Lectin staining for urine cytologic monitoring after kidney transplantation

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Abstract

Background. Urine cytology, although considered a valuable diagnostic tool in the monitoring of kidney graft function, is hampered by difficulty in differentiating the nucleated non-squamous cells in urine using conventional techniques. We have now developed a method for the simple identification of urinary cell types by lectin staining.

Methods. Acetone-fixed cytopreparations of urinary sediments were incubated with the lectin combination Sophora Japonica agglutinin (SJA; rhodamine-labelled) and Erythrina cristagalli agglutinin (ECA; fluorescein isothiocyanate (FITC)-labelled) for 15 min, followed by staining of the nuclei with 4,6-diamidino-2-phenylindole (DAPI). The courses of 38 patients were serially monitored after kidney transplantation during the period in hospital.

Results. Nucleated urinary cell types could be easily identified from one specimen by their characteristic lectin-binding pattern using triple-immunofluorescence microscopy (FITC + rhodamine + ultra violet), permitting a differentiation between proximal (SJA + / ECA +) and distal tubules (SJA − / ECA +), collecting ducts (SJA + / ECA −) and lymphocytes (SJA − / ECA −). Stable graft function was characterized by low numbers of lymphocytes, tubular cells and urothelia. During rejection episodes, but not graft dysfunction unrelated to rejection, urinary excretion of lymphocytes as well as of distal tubular cells (from 1.0 to 6.0 and from 1.4 to 4.0 per 10 high-power fields, respectively) increased significantly up to 3 days prior to clinical diagnosis.

Conclusions. Lectin staining facilitates unambiguous differentiation of the urinary cell types, in particular the various tubular epithelial cells, which are otherwise difficult to identify. This technique provides a rapid and easily applicable tool to evaluate the significance of the respective cell types in the monitoring of kidney graft function.

Keywords: graft dysfunction; kidney transplantation; lectin; rejection; Tx monitoring; urine cytology

Introduction

Allograft dysfunction is the most common complication of renal transplantation. The early detection and classification of this dysfunction, together with the immediate adoption of appropriate measures is necessary to prevent further deterioration of graft function. Monitoring of graft function should be effective and should not involve undue stress for the recipient, i.e. it should be non-invasive. It normally comprises evaluation of clinical symptoms, laboratory parameters such as plasma creatinine, and the examination of graft perfusion by Doppler ultrasound. Several studies have also suggested that urine cytology, considering either the whole panel of urinary cells or selected cellular subsets, is a useful, non-invasive approach to evaluate allograft function. For example, early detection of graft rejection by this technique has been reported [1–10]. Although these studies have suggested urine cytology as an efficient, non-invasive tool to monitor kidney graft function after transplantation, its broad clinical application has been predominantly hampered by the difficulty in the differentiation of the various cell populations. This is primarily due to the fact that the differentiation of cellular elements in urine by conventional techniques such as bright field or phase-contrast microscopy, even after Papanicolaou staining, is relatively difficult and requires skilled examiners [11]. Therefore, this technique has so far found application as a routine diagnostic tool only in a limited number of specialized centres. We have now evaluated the use of lectin staining for the
differentiation of nucleated cells in the urinary sediment. The lectin combination of *Sophora Japonica* agglutinin (SJA) and *Erythrina cristagalli* agglutinin (ECA) permitted the easy differentiation of lymphocytes and the cells of the proximal tubule, distal tubule and collecting duct.

Examination of the course of 38 patients after kidney transplantation with lectin staining allowed an easily applicable and unambiguous differentiation of urinary cellular elements, especially of the otherwise difficult to discern tubular epithelial cells.

**Subjects and methods**

**Patients**

A consecutive series of 38 renal allograft recipients (13 females, 25 males) with an age range of 14–76 years were studied. All were given cyclosporin or tacrolimus and steroids as basic immunosuppression. Rejection episodes were initially treated with methylprednisolone pulse therapy, followed by OKT3 (anti-CD3; Ortho, NJ, USA) and/or ATG if the initial therapy proved ineffective.

Allograft biopsies were taken in all cases of unexplained rises in plasma creatinine. Rejections were classified according to the Banff scheme [12]. Five rejection episodes were classified as Banff 4.1 and four as Banff 4.2; in three cases a classification was not possible due to the lack of arteries. Rejection episodes were clinically defined as the period between the first rise in plasma creatinine (>0.2 mg/dl within 24 h; the biopsy was usually taken at this time-point) and the return to a stable creatinine value.

