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ORIGINAL ARTICLE

Urinary cytotoxic molecular markers for a noninvasive diagnosis in acute renal transplant rejection*

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Summary

Perforin (P), Granzyme B (GB) and Fas-Ligand (FAS-L) are cytotoxic molecules involved in acute rejection (AR) after renal transplantation. A noninvasive diagnostic test to monitor AR and other complications could improve clinical management. We investigated the predictive and diagnostic interest of target mRNA measurements, with a quantitative PCR assay, in AR, as well as in other clinical complications recurrent in kidney transplantation. One hundred and sixty-two urine specimens from 37 allograft recipients were investigated. Clinical settings were AR, urinary tract infection (UTI), cytomegalovirus infection (CMVi) or disease (CMVd), chronic allograft nephropathy (CAN), delayed graft function (DGF) and stable graft course (controls). In the case of AR, mRNA levels of all three molecules were significantly higher than in recipients not showing any clinically evident signs of complication. Indeed, it was observed that expression levels of P, GB and Fas-L mRNA also increase in other clinical situations such as UTI, CMV and DGF. Finally, kinetic studies in three patients with AR revealed that increased P, GB and Fas-L mRNA levels could precede or were concomitant with increased serum creatinin levels. P, GB and Fas-L gene expression in urine specimens were upregulated in AR episodes but also in UTI, CMV infection and DGF. Therefore, this technique would appear to be of limited clinical value as a noninvasive method of diagnosing AR.

Introduction

Acute rejection (AR) during the first month following kidney transplantation, is the major immunological event that may influence the longer term outcome of the transplant [1–6]. Clinical and routine biological signs are not always sufficient to differentiate AR from other causes of graft dysfunction, such as acute tubular necrosis, drug toxicity, viral or bacterial infection.

Therefore, kidney biopsy remains the major implement for the diagnosis and evaluation of acute graft dysfunction. Recent refinements have reduced but not eliminated biopsy-associated complications such as haematuria, anuria, perirenal haematoma, bleeding, shock, arteriovenous fistula and graft loss [7,8]. Inaccessible localization for allograft biopsy, sampling errors and limitations of AR diagnosis by noninvasive Doppler imaging [9] are additional problems. The development of an accurate noninvasive diagnostic test allowing a specific diagnosis of AR would be of considerable value.

Cytotoxic lymphocyte (CTL)-mediated apoptosis is thought to play a major role in the rejection of renal

allograft following transplantation [10–12]. Antigen-triggered T-cell activation and the subsequent infiltration of activated $CD4^+$ and $CD8^+$ T cells, macrophages [13] and natural killer (NK) cells into the graft are key events in acute allograft rejection [14]. The two major effector pathways of CTL killing/mediating apoptosis involve the Fas/Fas-L lytic pathway and the Perforin/Granzyme degranulation pathway.

Although not evaluated in urine samples, Perforin (P), Granzyme B (GB) and/or Fas-L expression levels have already been evaluated in peripheral blood samples [15–17] and in graft specimens [18–21], where they have been shown to be significantly increased during AR. Indeed, Li *et al.* have reported that P and GB messenger RNA (mRNA) expression levels, tested in noninvasive urine samples, were significantly increased during AR episodes [22,23]. The same group reported that levels of CD103 mRNA, encoding a protein expressed on the cell surface of CTLs were increased in urinary cells in cases of AR [24]. Measuring mRNA encoding cytotoxic proteins in urinary cells, in conjunction with the proteomic-based detection profiling method [25], may be a novel aid in AR diagnosis and could add to or even replace standard methods. Finally, a more recent study showed an inverse correlation between the levels of FOXP3 mRNA in urine samples of AR patients [26]. Most of these studies are in favour of AR diagnosis molecular markers relevance in noninvasive samples after renal transplantation.

Nevertheless, previous investigations have not resolved the important issue of whether other clinical events such as urinary tract infection (UTI), CMV infection (CMV_i) or disease (CMV_d) and delayed graft function (DGF) also bring about increased mRNA levels of these cytotoxic molecules and therefore possibly limit the overall diagnostic value of such determinations [27].

In this study, we developed a real-time PCR assay, using hybridization fluorescent target specific probes to obtain a sensitive and rapid quantitative assessment of RNA expression levels of Perforin, Granzyme B and Fas-L mRNA in urinary cells. This method aimed at measuring expression levels of these molecules in the different relevant clinical settings mentioned above and at determining whether increased levels of these markers in urine is specific to acute renal allograft rejection.

Patients and methods

Patients

Sequential urine specimens were obtained from 37 cadaveric kidney allograft recipients, transplanted over a period of 9 months from November 2001 to July 2002. Patient clinical characteristics (Banff classification, sex, induction treatment) are reported in Table 1.

The immunosuppressive regimen combined polyclonal anti-lymphocyte globulins (ATG 9 mg/kg at day 0, then 3 mg/kg on days 1–4, Fresenius, Taunusstein, Germany) with tacrolimus (Fujisawa, La Celle Saint Claud, France), azathioprine (GlaxoSmithKline, Marly Le Roi, France) and steroids. Polyclonal globulins were replaced by basiliximab (Novartis, Switzerland) in patients older than 60 years. Patients with pretransplant panel reactive antibodies received mycophenolate mofetil (Roche, Bâle, Switzerland) instead of azathioprine. Acute rejection treatment consisted in steroid-based regimen and a switch of Azathioprine to Mycophenolate mofetil. In compliance with the declaration of Helsinki, patients were informed that their urine samples would be used in molecular studies and were included after giving their written consent.

