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Urine Immunocytology as a Noninvasive Diagnostic Tool for Acute Kidney Rejection: a Single Center Experience

Karlo Mihovilović[1], Ika Kardum-Skelin[2,5], Danica Ljubanović[3,5], Mirjana Sabljar-Matovinović[1,5], Željko Vidas[4] and Mladen Knotek[1,5]

[1] Department of Medicine, University Hospital »Merkur«, Zagreb, Croatia
[2] Laboratory for Cytology and Hematology, University Hospital »Merkur«, Zagreb, Croatia
[3] Department of Pathology, University Hospital Dubrava, Zagreb, Croatia
[4] Division of Urology, University Hospital »Merkur«, Zagreb, Croatia
[5] University of Zagreb, School of Medicine, Zagreb, Croatia

A B S T R A C T

Renal biopsy is a gold standard for establishing diagnosis of acute rejection of the renal allograft. However, being invasive, renal biopsy has potential significant complications and contraindications. Therefore, possibility to noninvasively diagnose acute rejection would improve follow-up of kidney transplant patients. The purpose of this study was to evaluate urine immunocytology for T cells as a method for noninvasive identification of patients with acute renal allograft rejection in comparison to renal biopsy. In this prospective study a cohort of 56 kidney, or kidney-pancreas transplant recipients was included. Patients either received their transplant at the University Hospital »Merkur«, or have been followed at the »Merkur« Hospital. Patients were subject to either protocol or indication kidney biopsy (a total of 70 biopsies), with simultaneous urine immunocytology (determination of CD3-positive cells in the urine sediment). Acute rejection was diagnosed in 24 biopsies. 23 episodes were T-cell mediated (6 grade IA, 5 grade IB, 1 grade IIA, 1 grade III and 10 borderline), while in 1 case acute humoral rejection was diagnosed. 46 biopsies did not demonstrate acute rejection. CD3-positive cells were found in 21% of cases with acute rejection and in 13% of cases without rejection (n.s.). A finding of CD3-positive cells in urine had a sensitivity of 21% and specificity of 87% for acute rejection (including borderline), with positive predictive value of 45% and negative predictive value of 68%. Although tubulitis is a hallmark of acute T cell-mediated rejection, detection of T cells in urine sediment was insufficiently sensitive and insufficiently specific for diagnosing acute rejection in our cohort of kidney transplant recipients.

***Key words**: renal transplantation, acute rejection, immunocytology, urinary sediment*

Introduction

Kidney transplantation is a standard replacement therapy for end stage kidney disease, with approximately 150 kidneys annually transplanted in Croatia[1]. Despite modern immunosuppressive therapy, acute rejection (AR) still remains an important problem. In the first year after kidney transplantation incidence of AR is 10–15%[2]. Unrecognized and untreated AR adversely impacts graft function and graft survival[2]; therefore it is of paramount importance to recognize AR and start treatment early and adequately.

Detection of AR is presently based on monitoring serum creatinine with kidney biopsy as a gold standard. However, serum creatinine is an insufficiently sensitive and late indicator of kidney function, because glomerular filtration rate (GFR) can decline more than 50% without significant changes in concentration of the serum creatinine[3]. Unfortunately, percutaneous renal biopsy under ultrasound guidance, although very sensitive and specific for diagnosis of renal pathology, is invasive procedure and therefore has some contraindications and side ef-

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fects. In addition, although quite safe, it is associated with some morbidity and even graft loss.[2,4] Another problems associated with renal pathohistology are focality of AR, with consequent sampling error, and interpathologist variation in interpretation of pathohistology.[5,6] Therefore, investigators have been searching for biomarkers that would be noninvasive and highly sensitive and specific for diagnosis of AR. Many molecules, from blood and urine, that reflect activation of the immune system, have been proposed and evaluated as potential biomarkers for AR.[7–10] Immunocytological staining of urinary sediment has also been evaluated as a method for detection of AR in transplant patients in several studies.[7,11,12] In some of these studies high sensitivity and specificity of immunocytological staining for CD3-positive cells for AR was reported.[7,11]

The aim of the present study was to evaluate our experience in using urine immunocytology for diagnosing AR in kidney transplant patients.

Participants and Methods

Patients

This study is a part of the prospective, single center study on immune monitoring of the kidney transplant recipients at the University Hospital »Merkur«, Zagreb, Croatia. The study was approved by the University Hospital »Merkur« ethical committee, and patients gave their consent. For the present analysis we included 56 renal transplant patients who had renal biopsy in parallel with urine immunocytology. Biopsy and urine cytology were performed either per protocol (1, 3 and 6 months post transplant), or for cause, in case of not otherwise explained impairment of graft function. One patient could have more than one analysis. Therefore, number of urine cytology analyses and renal biopsies (70) is greater than the number of patients.

