Transplant Int (1992) 5 [Suppl 1]: S 8–S 12

TRANSPLANT
International

Flow cytometry evaluation of urinary sediment in renal transplantation

A. Nanni-Costa, S. Iannelli, A. Vangelista, A. Buscaroli, G. Liviano, C. Raimondi, P. Todeschini, G. Lamanna, S. Stefoni, and V. Bonomini

Institute of Nephrology, University of Bologna, Italy

Abstract. The value of exfoliative urinary cytology for the diagnosis of different pathological conditions in renal transplantation is widely recognized. The method, however, has not yet gained full acceptance, mainly because identification of the different cells is not always possible by means of standard staining techniques. In view of its characteristics, flow cytometry (FC) seems to represent a consistently reliable, rapid and innovative approach for differentiating the various cells present in the urinary sediment and assessing their number. This study gives the examination result of 223 urinary specimens from 127 transplanted patients selected according to pathology. Sediment cells, collected from fresh urine samples, were washed, treated with a lysing solution, resuspended in saline solution and directly analysed in a FACSCAN cytometer. Morphological evaluation showed: a small number of cells in patients with stable renal function; a larger number of cells, with predominance of lymphocytes, during acute rejection episodes; an absolute predominance of neutrophils during bacterial infection; large-sized cellular debris in cases of post-transplant tubular necrosis; and small cell debris in cases of cyclosporine cytotoxicity. Lymphocyte surface-marker evaluation made it possible to differentiate lymphocyte populations observed during acute rejection episodes (cytotoxic T-cell, CD8 and HLA class II and NK cells) from those detected during bacterial infection (T-cell CD4 positive). These results suggest that urinary FC may be a reliable diagnostic tool in clinical renal transplantation.

Key words: Renal transplantation – Urinary sediment – Flow cytometry – Lymphocytes

The value of exfoliative urinary cytology for the diagnosis of various pathological conditions in renal transplantation

has been suggested by various investigators [1, 2, 4, 9]. In particular the detection of lymphocyturia has been indicated as a sign of acute rejection [10].

The main features of standard urinary cytology are its non-invasive nature, ease of execution (usually by Giemsa staining) and the possibility it affords to realize a simple serial monitoring of the patient's condition. Preparing the urinary sediment may, however, give rise to methodological difficulties connected with the quality of material used. At least in some cases identification and quantification of the cells examined may not be possible.

This considerable drawback may be overcome by the use, in this case on urinary sediment, of flow cytometry (FC [6], an up-to-date technique which combines light microscopy examination characteristics, such as multiparametric analysis, with high precision for rapid analysis of individual cells. FC represents a rapid, objective and reliable approach to differentiating and assessing the number of any kind of cell population on the basis of the scatter of a laser beam focused on the cells running through a microscopic capillary called a flow chamber. The light impulses are later processed by a computer which gives a quantitative evalution and a visual picture of the cells examined.

Analysing the results of applying cytometry to urinary sediment diagnosis in renal transplantation may yield interesting results both from the clinical point of view and as regards graft pathophysiology. In clinical terms, it may be possible to identify and quantify sediment cell populations and correlate findings with the varying clinical conditions of the transplant patient, and on an immunological level, it may be possible to identify the profile of the main lymphocyte subpopulations present in the sediment, to compare this with the peripheral circulating subpopulations and to correlate these observations with the immunopathological mechanisms acting in the allograft and/or in the urinary tract.

In this study, a wide range of FC urinary tests were carried out on patients with a clear-cut clinical picture. The aim was to establish whether a correlation exists between urine cytometric results and individual pathology, in

Offprint requests to: Alessandro Nanni-Costa, M.D., Institute of Nephrology, St. Orsola University Hospital, Via Massarenti 9, 40138 Bologna, Italy

which case FC of the urinary sediment would indeed become a valuable diagnostic tool in transplantation.