Collection of urine samples

Urines emitted between midnight and 07:00 a.m. were collected at regular intervals during initial hospitalization and then at each outpatient visit, except for patients presenting chronic allograft nephropathy (CAN), from whom we obtained only one urine sample. Among the 311 urine specimens collected, 162 (52%) taken from 37 patients, were suitable for cDNA synthesis; 69 specimens were collected in the first month following transplantation, 53 during the second and third months, 20 from the fourth and sixth months and 20 were taken at a later interval.

The distribution of analysable urine specimens, according to the clinical situations described above are reported in Table 1a, and creatinin serum levels are shown in Table 1b. Rejection was diagnosed by standard pathological parameters and classified according to the Banff criteria [28] making it possible to confirm seven cases of AR [$n = 8$ specimens, two collected from the same patient at time of AR; four rejections occurred during the first month (median = 19 days post-transplantation) while three rejections were late acute rejections occurring 1 year or more post-transplantation]. Patients underwent needle biopsies only in cases of increased serum creatinin level in order to identify the basis of graft dysfunction. At the time of AR diagnosis, six of the seven AR patients, were found to have their immunosuppressive drugs in the therapeutic range and one had stopped his immunosuppressive treatment. Six out of seven patients responded to anti-rejection treatment with a return to a baseline serum creatinin levels, whereas one did not respond and was referred for haemodialysis. The distribution of other specimens was as follows: 14 specimens of bacterial UTI (positive urinary culture and clinical manifestations; treated with antibiotics until negative urinary culture), eight specimens of CMVi (Clinical manifestations, Ag pp65 positive), six

Table 1. (a) Clinical characteristics of patients and Banff classification. (b) Creatinin level (in μmol/ml) within clinical situations.

	Acute rejection ($n = 7$)	No acute rejection ($n = 30$)	*P*-value
(a)			
Recipient age (year)	46.7 (±11.9)	47.2 (±13.4)	NS
Donor age (year)	34.14 (±12.1)	40.7 (±16.5)	NS
Recipient gender (M/F)	6/1	17/13	NS
Mismatches	3.86 (±1.21)	3.8 (±1.0)	NS
Cold ischemia time (min)	960 (±171)	1333 (±441)	$P = 0.014$
Rejection day postoperative (day)	485.85 (±710.6)		
Banff classification			
Suspicious (mild cellular infiltrate)	3		
IA	2		
IB	0		
IIA	2		
IIB	0		
ATG induction	7	23	

	Min	Max	Mean	SD
(b)				
Controls (CONT)	57	610	159.93	±127.01
Acute rejection (AR)	111	1037	343.5	±320.83
Delayed graft function (DGF)	280	794	531.8	±216.66
Urinary tract infection (UTI)	66	275	134.57	±46.85
Chronic allograft nephropathy (CAN)	110	480	272.33	±124.93
Cytomegalovirus infection (CMVi)	83	117	100	±10.86
Cytomegalovirus infection (CMVd)	105	143	121.16	±13.96

specimens of CMVd (Ag pp65 positive, positive CMV PCR, and treatment with Gancyclovir, clinical manifestations), nine specimens of CAN and five specimens of DGF. The remaining 81 specimens were collected from patients with stable graft function and were defined as controls (CONT). In our population, there was no biopsy-proven BK virus nephropathy.

We also collected urines from nontransplanted healthy donors ($n = 5$); CD3/CD4/CD8 phenotype revealed very low levels of $CD3^+$ T-cells representing $0.06 \pm 0.02\%$ (among which, $33.1 \pm 32.7\%$ were $CD4^+$ and $18.64 \pm 18.58\%$ were $CD8^+$). Because of the low level of cytotoxic T-cells, these samples were not included in the RNA extraction and QRT–PCR analysis. In comparison, CONT samples contain $1.05 \pm 3.42\%$ (among which, $41.18 \pm 33.19\%$ were $CD4^+$ and $20.76 \pm 24\%$ were $CD8^+$).

In addition to the AR samples, 30 other urine specimens from three patients suffering from AR were analysed prior to or after biopsy-proven samples in the aim of performing a kinetic study of P, GB and Fas-L mRNA expression. The kinetic study was completed by a kinetic expression analysis of the three genes in UTI ($n = 3$ patients) and CMVi ($n = 2$ patients) clinical situations as well as in CONT.

Isolation of RNA and cDNA preparation

Urine samples (500 ml) were centrifuged at 2500 *g* for 30 min at 4 °C. Total RNAs were extracted using a commercial kit (RNeasy®, Qiagen, Courtaboeuf, France) with the addition of a polyA RNA carrier, according to the manufacturer's recommendations. In our study, 52% of RNA extractions were suitable for cDNA synthesis and PCR. Thirteen of 40 μl extraction column-eluted RNA were reverse transcribed as previously described [29] in a final volume of 20 μl. The presence of cDNA and genomic DNA contamination was checked by standard PCR amplification of an ubiquitous gene cRaf, as previously described [30], before QRT–PCR.

Quantification of gene expression by real-time PCR assay

TaqMan probes and primers

Primers and fluorescent probes for target genes and a housekeeping gene HPRT (hypoxanthine phosphoribosyl transferase) were designated using LightCycler Probe Design software (Roche diagnostics, Meylan, France) or Oligo 5.0 (MedProbe SA, Oslo, Norway). All primers were chosen to specifically target mRNA (spanning intron/exon junctions) and to avoid any genomic DNA

amplification. Further information about these primers and probes are provided in Table 2.

