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Early diagnosis of kidney transplant rejection and cyclosporin nephrotoxicity by urine cytology

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Abstract. A total of 2000 urine samples from 53 kidney transplant recipients were studied to develop a routine method for the early diagnosis of rejection and cyclosporin (CSA) nephrotoxicity in urine. New-Sternheimer staining and an immunocytochemical technique were used together with classical Papanicolaou staining to differentiate cells in the urine. Af r cell count and differentiation of second morning urine samples with New-Sternheimer and Papanicolaou stains, immunocytochemistry was performed using antibodies against the following antigens: CD2, CD4, CD8, CD25, CD71 (transferrin receptor), HLA-DR and cytokeratin (Lu-5). Cell counts were obtained for the positively-reacting cells per millilitre of urine. By New-Sternheimer and Papanicolaou staining, CSA nephrotoxicity was characterized by the predominance of proximal tubular cells. During rejection episodes, increased numbers of mononuclear cells and renal epithelial cells were found. Immunocytochemical analysis showed a significant increase in CD2-, CD4-, CD8-, CD25-, CD71-, and HLA-DR-positive epithelial cells and in the ratio HLA-DR/cytokeratin-positive epithelial cells in rejection. CD25-positive cells had the highest sensitivity and specificity for the diagnosis of rejection. Our urine cytology technique proved to be a useful and non-invasive method for the early diagnosis of rejection and CSA nephrotoxicity.

Key words: Urine cytology – Rejection – Cyclosporin nephrotoxicity – Immunocytochemistry – New-Sternheimer staining

The introduction of cyclosporine (CSA) has significantly improved the graft survival rate in renal transplantation [2]. The clinical diagnosis of rejection, however, has become more difficult due to nephrotoxic side-effects of the drug. A non-invasive method is thus needed for daily graft monitoring to complement renal biopsy.

This study was conducted to develop a routine method for the early differential diagnosis of kidney transplant rejection and CSA nephrotoxicity.

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Materials and methods

From June 1988 to March 1991, 53 renal transplant patients (19 males, 34 females) with a mean age of 41.8 years (range 9–69) were studied in Basel (n = 37) and Nishinomiya (n = 16). All patients received CSA and steroids as basic immunosuppression. During the study, 23 rejection episodes in 20 patients and 21 episodes of CSA nephrotoxicity in 18 patients were observed. Rejection was diagnosed by biopsy (n = 20) or clinically (n = 3). CSA nephrotoxicity cases were also diagnosed by biopsy (n = 9) or improvement of renal function after CSA dose reduction (n = 12). Two biopsy-proven rejection cases also showed tubular CSA nephrotoxic patterns.

Fresh second-morning urine samples were studied every second day during hospitalization, and after discharge at each medical examination until day 60. Urine samples (25–50 ml) were centrifuged for 10 min at 2000 rpm (700 g), washed in Hank's solution, and counted in a Neubauer's chamber. The cell number was adjusted to about 10000 cells/ml Hank's solution. A cytospin preparation was made in 2 min (for Papanicolaou staining) or 6 min (for immunocytochemistry) at 600 rpm (55 g). For Papanicolaou staining, preparations were fixed immediately with fixspray, Cytostat 400 (Simat AG, Switzerland). The total number of monoclear cells, i. e. lymphocytes and monocytes, and renal epithelial cells (tubular cells and collecting duct cells) was counted.

New-Sternheimer staining was performed for 16 patients in Nishinomiya. After centrifugation for 5 min at 1500 rpm (500 g), lymphocytes/monocytes and renal tubular cells were counted using a simple cell-counting chamber, Kova-System (Miles-Sankyo, Japan). Finally, cell concentration per millilitre of urine of these cells was calculated for each patient.

Immunocytochemical staining was performed by a three-layer alkaline phosphatase anti-alkaline phosphatase (APAAP) method. The monoclonal antibodies used were directed against CD2, CD4, CD8, CD25, CD71, HLA-DR and cytokeratin (Lu-5).

