Clinical Graft Evolution of Lymphocytes, Polymorphonuclear Cells, and Antigen Expression in Tubular Renal Cells in the Urine Sediment of 20 Renal Allograft Recipients

J. Péfaur, R. Triviño, C. Navarrete, E. Oberhauser, M. Melys, I. Morales, P. Salinas, and A. Mocarquer

ABSTRACT

Urinary samples from 20 kidney transplant recipients were studied to determine the cellular composition of the sediments using an immunocytological (IC) technique. The expression of HLA class I (A, B, C) and class II (DR, DQ, DP), CD2, CD3, CD4, CD8, and interleukin (IL)-2 receptor (IL-2R) on lymphocytes was assessed using a panel of monoclonal antibodies. The results were correlated with graft function and with the number of episodes of acute renal graft rejection (AR) during a period of 6 months posttransplantation. The cellular infiltration of lymphocytes (LC) and polymorphonuclear cells (PMNC) also was studied using a standard cytology (SC) technique. During this period, 17 of 30 episodes of graft dysfunction due to AR occurred in 12 patients: 8 to acute tubular necrosis (ATN) (n = 8); 4 to cyclosporine (CsA) toxicity (n = 4) and 1 to amphotericin toxicity (n = 1). The diagnosis of AR was made clinically by 3 independent observers, using biopsy in some cases. The immunocytology showed a significantly increased expression of HLA-DR, DO, and DP namely, greater than 20% positivity in 10% of samples on the tubular epithelial cells (TEC) of patients presenting with versus without AR ($P \leq .001$). In addition, a high correlation was observed between the expression of IL-2R and the presence of AR ($p \leq .002$). The standard cytology results showed a significantly increased percentage of LC and decreased percentage of PMNCs in samples obtained 2 days prior to the clinical manifestations of patients who developed AR ($P = .001$). A greater level of expression of antigen determinants was observed prior to AR. These results suggest that immunocytology of urinary sediments, which is a noninvasive technique, has enormous clinical potential for the differential diagnosis of AR, ATN, and CsA toxicity. In our study, the use of HLA class IL–specific monoclonal antibodies (Abs) gave a 100% specificity, 95% sensitivity, and 95% predictability. Although our results also indicate a potential value in the increased IL-2R expression, these findings must be confirmed by further studies. Furthermore, the combination of both immunologic and SC techniques in urinary sediments allows early detection of AR and is cost effective and simple features that could be used routinely for follow-up of renal transplant recipients.

ACUTE CELLULAR allograft rejection is one of the most frequent complications in renal transplantation. Its speed, intensity, and frequency has a direct impact on the long-term outcome of the graft. It is, therefore, crucial that a clear diagnosis be established as early as possible.

Standard urine cytology may provide a simple tool to diagnose acute renal allograft rejection (AR) because it is a noninvasive technique that can be performed sequentially at no risks to patients. Using this technique, a number of different findings have been described to diagnose graft-related pathologies of acute cellular rejection, acute tubular necrosis (ATN), and cyclosporine (CsA) toxicity.[1,2]

From the Department of Nephrology (J.P., M.M., I.M., P.S., A.M), Hospital Barros Luco Trudeau, Department of Experimental Medicine (R.T., E.O.), University of Chile, and North London Blood Transfusion Centre, London, England.

Supported by Fondecyt Grant No. 1930264.

Address reprint requests to J. Péfaur, Department of Nephrology, Hospital Barros Luco Trudeau. E-mail: jpefaur@tie.cl

The use of monoclonal antibodies (MoAbs) reactive with cell-specific immunocytology markers, such as CD2, CD3, CD4, and CD8, in the urinary sediments (US) has resulted in the development of a more sensitive technique, immunocytology (IC) of urinary sediments. This technique, similar to the IC of fine-needle aspirates, provides a better characterization of the cellular components in urinary sediments including polymorphonuclear cells (PMNC), lymphocyte cells (LC), and tubular epithelial cells (TEC).[1,3,4] However, these cells are also present in a variety of other pathological conditions associated with graft dysfunction, therefore, the application of more specific markers is required to provide a differential diagnosis of AR.

