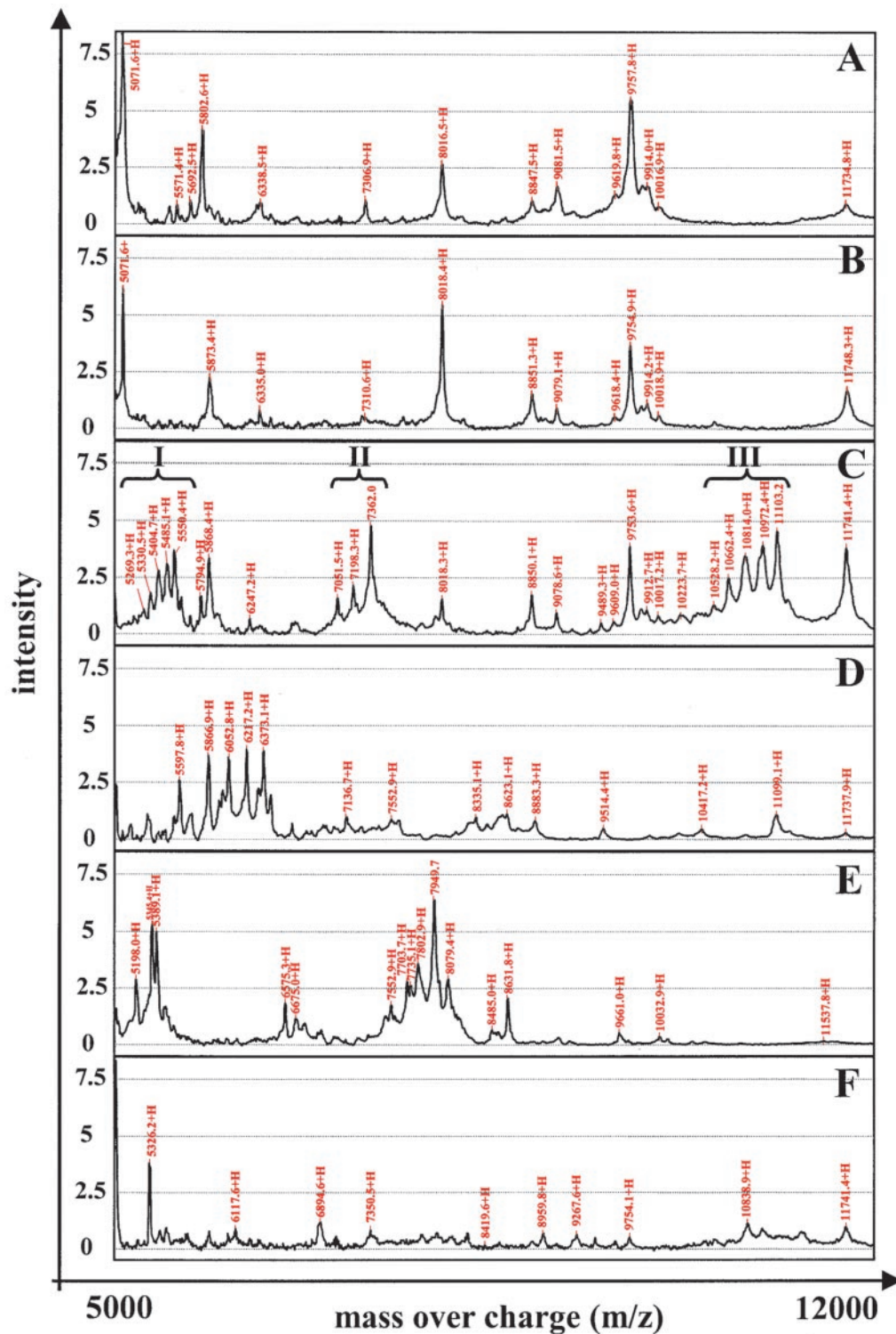


Figure 1. Representative urine protein profiles. (A) Normal control with normal pattern. (B) Stable transplant with normal pattern. (C) Acute clinical rejection with rejection pattern. (D) Glomerulopathy. (E) ATN. (F) UTI. The rejection pattern had prominent peak clusters in three regions corresponding to m/z values of 5270–5550 (region I; 5 peaks), 7050 to 7360 (region II; 3 peaks), and 10530 to 11100 (region III; 5 peaks).

