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Renal graft rejection or cyclosporin toxicity?

Early diagnosis by a combination of Papanicolaou and immunocytochemical staining of urinary cytology specimens

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Abstract. A method is described for distinguishing between graft rejection and cyclosporin nephrotoxicity in renal allograft recipients by analyzing fresh morning urine samples. The technique combines classic Papanicolaou with immunocytochemical staining and was performed in urine specimens from a series of 42 patients. Early-stage cyclosporin toxicity was usually associated with increased numbers of proximal tubular cells only, whereas in rejection and late-stage toxicity there were increases in both tubular cells and in lymphocytes and monocytes (> 2000 cells/ml urine). Differentiation between these two clinical conditions was achieved by immunostaining, which revealed that increased numbers of $CD25^+$ and $CD8^+$ cells, as well as an increase in the HLA-DR/Lu5 ratio, were typical of rejection. CD25 positivity proved to be the best indicator of rejection, with a sensitivity and specificity of more than 90%. A cytodiagnostic algorithm is presented that is based on cell numbers and types, including immunophenotypes. The proposed method has the advantage of being noninvasive and appears to represent a reliable and rapid adjunct for the monitoring of graft function, especially in high-risk patients on cyclosporin immunosuppression.

Key words: Rejection, kidney, cyclosporin toxicity – Cyclosporin toxicity, rejection, kidney – Urinary cytology, in cyclosporin – Immunocytochemistry, in urine, cyclosporin

While the introduction of cyclosporin has resulted in a significant improvement in the rate of renal allograft survival [2, 3], the clinical diagnosis of rejection has become more difficult, due to the renal side effects of the drug. Reliable diagnosis is possible by means of biopsy, but a less invasive method for the routine monitoring of graft function would be preferable, particularly one that would permit rapid differentiation between graft rejection and cyclosporin toxicity.

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The purpose of the present study was to develop a rapid and reliable procedure involving urinary cytology and immunocytochemical techniques for the diagnosis of a graft rejection as opposed to cyclosporin toxicity. More than 700 specimens from 42 kidney transplant recipients were investigated and the results correlated with both clinical and biopsy findings.

Materials and methods

Patients

A consecutive series of 42 renal allograft recipients (16 males, 26 females) with an age range of 9–69 years were studied in the period from June 1988 to February 1989. All were given cyclosporin, steroids, and azathioprine as basic immunosuppression and 11 were started on antithymocyte globulin (ATG; Fresenius, Oberursel, FRG) immediately after transplantation. Rejection episodes were initially treated with methylprednisolone pulse therapy, followed by ATG ($n = 8$) and/or OKT3 (anti-CD3; Ortho, N.J., USA) where this proved ineffective. The study was performed blindly.

At the end of the study the following patient groups were identified:

Rejection group: patients with impairment of renal function, followed by rapid improvement in response to antirejection therapy ($n = 12$). Rejection was confirmed by biopsy in nine cases, two of which also showed tubular cell patterns consistent with cyclosporin toxicity.

Cyclosporin toxicity group: patients with impairment of renal function, followed by rapid improvement in response to cyclosporin dose reduction ($n = 17$). Toxicity was confirmed by biopsy in six cases, five of which showed tubular and one vascular interstitial toxicity.

Control group: patients without impairment of renal function belonging to neither the rejection group nor the cyclosporin toxicity group.

Episodes of urinary tract obstruction occurred in 2 of the 42 patients.

Preparation of urine specimens

Urine samples were collected from the second morning voiding every other day during hospitalization, and prior to each medical examination after discharge until day 60. A work-up was performed on the samples within 1 h of collection since any other procedure re-

Fig. 1a–d. Various cell types in a Papanicolaou-stained preparation derived from a urine sample: **a** lymphocyte; **b** monocyte; **c** proximal tubular cell; **d** collecting duct cell (× 950)