Real-time PCR assay

Real-time PCR assay is performed using fluorescence resonance energy transfer hybridization probes (FRET) that binds to the PCR product in a head-to-tail fashion. When the two oligonucleotides bind, their fluorophores come into close proximity, allowing a transfer of energy from a donor (fluoresceïn) to an acceptor dye (Red640) (Roche Diagnostics, Meylan, France). The fluorescence thus generated, which is directly proportional to the target starter amount, is measured in real-time on the Lightcycler® (Roche, Meylan, France) thermocycler.

For each RNA sample and for each gene, 2 μl of cDNA were amplified, in duplicate, in a reaction mixture containing 10 μl of premix reaction PCR buffer (Quantitec probe PCR Mastermix, Qiagen), 10 μM each of forward and reverse primer, 5 μM of each fluorescent probe in a volume of 20 μl. and added to the mix. For all targets studied, the program consisted in a *Taq* DNA polymerase activation at 95 °C for 15 min and then heating at 95 °C for 5 s, 62 °C for 40 s and 72 °C for 40 s.

The accumulation of PCR products was detected by monitoring the increase in fluorescence. The cycle number (C_t) of a sample, defined as the moment when the fluorescence became three times greater than the background was directly compared with C_t of a standard curve, consisting of a plasmid-DNA dilution, where the number of copies of a target gene is known. PCR products were cloned in a pGEMT-easy vector (Promega, Charbonnières, France) according to the T/A cloning procedure in order to prepare plasmid dilutions for each target and housekeeping gene. The plasmids were then serially diluted one hundred fold in a salmon sperm DNA solution (100 ng/μl). The data collected were automatically analysed at the end of the thermal cycling using Lightcycler® v3.5.2 software. As we did not estimate the quantity of starter RNA, or the efficiency of the reverse transcription reaction, we used the HPRT housekeeping gene (as described in Kotsch *et al.* [31]), with a comparable level of expression than the target genes [32], to normalize the QRTPCR data.

Table 2. Primers and fluorescent probes of quantitative real-time PCR (QRT–PCR).

	Primers		Probes		Genebank accession number		PCR products size (bp)
	Location	Sequence (5–3′)	Location	Sequence (5–3′)	mRNA	gDNA	
Perforin	528–545	CCGCTTCTACAGTTTCCA	776–793	CTGTCGAGGCCCAGGTCA-f	M28393	M31951	367
	880–895	GCTCCCGGTAGGTTTG	793–813	x-ATAGGCATCCACGGCAGC-p			
Granzyme B	329–347	GCTACTGCAGCTGGAGAGA	505–527	AAGATCGAAAGTGCGAATCTGAC-f	XM012328	NT019583	280
	590–609	CAGAVTCCCCCTTAAAGGAA	530–553	x-ACGCCATTATTACGACAGTACCA-P			
Fas-L	333–348	GCAGAACTCCGAGAGT	469–486	CCATGCCTCTGGAATGGG-f	U11821	NT004668	312
	624–644	GTTCCTCATGTAGACCTTGTG	489–511	x-GACACCTATGGAATTGTCCTGCT-p			
HRPT	180–197	CACATTGTAGCCCTCTG	221–240	CTTTGCTGACCTGCTGGAT-f	M31642	M26434	159
	322–338	GTCCCCTGTTGACTGG	242–276	x-CATCAAAGCACTGAATAGAAATAGTGATAGATCC-p			

Location of primers and fluorescent probes is given from the first base of the start codon ATG.
Fluorescent dyes: f, fluorescein; x, Red 640; p, phosphate.

Statistical analysis

Statistical analysis was performed using nonparametric tests such as the Mann–Whitney *U*-test for comparing quantitative variables and Fishers exact test for qualitative variables. For significant statistical determination, data from a given group were compared with data from each other group. Spearman's rank correlations were used to test for a monotonic association of P, GB and Fas-L

mRNA levels with serum creatinin levels, in different clinical settings.

Results

Isolation of RNA and cellular mortality

In total, we collected 311 urine specimens but successful QRT–PCR RNA extraction and cDNA synthesis was achieved in only 162 samples (52%). The mean number of lymphocytes in the urine cellular pellets in successful and failed extraction pellet, stood at 5.8×10^6 and 4.7×10^6, respectively, and were not statistically significant ($P = 0.11$). However, cellular mortality was higher, in successfully extracted cellular pellets versus pellets with failed RNA isolation (65.37% vs. 44.3%, $P < 0.001$). Quality/quantity of RNA/cDNA were compatible with QRT–PCR, with no difference between target and reference genes, as attested by the mean of Ct levels for both CTL markers [mean 32.02 ± 2.89 (min 26–max 38), 34.04 ± 3.84 (min 32–max 39), 35.06 ± 1.96 (min 28–max 39), respectively, for GB, P and Fas-L] and housekeeping gene [HPRT, mean 35.76 ± 2.15 (min 32–max 39)]. Finally, no evidence of specific failed RNA extractions was seen within the different clinical situations.