18 patients who originally had a normal pattern. One patient went on to have stable allograft function and two normal protocol biopsies, but the urine profile could not be classified.

One patient developed acute clinical rejection (Banff type IA) and the urine protein profile changed from the normal to the rejection pattern. In ten patients, stable allograft function per-

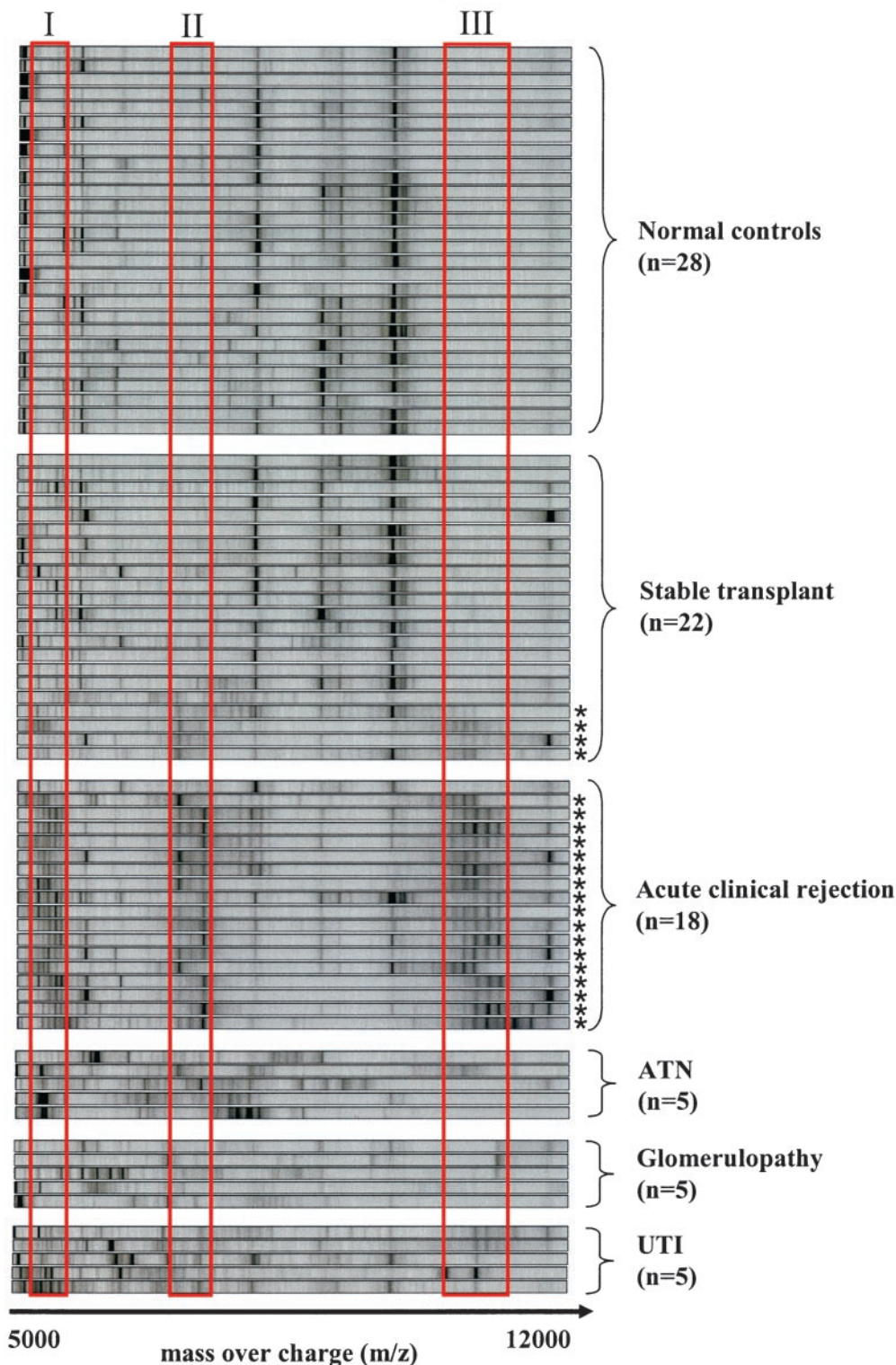


Figure 2. Software generated gel-view of urine protein profiles from all groups. Box frames represent the three regions corresponding to m/z values of 5270 to 5550 (region I), 7050 to 7360 (region II), and 10530 to 11100 (region III). * a urine sample with the rejection pattern.

sisted and 20 subsequent protocol biopsies were interpreted as normal ($n = 18$) or borderline rejection ($n = 2$). Eight of these ten patients showed the normal pattern throughout (Figure 3A),

whereas two patients exhibited the rejection pattern in a single urine sample that subsequently reverted to the normal pattern.