Fig. 2. $CD8^+$ cells in a urine sample as demonstrated by the APAAP method (× 1250)

sults in loss of cells, loss of cell integrity with increased numbers of unclassifiable cells, and loss of antigen preservation. A total of 30–50 ml of urine was centrifuged for 10 min at 2000 rpm (700 *g*), before being washed twice with Hank's solution (Animed, Muttenz, Switzerland). Cell counts were then determined using a Neubauer chamber and adjusted to about 10000 cells/ml Hank's solution. Cytospin preparation (Cytospin 2; Shandon, Astmoor, UK) was done for 2 min (Papanicolaou staining) or for 6 min (immunocytochemical staining) at 600 rpm (55 *g*), preparations for Papanicolaou staining being immediately fixed with Cytostat 400 spray (Simat, Zürich, Switzerland). Total counts of mononuclear cells, i.e., lymphocytes and monocytes (Fig. 1 a, b), and renal epithelial cells (tubular cells and collecting duct cells) were determined (Fig. 1 c, d), but squamous cells and transitional cells were ignored. Finally, the concentration of all cells per ml urine was calculated for each sample.

Immunocytochemical staining

The air-dried cytospin preparations were fixed in acetone for 10 min at room temperature, then stained immediately or, if this was not possible, sealed in plastic and stored at − 70°C.

Immunocytochemical staining was performed via a three-layer alkaline phosphatase/anti-alkaline phosphatase (APAAP) method. The monoclonal antibodies used are shown in Table 1. Cytospin preparations dried by a fan for 30 min were incubated with the first monoclonal antibody for 30 min at room temperature, then washed for 30 min with 0.05 mol TRIS-NaCl buffer before being incubated for a second time with rabbit anti-mouse globulin (Dakopatts) diluted 1:30 with human serum. Incubation with APAAP complex (Dakopatts), diluted 1:80 with 0.05 mol TRIS-NaCl, was performed for 30 min. After washing, the preparations were stained with new fuchsin for 20 min in a desiccator. They were then counterstained with hematoxylin and the slides finally covered with Aqua-Mount. A slide incubated with 0.05 mol TRIS-NaCl buffer and rabbit anti-mouse globulin, instead of the specific antibody, was used as a control. Although duplicate incubations were performed with the various antisera in some cases, this was not done systematically. No relevant differences were observed between samples from the same urine specimen.

Four hundred mononuclear cells were counted (Fig. 2) and the concentration of positive cells per milliliter urine was calculated.

Statistical analysis

Statistical analysis was performed using Wilcoxon's U-test.

Results

Cell counts as determined by Papanicolaou staining

The mean curve for total mononuclear cells (lymphocytes, monocytes, renal tubular and collecting duct cells), based on 228 urine samples from patients showing no clinical evidence of rejection or toxicity at the time of sampling, reached a peak in the immediate postoperative period, before settling at below 2000 cells/ml on about day 13 (Fig. 3). Counts on days 1–12 varied widely from day to day and patient to patient and were, therefore, not included in the subsequent analysis. During episodes of rejection, cyclosporin toxicity or obstruction cell counts increased sharply, commencing prior to clinical diagnosis (Fig. 3). In the 12 cases of rejection, mean increases at the time of clinical diagnosis were between 1.6 (SD 1.2) and 7.6 (SD 6.5) $\times 10^3$/ml, while in episodes of cyclosporin toxicity two different patterns were found: cases with more than 2000 cells/ml ($n = 8$) and cases with fewer than 2000 cells/ml ($n = 9$). Particularly in the cases with fewer than 2000 cells/ml there was a predominance of proximal tubular cells, which are characterized by their size, indistinct border, micro- and macrovacuolization, granular cytoplasm with intracytoplasmic inclusion bodies, and eccentric and pyknotic nuclei [15, 19]. A similar predominance was found in those with more than 2000 cells/ml, but it was less pronounced (Fig. 4). As a general rule, therefore, cyclosporin toxicity should be suspected if the proportion of proximal tubular cells exceeds 50%. Later in the course of cyclosporin toxicity, lymphocytes and monocytes were also increased.

Renal epithelial cell counts were also increased during the episodes of obstruction that occurred in two patients. Proximal tubular cells predominated in one case due to concomitant cyclosporin toxicity, while in the other case there was a predominance of collecting duct cells.