Real-time PCR validation

The sensitivity of each QRT–PCR allowed the detection of one copy of target mRNA diluted in 100 ng of gDNA, the equivalent of 10^5 cells. Standard curves were generated after the amplification of triplicate plasmid-DNA dilutions by plotting C_t (Threshold cycle: the cycle at which the amplification plot crosses the threshold, i.e. the moment at which there is a clearly detectable increase in fluorescence) values against Log of number of gene copies. The dynamic range for all QRT–PCR covered 5 Log of dilution (Fig. 1). Calculated regression co-efficients were all >0.95, indicating good linear correlation. QRT–PCR efficiencies were calculated from regression line slopes (curves?) using the $[10^{(1/-S)}-1]$ equation and which stood at 97.7%, 93.7%, 93.8% and 91.4% for P, GB, Fas-L and HPRT QRT–PCR respectively. Comparable results were found after amplification of serial dilutions of cDNA synthesized from urine sample extracted RNAs (data not shown).

Assay reliability was assessed by studying the reproducibility of intra- and inter- QRT–PCR analysis. The intra-assay reproducibility was estimated by repeating the analysis of the same sample (each plasmid dilution) three times in the same assay. For 5 Log of dilution, co-efficient of variance (CV) mean values stood at 0.452% (range: 0.1010–0.7880), 0.434% (0.2528–0.9676), 0.475% (0.1203–1.2755) and 0.657% (0.015–1.854) for P, GB, Fas-L and HPRT QRT–PCR respectively.

Inter-assay reproducibility of all four QRT–PCR was achieved by amplifying three different cDNA samples, in duplicate, in three different experiments, on three different days. CV mean values stood at 6.12%, 5.24%, 8.39% and 3.97% for P, GB, Fas-L and HPRT QRT–PCR respectively.

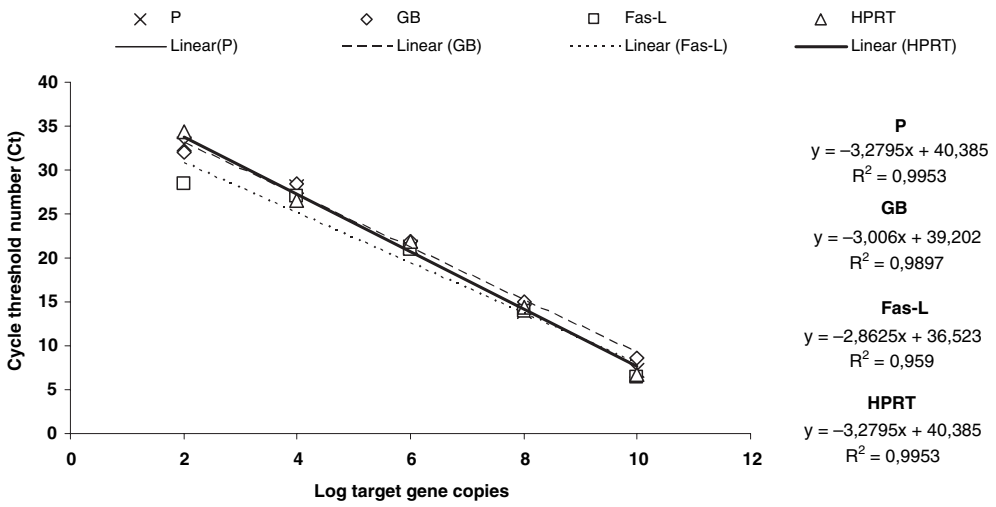

Figure 1 Linear relationship between C_t and Log number target gene copies for the three target genes P, GB and Fas-L and the constitutive HPRT gene. Each plotted value corresponds to the triplicate mean value. Serial dilutions of plasmid DNA standard were amplified to detect the dynamic range of the four QRT–PCR. Equations of the linear curve (where the slope represents the PCR efficiency) as well as the linear correlation (r^2) co-efficients are indicated. PCR efficiency (E) can be calculated from the slope (S) of the regression line using the mathematical equation $E = 1 - 10^{-1/S}$.

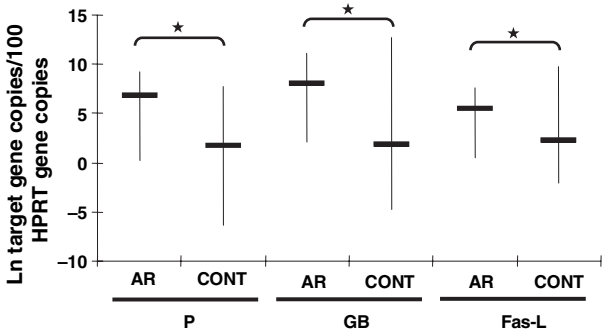

Figure 2 Median (horizontal thick bars) and min/max values (vertical thin bars) of gene expression level for the three target genes P, GB and Fas-L in urine samples from patients in renal acute rejection (AR, $n = 8$) compared with transplanted recipients with stable graft course after transplantation (controls: CONT, $n = 81$). Results are expressed in Ln number target gene copies/100 copies HPRT. The numbers of urine samples are shown in brackets. Statistical significance is indicated for expression of a target gene between two groups: (★) represent $P < 0.01$.

Analysis of cytotoxic molecular mRNA levels in episodes of acute allograft rejection

As shown in Fig. 2, P, GB and Fas-L gene expression levels were significantly heightened in the urine samples collected over the course of AR ($P < 0.01$ for all three gene expressions) compared with controls. Surprisingly, there was a major overlap between AR specimen values and those observed in control patient urine samples. For example, in some urine specimens collected from patients with stable graft function gene expression levels were higher than the lowest values found in AR specimens. Overall, there were 60, 37 and 64 such cases for P, GB and Fas-L respectively.