In the acute clinical rejection group, we had sufficient urine

Table 2. Correlation between CMV-viremia and urine protein pattern

CMV-Viremia	Normal Pattern (<i>n</i> = 19) ^a	Rejection Pattern (<i>n</i> = 21) ^b	<i>P</i> -value
CMV-DNA positive, <i>n</i>	3	2	<i>P</i> = 0.83
CMV-DNA negative, <i>n</i>	10	12	
No CMV-PCR available, <i>n</i>	6 ^c	7 ^d	

^a Consists of 18 patients from the stable transplant group plus 1 patient from the acute clinical rejection group.

^b Consists of 4 patients from the stable transplant group plus 17 patients from the acute clinical rejection group.

^c CMV-PCR was not performed for the following reasons: CMV sero-negativity of both donor and recipient (*n* = 2); test was not ordered (*n* = 3); or only CMV pp65-antigen was evaluated (*n* = 1; patient tested negative).

^d CMV-PCR was not performed for the following reasons: CMV sero-negativity of both donor and recipient (*n* = 3); test was not ordered (*n* = 3); or only CMV pp65-antigen was evaluated (*n* = 1; patient tested negative).

and histology samples for sequential analysis to evaluate 12 of the 17 patients who originally had a rejection pattern. One patient had two subsequent normal protocol biopsies, but the creatinine remained elevated at the level seen during the acute rejection episode (20% above baseline), and the urine always showed the rejection pattern. In six patients, the allograft function returned to baseline and subsequent protocol biopsies were interpreted as normal (*n* = 3) or borderline rejection (*n* = 3). All urine samples from these patients changed to the normal pattern (Figure 3B). Five patients had further episodes of acute clinical rejections, and all of them kept the rejection pattern throughout (Figure 3C).

Discussion

We used a proteomic technique to determine whether the urine of renal transplant patients undergoing acute allograft rejection had a characteristic profile. As urine can be very heterogeneous, standardization of urine collection and storage is critical. We have recently reported those factors that influence the reproducibility and peak detection in urines analyzed by SELDI-TOF-MS (24). In the design of the current study, we therefore required midstream urines that were collected immediately before the allograft biopsy and were stored the same day at -70°C . Next it was necessary to determine the urine protein profile of a “normal” kidney transplant, and this was done by selecting urines from patients with immediate and persistent good graft function that had normal graft histology on protocol biopsy. This stringently defined control group is unique as it includes histology; other groups attempting similar studies have inferred normal histology from a stable serum creatinine (12,13,16). Indeed, adherence to this stringent definition of “normal” demonstrates that the urine protein profile from 18 of 22 patients (82%) in the stable transplant group was

similar to the urine profile of normal non-transplanted individuals.

The reliable identification of the urine protein pattern of the normal kidney transplant allowed for the clear differentiation, on visual inspection alone, of a distinct urine protein profile in the group with acute rejection (Figure 2). Other groups have used SELDI-TOF-MS to compare the protein profiles between different clinical outcomes, but required bioinformatic analysis to assign protein peaks to a specific outcome (25,26). In a similar study to ours, Clarke *et al.* (25) reported differences in the urine profiles between rejection and stable transplants; however, their requirement of bioinformatics to do so may relate to the fact that their definition of “stable” transplants was less stringent than ours (*i.e.*, based on serum creatinine alone). Interestingly, the protein peaks reported in their paper as specific to rejection are different from those found by our group. This may be related to the different protein chip surfaces and experimental conditions that were utilized; but also, to the fact that Clarke *et al.* (25) failed to include any control populations (*e.g.*, ATN, recurrent or *de novo* glomerulopathies, UTI, CMV) in the analysis, the importance of which is discussed below. In another study, Petricoin *et al.* (26) have used SELDI-TOF-MS to compare the protein profiles between different clinicopathologic diagnoses in cases of ovarian cancer, but also required bioinformatic analysis to assign peaks to specific outcomes. In their study the analysis involved serum samples, which is clearly a more complex biologic fluid than urine. Indeed, the urine-based proteomics has the advantage of excluding most of the serum proteins from the urine due to the size/charge selectivity of the glomerular basement membrane.