Acetone-fixed cytospin preparations were incubated with the first monocloncal antibody for 30 min at room temperature. After washing with 0.05 M Tris-NaCl buffer, the second incubation with rabbit anti-mouse globulin and the third incubation with the APAAP-complex were performed. After washing, reaction with New-Fuchsin was carried out. Hemalaun was used as counterstain,

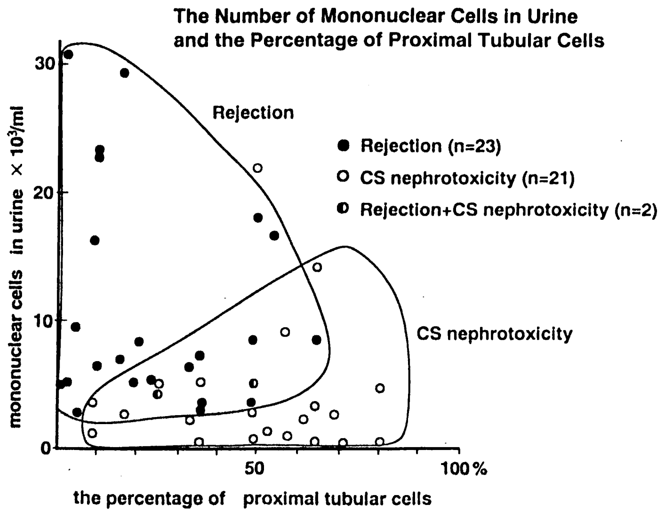

Fig. 1. The number of mononuclear cells in urine and the percentage of proximal tubular cells in all mononuclear cells are shown. Rejection cases showed more mononuclear cells than CSA nephrotoxicity, and in CSA nephrotoxicity proximal tubular cells predominated

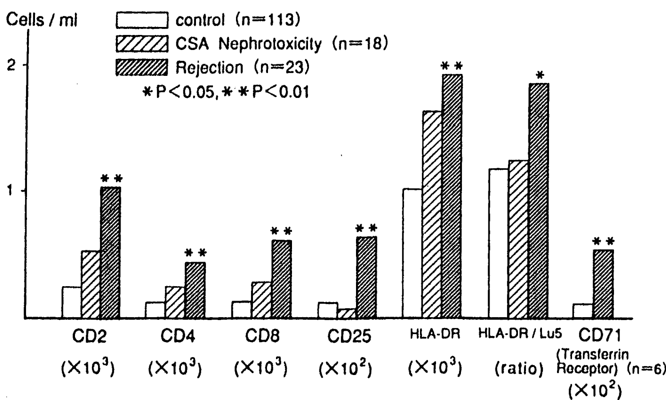

Fig. 2. Immunocytochemical study shows increased numbers of CD2-, CD4-, CD8-, CD25-, CD71-, and HLA-DR-positive cells and the increased ratio of HLA-DR/Lu-5 in rejection. Patients with CSA-nephrotoxicity did not differ from the control group

then 400 mononuclear cells were counted and the cell concentration of positive cells per millilitre of urine was calculated.

Results

Papanicolaou staining

Because of the considerable variation in the number of cells from day to day in the early postoperative days, the first 12 days were not considered in the analysis. Patients with rejections and CSA nephrotoxicity showed increased excretion of mononuclear cells before the clinical diagnosis. All rejection episodes were characterized by an increase in mononuclear cells (mean +/− SD): 1.8 +/− 1.5 to 8.4 +/− 7.5×10^3/ml). CSA nephrotoxicity cases also showed a slight increase in monocucléar cells (1.7 +/− 2.4 to 6.1 +/− 9.1×10^3/ml). In cases of CSA nephrotoxicity proximal tubular cells predominated. These were characterized by size, indistinct cell border, micro- and macro-vacuolization, granular cytoplasm with intracytoplasmic inclusion bodies and eccentric and pyknotic nuclei [14]. The number of mononuclear cells in urine and the percentage of proximal tubular cells among all mononuclear cells at diagnosis of rejection of CSA nephrotoxicity are shown in Fig. 1. Rejection cases showed more mononuclear cells than CSA nephrotoxicity cases ($P < 0.01$), and in CSA nephrotoxicity significantly more proximal tubular cells were found than in rejection ($P < 0.01$).

Immunocytochemical staining

Immunocytochemical staining was performed on 23 urine samples at the time of diagnosis of rejection and 18 urine samples at diagnosis of CSA nephrotoxicity. As control group, 113 urine samples not associated with rejection or CSA nephrotoxicity were used. The mean cell count in rejection cases was significantly higher than in the control group for cells expressing CD2, CD4, CD8, CD25, CD71, HLA-DR and there was also an increased HLA-DR/Lu-5 ratio in rejection (Fig. 2). The CSA nephrotoxicity group showed no significant differences from the control group. Differentiation between rejection and CSA nephrotoxicity cases was possible with the help of CD2*, CD4*, CD8*, CD25** and the ratio HLA-DR/Lu-5* (* $P < 0.05$; ** $P < 0.01$).

For the calculation of sensitivity and specificity, the 75% value of all patients was considered as the upper limit of the normal cell count in urine. The best results for sensitivity and specificity were found in CD25 (Table 1).