Increased or de novo expression of HLA class II cell surface markers is the direct result of immune activation of the posttransplantation endothelium.[5] For these reasons, HLA class II molecules may provide an additional marker to assess the cellular composition of urinary sediments. Furthermore, published data suggest that the expression of HLA class II antigens in association with lymphocytosis in the US of kidney allograft recipients can contribute to the diagnosis of AR.[6,7] The expression of interleukin 2 (IL)-2 and IL-2 receptor (IL-2R) in plasma and urine of transplant recipients also has been postulated to be useful marker to monitor cellular rejection.[1]

In our study, both standard cytology (SC) and IC techniques were used to study the cellular composition of US samples obtained from 20 renal transplant recipients. The expression of HLA class I (A, B, C) and class II (DR, DQ, DP) markers was determined on TEC as well as the expression of CD2, CD3, CD4, CD8, and IL-2R on infiltrating cells. The results were correlated with graft function and with the number of AR episodes during the first 6 months posttransplantation.

MATERIALS AND METHODS

Patient Selection

Twenty-two patients who underwent transplantation during a 10-month period from November 1993 to August 1994 (minimum follow-up period of 6 months) were studied prospectively. Fifteen patients received a cadaveric and 5 live related donors graft. Twelve were men and 8 women of mean age of 39.8 years (range 25–59 years). All patients were treated with the same immunosuppressive regimen of prednisolone, azathioprine, and CsA, according to our local protocol.

Samples

Urine samples were sequentially collected from each patient 3 times a week from day 3 posttransplantation until the end of the third month as well as weekly sampling until month 6. The patients follow-up included laboratory and clinical parameters according to the local protocol.

Renal dysfunction was defined when the serum creatinine level increased 25% over the baseline levels. The clinical diagnosis of rejection episodes was performed by 3 independent observers. Renal biopsy specimens, when available, were evaluated blindly by 2 independent observers. The final diagnosis was determined based upon the biopsy specimen, when available, or the clinical consensus of 3 clinical observers.

Renal biopsy was only performed when other etiologies for renal dysfunction were suspected. All AR episodes were treated with intravenous administration of 0.5 to 1.0 g/d of methylprednisolone for 3 days, according to the age and weight of the patient. The diagnosis of ATN was considered when the renal dysfunction was associated with a persistently low urinary output in the immediate posttransplantation period with or without dialysis support, or in biopsy-confirmed cases performed on day 5.

CsA toxicity was diagnosed when renal dysfunction was accompanied by CsA trough levels >300 ng/dL, and/or in the presence of suspicious clinical symptoms or laboratory findings, eg, tremor, gingival hypertrophy hyperbilirubinemia, hyperuricemia and hyperglycemia, after a clinical response to reduced CsA doses, and/or biopsy findings.

Amphotericin toxicity was diagnosed when the renal dysfunction appeared following administration of high drug doses and when the biopsy failed to reveal AR and CsA toxicity.