Urine profiles of the various groups could have been altered by the procedures of urine collection and storage. Due to the fact that all urine samples were stored non-centrifuged, the rejection pattern may have derived from intracellular proteins of leukocytes, red blood cells (rbc), or epithelial cells released after a freeze-thaw cycle. Interestingly, in one of the rejection cases, we found that lysis of rbc prevented the detection of the rejection pattern due to ion suppression. However, pre-centrifugation to remove the rbc before freeze-thawing of this sample allowed the rejection pattern to be detected (data not shown). Therefore, this argues that the pattern is not necessarily derived from cell lysis associated with a freeze-thaw cycle.

Although there were significant differences in the urine profiles between the stable transplant and the acute clinical rejection groups, there were also one “false negative” and four “false positives” samples. The only patient with the “false negative” urine profile in the acute clinical rejection group had no specific clinical or demographic feature. He had a course of a subclinical rejection (ai3t3g0v0) followed by a clinical rejection (ai3t3g0v1)—both treated with oral high dose steroids—and returned to normal histology (ai0t1g0v0) 15 wk later. We found no obvious explanation for this “false negative” result. Theoretically, a low protein concentration in dilute urine may influence the ability to detect a rejection pattern. However, the protein concentration of the urine samples from the stable transplant and the acute clinical rejection group were similar, making inadequate protein load an unlikely explana-