New-Sternheimer staining

For screening purposes, New-Sternheimer stain was used. Since 1990, cell counting has been performed on 1500 urine samples at Nishinomiya Hospital. In 10 rejection episodes, 10^3lymphocytes per millilitre of urine on two consecutive days, or more than 2×10^3lymphocytes per millilitre of urine were observed 3.8 days, on average, before clinical or pathological diagnosis (Fig. 3). Three CSA nephrotoxicity patients excreted proximal tubular cells in the urine at a mean of 4 days before clinical diagnosis.

Table 1. Sensitivity, specificity and predictive value of different antigens for the diagnosis of rejection (n = 23)

	CD2	CD4	CD8	CD25	HLA-DR	HLA-DR/Lu5
SE	0.52	0.34	0.52	0.65	0.41	0.58
SP	0.85	0.85	0.86	0.93	0.86	0.76
PVp	0.34	0.24	0.35	0.60	0.20	0.17
PVn	0.92	0.90	0.92	0.95	0.94	0.95

SE, sensitivity; SP, specificity; PV, predictive value of positive (p) or negative (n) cases

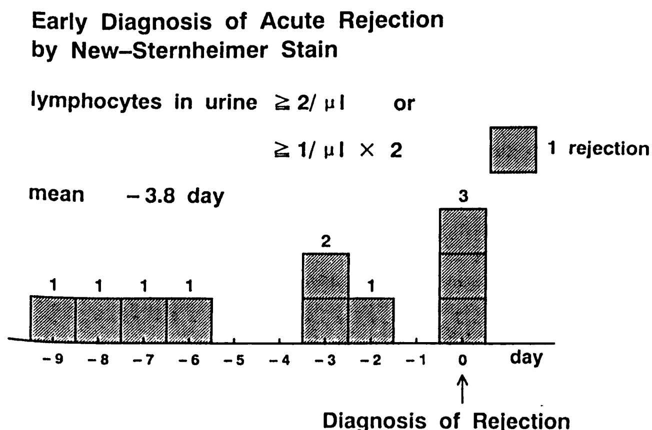

Fig. 3. Results of New-Sternheimer stain in patients with rejection. Rejection episodes were diagnosed on average 3.8 days before the clinical or biopsy diagnosis

Discussion

Objective and if possible non-invasive methods are necessary for the differentiation of rejection and CSA nephrotoxicity. Thick needle biopsy is the standard which allows evaluation of all renal compartments. Fine needle aspiration biopsy permits an analysis of tubulo-interstitial lesions, mainly infiltrating cells and tubular cells [3]. One problem of this technique is the uncertainty of the origin of the aspirated cells, which may come from blood vessels. Despite the use of a thin needle, it is still an invasive method and uncomfortable for the patients. The third method, urine cytology, is the least invasive method for monitoring cellular reacton in the graft. Urine is the easiest material to obtain, and it is the best routine method for graft monitoring, even though urine cytology has the same limitations as fine needle aspiration biopsy in that only tubulo-interstitial lesions can be evaluated.

Several studies on urine cytology have reported the value of lymphocytes [15] or collecting duct cells [4] for diagnosis of rejection. In CSA nephrotoxicity, damaged proximal tubular cells have been found in the urine [13, 14]. A percentage of more than 50% of proximal tubular cells is highly indicative of CSA nephrotoxicity. Of our CSA nephrotoxicity cases, 65% showed this predominance of proximal tubular cells, especially those with less than 2000 cells/ml. In cases of more than 2000 cells/ml a gradual increase in lymphocytes and monocytes was also found. In the latter cases, it was difficult to differentiate between CSA toxicity and rejection on the basis of Papanicolaou staining alone. In such cases immunocytochemical studies should also be performed.

Immunotyping of mononuclear cells using monoclonal antibodies has been widely used in recent studies on infiltrating cells in kidney graft biopsies [1]. However, only a few studies have been made in urine cytology. Vangelista et al. [17] found an increase in CD2- and CD8-positive cells in urine during acute rejection. This was confirmed by our study. However, in our study, the highest sensitivity and specificity for rejection was found for CD25-positive cells. Simpson et al. [12] showed that soluble urinary IL-2 and IL-2-receptor levels increased in acute rejection, whereas in CSA nephrotoxicity, they were not detected. T-cell activation markers are most helpful for the differential diagnosis of rejection and CSA nephrotoxicity. Another marker indicating T-cell activation is the demonstration of HLA-DR. In normal tissue, HLA-DR antigen is confined to macrophages, dendritic cells, B cells and vascular endothelium [7]. In rejection, however, the expression of HLA-DR antigens increases on renal tubular cells [8]. The ratio HLA-DR/Lu-5 gave better results in the diagnosis of rejection than the absolute number of HLA-DR-positive cells. Lu-5 is a marker of pancytokeratin [5], and in the case of renal damage, the antigen was strongly expressed in tubular cells, but additional HLA-DR expression only occurred in rejection, and not in CSA nephrotoxicity. A positive correlation between the total number of infiltrating cells and anti-transferrin receptor-positive cells in rejection has been reported [8]. Metabolically active cells express transferrin receptor [6]. We found that in all six rejection episodes studied up to now, transferring receptor-positive cells were increased to a similar extent to CD25-positive cells.