Renal pathohistology

Renal tissue was analyzed by the light microscopy and by the immunoflouorescence for c4d staining of the peritubular capillaries. Pathohistological changes were classified according to the Banff 97 classification and its later updates (Table 1).[5,6]

Urine cytology

Urine samples were collected within two days before or after renal biopsy, but prior to any antirejection treatment. Freshly voided urine samples were centrifuged for 5 min at 600 rpm in a cytocentrifuge. Air-dried sediments were fixed in cold acetone for 2 min followed by washing in TBS (pH 7.6) for 1 min After that they were incubated with primary anti-CD3 monoclonal antibody (Dako) for 15 min at room temperature. After further washing in TBS for 15 min, sediment was incubated with streptavidin for 15 min, washed in TBS for 5 min, and than incubated with chromogen for 4 min.

Statistical analysis

Statistica software version 7.1 (StatSoft, Tulsa, USA) was used for statistical analysis. Medians and ranges were used for continuous data that did not have normal distribution. Differences between the groups were analyzed by the Fisher's exact test for categorical data and by the Mann-Whitney U-test for continuous and ordinal data. p-value of <0.05 was considered to be statistically significant.

Results

Patients characteristics are shown in Table 2. Among total of 70 kidney biopsies there were 24 cases of AR, while in 46 cases other pathohistological diagnosis was obtained. Characteristics of patients with and without AR are shown in Table 3.

TABLE 1
BANFF CLASSIFICATION OF RENAL ALLOGRAFT REJECTION- BANFF 07 UPDATE

Normal
Antibody mediated changes
I. Acute tubular necrosis-like mediated changes
II. Capillary and or glomerular inflammation and/or thrombosis
III. Arterial inflammation
Borderline changes
T-cell mediated rejection
1. IA. Cases with significant interstitial in?ltration (>25% of parenchyma affected) and foci of moderate tubulitis
2. IB. Cases with significant interstitial in?ltration (>25% of parenchyma affected) and foci of severe tubulitis
3. IIA. Cases with mild-to-moderate intimal arteritis
4. IIB. Cases with severe intimal arteritis comprising >25% of the luminal area
5. III. Cases with 'transmural' arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation
Other: Changes not considered to be due to rejection – acute and/or chronic

The distribution of cases in AR rejection group according to the Banff classification was as follows: T-cell--mediated acute rejection in 23 cases (borderline 10, grade IA 6, grade IB 5, grade IIA 1, grade III 1) and acute antibody-mediated rejection in 1 case. There was no statistically significant difference between AR and non rejection patients regarding total HLA mismatch, as well as regarding HLA-B and HLA-DR mismatch (p= n.s. for all comparisons, Mann Whitney U test). In AR group 21% of the cases had CD3-positive cells in urine, whereas in the non-rejecting group that percentage was 13 (p= n.s., Fisher's exact test). Specificity and sensitivity for establishing diagnosis of AR based on CD3 positive cells (Figure 1) is shown in Table 4.

Exclusion of patients with borderline rejection from the AR group did not influence results (data not shown). Although CD3-positivity was associated with shorter time after transplant, as compared with CD3-negative cases in both groups, this did not reach statistical significance. In AR group CD3-positivity occurred 30 (10–109)

TABLE 2
PATIENTS CHARACTERISTICS

Number of patients	56
Gender (m/f)	32/24
Age at transplant (years); median (range)	43 (16–69)
HLA mismatch; median (range)	3 (0–6)
HLA-B MM	1 (0–2)
HLA-DR MM	1 (0–2)
Donor type	
Deceased	38
Living	18
Age of donor (years) mean (range)	41 (17–73)
Cause of ESRD	
Diabetes mellitus	15
Glomerulonephritis	19
Hypertension	2
Other/unknown	20

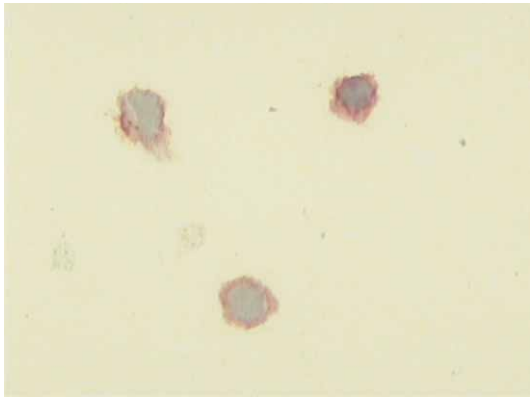

Fig. 1. CD3-positive lymphocytes in urine (LSAB, x1000).