Renal dysfunction unrelated to acute rejection was due to cytomegalovirus (CMV) infection (two patients, diagnosed by pp65 immunostaining and reverse transcription polymerase chain reaction), acute tubular necrosis (ATN; four patients, diagnosed by biopsy) and stenosis of the lower urinary tract (one patient).

**Preparation of urine specimens**

Starting on day 4 after transplantation, urine samples were collected from freshly voided morning urine every other day during the hospital stay. Samples were prepared within 4 h of collection. From each sample three specimens were prepared in parallel, and the results were averaged. Cytopreparations of urinary sediments were obtained by concentrating the cellular elements in 10 ml urine by centrifugation at 460 g for 10 min. After carefully decanting the supernatant, the pellet was resuspended in 100 µl phosphate-buffered saline (PBS). Sixty microlitres of this suspension were spun by a cytocentrifuge (Shandon Cytospin 2; Astmoor, UK), at 164 g for 10 min, onto a glass cover slide. Subsequently specimens were fixed with acetone (~20°C, 10 min), washed with PBS, and incubated with a combination of the lectins SJA (rhodamine conjugated) and ECA (fluorescein isothiocyanate (FITC)-conjugated) dissolved in PBS at a concentration of 75 µg/ml for 15 min at room temperature in the dark. After a washing step with PBS, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) (5 µg/ml stock solution in methanol, diluted 1:50 with PBS before use) for 7 min at room temperature. To prevent bleaching, specimens were finally embedded with Mowiol (Calbiochem, La Jolla, CA, USA) (10% (w/v) in 25% (v/v) glycerol pH 8.5 with Tris–HCl).

### Table 1. Binding pattern of the lectins *Sophora Japonica* agglutinin (SJA) and *Erythrina cristagalli* agglutinin (ECA)

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specificity</th>
<th>Gl</th>
<th>PT</th>
<th>tH</th>
<th>TalH</th>
<th>DT</th>
<th>CD</th>
<th>Uro</th>
<th>T-ly</th>
<th>Gr/Mo</th>
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</thead>
<tbody>
<tr>
<td>SJA</td>
<td>β-D-galNAc</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ECA</td>
<td>β-D-gal(14)DglcNAc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Staining urinary sediments with the combination of SJA and ECA, coupled to the fluorescence markers rhodamine and FITC, permitted the differentiation between the major nucleated cell types occurring in the urinary sediment by immunofluorescence microscopy. Gl = glomerulum, PT = proximal tubule, tH = thin limb of Henle, talH = thick ascending limb of Henle, DT = distal tubule, CD = collecting duct, Uro = urothelium, T-ly = T-lymphocytes, Gr = granulocytes, Mo = monocytes. Granulocytes and monocytes exhibited a similar binding pattern with respect to these lectins. +, lectin binds to the indicated structure; -, no lectin binding; (-), very faint lectin binding.
binding pattern as distal tubular cells (SJA +, ECA −). These cells, however, could be relatively easily differentiated from each other. Granulocytes were identified by their segmented nuclei as revealed by DAPI staining with the UV filter, as reported above. Cells of the distal tubule and urothelia could be clearly discriminated by morphological criteria as outlined, for example, by Schumann and Weiss [16] and Piccoli et al. [17]. Distal tubular cells are irregularly polygonal, cuboid or faceted, whereas cells of the urothelium are rounded, raquet-shaped or flat, umbrella-shaped.

For each specimen, the numbers of cells in 10 high-power fields (HPF) were counted at 300× magnification. Numbers are given as cells per 10 HPF.

**Statistics**

Because of the skew distribution of the data, cell numbers are given as median and 25% and 75% quantiles. Data were statistically analysed by the Mann–Whitney U-test. Statistical software (Statistica version 5.1; Statsoft, Hamburg, Germany) was used. Receiver operating characteristic (ROC) curve analysis was carried out to test the ability of the various cell types to discriminate between cases of acute rejection and cases without rejection. Urine samples were considered as true positive if they were taken during a biopsy proven rejection episode as defined above, or during a time interval of 3 days prior to the clinical definition of the rejection episode, since an increase in urinary cells had already been observed during this period. All other samples obtained either during stable graft function or dysfunction unrelated to rejection were regarded as true negative. The ROC curve was constructed by plotting diagnostic sensitivity (true positives) against 1-specificity (true negatives) over all possible decision thresholds. The areas under the individual ROC curves were computed using the non-parametric method of Hanley and McNeil [18]. ROC curve analysis was performed using the software package Analyse-IT version 1.44 (Analyse-IT Software Ltd, UK).