In summary, urine cytology is reliable method after the first 2 weeks when urine and cell excretion are less variable than immediately after renal transplantation. The use of New-Sternheimer, Papanicolaou and immunocytochemical stains in combination makes it possible to diagnose rejection and CSA nephrotoxicity earlier than by other clinical means and to differentiate between them.

References

1. Bishop GA, Hall BM, Duggin GG, Horvath JS, Sheil AGR, Tiller DJ (1986) Immunopathology of renal allograft rejection analyzed with monoclonal antibodies to mononuclear cell markers. Kidney Int 29: 708–717
2. Calne RY, Wood AI (1985) Cyclosporin in cadaveric renal transplantation: 3 year follow-up of a European multicentre trial. Lancet 8454: 549
3. Droz D, Campos H, Noel LH, Adafer E, Kreis H (1984) Renal transplant fine needle aspiration cytology: Correlations to renal histology. In: Renal transplant cytology. Proceedings of the Second International Worskhop. Paris Milan Italy Wichitg, pp 59–65
4. Eggensperger D, Schweitzer S, Ferriol E, O'Dowd G, Light JA (1988) The utility of cytodiagnostic urine analysis for monitoring renal allograft injury. Am J Nephrol 8: 27–34
5. Franke WW, Winter S, von Overbeck J, Gudat F, Heitz PU, Staehli C (1987) Identification of the conserved, conformation-dependent cytokeratin epitope recognized by monoclonal antibodies (Lu-5). Virchows Arch A 411: 137–147
6. Gatter K, Brown G, Trowbridge IS, Woolston RE, Mason DY (1983) Transferrin receptors in human tissue: their distribution and possible clinical relevance. J Clin Pathol 36: 539–544
7. Hall BM, Bishop GA, Duggin GG, Horvath JS, Philips J, Tiller DJ (1984) Increased expression of HLA-DR antigens on renal tubular cells in renal transplants: relevance to the rejection response. Lancet 8397: 247–251
8. Henny FC, Weening JJ, Baldwin WM, Oljans PJ, Tanke HJ, Van ES LA, Paul LC (1986) Expression of HLA-DR antigens on peripheral blood T lymphocytes and renal tubular epithelial cells in association with rejection. Transplantation 42: 479–483

9. Mihatsch MJ, Thiel G, Basler U, Ryffel B, Landmann J, Overbeck J, Zollinger HU (1985) Morphological patterns in cyclosporine-treated renal transplant recipients. Transplant Proc 17: 101–116
10. Sanfilippo F, Kolbeck PC, Vaughn WK, Bollinger RR (1985) Renal allograft cell infiltrates associated with irreversible rejection. Transplantation 40: 679–685
11. Seron D, Alexopoulos E, Raftery MJ, Hartey RB, Cameron JS (1989) Diagnosis of rejection in renal allograft biopsies using the presence of activated and proliferating cells. Transplantation 47: 811–816
12. Simpson M, Madras P, Cornaby AJ, Etienne T, Dempsey RA, Clowes GHA, Mónako P (1989) Sequential determination of urinary cytology and plasma and urinary cytokines in the management of renal allograft recipients. Transplantation 47: 218–223
13. Simpson M, Madras P, Monaco A (1989) Cytologic examination of urinary sediment in renal allograft recipients. Transplant Proc 21: 3578–3580
14. Stella F, Stella C, Battistelli S, Alfani D, Famulari A, Berloco E, Molajoni ER, Pretagostini R, Rossi M (1986) Monitoring of ciclosporin toxicity by exfoliative urinary cytology in renal transplantation. Contrib Nephrol 51: 152–155
15. Taft PD, Flax MH (1966) Urinary cytology in renal transplantation: association of renal tubular cells and graft rejection. Transplantation 4: 194–204
16. Uchiyama T, Broder S, Waldmann TA (1981) A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. J Immunol 126: 1393–1397
17. Vangelista A, Nanni-Costa A, Fatone F, Buscaroli A, D'Atena T, Bonomini V (1987) Flow cytometry analysis of urinary cytology in renal transplantation. Transplant Proc 19: 1665–1666