TABLE 3
CHARACTERISTICS OF AR AND NON-REJECTING GROUP

	AR	no rejection
Number of patients	23	33
Gender (m/f)	14/9	18/15
Age at transplant (years) mean (range)	46 (16–67)	42 (26–69)
Time of first biopsy after Tx (days)	59 (10–698)	97 (2–1071)
HLA mismatch of patients; median (range)	4 (1–6)	3 (0–6)
HLA-B mismatch	1 (0–2)	1 (0–2)
HLA-DR mismatch	1 (0–2)	1 (0–2)
Donor type		
Deceased	13	25
Living	10	8
Age of donor (years); median (range)	43 (18–71)	40 (17–73)
Cause of ESRD		
Diabetes mellitus	6	9
Glomerulonephritis	7	13
Hypertension	2	0
Other/unknown	8	11
Immunosuppressant drugs		
Induction (ATG/daclizumab)	2/21	4/29
Maintenance (MPA/tacrolimus/ciklosporin/corticosteroids)	21/13/8/18	33/20/12/25

TABLE 4
SENSITIVITY, SPECIFICITY AND POSITIVE AND NEGATIVE PREDICTIVE VALUE OF CD-3 POSITIVITY OF URINARY LYMPHOCYTES FOR DIAGNOSIS OF AR

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
CD3-positive cells	21	87	45	68

days after transplantation, while CD3-negative cases were 101 (12–698) days after transplantation (p=0.086, Mann Whitney U-test). Similarly, in non-rejection group CD3-positivity occurred 33 (9–366) days after transplantation and CD3-negative samples were 106 (2–1071) days after transplantation (p=0.313, Mann Whitney U test).

Discussion

Kidney transplant patients who suffer from AR are at increased risk for decreased graft function and graft loss.[13,15] However, early detection and treatment of AR has been shown to decrease renal allograft injury, leading to non- -inferior long-term graft survival, if after an AR episode renal function returned to the baseline value.[15] Although it is highly sensitive and specific, renal biopsy is invasive, and it is impractical to perform it frequently due to associated inconvenience, morbidity and cost.[4,16] To overcome these limitations of allograft biopsy many attempts have been made to establish surrogate markers for AR. However, none of these new biomarkers have made their way into clinical practice as a universally accepted diagnostic tool, because of inferior performance in comparison to biopsy.[8]

Native urine cytology was previously shown to detect an increased frequency of lymphocytes and monocytes in urine during AR[17–19]. However, it has been abandoned as a screening method for AR, because it showed poor sensiivity and specificity[11]. An improved performance was achieved by employing urine immunocytology with identification of different subpopulations of lymphocytes in urine. Since it has been reported that 30–50% of cells that infiltrate the kidney during AR are CD3-positive lymphocytes, with intratubular infiltration (tubulitis) as a hallmark, several studies have been published on the performance of urine immunocytology for CD3 in diagnosing AR. Some of the previous studies showed sensitivity and specificity for that method of 79–87% and 95–97%, respectively.[7,11] Authors of these studies proposed that method as a fast, simple and non-invasive procedure that allows easy follow up of transplanted patients.

Based on these results we started immune monitoring program of kidney transplant recipients based on urinary cytology and immunocytochemistry at our center. However, in our cohort of patients with transplanted kidney or kidney and pancreas, sensitivity of only 21% with specificity of 87% of CD3 staining of urinary lymphocytes for diagnosis of AR was observed. From these results it is evident that presence of CD3 lymphocytes in urine may not correlate well with AR based on Banff criteria. Our results are different from those in other reported studies.[7,11] Reasons for that discrepancy are unknown but maybe attributed to several technical factors, besides the possibility that urinary immunocytology for CD3 cells is in fact insufficiently sensitive. Key technical issue that might have contributed to lower sensitivity in our study was somewhat different processing of urine for immunocytological staining, using smaller urine volume, than in some other reported studies. However, increased urine volume would probably further decrease specificity of CD3 staining for diagnosis of AR. Namely, it has been reported that CD3-positive lymphocytes can be present in urine even in normal graft during the first week after transplantation, due to ischemia-reperfusion injury, associated with transplant procedure, which could contribte to a decreased specificity.[7] Although in our study there was similarly a clear trend that patients with CD3-positive cells without AR were earlier after transplantation than those with negative CD3 cells in urine, CD3-positivity occurred well after the first week after transplantation. Another reason for a low specificity may result from urinary infections, which may also be associated with findings of CD3-positive lymphocytes in urine.[7] That was, however, not a case in our study, as there were no patients with active urinary infection included in the study. Thus, relatively low specificity of urinary CD3 staining is probably intrinsic limitation of the test. Attempts to increase performance of urine immunocytology consist of inclusion of more markers, such as a panel of three or more antibodies for specific subpopulations of T lymphocytes. A recent study reported highly increased sensitivity and specificity with such approach.[11] It may also prove that a combination of urine lymphocyte subset analysis with some of the recently investigated urinary biomarkers, such as perforin, granzyme B, or FOXP3, among the others, would demonstrate sufficient diagnostic and prognostic value in kidney transplant patients with AR[20–22]. However, further prospective studies are necessary to confirm this.