Patients and methods

The study included 223 urine sediments from 127 transplant patients. All were examined by FC and by normal light microscopy techniques. Samples were divided into five categories selected according to clinical conditions:

1. normal renal function with no clinical or laboratory signs of bacterial infection (41 samples);
2. acute rejection diagnosed from clinical, laboratory, instrument and immunological signs (93 samples);
3. acute infection of the urinary tract diagnosed from clinical signs and culture isolation (57 samples);
4. acute post-transplant tubular necrosis diagnosed via clinical signs (oliguria) and/or laboratory investigations (creatinine clearance < 10 ml/min) (32 samples); and
5. tubular toxicity from cyclosporine (increase in serum creatinine with no sign of rejection, serum cyclosporine > 600 ng/ml (18 samples).

Urinary sediment from 25 normal subjects was examined as a negative control for reference purposes.

Urinary sediment preparation

Fresh urine (10 ml) from the first micturition of the morning was centrifuged at 200 *g* for 10 min. After removal of the supernatant, the sediment was treated at 22 °C for 10 min with a hypertonic solution (8.3% NH_4Cl, 1% $KHCO_3$ and 0.037% EDTA tetrasodic) in order to lyse out any erythrocytes, after which it was twice washed in phosphate buffer and resuspended in a final volume of 1 ml.

Morphological assessment

Cytomorphometric evaluation of samples was performed using a FACSCAN cytofluorograph (Becton Dickinson, Mountain View, USA). For each sample a cytogram was obtained based on the size and density of each individual cell element, calculated according to the spread of rays emitted by a laser source at a wavelength of 488 nm. From this computerized picture, identification was made of the various cell populations present in the sediment, and for each of these the percentage distribution and overall number was computed.

Analysis of surface markers

In 52 patients suffering from acute rejection (31 cases) or bacterial infection (21 cases) showing a lymphocyturia higher than 500 per ml, determination was made of lymphocyte surface markers on urinary sediment. Peripheral venous blood samples were taken simultaneously. The technique used was that of double immunofluorescence, employing mouse monoclonal antibodies conjugated with fluorescein and phycoerythrin (Table 1), which, because of the simultaneous use of the two fluorochromes allows simultaneous identification of two different markers on the same cells [3].

Table 1. Membrane markers and lymphocyte subpopulations

Membrane markers	Monoclonal antibodies	Lymphocyte population
CD3 + TCR	Leu 4 + alpha-beta TCR	T cell
CD3 + HLA class II	Leu 4 + HLA-DR	T-activated cell
CD3 + CD4	Leu 4 + Leu 3	T-helper cell
CD3 + CD8	Leu 4 + Leu 2	T-cytotoxic, T-suppressor (?)
CD16 + CD56	Leu 11 + Leu 19	NK cells

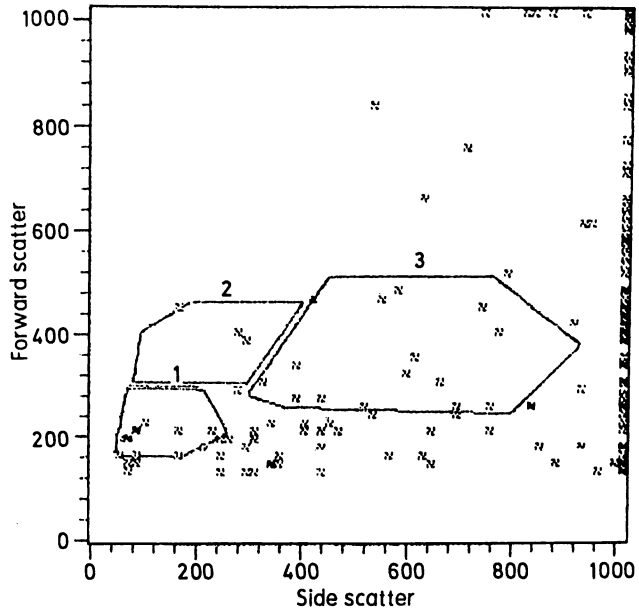

Fig. 1. Cytogram of urinary sediment in a normal subject. The sediment is poor in cells and debris