Table 1. Monoclonal antibodies used in the study

Monoclonal antibody (Source)		Working dilution
CD2-T 11:	Pan T cell (Dakopatts)	1:20
CD4-T 4:	Helper/inducer T cell (Dakopatts)	1:2
CD8-T 8:	Suppressor/cytotoxic T cell (Dakopatts)	1:20
CD25-Tac:	Interleukin 2 receptor (Dakopatts)	1:10
HLA-DR:	HLA class II antigen (Becton-Dickinson)	1:10
Lu5:	Pankeratin (Roche Diagnostika)	1:100

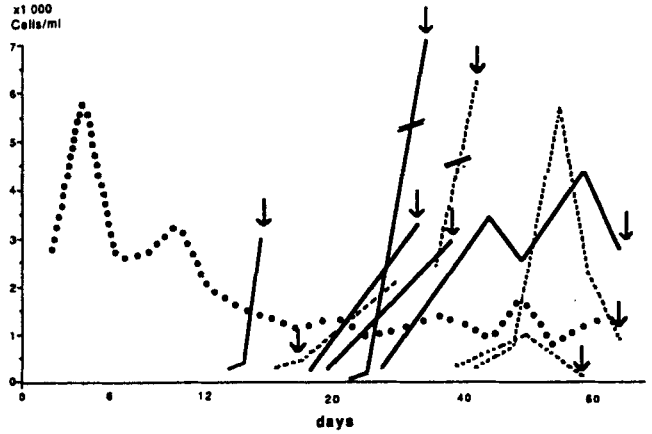

Fig. 3. Increases in urinary cell counts in rejection and cyclosporin toxicity, the *dotted line* representing the mean cell count derived from 228 urine samples obtained from patients showing no clinical evidence of either rejection or toxicity at the time of sampling. The starting point of the episode was taken as the day on which two out of three immunocytochemical rejection indices were present or more than 50% of the mononuclear cells in the urine were proximal tubular cells. ● Total number of cells; — rejection; ---- CyA toxicity; → day of diagnosis

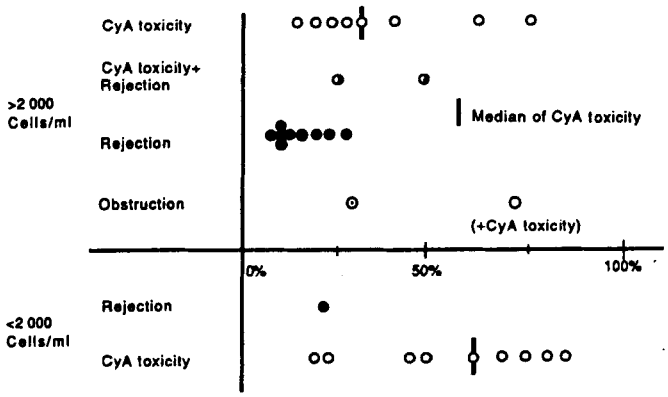

Fig. 4. Percentage of proximal tubular cells in rejection and cyclosporin toxicity

Cell counts exceeding 2000/ml (mainly polymorphonuclear leukocytes) also occurred in 7 of the 13 control patients showing no clinical or cytological evidence of rejection, cyclosporin toxicity, or obstruction, but in a single or in two consecutive urine samples only. This was interpreted as being due to infection, although no clinical signs of urinary tract infection were, in fact, found.

Immunocytochemical findings

Of a total of 733 urine samples collected, 190 collected after day 13 with more than 2000 cells/ml or from patients with clinical evidence of kidney failure were studied using immunocytochemical methods. They were obtained from 12 patients during acute rejection episodes and from 17 patients during episodes of cyclosporin toxicity. The highest cell counts found during the rejection episodes were used in the statistical analysis. The mean counts per ml urine, together with the standard deviation, median, and the 75% value, are presented in Table 2.