Processing of Samples

Fifteen milliliters of the second stream urine samples were processed within 2 hours of collection. Samples were centrifuged for 10 minutes at 1500 rpm. The supernatant was discarded and the cell pellet washed twice in PBS, pH 7.4. In the final wash, the cell pellet was resuspended at a concentration of 10^6 cells/mL. The final volume was then divided into 2 aliquots. One was used to prepare 2 slides, which were stained using May Grunwald-Giemsa/Wright for SC examination. The second aliquot was used for immunocytochemistry after 40 μL of cell suspension was distributed into a 10-well glass slide and left to air dry. The cells were then fixed with cold acetone (4°C/5 min) and left to air dry again. The slides were wrapped in foil and frozen at −20°C. Immunocytochemical staining was performed by adding 40 μL of the relevant MoAb to each well for 1 hour, followed by 3 washes in TBS at pH 7.6 for 2 minutes each wash. Each slide was incubated with 40 μL of rabbit anti-mouse immunoglobulin (RAM Ig) for 30 minutes. After 3 more washes, the slides were incubated with APAAP complexes (Dako APAAP kit System) for a further 30 minutes and then washed in TBS. The reaction was developed using alkaline phosphatase for 20 minutes at room temperature (RT). (Naphtol AS MX phosphate, fast red, TS, levamisol and 15 mL 0.1 mol/L substrate buffer). The final wash was performed in tap water. The slides were then counter-stained with hematoxylin, mounted in glycerol buffer, and read using a light microscope. All incubations were carried out at RT in a humidified chamber with MoAbs to detect monomorphic HLA class II (DR, DQ, and DP) L-227 monomorphic HLA-DR L-243; monomorphic HLA class I (A, B, C) W6/32; CD2 (OKT11) CD3 (OKT3); CD4 (OKT4); CD8 (OKT8); IL-2(P-55); anti-keratin type 5 and 8 (RCK 102), and epithelial cells (LP34).

Analysis of the Samples: SC

After a minimum of 100 cells or at least 10 fields were assessed in each slide the results were expressed as percentage positive cells. Samples with <25 cells or those with bacterial or red blood cell contamination were excluded from the analysis. Epithelial cells from the lower urinary tract, urothelial cells, and red blood cells also were excluded. All samples were assessed by 2 independent observers with no knowledge of the clinical data.

The analysis included the percentage of LC, eosinophils, PMNC, and renal TEC types I and II, including cytoplasmic characteristics

Table 1. Clinical Parameters and Evolution of Transplanted Recipients Who Experienced AR

Patient No.	DV/C	Compatibility	% PRA	No. of AR Episodes	Creatinine 6 mon	Biopsy	AR Post Observation Period	Creatine Level
1	V	2A1B-2DR	0	3	4.3	1/AR	0	6.4
3	C	1A-1B-1DR	27	3	1.75	1/TxCsA	0	2.6
5	V	1A-2B-1DR	0	1	1.5	0	1	1.5
6	C	1A-1DR	8	1	1.5	0	0	1.2
8	C	1A-1B-1DR	2	2	1.4	1/AR	0	1.6
10	V	2A-2B-2DR	3	1	1.5	0	3	3
12	C	1DR	0	1	1.4	2/NTA	0	1.5
13	C	1A-1B-1DR	0	1	1.3	1/NTA	0	1.3
14	C	1-DR	3	1	1.36	1/NTA + AR	1	1.3
15	C	1A-2DR	4	1	1.2	1/NTA + AR	0	1.2
18	C	1A-1DR	20	1	1.5	1/NTA	1	2.5
20	V	1A-1B-1DR	0	1	1.1	1/AR	0	0.9
Average			5.58	17	1.65			1.39

of the latter. Samples were considered to indicate rejection in the presence of >20% LC and <55% of PMNC as previously described.[2,8]

IC

Samples with <10 cells/well were excluded from the analysis. Similar to the method for the SC, a minimum of 100 cells or ≥10 different fields (100 HPF) were required in each well and the results expressed as percentage of cells staining positive. As previously reported, the cytoplasmic staining of TEC was clearly distinguished from the membrane staining of lymphoid cells.[7,9]

RESULTS

During the follow-up, 30 episodes of graft dysfunction were observed in 20 patients. Seventeen were due to AR; 8 to ATN; 4 to CsA toxicity; and 1 to amphotericin toxicity. The 17 episodes of AR were observed in 12 patients based upon a clinical diagnosis in 15 cases, and both clinical and biopsy features in 5 cases.

Of the 12 patients with AR, 9 experienced only 1 episode of rejection, 1 patient had 2 episodes and 2 patients had 3 episodes. An additional mean follow-up of these patients of 218 days (range, 53–519 days) showed 6 more episodes of AR, 3 of these occurring in 1 patient. A further AR episode was observed in 1 patient or on 6 days after completing the observation period. All the AR episodes were treated with methylprednisolone (Table 1).