In contrast, no significant difference in P, GB and Fas-L gene expression was found between AR urine samples and urine specimens collected in the course of UTI ($n = 14$), CMV infection urine specimens ($n = 8$) or those collected during DGF ($n = 5$; Fig. 2). Overall, while the level of expression for each of all the three genes remains similar when comparing AR with other clinical situations, we noted a discrepancy between the three gene expressions and only P expression, but not that of GB and Fas-L, was significantly higher ($P < 0{,}01$) in AR than in CMVd ($n = 6$) urine samples.

We did not observe a significant association between GB, P, and Fas-L mRNA and serum creatinin levels in the AR group ($r_s = -0.7$, $P = 0.85$, $r_s = -0.23$, $P = 0.52$, $r_s = 0.05$, $P = 0.9$ respectively for GB, P and Fas-L) nor within the control group ($r_s = -0.02$, $P = 0.82$, $r_s = -0.032$, $P = 0.74$, $r_s = -0.02$, $P = 0.81$, respectively, for GB, P and Fas-L).

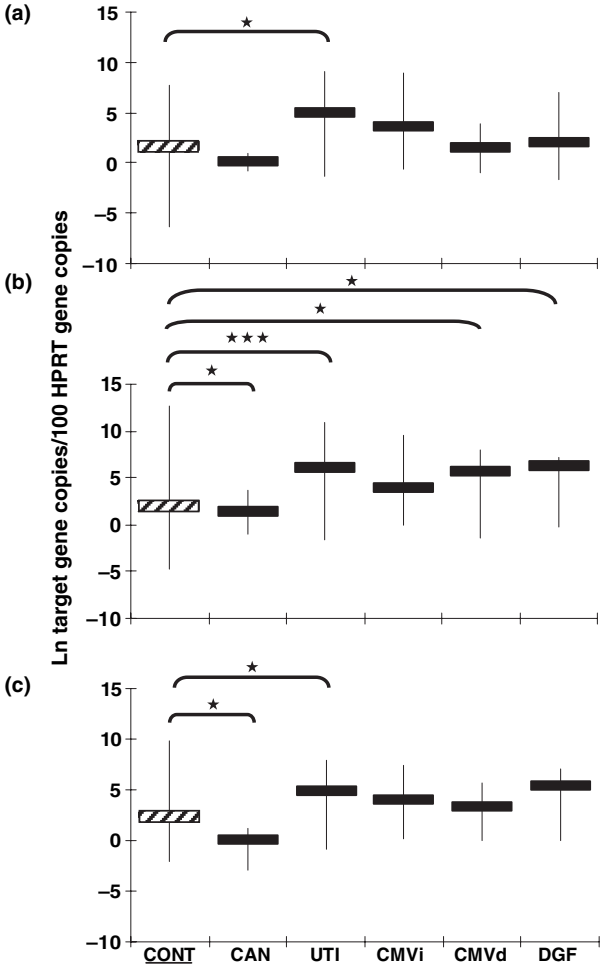

Figure 3 Median (horizontal thick hashed and black bars, respectively for CONT and others) and (min/max) values (vertical thin bars) of gene expression level for the three target genes P (a), GB (b) and Fas-L (c) in urine specimens from patients in chronic allograft nephropathy (CAN, $n = 9$), with urinary tract infection (UTI, $n = 14$), with CMVi, ($n = 8$) or CMVd, ($n = 6$) and with delayed graft function (DGF, $n = 5$) compared with control (CONT, $n = 81$) group. Results are expressed in Ln number target gene copies/100 copies HPRT. Statistical significance is indicated for expression of a target gene between two groups: (★) and (★★★) represent $P < 0.05$ and $P < 0.01$ respectively.

Analysis of other pertinent clinical complications following renal transplantation

The expression of all three target gene expression levels was significantly higher in urine samples collected in the course of UTI – as observed in AR specimens – than in control urine samples ($P < 0.01$ for all; Fig. 3). Similarly, as in the case of CMVi, no difference was found in any of the three gene expression levels when compared with controls.