In 11 of the 12 rejection episodes, counts of the critical antigen-bearing cells were highest before rejection was diagnosed on clinical grounds, 1–3 days in most cases, but in two cases as long as 7 or more days before clinical diagnosis (Fig. 3). In cyclosporin toxicity, cell counts continued to increase even after dose reduction (Fig. 3). The values used in the analysis were those at the time of diagnosis (clinical or biopsy).

In the rejection group, the mean counts of cells expressing CD2, CD4, CD8, CD25 ($P < 0.005$), HLA-DR ($P < 0.025$), and the HLA-DR/Lu5 ratio ($P < 0.05$) were significantly higher than the corresponding mean counts for all samples, but no significant difference was found for Lu5. The cyclosporin toxicity group did not differ from the study population as a whole with respect to any of the antigens tested. Differences between the rejection group and

Table 2. Results of immunotyping (positive cells/ml urine)

		CD2 (×10²/ml)	CD4 (×10²/ml)	CD8 (×10²/ml)	CD25 (×10/ml)	Lu5 (×10²/ml)	HLA-DR (×10²/ml)	HLA-DR/Lu5 (ratio)
All samples[a]	Mean	4.7	2.0	2.2	1.6	18.0	18.5	1.27
day > 12	SD	6.0	3.0	3.1	3.3	36.3	32.7	0.72
(n = 190)	Median	2.4	0.9	0.9	0.0	6.2	7.0	1.15
	75%	6.2	3.0	3.4	2.4	16.6	21.8	1.56
CyA Toxicity	Mean	4.2	1.6	1.9	0.5	19.5	19.8	1.19
(n = 17)	SD	5.9	2.2	3.1	1.3	44.4	42.6	0.50
	Median	2.0	0.5	0.2	0	4.2	6.4	1.05
Rejection	Mean	13.1**	5.6***	7.6***	9.0***	14.2	24.2**	2.04*
(n = 12)	SD	20.7	8.9	12.0	6.0	12.6	21.7	1.53
	Median	6.1	2.7	3.4	8.6	9.8	23.1	1.67
Control samples[b]	Mean	1.4	0.6	0.5	0.1	4.1	4.8	1.3
(n = 13)	SD	1.6	0.7	0.8	0.3	6.5	7.3	0.67
	Median	0.7	0.2	0.3	0	0.3	1.6	1.17

* $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$ Wilcoxon's test: all samples vs CyA toxicity or rejection cases

[a] All samples with > 2000 cells/ml from patients belonging to the CyA toxicity or rejection group, day > 12

[b] All samples from patients in the control group with < 2000 cells/ml

the controls (i.e., patients with no impairment of renal function) were even more highly significant than between the rejection group and the total study population, including for Lu5, whereas the cyclosporin toxicity group did not differ significantly from the control group, even where $Lu5^+$ cells were concerned.

For the calculation of sensitivity and specificity, the 75% value for all samples was taken as the upper limit of the urinary cell count. The best results for sensitivity and specificity were found for CD25, with a sensitivity and specificity of more than 90%, whereas most of the other antigens (CD2, CD4, CD8, HLA-DR) ranged between 40% and 50% for sensitivity and between 75% and 80% for specificity (Table 3). In addition to CD25, HLA-DR/Lu5, CD8, and CD2 showed greater sensitivity and/or specificity than Lu5, HLA-DR, and CD4. The negative predictive value showed that in the absence of a significant increase in $CD25^+$ cells, rejection can be excluded (PV 0.99), whereas in the presence of such an increase, a diagnosis of rejection will be correct in 50% of all cases.

Discussion

The diagnosis of renal graft rejection is normally based on findings such as an increase in serum creatinine, local graft tenderness, and various other nonspecific symptoms. This means that diagnosis is often difficult, especially where rejection takes an oligosymptomatic course, and rejection diagnosed in this way is often shown by subsequent renal biopsy to be graft failure due to some other cause. Thus, more objective and, if possible, non-invasive methods are needed for early differentiation between rejection and other causes of graft dysfunction.

Three procedures are currently available: core biopsy (thick-needle biopsy), fine-needle aspiration, and urinary cytology. Core biopsy is the standard procedure, permitting evaluation of all renal structures, i.e., glomeruli, arterioles, arteries, and the tubulointerstitial space, and thus diagnosis of the widest possible range of lesions.