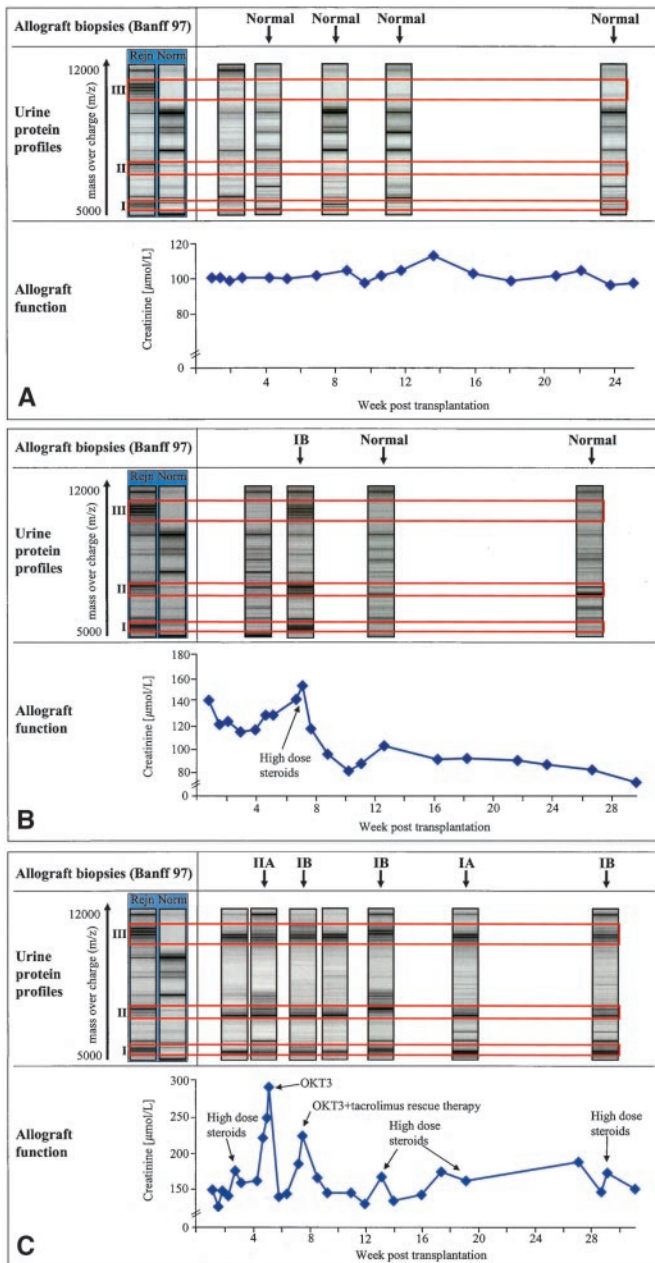


Figure 3. Sequential urine protein profiles in representative patients. Urine protein profiles highlighted in the blue box are examples of the rejection (Rejn) and the normal (Norm) pattern for comparison. Red box frames indicate the three regions corresponding to m/z values of 5270 to 5550 (region I), 7050 to 7360 (region II), and 10530 to 11100 (region III). (A) Patient with stable allograft function, normal protocol allograft biopsies, and normal pattern urine protein profiles throughout the posttransplant course. (B) Patient with acute clinical rejection (Banff IB) on week 7 posttransplant. After treatment with high-dose oral steroids, the serum creatinine normalized and remained stable. Subsequent allograft biopsies were normal. The urine protein profile showed the normal pattern 3 wk before the rejection episode, changed to the rejection pattern at the time of rejection, and returned to the normal pattern consistent with the subsequent allograft biopsies and the allograft function. (C) Patient with recurrent acute clinical rejection episodes (Banff IA to IIA). Despite treatment with OKT3, high-dose steroids and increased baseline immunosuppression, the patient always exhibited the rejection pattern.

tion for the absence of the rejection pattern. The four patients with “false positive” urine profiles in the stable transplant group also had no specific clinical or demographic features at the time of the biopsy. However, one of them went on to subclinical rejection (ai1t3g0v0) 9 wk later, and one experienced an acute clinical rejection and polyomavirus-type BK-nephropathy 13 wk later. The other two patients had a normal transplant course with stable graft function. There are mainly two possible explanations for these “false positive” results: first, they are true “false positives” and we cannot explain why; second, they are not “false positives,” as the urine profile may be detecting an early rejection process that was missed by the allograft biopsy (*i.e.* sampling error) (10,11).

The urine protein profile in the ATN and glomerulopathy groups did not show the pattern of rejection. Both ATN and glomerulopathies are important in the differential diagnosis of allograft dysfunction and may represent pathophysiologic models of allograft injury distinct from that due to the alloimmune response. Whereas ATN can be regarded as a model of injury to the tubules due to ischemia-reperfusion, in the glomerulopathies, the injury, although presumably immune in nature, is largely centered on the glomerular capillary. As these two pathologic states did not show the characteristic pattern of rejection, we infer that the urine proteins detected in acute rejection are related to recipient immune cells infiltrating the graft and/or to tubular epithelial cells that are involved in the allo-directed inflammation. We acknowledge, however, that we cannot exclude the possibility that the urine proteins associated with rejection may also be found in other causes of tubular-based pathology (*i.e.*, calcineurin-inhibitor-toxicity, polyomavirus type BK-nephropathy, pyelonephritis). These latter outcomes are of relatively lower frequency in our patient population, such that we were unable to generate pure examples of each in sufficient number to make any reliable conclusions. Indeed, it is notable that only one patient (0.5%) in our patient population ($n = 212$) developed polyomavirus type BK-nephropathy, which is a much lower incidence than reported from another center (8%) (27).

An additional potential confounder of the diagnostic specificity of the urine protein profile observed in allograft rejection is systemic inflammation that could lead to the filtration of inflammatory proteins (*e.g.*, chemokines, cytokines) by the transplant kidney. Posttransplant CMV viremia, which has a high incidence in kidney transplant recipients (28,29) but very rarely infects the allograft (30,31), is one of the most common causes of systemic inflammation posttransplant. Indeed, our group has previously reported that CMV viremia is a significant confounding variable when examining activated T cells in the circulation as a possible non-invasive correlate of biopsy-proven allograft rejection (32). In the current study, we found no correlation between CMV viremia and the urine profile of rejection, which argues against systemic inflammation associated with CMV viremia as a significant confounding factor. While this does not rule out the possibility that other systemic inflammatory processes may mimic the urine profile seen in allograft rejection, it suggests that this is probably less likely.

It was of interest that the protein profile of rejection was

similar regardless of the histologic severity (Banff IA *versus* IB) or type (Banff IA/B *versus* IIA). This finding might represent a relative limitation of the technique of urine proteomics in identifying biomarkers specific for tubulointerstitial *versus* vascular rejection. However, because the assignment of histologic severity/type of acute rejection is based on a small biopsy sample of a large organ, urine profiling, which is representative of the entire allograft, may be pointing to the extent of heterogeneity of inflammation within the allograft, a fact that renal transplant pathologists are well aware of (11).