The group of patients who did not show AR (8 patients) within 6 months were observed for a further mean of 174 days (range, 55–319 days). During this period, 1 patient (number 9) suffered 2 episodes of AR at days 116 and 206. The rest of the patients remained well (Table 2).

The results of 13 biopsies performed in 12 patients showed 7 with ATN, 3 with AR, 1 with CsA toxicity and 2 with AR associated with ATN.

Regarding IC, of the 970 urine samples, 335 of them fulfilled the criteria for analysis because 621 were too small and 14 were inadequate. Each sample was repeated in duplicate in plates of 10 small wells each with 10 different markers, including both a positive and a negative control. The percentage of cells that express HLA class I (ABC), class II DR and DR DP DQ, CD2, CD3, CD4, CD8, and IL-2 were correlated with the presence or absence of clinical diagnosis of AR.

A greater expression of HLA class II markers was seen in the group of 12 patients who did versus the 8 who did not experience AR ($P < .01$) (Fig 1 and Fig 2).

Figure 1 shows the individual patient data with the number of AR (in red squares) and the percentage of antigenic expression (green dots) for each sample. Using a chi-square analysis we documented a breaking point of significance at 20% for DR, DP, DQ and at 15% for DR, as shown by Figure 2 ($P < .02$).

Table 2. Clinical Parameters and Evolution of Transplanted Recipients Who Did Not Show AR at 6 Months

Patient No.	DV/C	Compatibility	% PRA	No. of AR Episodes	Creatinine 6 mo	Biopsy	AR Post Observation Period	Creatinine Level
2	C	1[a]A-1B-2DR	2	0	1.2	0	0	1.4
4	V	2[a]A-1B-2DR	0	0	1.3	0	0	1.28
7	C	1[a]-A-1B-1DR	5	0	1.5	1/NTA	0	1.5
9	C	1[a]-A-2DR	14	0	1.4	0	2	1.85
11	C	1B	0	0	0.99	0	0	1.5
16	C	1B-1DR	2	0	1.1	1/NTA	0	1.2
17	C	1B-1DR	7	0	1.05	1/NTA	0	1.2
19	C	1[a]A-1B-2DR	2	0	1.1	0	0	1.2
Average			4		1.2			1.39

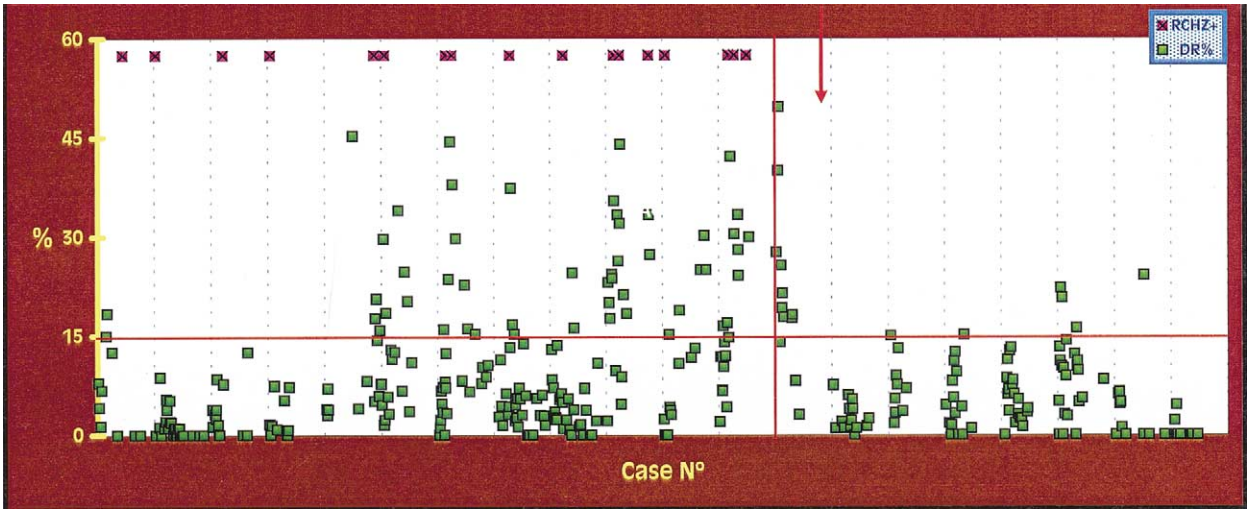

Fig 1. PR-OP-DQ percentual positivity of urinary immunocytology.