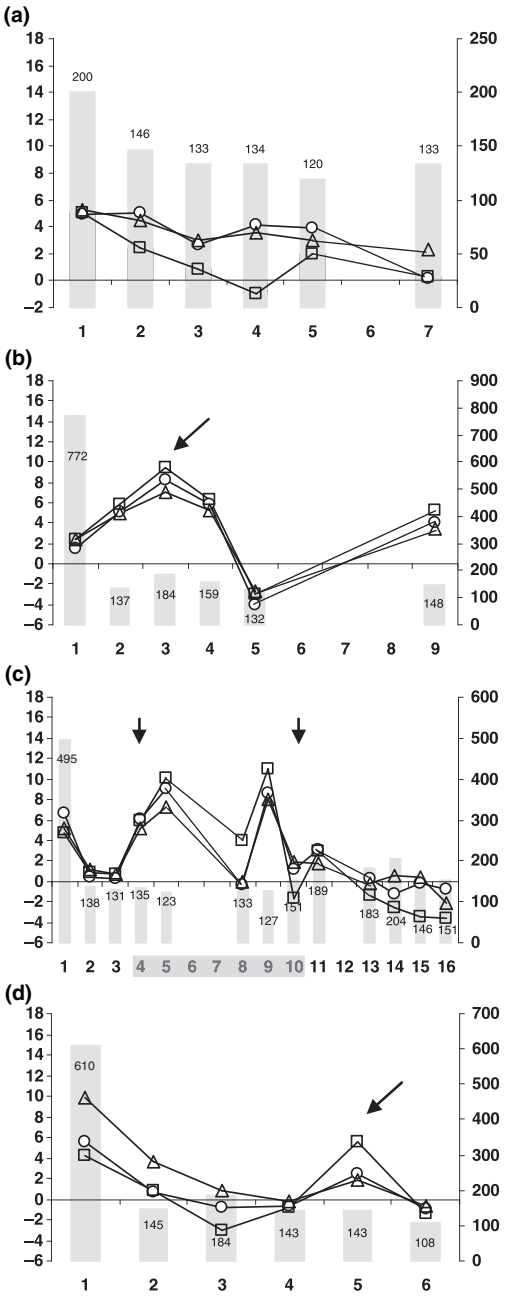

Figure 4 Representative kinetic (out of three AR, three UTI and two CMVi) of expression level for the three target genes Perforin (○), Granzyme B (□) and Fas-L (△) in four clinical situations from four different patients as CONT (a), AR (b), with UTI (c) and CMVi (d) expressed as the Ln of target gene copies/100 HPRT gene copies (left *y*-axis). The number of weeks post-transplantation is presented on the *x*-axis. The serum creatinin level, with the value reported as vertical bars and expressed in μmol/ml (right *y*-axis). The arrows correspond to the time of significant clinic status, such as acute rejection (a) or CMV infection (d). The shaded area (graph c) is the period (weeks 4–10 post-transplantation) of UTI; vertical arrows represent the start (diagnosis of UTI) to the end of the antibiotic treatment (disappearance of clinical signs of UTI).

In contrast, discrepancies between the three gene expression levels were observed in other comparisons. When compared with controls, only GB gene expression is significantly elevated in DGF ($P < 0.01$) and CMVd ($P < 0.05$) whereas P and Fas-L gene expression remain stable. GB and Fas-L, but not P, gene expressions were statistically lower in CAN urine specimens than in CONT samples ($P < 0.05$ for both).

Kinetic analysis during AR, CMVi and UTI and CONT

In three patients presenting an AR episode, sequential urine specimens were obtained before and after AR onset or in other patients at time of clinical events (UTI or CMVi) as well as in controls. As shown in Fig. 4, for representative examples, expression levels for the three target genes remained stable in urine specimens of patients with stable graft function and without any clinical event (Fig. 4a). Otherwise, we observed a simultaneous increase in all three genes in cases of AR. In only one case of AR (Fig. 4b), however, did the expression of target genes precede the increase of serum creatinin by 9 days. In the two other cases available for this study, these events were concomitant. Moreover, a kinetics study revealed that expression of the three genes remained constantly elevated during UTI and CMVi even in the absence of an increase in serum creatinin levels (Fig. 4c).

Discussion

Acute renal allograft rejection is a major risk factor for chronic allograft nephropathy and allograft failure. A rapid and reliable diagnosis of AR, without biopsy, would expedite AR diagnosis and thus may be of value in CAN prevention and increasing chances of renal graft survival.

P, GB and/or Fas-L mRNA expression have previously been reported as being upregulated during acute renal allograft rejection. Upregulation was observed in histology fragments as well as in transplant recipients' blood or urines. The report of Li *et al.* [22] on AR diagnosis in urine samples was particularly interesting. Nevertheless, the clinical interest of this test relies on its ability to differentiate AR from other acute signs of allograft dysfunction such as UTI, DGF, or CMV disease. Unfortunately, these relevant situations were incompletely investigated in Li's work, as Soulillou [27] has already pointed out.

In order to elucidate the clinical interest of measuring P, GB and Fas-L mRNA expression in the urines of kidney allograft recipients, we developed a real-time QPCR assay, using the FRET-hybridization-probe technique, on a LightCycler, to measure the expression of these three genes in urinary cells. We studied P, GB and Fas-L gene expression during acute rejection

episodes and in other situations of graft dysfunction, such as UTI, CMVd, CMVi, DGF and CAN. Moreover, serial urine collection made it possible to analyse the development of gene expression in these various clinical settings.

Our work confirms the findings of Li's work [22] by showing that P and GB expression levels are significantly higher in urine specimens of recipients presenting AR, than in the urines of recipients having a stable graft course after transplantation. Moreover, our results demonstrate that Fas-L gene expression is also upregulated in urine CTLs during AR episodes. This confirms Kotsch *et al.*'s results [31]. Nevertheless, our results were notably different from those of Li *et al.* [22] insofar as we observed a major overlap between gene expression measurements in the different clinical settings studied. Therefore, it was not possible to establish a threshold value for acute rejection diagnosis. For example, in some urine specimens collected from patients with stable graft function gene expression levels were higher than the lowest values found in AR specimens.

In our experience P, GB and Fas-L gene expression measurements were not sufficiently significant as to differentiate AR from other common clinical complications following renal transplantation, such as UTI. The 3 genes appeared to be upregulated in cases of UTI as well as in AR episodes. These findings contradict those published by Dadhania *et al.* [23], that reported low GB expression levels in UTI cases as well as those published by Kotsch *et al.* [31], which similarly showed that T-cell (CD3) and cytotoxic (granulysin) marker expressions were not increased in patients with bacterial UTI in the absence of AR. However, the type and duration of bacterial infection should be taken into account. We observed significant upregulations of GB gene expression in the course of DGF, CMVd and CAN, and of Fas-L gene in CAN urine specimens. Our results are consistent with those obtained in nontransplanted patients demonstrating heightened expressions of P, GB and Fas-L genes during bacterial [33–35] or viral infections [36–38]. They are also in accordance with those obtained in kidney transplant recipients showing increased GB plasma concentrations during CMV infection [11,39,40].