**Results**

On examining urinary specimens (n = 1258) using the lectin combination SJA/ECA after kidney transplantation, a definitive identification of nucleated cells in the urinary sediment could be made in 1164 specimens (92.5%). In the remaining specimens, an evaluation was not possible due to the following reasons: massive granulocyturia 3.2%, mucous 1.3%, overlaying by squamous epithelial cells (predominantly in females) 1.2%, complete degeneration of the cells 1.2%, and massive haematuria 0.6%.

With the exception of granulocytes, the excretion of nucleated cells in the urine was generally low during periods of stable transplant function as shown in Table 2. In contrast, during rejection episodes an enhanced excretion of lymphocytes as well as of tubular and initially also of urothelial cells was observed. The increased excretion of tubular cells was primarily due to distal tubular cells rather than proximal tubules or collecting ducts. The time course of the excretion rate of the respective cell types during rejection episodes is shown in Figure 1. As can be seen, excretion of lymphocytes and distal tubular cells showed an increase starting 3 days before the clinical diagnosis of rejection. Both the excretion of lymphocytes and distal tubular cells exhibited their maxima 1 day after the diagnosis of rejection. In contrast to phases of acute rejection, no increased lymphocyturia was observed in periods of graft dysfunction unrelated to acute rejection. The relative frequency of lymphocytes per specimen for periods of stable graft function, acute rejection and impairment of renal function unrelated to acute rejection is shown in Figure 2. During acute rejection episodes a shift of lymphocytes to higher cell numbers was observed. The abilities of either lymphocytes or distal tubular cells to discriminate acute rejection, as defined in Subjects and Methods, from cases without acute rejection were compared using ROC curve analysis (Figure 3). Lymphocytes proved to be highly effective in discriminating acute rejection episodes as shown by the ROC curve, which climbs rapidly towards the upper left-hand corner of the graph, and by the area under the ROC curve of 0.868 (95% confidence interval (CI) 0.789–0.947; P < 0.001). Distal tubular cells were less effective, with an area under the ROC curve of 0.713 (95% CI 0.595–0.832; P = 0.002). When considering only those data points related to an acute rejection episode obtained on days −3 to 0, which are (from a diagnostic point of view) of particular interest, a highly significant association with rejection was still found for lymphocytes (area under the ROC curve 0.845; 95% CI 0.697–0.992; P < 0.0001) but not for distal tubular cells (area under the ROC curve 0.647; 95% CI 0.413–0.881; P = 0.109). With regard to the discrimination of acute rejection, all other cell types investigated were inferior (data not shown). Taking a cutoff of three or more lymphocytes per 10 HPF as indicative of an acute rejection episode, a sensitivity of 80.6% and a specificity of 77.9% was obtained.

### Table 2. Urine cytologic excretion pattern during stable graft function

<table>
<thead>
<tr>
<th>Stable graft function</th>
<th>25% quantile</th>
<th>Median</th>
<th>75% quantile</th>
</tr>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>0.5</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Proximal tubules</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Distal tubules</td>
<td>0.5</td>
<td>1.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Collecting duct cells</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Urothelium</td>
<td>0.3</td>
<td>1.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>18</td>
<td>51</td>
<td>129</td>
</tr>
<tr>
<td>Squamous epithelial cells</td>
<td>0</td>
<td>0.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Excretion pattern of various nucleated cell types, identified by staining the sediment with the lectin combination SJA/ECA, during stable graft function is shown. With the exception of granulocytes, only small numbers of cells were observed per specimen. Numbers are given as cells per 10 HPF.
The increase in cell numbers during the rejection episodes was not due to a concentrating effect of the cells in the urine, since diuresis decreased only moderately from $3213 \pm 70$ ml/day (mean ± SEM) to $2730 \pm 145$ ml/day during acute rejection episodes, whereas the number of lymphocytes and distal tubular cells increased from $1.0/0.5/2.5$ and $1.4/0.5/3.0$ (median/25% quantile/75% quantile) during stable graft function to $6.0/3.8/11$ and $4.0/1.8/9.3$, respectively ($P<0.01$). A similar decrease of diuresis ($2808 \pm 146$ ml/day) was observed in graft dysfunction unrelated to acute rejection without major changes in the excretion rate of lymphocytes and distal tubular cells ($1.6/0.7/3.0$ lymphocytes per 10 HPF, $1.8/0.6/3.0$ distal tubular cells; not significant compared with stable graft function).