Fine-needle aspiration permits evaluation of tubulointerstitial lesions, in particular those involving infiltrating and tubular cells [4], although one problem with this technique is the uncertainty as to the origin of the aspirated cells, which may come from blood vessels. However, it is extremely useful in anuric patients early post-transplantation to differentiate between acute tubular necrosis (ATN) and rejection. However, while it is less uncomfortable for the patient than core biopsy, due to the use of a thin needle, it is nevertheless an invasive and unpleasant experience.

By comparison, the third method, urinary cytology, is a noninvasive routine method for monitoring cellular reactions in organ grafts, although it does suffer from the same limitation as fine-needle biopsy in that only tubulointerstitial lesions can be evaluated. Other limitations are that it cannot be used in patients with early post-transplant nonfunction and that it yields unreliable results if only one urine sample per day is analyzed, due to the variability of cell excretion in the first few days. There is also the serious problem of cell preservation, which can, however, be overcome by processing freshly voided urine within 1 h of collection, as well as the possibility of interference from cells deriving from the native kidney.

Several urinary cytology studies using Wright [10], Papanicolaou [15, 20], or Giemsa [18] staining have demonstrated the value of urinary lymphocytes [10, 20] or collecting duct cells [15] in the diagnosis of rejection in renal allograft recipients receiving conventional immunosuppression. The value of lymphocytes [13] and collecting duct cells [5] in the diagnosis of rejection has been confirmed in cyclosporin-treated patients. Damaged renal tubular epithelial cells were found in the urine in cases of cyclosporin nephrotoxicity [19]. However, in our experience, a high percentage ($> 50\%$) of proximal tubular cells in the urine is more indicative of cyclosporin nephrotoxicity than is the presence of morphologically altered proximal tubular cells. The morphological features of tubular cyclosporin toxicity found by renal biopsy – such as isometric vacuolization or tubular inclusion bodies – have rarely been found by urinary cytology, with isometric tubular vacuolization present in only two of five biopsy-confirmed cases of cyclosporin toxicity and cytoplasmic inclusion bodies in only one [11]. The diagnosis of tubular cyclosporin toxicity by urinary cytology was thus made exclusively on the basis of the presence of exfoliated proximal tubular cells. Where fewer than 2000 cells/ml are present, which is usually the case in early-stage toxicity, a predominance of proximal tubular cells suggests a diagnosis of toxicity. In cases with more than 2000 cells/ml, a possible predominance of proximal tubular cells might be masked by the presence of lymphocytes and monocytes, making immunocytochemical studies necessary for further evaluation.

The immunotyping of mononuclear cells using monoclonal antibodies has been widely used in recent studies of infiltrating cells in kidney graft biopsies [1, 8, 14]. Severe rejection episodes were often characterized by a predominance of $CD8^+$ over $CD4^+$ cells [1, 14], with the former indicating a diffuse type of infiltration and the latter occurring predominantly in focal infiltration [1]. Results are conflicting, however, and there is considerable overlap between focal and diffuse forms, even in residual infiltration after successfully treated rejection episodes. More accurate information was obtained by using monoclonal antibodies to T-cell activation markers, one of the most interesting of which is Tac antigen (CD25, IL2 receptor) [21], and anti-Tac monoclonal antibody has been reported to prolong renal allograft survival in monkeys [12]. Increased numbers of anti-Tac^+ cells were found in biopsies showing evidence of severe rejection [8, 16].