It is interesting to note that 1 patient (patient no. 9), who did not have AR during the observation period, behaved differently from the rest of the group without rejection. If one excludes this subject from the group of nonrejecting patients, the significance increases ($P < .001$). We think that this patient was experiencing subclinical rejection episodes during the 6-month observation period, which lead to 2 late AR episodes, as opposed to the rest of the group who did not show any AR episodes.

The other antigenic determinants were not significantly different among both groups. With the criteria of positivity of >20% for the cellular markers DR, DP, DQ, and >15% for DR, this technique showed a positive trend since day −2; however, it was not statistically significant due to the small number of samples.

We found that the HLA class II antigenic expression on epithelial cells was significantly lower between patients who did not have a rejection episode during the period of observation versus the patients who subsequently rejected only after 5 days before the diagnosis of rejection (Table 3).

The results in Table 3 show that there is a significant difference in the basal status of immune activation in both groups of patients.

The expression of IL2-R was positive in 26 samples correlating with AR in 17 of them. Among the remaining 9 samples, 3 belonged to patient no. 9 who experienced ATN during the immediate posttransplanation period, which was statistically significant ($P < .02$).

If we consider as a criterion of 20% increased positivity in the expression of DR-DP-DQ in >10% of the samples, we yield a specificity of 87.5%, a sensitivity of 66.7%, and a predictive value of 75%. If we correlate the expression of DR-DO-DQ with DR we obtain a specificity of 87.5%, a sensitivity of 83.3%, and a predictive value of 85%.

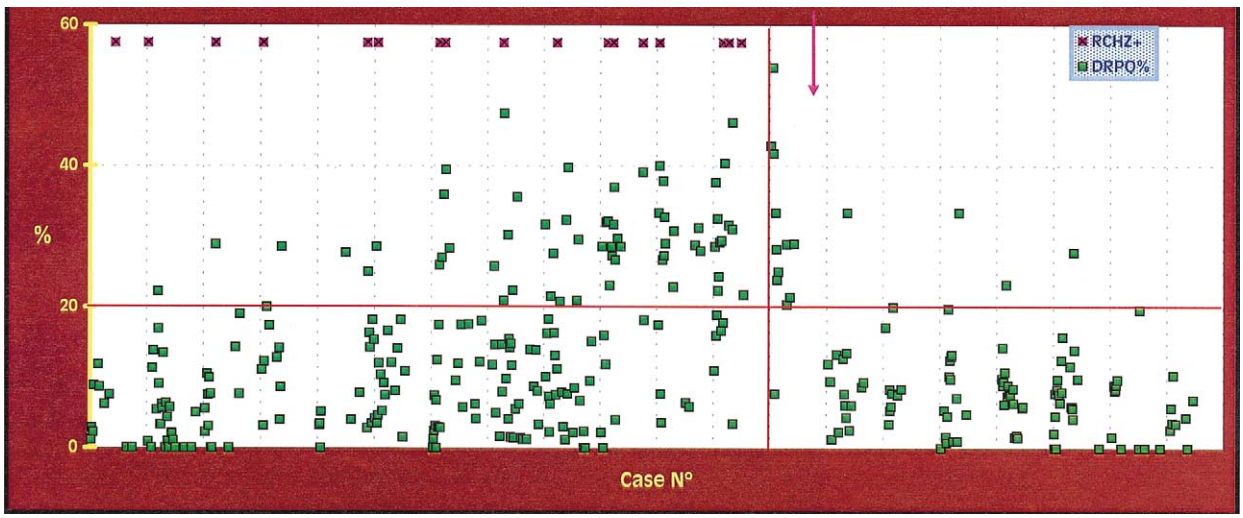

Fig 2. DR-DP-DQ Percentual positivity of urinary immune cytology.