The correlation between the changes in serial urine profiles and the clinicopathologic course of the patients provided additional support that the detected proteins are related to acute allograft rejection. However, we do not propose the SELDI-TOF-MS spectra as a diagnostic test, but rather as a tool to detect proteins that are specifically involved in the pathogenesis of rejection. In addition, the patient selection criteria set for this study reflect the extremes of the rejection spectrum (stable transplant *versus* acute clinical rejection) rather than the whole spectrum seen in regular clinics. Therefore, we have avoided calculation of parameters that characterizes a clinical test (*e.g.*, sensitivity, specificity, positive and negative predictive value) because we regarded this as potentially misleading.

Clearly the isolation and identification of the urine proteins associated with acute clinical rejection is the next step. In terms of diagnostics, once they are identified, simple specific assays (*e.g.*, ELISA) may be developed to monitor the graft. In our study, the fact that the protein profiles were visually distinct between the normal and rejection pattern supports the possibility that an ELISA may detect significant quantitative differences. Finally, once validated in a larger patient population, the greatest utility of such a non-invasive biomarker may be to determine that the urine profile is normal, and by inference that the allograft is devoid of rejection. This may allow for tapering of immunosuppression, whereas an abnormal urine profile may warrant further investigation.

In conclusion, we have demonstrated that a proteomic technique, together with stringent patient selection based on allograft histology, allograft function, and clinical course, has the potential to detect a urine protein profile associated with acute renal allograft rejection. Our current efforts will concentrate on the identification of these proteins to develop a clinical test to non-invasively monitor the renal allograft post-transplant.

Acknowledgments

We thank Elzbieta Stern, Iga Dembinski, and the staff of the renal transplant unit for their help with urine specimen and clinical data collection. This work is supported by grants from the Canadian Institute for Health Research (PN, JW, DR). SS is supported by training awards from the Swiss National Foundation (81-BS-68421) and the Novartis Stiftung (Basel, Switzerland).

References

- 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* 342: 605–612, 2000
- Agodoa L, Eknoyan G, Ingelfinger J, Keane W, Mauer M, Mitch W, Striker G, Wilcox C: Assessment of structure and function in progressive renal disease. *Kidney Int Suppl* 63: S144–S150, 1997
- Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB: Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 346: 580–590, 2002
- Meier-Kriesche HU, Ojo AO, Hanson JA, Cibrik DM, Pugh JD, Leichtman AB, Kaplan B: Increased impact of acute rejection on chronic allograft failure in recent era. *Transplantation* 70: 1098–1100, 2000
- Almond PS, Matas A, Gillingham K, Dunn DL, Payne WD, Gores P, Gruessner R, Najarian JS: Risk factors for chronic rejection in renal allograft recipients. *Transplantation* 55: 752–756, 1993
- Wilczek HE: Percutaneous needle biopsy of the renal allograft. A clinical safety evaluation of 1129 biopsies. *Transplantation* 50: 790–797, 1990
- Kolb LG, Velosa JA, Bergstralh EJ, Offord KP: Percutaneous renal allograft biopsy. A comparison of two needle types and analysis of risk factors. *Transplantation* 57: 1742–1746, 1994
- Rush DN, Henry SF, Jeffery JR, Schroeder TJ, Gough J: Histological findings in early routine biopsies of stable renal allograft recipients. *Transplantation* 57: 208–211, 1994
- Shapiro R, Randhawa P, Jordan ML, Scantlebury VP, Vivas C, Jain A, Corry RJ, McCauley J, Johnston J, Donaldson J, Gray EA, Dvorchik I, Hakala TR, Fung JJ, Starzl TE: An analysis of early renal transplant protocol biopsies—the high incidence of subclinical tubulitis. *Am J Transplant* 1: 47–50, 2001
- Sorof JM, Vartanian RK, Olson JL, Tomlanovich SJ, Vincenti FG, Amend WJ: Histopathological concordance of paired renal allograft biopsy cores. Effect on the diagnosis and management of acute rejection. *Transplantation* 60: 1215–1219, 1995
- Nicholson ML, Wheatley TJ, Doughman TM, White SA, Morgan JD, Veitch PS, Furness PN: A prospective randomized trial of three different sizes of core-cutting needle for renal transplant biopsy. *Kidney Int* 58: 390–395, 2000
- Li B, Hartono C, Ding R, Sharma VK, Ramaswamy R, Qian B, Serur D, Mouradian J, Schwartz JE, Suthanthiran M: Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* 344: 947–954, 2001
- Ding R, Li B, Muthukumar T, Dadhania D, Medeiros M, Hartono C, Serur D, Seshan SV, Sharma VK, Kapur S, Suthanthiran M: CD103 mRNA levels in urinary cells predict acute rejection of renal allografts. *Transplantation* 75: 1307–1312, 2003
- Roberti I, Reisman L, Burrows L, Lieberman KV: Urine cytology and urine flow cytometry in renal transplantation—a prospective double blind study. *Transplantation* 59: 495–500, 1995
- Gebauer BS, Hricik DE, Atallah A, Bryan K, Riley J, Tary-Lehmann M, Greenspan NS, DeJelo C, Boehm BO, Hering BJ, Heeger PS: Evolution of the enzyme-linked immunosorbent spot assay for post-transplant alloreactivity as a potentially useful immune monitoring tool. *Am J Transplant* 2: 857–866, 2002
- Najafian N, Salama AD, Fedoseyeva EV, Benichou G, Sayegh MH: Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: Potential novel assay for prediction of outcomes for renal transplant recipients. *J Am Soc Nephrol* 13: 252–259, 2002
- Scherer A, Krause A, Walker JR, Korn A, Niese D, Raulf F: Early prognosis of the development of renal chronic allograft rejection by gene expression profiling of human protocol biopsies. *Transplantation* 75: 1323–1330, 2003