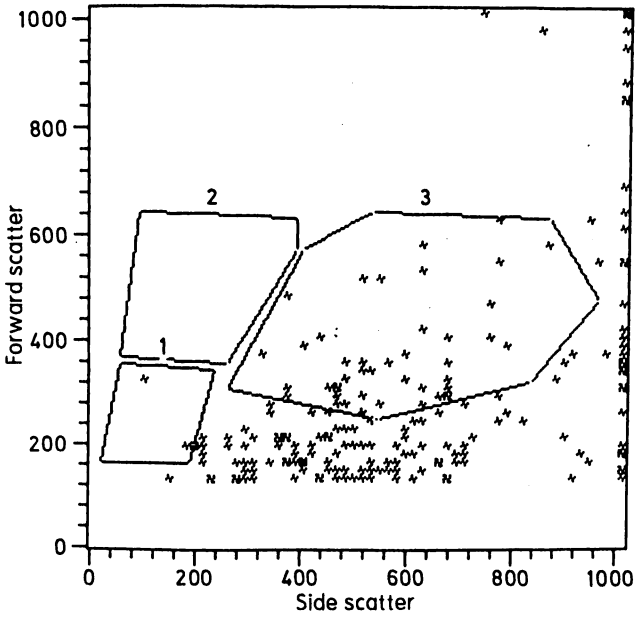

Fig. 2. Cytogram of urinary sediment in transplant patient with stable clinical condition. Cell count appears low

To determine lymphocyte surface markers, 0.1 ml of urinary sediment suspension was incubated with 20 µl monoclonal antibody at 22 °C for 15 min, lysed with hypertonic solution and washed twice in buffer solution (PBS) before being brought to a final volume of 0.5 ml.

The same procedure was repeated on samples of venous blood treated with EDTA in order to identify lymphocyte subpopulations in the peripheral blood. Samples were analysed by FACSCAN, obtaining fluorescence cytograms of the antibody-reacting lymphocyte populations. The percentage of positive lymphocytes was calculated for each membrane marker. Lymphocyte viability was assessed, after staining with ethidium bromide, immediately before cytometric analysis. The percentage of viable cells ranged from 55% to 90%.

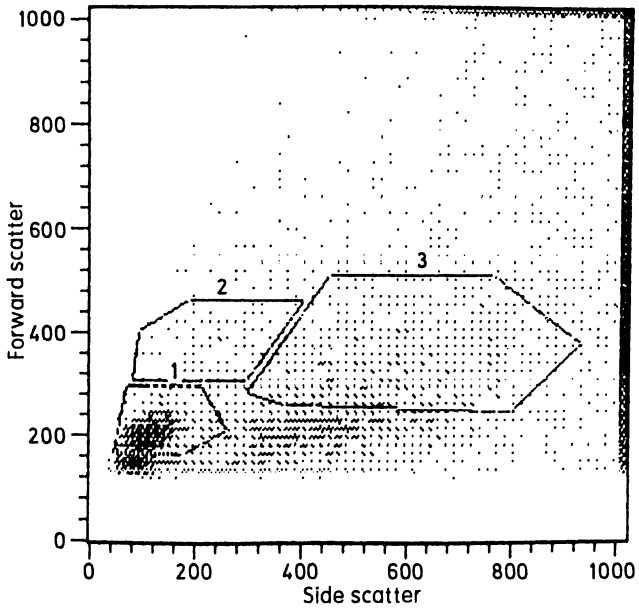

Fig. 3. Cytogram of urinary sediment in transplant patient during an acute rejection episode. Lymphocytes *(bottom right)* clearly predominate

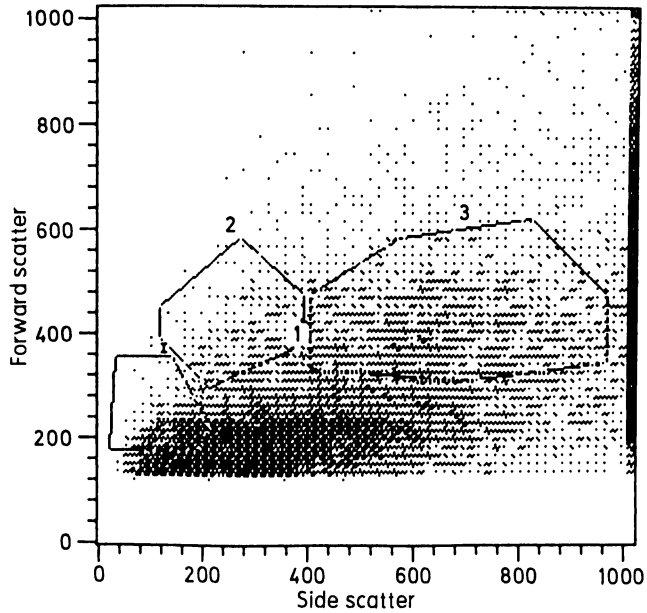

Fig. 5. Cytogram of urinary sediment in patient with post-transplant tubular necrosis. The debris *(right)* has a 'high scatter pattern', i.e. high density and large particles

