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IL-17 expression as a possible predictive parameter for subclinical renal allograft rejection

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Abstract In the present study we have tried to establish the role of IL-17 in subclinical renal allograft rejection. In this animal model, renal grafts from BN ($RT1^n$) were transplanted heterotopically into LEW ($RT1^l$) rats. As controls, LEW grafts were transplanted into LEW rats. The histopathological examination demonstrated that the changes in the allograft kidney on day 2 were similar to those ranked as borderline changes according to the Banff classification scale. On day 2, the serum level of blood urea nitrogen (BUN) and creatinine were the same as on day 1. The examination of allograft cytokines mRNA showed that IL-17 mRNA expressed earlier on the second postoperative day, peaked at day 5, and then declined, becoming almost undetectable at day 9, when most rats died. IL-17 antigen was also proven, by histochemical staining, to be expressed early, however we could not find the same early appearance on other Th1/Th2 cytokines. In human renal biopsy samples, the IL-17 antigen could be found scattered around in the borderline changed rejected renal allografts without evidence of a serum creatinine increase, but was undetectable both in normal controls and in renal transplant tissue without signs of rejection. IL-17 mRNA was detected in the mononuclear cells of the urinary sediment of patients suffering from borderline subclinical rejection. From the above results we can hypothesize that IL-17 could serve as a predictive parameter for borderline subclinical renal allograft rejection in the future.

Keywords IL-17 · Borderline-, subclinical-, renal allograft rejection

Abbreviations *BUN* Serum levels of blood urea nitrogen · *CTLA 8* Cytotoxic T lymphocyte associated antigen-8 · *GCSF* Granulocyte stimulating factor · *MNCs* mononuclear cells · *RECs* Renal tubular epithelial cells

Introduction

The most common complication after transplantation is rejection. Even in long-term surviving renal transplant patients, rejection is still the major cause of graft failure [1]. Pathologically, acute rejection manifests itself in a rise in inflammatory cells infiltrating especially the monocytes and lymphocytes. Various cytokines are secreted from these cells and can be classified as Th1 cytokines (Interleukin-2 [IL-2] and tumor necrosis factor-α [TNF-α]) and Th2 cytokines (IL-4, IL-6 and IL-10) [18]. We previously reported that both Th1 and Th2 cytokines might be involved in the acute rejection process, however the Th1 cytokines were not involved in the chronic rejection process [16]. Not only infiltrating cells in the renal allograft but also renal tubular epithelial cells (RECs) play an important role during the inflammatory response. *In-vitro* studies with RECs have dem-

onstrated that these cells can produce large amounts of pro-inflammatory cytokines (such as IL-6, TNF-α) [4, 37] as well as chemokines (IL-8, monocyte chemoattractant protein-1 [MCP-1], RANTES) [19, 20, 29]. So RECs actively participate in the local inflammatory response via the production of pro-inflammatory cytokines and chemokines and contribute to the regulation of interstitial infiltration [30].

Interleukin-17 (IL-17) is a recently identified cytokine, which is specifically produced by activated T cells [9, 11, 35]. It has been shown that IL-17 is produced mainly by the Th1/Th0 cells. Its biological activities are restricted to the activation of non hematopoietic stromal cells including RECs [9]. In vitro, IL-17 can induce RECs to secrete high levels of IL-6, IL-8 and MCP-1 [12]. Since IL-17 stimulates monocytes and RECs to secrete proinflammatory cytokines and promote the maturation of dendritic cells progenitors [3], it may influence further allogenic T cell proliferation. Consequently we established an acute rat renal allograft rejection model. Using this model we tested whether IL-17 appears early during acute renal transplant rejection even if it is undetected clinically. If it does, then the detection of an early expression of IL-17 may be of value for an early diagnosis of renal allograft rejection.

Materials and methods

Animals

In this model used 8–10 week-old LEW ($RT1^l$) and BN ($RT1^n$) male rats weighting 250–300 g. The rats (from The National Animal Breeding and Research Center, National Science Council, Taiwan) were housed in the Animal Center of Taipei Veterans General Hospital prior to renal transplantation. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" published by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" proposed by the National Institute of Health (National Institutes of Health Publication No.86–23, revised 1988). The rats were divided into three groups, the allograft group, in which LEW rats were used as recipients and the BN rats were donors; the isograft group, in which the LEW rats were both donors and recipients; and the sham operation group, in which LEW rats were explored without undergoing transplantation.

Group A (Allograft Group): Fifty-four rats underwent renal allograft transplantation without immunosuppressant.

Group B (Isograft Group): Eighteen rats underwent renal isograft transplantation without immunosuppressant.

Group C (Sham Operation group): Eighteen rats underwent abdominal opening without transplantation.

Rat renal transplantation

The heterotopic renal transplantation was carried out as usual. Briefly, the native left kidney was removed first, then the donor's graft renal artery was anastomosed to the recipient's infrarenal aorta, and the donor's renal vein was anastomosed to the recipient's infrarenal inferior vena cava, both by the end-to-side method. The donor's ureter was anastomosed to the recipient's ureter by the end-to-end method. The recipient's right kidney was removed immediately after the transplantation. The allograft- (6 rats/day), isograft- (2 rats/day) and sham operation (2 rats/day) recipients were killed from postoperative day 1 to day 9. The graft kidney was excised and dissected into several parts, some immersed in 10% formalin, others in OCT compound (Tissue-Tek, USA), then snap frozen and stored at –20 °C. The remaining parts were stored at –70 °C for further RNA extraction. The blood withdrawn from the recipient's inferior vena cava and the sera was stored in the refrigerator for further assay. The serum levels of blood urea nitrogen (BUN) and creatinine were checked. The diagnosis of borderline subclinical rejection was made mainly from histopathological evaluation and associated with a change in serum creatinine levels under 10% of the first day baseline.