18. Eikmans M, Sijpkens YW, Baelde HJ, De Heer E, Paul LC, Bruijn JA: High transforming growth factor-beta and extracellular matrix mRNA response in renal allografts during early acute rejection is associated with absence of chronic rejection. *Transplantation* 73: 573–579, 2002
19. Stegall M, Park W, Kim D, Kremers W: Gene expression during acute allograft rejection: Novel statistical analysis of microarray data. *Am J Transplant* 2: 913–925, 2002
20. Chua MS, and Sarwal MM: Microarrays: New tools for transplantation research. *Pediatr Nephrol* 18: 319–327, 2003
21. Knepper MA: Proteomics and the kidney. *J Am Soc Nephrol* 13: 1398–1408, 2002
22. Hanash S: Disease proteomics. *Nature* 422: 226–232, 2003
23. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Yamaguchi Y: The Banff 97 working classification of renal allograft pathology. *Kidney Int* 55: 713–723, 1999
24. Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney Int* in press
25. Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP: Characterization of renal allograft rejection by urinary proteomic analysis. *Ann Surg* 237: 660–665, 2003
26. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA: Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359: 572–577, 2002
27. Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, Steiger J: Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med* 347: 488–496, 2002
28. Sagedal S, Nordal KP, Hartmann A, Degre M, Holter E, Foss A, Osnes K, Leivestad T, Fauchald P, Rollag H: A prospective study of the natural course of cytomegalovirus infection and disease in renal allograft recipients. *Transplantation* 70: 1166–1174, 2000
29. Abecassis MM, Koffron AJ, Kaplan B, Buckingham M, Muldoon JP, Cribbins AJ, Kaufman DB, Fryer JP, Stuart J, Stuart FP: The role of PCR in the diagnosis and management of CMV in solid organ recipients: what is the predictive value for the development of disease and should PCR be used to guide antiviral therapy? *Transplantation* 63: 275–279, 1997
30. Andersen CB, Ladefoged SD, Lauritsen HK, Hansen PR, Larsen S: Detection of CMV DNA and CMV antigen in renal allograft biopsies by in situ hybridisation and immunohistochemistry. *Nephrol Dial Transplant* 5: 1045–1050, 1990
31. Kashyap R, Shapiro R, Jordan M, Randhawa PS: The clinical significance of cytomegaloviral inclusions in the allograft kidney. *Transplantation* 67: 98–103, 1999
32. Karpinski M, Rush D, Jeffery J, Pochinco D, Milley D, Nickerson P: Heightened peripheral blood lymphocyte CD69 expression is neither sensitive nor specific as a noninvasive diagnostic test for renal allograft rejection. *J Am Soc Nephrol* 14: 226–233, 2003