An interesting side finding in our study is a lack of associatian of HLA mismatch with occurrence of AR. This probably reflects diminished significance of HLA matching in face of modern immunosupression and further justifies our approach to kidney transplantation by not absolutely observing histocompatibility, especially in living donor kidney transplantation.

Conclusion

Based on our results, early enthusiasm about using urine cytology to diagnose AR seems unjustified, especially because of insufficient sensitivity of urine immunocytology. While it is vital to achieve high sensitivity, somewhat lower specificity may not represent a problem

in screening purposes. In that case finding of CD3-positive cells (or other biomarker) in urine should lead to renal biopsy, if urinary infection is excluded. Kidney biopsy remains gold standard for detection of AR. Urine immunocytology may have potential to become one of the screening methods for detection of AR in kidney transplant patients, if substantial improvements in sensitivity of this method could be made.

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M. Knotek

University Hospital »Merkur«, Zajčeva 19, Zagreb 10000, Croatia
e-mail: mladen.knotek@zg.t-com.hr

IMUNOCITOLOGIJA URINA KAO NEINVAZIVNI DIJAGNOSTIČKI POSTUPAK ZA OTKRIVANJE AKUTNOG ODBACIVANJA BUBREGA: ISKUSTVO KB »MERKUR«

SAŽETAK

Biopsija bubrega je zlatni standard za postavljanje dijagnoze akutnog odbacivanja bubrega u transplantaciji bubrega. Zbog svoje invazivnosti, biopsija bubrega ima kontraindikacije i značajne nuspojave. Pronalazak neinvazivne metode za dijagnosticiranje akutnog odbacivanja unaprijedilo bi praćenje transplantiranih pacijenata. Cilj ove studije je bio procjena pouzdanosti immunocitologije urina na T-limfocite,u usporedbi sa biopsijom bubrega, u postavljanju dijagnoze akutnog odbacivanja u pacijenta s transplantiranim bubregom. U ovu prospektivnu studiju uključeno je 56 pacijenata s transplantiranim bubregom ili bubregom i gušteračom. Pacijenti su ili transplantirani u Kliničkoj bolnici »Merkur«, ili su samo praćeni u Kliničkoj bolnici »Merkur« nakon transplantacije u drugoj bolnici. Pacijentima su rađene protokol ili indikacijske biopsije (ukupno 70 biopsija) uz istovremenu imunocitologiju urina (određivanje CD3--pozitivnih stanica u sedimentu urina). Akutno odbacivanje je nađeno u 24 biopsije. 23 epizode odbacivanja su bile T-stanično posredovane (6 stupanj IA, 5 stupanj IB, 1 stupanj IIA, 1 stupanj III i granično 10 epizoda), dok je u jednom slučaju dijagnosticirano akutno humoralno odbacivanje. U 46 bioptičkih uzoraka nije dijagnosticirano akutno odbacivanje. CD-3 pozitivne stanice su nađene u 21% slučajeva s prisutnim akutnim odbacivanjem u biopsiji, dok je u skupini bez akutnog odbacivanja taj pozivitet je bio 13% (n.s.). Pronalazak CD-3 pozitivnih stanica u urinu imalo je osjetljivost od 21%, specifičnost od 87%, pozitivnu prediktivnu vrijednost od 45% i negativnu prediktivnu vrijednost od 68% za dijagnozu akutnog odbacivanja. Iako je tubulitis značajka akutnog odbacivanja posredovanog T-stanicama, detekcija CD-3 pozitivih stanica u sedimentu urina pokazala je nedostatnu osjetljivost i specifičnost za dokazivanje akutnog odbacivanja u našoj kohorti pacijenata sa transplantiranim bubregom.