We do not believe that the discrepancies between our results and those previously published are due to the methodological approach; we also used a sophisticated and perfectly reliable QRT–PCR technique, with high sensitivity and insignificant inter or intra-assay variability. The role of induction therapy with polyclonal antibodies is a more likely hypothesis in explaining the observed differences. Anti-lymphocyte globulins may alter the number of urine lymphocytes as well as lymphocytic functions, as described in peripheral blood lymphocytes [41]. Induction treatment could also explain the low rate of RNA extraction suitable for QRT–PCR. Indeed, this treatment can destroy target cells, which could increase the level of RNA damage, compared with live cells present in urine samples, carrying preserved RNA. Moreover, an overnight period of urine collection, in order to get sufficient cells and RNA, can contribute to RNA damage; however there is no correlation between RNA failed extractions and cellular mortality. Recommendations on the optimum treatment of urine cell pellets for RNA extraction, published after collection period of our sample should be taken into account for further studies [42]. Another hypothesis worthy of consideration is that we collected an average of five urine specimens from each transplanted recipient allowing us to obtain a kinetics study of the expression of the three genes involved, facilitating the reduction of individual intra- and inter- variations in expression.

In the reference work of Li *et al.*, although sequential urine samples were studied at an early stage after transplantation (first 9 days), the kinetics of gene expression, at the time of relevant clinical events was not investigated. Another study has suggested that serial measurements of P and GB gene expression in peripheral blood could be good predictors of early graft rejection [17]. In our experiment, serial urine samples were collected before, during and after AR episodes in three patients, and in only one did the elevation of molecular markers precede serum creatinin increase, making it impossible to consider the measurement of P, GB and Fas-L gene expression as a predictor of AR episodes.

In conclusion, we used a real-time PCR assay to quantify the expression of P, GB and Fas-L genes in urine specimens of renal transplantation recipients. This technique is sensitive, reproducible and rapid but in the context of urine samples, the RNA extraction step remains laborious, tricky and difficult. Even if a significant increase in P, GB and Fas-L gene expression was observed in the course of AR, the results of the current investigation demonstrate that this increase is not specific to AR and can be observed in other types of allograft dysfunction. In light of our results, although it concerns a limited number of patients and clinical settings, the transfer of P, GB and Fas-L gene expression QRT–PCR analysis in urinary lymphocytes to a routine laboratory for the clinical management kidney graft recipients warrants careful consideration.

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References

1. Ishikawa A, Flechner SM, Goldfarb DA, *et al.* Quantitative assessment of the first acute rejection as a predictor of renal transplant outcome. *Transplantation* 1999; **68**: 1318.
2. Matas AJ, Gillingham KJ, Payne WD, Najarian JS. The impact of an acute rejection episode on long-term renal allograft survival (t1/2). *Transplantation* 1994; **57**: 857.
3. Pelletier RP, Cosio F, Henry ML, *et al.* Acute rejection following renal transplantation. Evidence that severity is the best predictor of subsequent graft survival time. *Clin Transplant* 1998; **12**: 543.
4. Ferguson R. Acute rejection episodes–best predictor of long-term primary cadaveric renal transplant survival. *Clin Transplant* 1994; **8**: 328.
5. Gulanikar AC, MacDonald AS, Sungurtekin U, Belitsky P. The incidence and impact of early rejection episodes on graft outcome in recipients of first cadaver kidney transplants. *Transplantation* 1992; **53**: 323.
6. Tullius SG, Nieminen M, Bechstein WO, *et al.* Contribution of early acute rejection episodes to chronic rejection in a rat kidney retransplantation model. *Kidney Int* 1998; **53**: 465.
7. Benfield MR, Herrin J, Feld L, Rose S, Stablein D, Tejani A. Safety of kidney biopsy in pediatric transplantation: a report of the Controlled Clinical Trials in Pediatric Transplantation Trial of Induction Therapy Study Group. *Transplantation* 1999; **67**: 544.
8. Mengel M, Mueller I, Behrend M, *et al.* Prognostic value of cytotoxic T-lymphocytes and CD40 in biopsies with early renal allograft rejection. *Transpl Int* 2004; **17**: 293.
9. Sharma AK, Rustom R, Evans A, *et al.* Utility of serial Doppler ultrasound scans for the diagnosis of acute rejection in renal allografts. *Transpl Int* 2004; **17**: 138.
10. Wever PC, Boonstra JG, Laterveer JC, *et al.* Mechanisms of lymphocyte-mediated cytotoxicity in acute renal allograft rejection. *Transplantation* 1998; **66**: 259.
11. Wever PC, Spaeny LH, van der Vliet HJ, *et al.* Expression of granzyme B during primary cytomegalovirus infection after renal transplantation. *J Infect Dis* 1999; **179**: 693.
12. Suresh V, Carey BS, Shaw S, *et al.* A retrospective study of the prognostic impact of cytokine secretion in mixed lymphocyte culture on long-term graft function following allogeneic renal transplantation. *Transpl Int* 2005; **18**: 1067.
13. Srinivas TR, Kubilis PS, Croker BP. Macrophage index predicts short-term renal allograft function and graft survival. *Transpl Int* 2004; **17**: 195.
14. Poindexter NJ, Steward NS, Shenoy S, *et al.* Cytolytic T lymphocytes from human renal allograft biopsies are tissue specific. *Hum Immunol* 1995; **44**: 43.
15. Vasconcellos LM, Schachter AD, Zheng XX, *et al.* Cytotoxic lymphocyte gene expression in peripheral blood leukocytes correlates with rejecting renal allografts. *Transplantation* 1998; **66**: 562.
16. Rukavina D, Balen-Marunic S, Rubesa G, Orlic P, Vujaklija K, Podack ER. Perforin expression in peripheral blood lymphocytes in rejecting and tolerant kidney transplant recipients. *Transplantation* 1996; **61**: 285.
17. Simon T, Opelz G, Wiesel M, Ott RC, Susal C. Serial peripheral blood perforin and granzyme B gene expression measurements for prediction of acute rejection in kidney graft recipients. *Am J Transplant* 2003; **3**: 1121.
18. Strehlau J, Pavlakis M, Lipman M, *et al.* Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc Natl Acad Sci USA* 1997; **94**: 695.
19. Robertson H, Wheeler J, Kirby JA, Morley AR. Renal allograft rejection – *in situ* demonstration of cytotoxic intratubular cells. *Transplantation* 1996; **61**: 1546.
20. Porter CJ, Ronan JE, Cassidy MJ. fas-fas-ligand antigen expression and its relationship to increased apoptosis in acute renal transplant rejection. *Transplantation* 2000; **69**: 1091.
21. Pascoe MD, Marshall SE, Welsh KI, Fulton LM, Hughes DA. Increased accuracy of renal allograft rejection diagnosis using combined perforin, granzyme B, and Fas ligand fine-needle aspiration immunocytology. *Transplantation* 2000; **69**: 2547.
22. Li B, Hartono C, Ding R, *et al.* Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* 2001; **344**: 947.
23. Dadhania D, Muthukumar T, Ding R, *et al.* Molecular signatures of urinary cells distinguish acute rejection of renal allografts from urinary tract infection. *Transplantation* 2003; **75**: 1752.
24. Ding R, Li B, Muthukumar T, *et al.* CD103 mRNA levels in urinary cells predict acute rejection of renal allografts. *Transplantation* 2003; **75**: 1307.
25. Schaub S, Rush D, Wilkins J, *et al.* Proteomic-based detection of urine proteins associated with acute renal allograft rejection. *J Am Soc Nephrol* 2004; **15**: 219.
26. Muthukumar T, Dadhania D, Ding R, *et al.* Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med* 2005; **353**: 2342.
27. Soulillou JP. Immune monitoring for rejection of kidney transplants. *N Engl J Med* 2001; **344**: 1006.
28. Racusen LC. The banff schema and differential diagnosis of allograft dysfunction. *Transplant Proc* 2004; **36**: 753.
29. Ferrand C, Robinet E, Contassot E, *et al.* Retrovirus-mediated gene transfer in primary T lymphocytes: influence of the transduction/selection process and of *ex vivo* expansion on the T cell receptor beta chain hypervariable region repertoire. *Hum Gene Ther* 2000; **11**: 1151.