Fig. 1. Time course of the excretion of lymphocytes (A), proximal tubular cells (B), distal tubular cells (C), collecting duct cells (D) and urothelial cells (E) during acute rejection episodes. Day 0 is defined as the day on which a rise in plasma creatinine is observed. Values are given as median, 25% and 75% quantile ($n=12$ rejection episodes).
Discussion

In this study, a new technique for the differentiation of urinary cell types using lectin-staining, combined with DAPI staining of the nuclei, is reported. This technique permits rapid and easy differentiation of nucleated cells in urine. It offers several advantages compared with our previous experience with the staining of urinary cellular elements by the Papanicolaou technique [10]. The main advantage is the unambiguous identification of cellular elements, especially of tubuloepithelial origin, which allows even relatively unskilled examiners to quickly and easily differentiate between urinary cells. Furthermore, the preparation of specimens is simple and rapid, requiring only two short incubation and washing steps after specimen fixation. According to the outlined criteria, lectin staining permitted a differentiation of all of the major urinary cell types within one specimen, in contrast to staining with antibodies, which is restricted to few cell types even after double labelling. Lectin staining is more rapid and usually cheaper than using antibodies, which could be advantageous for the study of the excretion of a particular cell type or the expression of a special antigen. The clinical utility of this lectin staining procedure was examined in a series of 38 patients after kidney transplantation. The most common cause of graft dysfunction in this group was acute rejection. Evaluating the urinary sediment in this condition showed, in agreement with previous urine cytological findings [1,3,4,10,19,20], a significant increase of lymphocyturia up to 3 days prior to the clinical diagnosis of rejection. This technique allowed the clear differentiation of lymphocytes from tubuloepithelial cells. Large lymphocytes in particular can be easily confused with tubular cells when applying only morphological criteria [17]. No increase in lymphocyte excretion was observed in graft dysfunction unrelated to rejection episodes. A similar decrease in diuresis was observed in graft rejection both related and unrelated to rejection, which demonstrates that the increase of lymphocyturia during rejection was only to a minor extent due to a concentrating effect. A standardization
on daily urine volume was therefore not necessary, facilitating this diagnostic procedure. However, we also observed changes in excretion rates of cell types other than lymphocytes during rejection episodes. An increased excretion of urothelia was noted during the initial phase of rejection, a finding also reported by Segasothy and coworkers [9]. This might be explained by a concurrent rejection of the ureter.

We also found a significantly enhanced excretion of tubuloepithelial cells during rejection episodes. Increased excretion of tubuloepithelial cells during acute rejection episodes has been observed by Simpson et al. [20], who classified these predominantly as collecting duct cells. A tendency to an increased excretion of collecting duct cells was also observed in our studies. The discrepancy is most likely explained by the difficulty in differentiating the respective tubular cell types without specific staining. The pathophysiological significance of this increased excretion of distal tubular cells is still unclear.

Due to the relative low numbers of rejection episodes, it has not been possible so far to establish clear correlations between the cellular excretion pattern of the various cell types and the respective rejection types, e.g. interstitial/vascular or acute/chronic, or other causes of graft dysfunction unrelated to rejection. However, the technique presented here should provide a basis on which to address this question on a large scale, especially with respect to the significance of tubuloepithelial cells in graft dysfunction.

In conclusion, lectin staining of urinary sediments offers a useful tool for examining the significance of the various cell types in graft dysfunction, in particular those that have so far been difficult to identify, such as the different types of tubular epithelial cells. This technique could contribute to improved urine cytological monitoring with respect to the early detection and differentiation of graft dysfunction, its degree and course, and response to therapeutic interventions.

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References


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