Table 3. Significance in the Antigenic Expression Comparing Both Groups in the Absence of AR

	DRDPDQ	DR
Group with AR		
Average	13.7	9.2
ST error	0.76	0.68
Group without AR		
Average	8.0	5.6
ST error	0.66	0.54
P	<.05	.0047

Evaluating cases of CsA toxicity, there was only 1 false-positive for DR-DP-DQ and none for DR and IL2-R among 3 of the 4 patients who experienced it. We did not have any samples to evaluate the fourth patient. The low number of CMV infections in the study group did not allow us to evaluate the correlation of DR with DR-DP-DQ. One of the 2 patients showed a false-positive reaction for AR.

Results of Urinary Cytology

The average values for each day prior to the AR (day 0), showed a trend toward decreasing the PMNC percentage and increasing LC in urine cytology beginning on day −4. The increase between days −4 and −3 was significant compared with that from days −2 to 0 ($P < .0006$) (Fig 3).

DISCUSSION

The use of the US is a fast, simple and noninvasive procedure that permits sequential follow-up of the transplanted organ. An increase in the number of lymphocytes in the US of patients with AR linked with a simultaneous decrease in the percentage of PMNC represents a useful early diagnostic tool for AR. The introduction of IC techniques for surface antigen recognition of a large number of cells has proven its usefulness in the differential diagnosis of the causes of graft dysfunction.[10] Cytological and IC monitoring to diagnose AR has generated great interest in the recent literature, not only in renal transplantation,[11] but also in simultaneous kidney-pancreas transplantation.[2]

The ICO has been compared with both biopsy and clinical findings yielding similar results, showing high sensitivity and specificity namely, 97% and 83%, respectively, compared with 95% and 80%, respectively for histological diagnosis as compared with a clinical diagnosis.[7–9]

Although the expression of HLA-DR appears to be a marker during AR, some groups[12] have shown that in renal biopsies the ICAI that CET of transplant recipients without