30. Bonner TI, Kerby SB, Sutrave P, Gunnell MA, Mark G, Rapp UR. Structure and biological activity of human homologs of the raf/mil oncogene. *Mol Cell Biol* 1985; **5**: 1400.
31. Kotsch K, Mashreghi MF, Bold G, *et al.* Enhanced granulysin mRNA expression in urinary sediment in early and delayed acute renal allograft rejection. *Transplantation* 2004; **77**: 1866.
32. Steen AM, Luthman H, Hellgren D, Lambert B. Levels of hypoxanthine phosphoribosyltransferase RNA in human cells. *Exp Cell Res* 1990; **186**: 236.
33. Hashimoto S, Kobayashi A, Kooguchi K, Kitamura Y, Onodera H, Nakajima H. Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2000; **161**: 237.
34. van den Broek MF, Hengartner H. The role of perforin in infections and tumour surveillance. *Exp Physiol* 2000; **85**: 681.
35. Lauw FN, Simpson AJ, Hack CE, *et al.* Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria. *J Infect Dis* 2000; **182**: 206.
36. Jerome KR, Chen Z, Lang R, *et al.* HSV and glycoprotein J inhibit caspase activation and apoptosis induced by granzyme B or Fas. *J Immunol* 2001; **167**: 3928.
37. Balkow S, Kersten A, Tran TT, *et al.* Concerted action of the FasL/Fas and perforin/granzyme A and B pathways is mandatory for the development of early viral hepatitis but not for recovery from viral infection. *J Virol* 2001; **75**: 8781.
38. Sandberg JK, Fast NM, Nixon DF. Functional heterogeneity of cytokines and cytolytic effector molecules in human $CD8^+$ T lymphocytes. *J Immunol* 2001; **167**: 181.
39. ten Berge IJ, Wever PC, Wolbink AM, *et al.* Increased systemic levels of soluble granzymes A and B during primary cytomegalovirus infection after renal transplantation. *Transplant Proc* 1998; **30**: 3972.
40. ten Berge IJ, Wever PC, Rentenaar RJ, *et al.* Selective expansion of a peripheral blood $CD8^+$ memory T cell subset expressing both granzyme B and L-selectin during primary viral infection in renal allograft recipients. *Transplant Proc* 1998; **30**: 3975.
41. Simon T, Opelz G, Weimer R, *et al.* The effect of ATG on cytokine and cytotoxic T-lymphocyte gene expression in renal allograft recipients during the early post-transplant period. *Clin Transplant* 2003; **17**: 217.
42. Medeiros M, Sharma VK, Ding R, *et al.* Optimization of RNA yield, purity and mRNA copy number by treatment of urine cell pellets with RNAlater. *J Immunol Methods* 2003; **279**: 135.