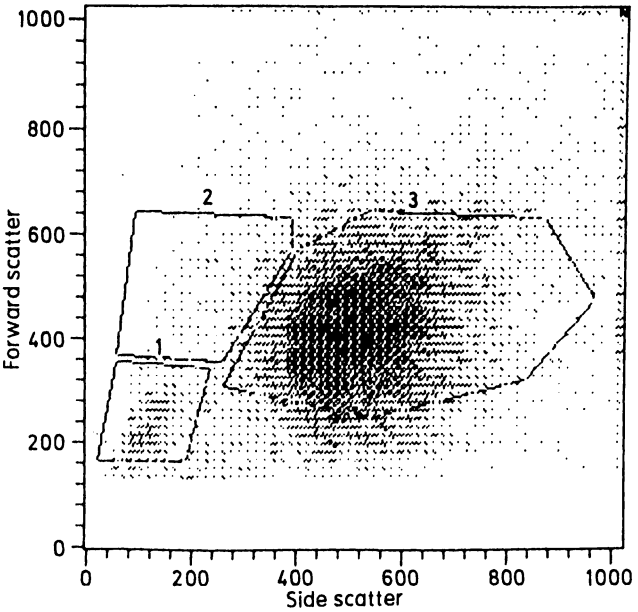

Fig. 4. Cytogram of urinary sediment in transplant patient with urinary infection. Clear prevalence of neutrophils *(middle of picture)*

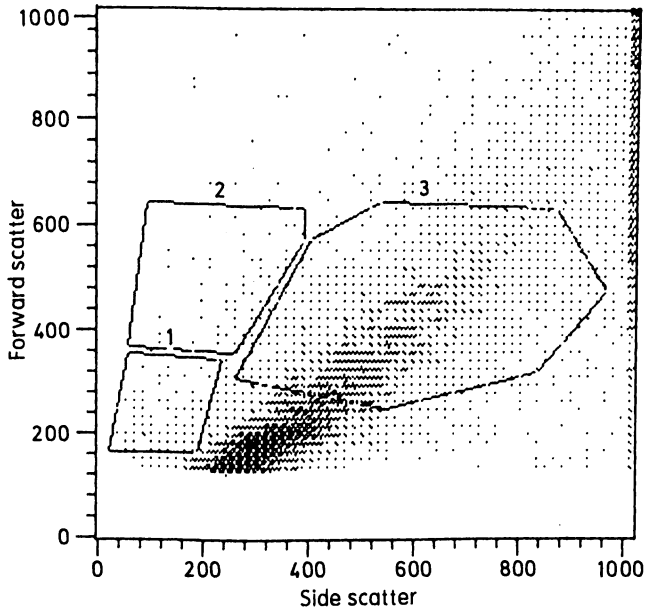

Fig. 6. Cytogram of urinary sediment in transplant patient suffering from cyclosporine toxicity. Debris *(bottom)* has a 'low scatter pattern', i.e. low density and small particles

Statistical analysis

Statistical analysis was performed according to Student's *t*-test for unpaired data.

Results

Figure 1 shows the graphic representation (cytogram), of the urinary sediment in a normal subject as a negative control: the sediment appears extremely poor both in cells and in debris.

Transplant patient cytograms showed a specific morphological pattern according to the clinical condition. Particularly evident are: low cell count in patients with stable renal function (Fig. 2), higher cell count (with lymphocytes clearly predominating) during acute rejection (Fig. 3), and clear prevalence of neutrophils during baterial infection (Fig. 4). Tubular pathology showed a morphological picture marked by the presence of cell debris. In acute post-transplant necrosis the debris had a high scatter pattern, i.e. high density and large particles (Fig. 5), while in cyclosporine toxicity (Fig. 6) debris had a low scatter pattern, i.e. low density and small particles.