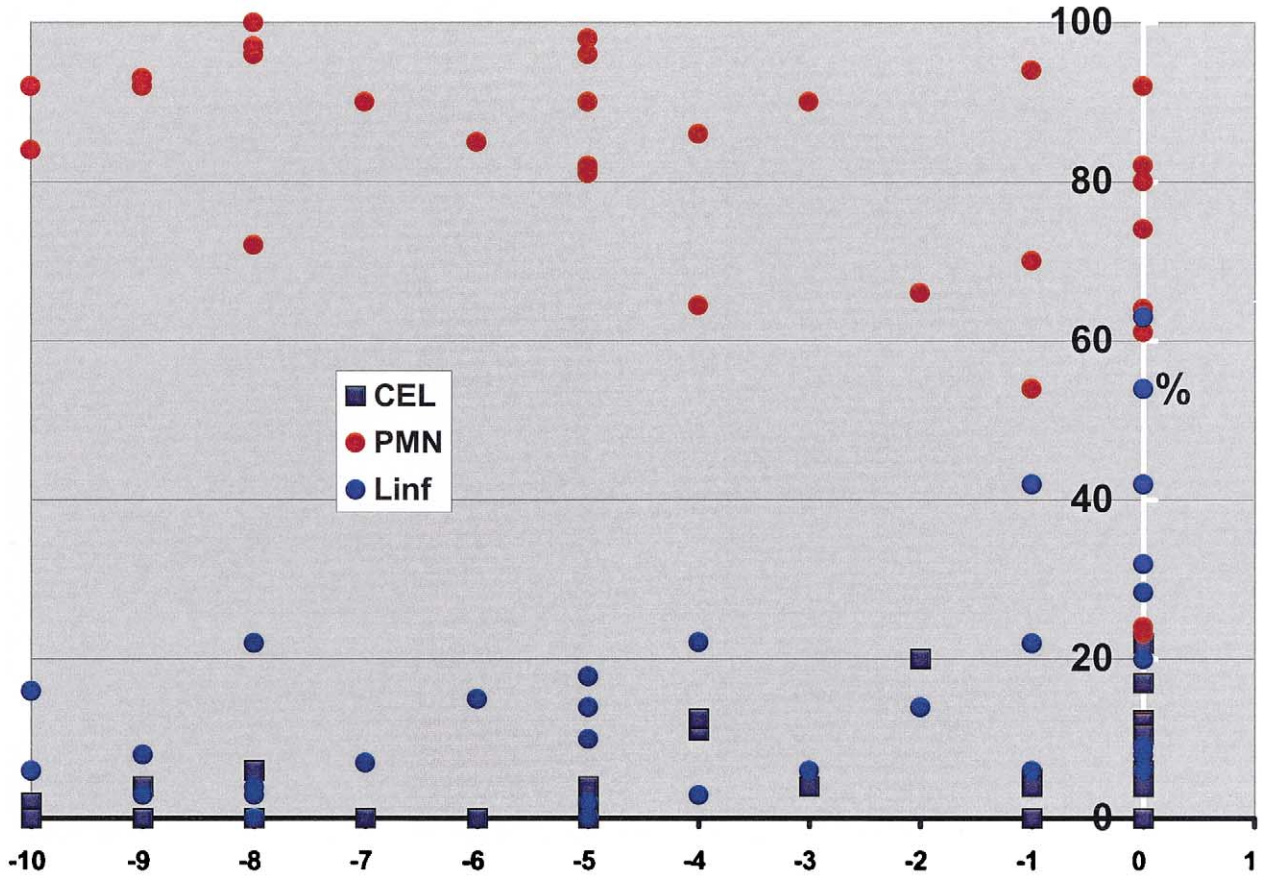

Fig 3. PMNC and tubular cells in the urine beginning 10 days prior to AR.

AR can also express DR. However, Miller (1988), using CAI, found that injury autotransplanted dogs did not induce the expression of antigen HLA-DR over CET as opposed to the findings with AR in allotransplantation. In addition, it has been reported that CsA and succesful antirejection therapy may decrease the expression of HLA-DR antigens, but Dooper et al[7] reported that it does not decrease its credibility as a diagnostic tool.

The sensitivity and specificity of CO as observed in our report covers concurs with the findings that an increase of LC >20% and a decrease in PMN <55% to be diagnostic criteria as previously described by other authors and closely correlating with the presence of AR. The high specificity observed herein must be confirmed in larger numbers of patients The finding of significant positivity of the test 2 days prior to the ACR (with a positive trend starting 4 days prior) represents a fundamental finding for the early therapy of AR.[10] If we add the low cost of the test, it becomes a highly useful method to follow transplant recipients. Our findings agree with previous reports for ICAI[7,9,13,14] and ICO,[7,15] revealing a close correlation of the expression of class II HLA antigens over renal CET and AR, showing significantly increased antigenic expression during AR.

The high specificity obtained by the ICO for the method, considering a value >20% of marked cells, may be considered to be an essential tool for the differential diagnosis of AR in the presence of a decreased graft function.

We observed an important increase in the sensitivity of the ICO when combining the percentage of DR-DP-DQ expression (20%) and DR (15%). Both parameters belong to class II HLA antigenic determinants. Thus, it is expected that its expression should be similar in all samples, suggesting that improvements in the methodology should increase the sensitivity using only 1 of the MOAC to decrease the cost. In relation to HLA II expression as a predictor of AR, we observed an increasing trend in expression starting on day −2, although we did not have a sufficient number of samples to reach statistical significance, a finding that agrees with conventional cytology. As reported in the literature,[10] both techniques are highly specific, allowing us to make a differential diagnosis of CsA toxicity in this pilot study.