Table 3. Sensitivity (SE), specificity (SP), and predictive value (PV) of different antigens for the diagnosis of rejection. p, positive; n, negative

		CD2	CD4	CD8	CD25	Lu5	HLA-DR	HLA-DR/Lu5
Diagnosis ($n = 12$)	SE	0.5	0.42	0.5	0.91	0.25	0.41	0.58
	SP	0.84	0.83	0.84	0.93	0.84	0.86	0.76
	PVp	0.21	0.16	0.21	0.52	0.11	0.2	0.17
	PVn	0.95	0.94	0.95	0.99	0.93	0.94	0.95

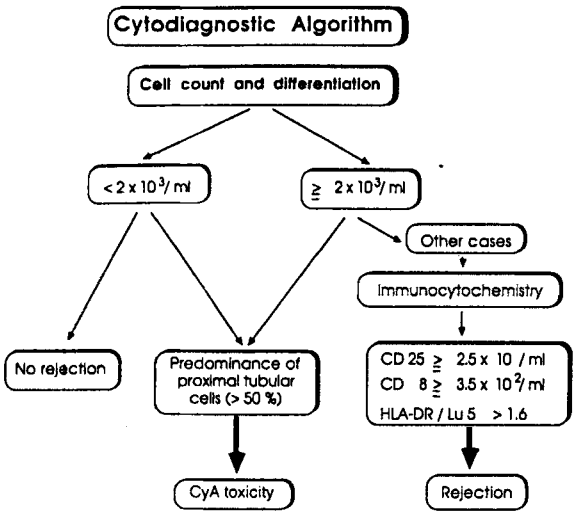

Fig. 5. Cytodiagnostic algorithm

To date, urinary cytology studies using immunotyping have been rare, but in one of the few, Vangelista et al. [22] found an increase in $CD2^+$ and $CD8^+$ cells in urine samples taken during acute rejection episodes. This is confirmed by the present study, with $CD2^+$ and $CD8^+$ cells showing much greater sensitivity as markers for rejection than $CD4^+$ cells, although $CD25^+$ cells were the most sensitive and specific in this respect. Simpson et al. [17] showed that soluble urinary IL2 and IL2 receptors were increased in renal allograft recipients during acute rejection but were not even found in cyclosporin toxicity. This T-cell activation marker might thus prove most useful in diagnosing rejection and distinguishing it from cyclosporin toxicity.

Another marker of T-cell activation is the HLA-DR antigen, which, in normal tissues, is confined to macrophages, dendritic cells, B cells, and vascular endothelium [7]. In rejection, however, there is increased expression of HLA-DR antigens on renal tubular cells [9]. Since urinary levels of $HLA\text{-}DR^+$ cells were generally so high, this variable was not helpful as a means of proving rejection. A better indicator of rejection than the absolute number of $HLA\text{-}DR^+$ cells is the ratio of HLA-DR to Lu5. Lu5 is a marker of pancytokeratin [6], which is strongly expressed on tubular cells in the presence of renal damage, while increased expression of HLA-DR occurs only in rejection and not in cyclosporin toxicity.

Based on the results of our investigations, the following cytodiagnostic algorithm is suggested (Fig. 5): Papanicolaou staining should first be performed to determine cell counts and types. If the number of lymphocytes, monocytes, and renal tubular cells is below 2000/ml, rejection is highly unlikely and cyclosporin toxicity must be considered, particularly if proximal tubular cells predominate (> 50%). The same applies if the urinary cell count exceeds 2000/ml, but immunocytochemistry is recommended where there is also mixed cellularity. If this shows that the number of $CD25^+$ cells exceeds 25/ml, $CD8^+$ cells exceed 350/ml, and the HLA-DR/Lu5 ratio is > 1.6, rejection is the more likely diagnosis. Two of these three criteria were present in 11 of the 12 rejection episodes in the present series. The absence of these criteria virtually excludes the possibility of interstitial rejection.

Urinary cytology is most reliable after the first 2 weeks following kidney grafting, when urine production and cell excretion are less variable than in the immediate post-transplantation period. Thus, it is an ideal method for monitoring high-risk patients both during this time and during subsequent outpatient follow-up. The use of a combination of conventional and immunocytochemical urinary cytology makes it possible to diagnose kidney transplant rejection and cyclosporin toxicity earlier than on clinical criteria. Sequential fine-needle aspiration cytology immediately after transplantation, followed by urinary cytology from the 2nd week onwards, is probably the methodology of choice in high-risk patients, but combined use of Papanicolaou staining and immunocytochemistry offers the possibility of making the diagnostic procedure reasonable in terms of cost and time.

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