Table 2 shows the number and percentage distribution of identified cell populations in certain of the patient groups (stable clinical condition, acute rejection, bacterial infection and acute tubular necrosis). Patients in a stable

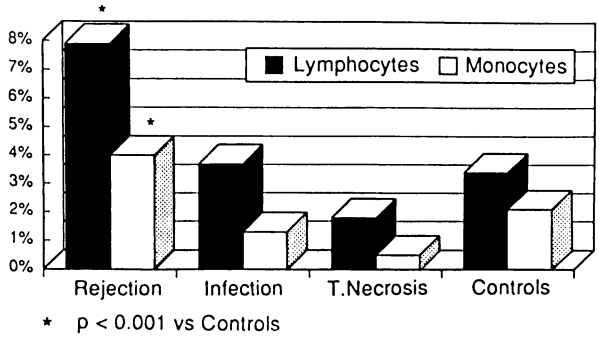

Fig. 7. Lymphocyte and monocyte percent distribution in the various patient groups

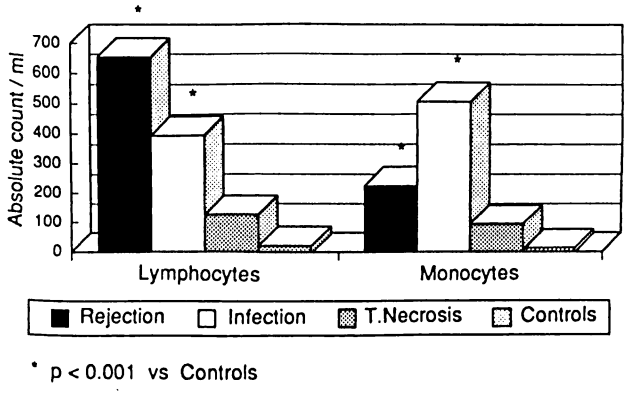

Fig. 8. Lymphocyte and monocyte counts in the various patient groups

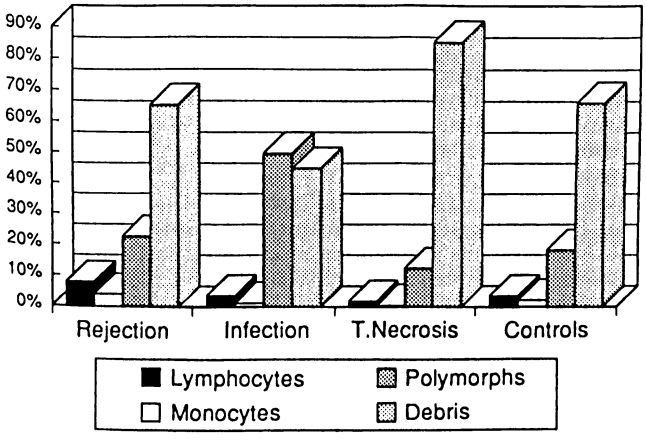

Fig. 9. Percent distribution of lymphocytes, monocytes, polymorphs and debris in the various patient groups

clinical condition showed a significantly lower cell count than the other groups ($P < 0.001$).

With regard to the lymphocyte and monocyte percent distribution and absolute count (Figs. 7 and 8), the acute rejection group showed the highest value ($P < 0.001$ vs other groups. Similarly, polymorphs and debris (Figs. 9 and 10) were typical of the bacterial infection and the tubular necrosis groups, respectively.