Patient no. 9, who constantly showed high levels of antigenic expression may represent a case of subclinical rejection, as suggested by the fact that he had 2 AR episodes immediately following the observation period. The significant difference between the period without AR (episodes) compared with patients who never had any AR, represents a situation of higher HLA expression in the group who had AR afterwards, suggesting an immunological activation state that predisposes to a higher risk of AR episodes and may be relevant both clinically and therapeutically.

We found no reports relative to the class I HLA Ag as a rejection marker and we did not observe significant differences comparing groups with versus without AR.

The T8/T4 ratio >1.5,[16–20] described by some as indicating AR, is controversial and its diagnostic value is questionable. Our results are inconclusive because most samples had a low cellularity. Helderman et al[21] reported that only 5% of the LC in patients with stable renal function showed IL2-R with a weaker tinction trend, and that 50% of the lymphoblasts were positive with a stronger tinction trend. Arndt et al[14] reported a peak of HLA-DR and IL2-R expression in LC among renal aspirates during and prior to AR. This observation confirms the importance of this marker of the clonal expansion of T LC and as a parameter to evaluate the immune activation state. In addition, it has been described that CsA qualitatively inhibit IL-2, but not its receptor.[21] Our study found that IL-2R expression was closely related to AR episodes, appearing almost exclusively in its presence, with a high specificity. On the other hand, the absence of IL-2R expression during AR was also frequent, showing that it was correlated with low sensitivity. We think that the techniques should be improved and/or combined with another parameter to improve both its sensitivity and specificity.

The ocurrence of abnormal OC prior to the clinical diagnosis of AR represents a high-yield and low-cost method as a clinical monitoring test. Urine immun-cology, due to its high specificity and sensitivity, represents a test with great clinical potential for the differential diagnosis and management of the transplanted recipient, allowing it to be implemented as a routine test. The role of IL-2R and other possible cellular immune activation markers represent potential areas of investigation.

REFERENCES

1. Madras P, Simpson M, Cornaby A, et al: Transplant Proc 21:1842, 1989
2. Radio SJ, Stratta RJ, Linder J, et al: Transplant Proc 26:529, 1994
3. Bishop G, Waugh J, Horvath J: Lancet 2:645, 1986
4. Bishop G, Hall B, Waugh J, et al: Lancet 2:645, 1986
5. Totterman T, Hanas E, Larsson E, et al: Transplant Proc 19:3570, 1987
6. Schumann G, Palmieri L, Jones D: Am J Clin Pathol 67:580, 1977
7. Dooper P, Bogman M, Maass C, et al: Transplant Proc 21:1873, 1989
8. Burrows L, Kupfer S, Shanzer H, et al: Transplant Proc 25:1052, 1993
9. Dooper Ine, Bogman J, Hoitsma A, et al: Transplantation 52:266, 1991
10. Kyo M, Gudat F, Dalquen P: Transplant Proc 24:1388, 1992
11. Bangui L, Hartono C, Ding R, et al: N Engl J Med 344:947, 2001
12. Raftery MJ, Seron D, Koffman G, et al: Transplantation 48:238, 1989
13. Gray DW, Richarson A, Hugles DW, et al: Transplantation 53:1226, 1992
14. Arndt R, Weiss M, et al: Transplant Proc 20:614, 1968
15. Lee PH, Huang MT, Lee CS: Transplant Proc 24:1543, 1992
16. Taube D, Welsh K, Hobby P, et al: Clin Nephrol 22:127, 198
17. González-Posada JM, García MC, Losada M, et al: Nefrología 9:99, 1989
18. González-Posada JM, García Castro C, Losada M, et al: Nephrol Dial Transplant 5:226, 1990
19. Waugh J, Bishop GA, Hall BM, et al: Transplant Proc 17:1701, 1985
20. Van Oers MHJ, Surachino S, Wilmink JM: Transplant Proc 19:1647, 198
21. Helderman J, Hernández J, Sagalowsky A: Kidney Int 32:376, 1988