The distribution of the main lymphocyte subpopulations during acute rejection and bacterial infection is shown in Figs. 11 and 12. During rejection the subpopulation profile differed between the urine and the peripheral

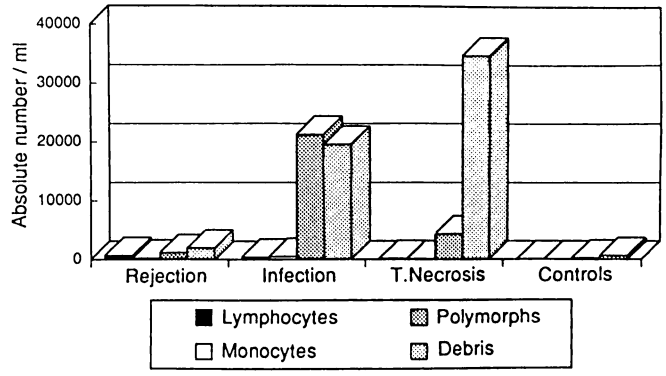

Fig. 10. Cell-count distribution of lymphocytes, monocytes, polymorphs and debris in the various patient groups

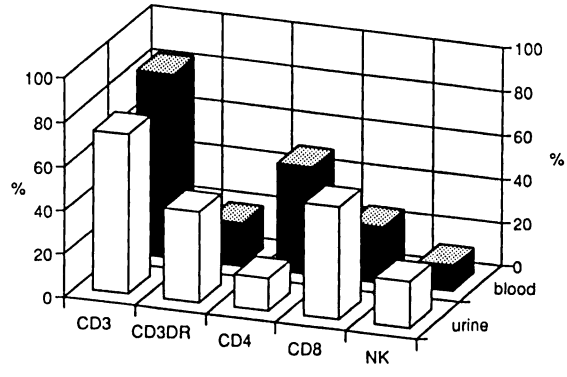

Fig. 11. Lymphocyte subpopulation profile in blood and urine during rejection

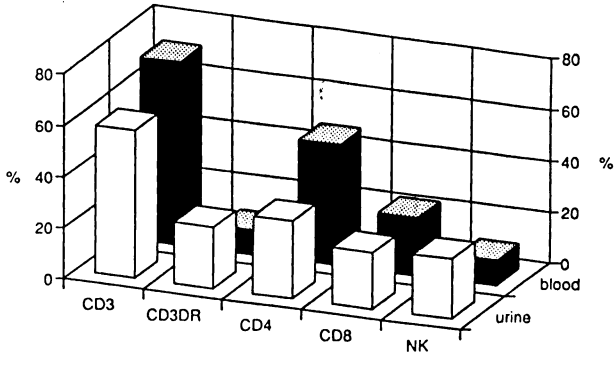

Fig. 12. Lymphocyte subpopulation profile in blood and urine during bacterial infection

blood. The main populations in the sediment were T cells (CD3 and TCR), positive for both CD8 and HLA class II antigens, combined with populations with NK markers and a CD4/CD8 ratio less than 1. In bacterial infection the subpopulation profile in the urine seemed similar to that of the peripheral blood. T populations were predominant, yet the percentage of CD4-positive lymphocytes seemed higher than in rejection, while T-DR-positive, CD8 and NK populations were significantly lower.

Discussion

Despite the noticeable increase in technical equipment currently used in clinical practice, an early and accurate diagnosis of the differing pathological conditions which

Table 2. Number and percent distribution of cellular elements in urinary sediments of renal transplanted patients

	Rejection (n = 93)		Infection (n = 57)		ATN (n = 32)		Controls (n = 41)	
	n	%	n	%	n	%	n	%
Lymphocytes	654* ±267	7.9	394* ±122	3.7	127* ±43	1.8	21 ±25	3.4
Monocytes	225* ±48	4.0	506* ±171	1.3	94* ±63	0.5	11 ±6	2.1
Polymorphs	1143* ±365	22.7	21276* ±5780	49.9	4351* ±794	12.4	184 ±37	18.3
Debris	1954* ±515	65.4	19591* ±6443	45.1	34788* ±13648	85.3	628 ±207	66.2

* $p < 0.001$ vs Controls
ATN, acute tubular necrosis

may affect a transplanted kidney is problematic for the physician, who sometimes needs time-consuming diagnostic protocols or invasive techniques in order to pinpoint the specific allograft pathology.

FC of the urinary sediment seems to be of real assistance. First of all, the advantage of urine over blood as the medium for patient cytological and immunological monitoring derives from the consideration that, as a product of the transplant organ, urine composition reflects actual intragraft events and is unlikely to be affected by irrelevant systemic events, which may condition blood cell populations. Furthermore, because of its peculiar technical features, urine cytomorphometric analysis offers various advantages over 'classical' cytology: first and foremost, greater simplicity in preparing samples, together with rapidity of execution; second, objectivity, reproducibility and reliability of measurement; third, the ability to obtain a graphic representation and statistical elaboration of the parameters observed [8].

Our results confirm that the diagnostic potential of urine FC is certainly interesting. It allows one to define lymphocyturia during rejection, to identify the morphological profile of urinary sediment in acute tubular necrosis and bacterial infection, to distinguish between a diagnosis of rejection or ischaemic damage in post-transplant oliguria, and to detect tubular damage during cyclosporine administration [7, 11].

In addition, by providing a serial, easily repeatable, non-invasive analysis of the cellular infiltrate of kidney transplants [5], FC may provide further insights for patient clinical monitoring. Our findings on lymphocyte surface markers (Table 1) lead to the identification of the various different lymphocyte subpopulations present in urinary sediment during rejection. The main populations are T cells (CD3 and TCR positive) showing both CD8 and HLA class II antigens, combined with populations with NK markers and a CD4/CD8 ratio less than 1. The antigen pattern of these lymphocytes, probably consisting of active cytotoxic cells, suggests that they come from cytotoxic clones placed in the allograft. These subpopulations also appear significantly different in profile from those circulating in the patient's peripheral blood.

During urinary infections CD4-positive T lymphocytes appear in the urine, probably expressing an immune response against bacterial antigens. Interestingly, the profile of these populations differs only slightly from that of the peripheral blood.

The results we have reported in this study suggest that monitoring of urinary cytology by means of FC may be a simple, reliable diagnostic tool offering the clinician rapid information on the allograft condition.

Acknowledgment. Supported in part by the Dott. Carlo Fornasini Foundation, Bologna.

References

1. Dooper MM, Bogman MJ, Maas CN, Vooys GP, Koene RA (1989) Immunocytology of urinary sediments in renal transplant patients with deteriorating graft function. Transplant Proc 21: 3596–3597
2. Eggensperger D, Dowd GO, Schweitzer S, Light J (1987) The clinical efficacy of cytodiagnostic urinalysis in the diagnosis of acute allograft rejection: clinico-pathological correlation of 122 specimens. Transplant Proc 19: 1787–1789
3. Fleischer TA, Hagengruber C, Marti GE (1988) Immunophenotyping of normal lymphocytes. Pathol Immunol Res 7: 305–318
4. Krishna GG, Fellner SK (1982) Lymphocyturia: an important diagnostic marker in renal allograft rejection. Am J Nephrol 2: 185–191
5. McWhinnie DL, Thompson JF, Taylor HM, Chapman JR, Bolton EM, Carter NP, Wodd RF, Morris PJ (1986) Morphometric analysis of cellular infiltration assessed by monoclonal antibody labeling in sequential human renal allograft biopsies. Transplantation 42: 353–359
6. Muirhead KA, Horan PK, Poste G (1985) Flow cytometry present and future. Biotechnology 3: 337–355
7. Nanni-Costa A, Stefoni S, Buscaroli A, Iannelli S, Borgnino LC, Stagni B, Raimondi C, Cotti P, Bonomini V (1989) Urinary cytology in renal transplantation. Flow cytometry evaluations. Third European Cytometry User's Meeting, Ghent 1989, pp 123–137
8. Shapiro HM (1988) Practical flow cytometry, 2nd edn. Alan R Liss, New York
9. Simpson MA, Madras PN, Cornaby AJ, Etienne T, Dempsey R, Clowes GH, Monaco AP (1989) Sequential determination of urinary cytology and plasma and urinary lymphokines in the management of renal allograft recipients. Transplantation 47: 218–223
10. Spohn B, Bauer H, Stoetter H, Guth R, Franz HE (1983) Comparison of urine cytology versus fine needle aspiration cytology in monitoring renal allograft disfunction. In: Touraine JL, Traeger J, Betuel H, Brochier J, Dubernard JM, Revillard JP, Triau R (eds) Transplantation and clinical immunology. Excerpta Medica, Amsterdam, pp 218–222
11. 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: 1665–1669