Histopathological evaluation

The fixed kidney tissues were dehydrated in graded ethanol and embedded in paraffin; they were then were sectioned to 3 μm and stained with hematoxylin and eosin for light microscopic examination. The slides were reviewed by pathologists, and the overall severity of rejection was determined by the pattern and intensity of intersititial inflammation and glomerular-, tubular-, and vascular abnormalities according to the Banff 97 working classification scale [21].

RT-PCR of Th1/Th2 Cytokines and IL-17 mRNA

The graft kidney samples were homogenized with the appropriate volume of RNAzol B solution and placed in centrifuge tubes. Then mRNA was extracted, and RT-PCR was performed as previously described [13]. The PCR products from each sample, including IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18 and β-actin were analyzed on a 2% agarose gel containing ethidium bromide. The final results from the gel were visualized on a UV light box and photographed on Kodak film. The density of each band was compared with a 600 bp band that expressed as β-actin, its relative data were calculated under the Laser Scanning Densitometer SLR-2D/1D (Biomed Instrument, Fullerton, Calif., USA). The intensity-ratio was calculated as the ratio of each cytokine over β-actin in the same gel. Every experiment was repeated three times. The intensity-ratio of the difference was later used to compare daily results after transplantation.

IL-17 expression in the human renal tissue

The human renal allograft biopsy samples from different patients and periods were collected and examined, the normal kidney tissues were obtained from a nephrectomy sample that was taken because of renal cell carcinoma. IL-17 Ag expression was later examined immunohistochemically.

Histopathological evaluation

The renal biopsy specimens were dehydrated in graded ethanol and embedded in paraffin, then sectioned to 3 μm and stained with hematoxylin and eosin for light-microscopy. The slides were reviewed by pathologists. The overall severity of rejection was determined by the pattern and intensity of intersititial inflammation

and glomerular-, tubular-, and vascular abnormalities according to the Banff 97 working classification [21].

In situ hybridization for IL-17 mRNA expression

The IL-17 cDNA fragments were radiolabelled employing the random primer method using [5-^{3}H]-dCTP (specific activity 25 Ci/mmol, New England Nuclear) and *in-situ* hybridization for IL-17 mRNA was done as before [6]. The specificity for *in-situ* hybridization was controlled in the following ways: 1, by obtaining a negative reaction when the IL-17 probe of the hybridization solution was substituted by plasmid pBR322 at the same concentration; 2, by obtaining a negative reaction when the probe was omitted from the hybridization mixture; 3, by obtaining a specific positive reaction for IL-17 mRNA in human mononuclear cells stimulated by lipopolysaccharide for 24 h; and 4, negative control was also performed in cultured human erythroleukimic cell lines K562. IL-17 mRNA was semiquantitatively analyzed employing the scoring system previously described, with slight modification [34]. Interstitium MNCs: 0 = weak- or no staining similar to that seen in negative control samples; 1 + = focal interstitial staining of mild intensity; 2 + = multi-focal, intense interstitial staining; 3 + = intense interstitial staining throughout the interstitium.

IL-17 immunohistochemical analysis

Anti hIL-17 monoclonal antibody (mAb) was bought from R&D systems (Minneapolis, Minn. USA). The block of OCT-embedded sample was cut with a cryostat into 4-μm sections and placed on the glass slide. The sections were fixed with cold 100% alcohol for 30 min under room temperature, washed with PBS for 5 min, then immersed in PBST (1X PBS; 0.2% Triton X-100) for 10 min also under room temperature. After PBS-washing three more times, the anti-IL-17 monoclonal antibody was added, placed at 4 °C for 8–12 h, and PBS-washed further 3 ~ 4 times. Then the second antibody was added, and the section was kept at room temperature for 30 min, PBS-washed three more times, and counterstained. The section was then examined under a microscope. A similar slide was also stained with anti-CD3 mAb. The semiquantitative evaluation scoring system was used as described previously [6, 7]. In brief, 40 or more glomeruli were examined on each slide and assigned a value from 0–3 + as follows: Mononuclear cells (MNCs): 0 = no staining, similar to that seen in negative control samples; trace, faint staining on MNCs; 1 + = distinct focal staining for IL-17 Ag on MNCs; 2 + = multifocal, intense MNCs staining; 3 + = intense staining throughout the MNCs. Then a total positive score was calculated for each specimen with the following equation: total positive score = (% MNCs staining 0 × 0) + (% MNCs staining trace × 0.5) + (%MNCs staining 1 + × 1) + (% MNCs staining 2 + × 2) + (%MNCs staining 3 + × 3). The values ranged from 0 to a maximum of 300.

IL-17 mRNA expression on the MNCs of urinary sediment

Urine was collected from renal transplant patients, irrespective of signs of rejection. Urine was also collected from patients with urinary tract infection and from normal volunteers. All urine samples were centrifuged and the sediment was placed under cytospin. Then the IL-17 mRNA expression on MNCs (Mononuclear Cells) was examined as previously stated, employing the *in-situ* hybridization method. The sediment slide was also stained with anti-CD3 monoclonal antibody as in the previous immnohistochemical method. The PBMC was isolated from the blood of healthy donors by Ficoll-Hypaque centrifugation. Then the enriched portion was cultured in RPMI medium supplemented with 10% fetal calf serum and activated with a mixture of 5 ng/ml PMA and 3 μg/ml ionomycin. The stimulated PBMC was also placed under cytospin and examined with *in-situ* hybridization as positive control.

Statistical analysis

Statistical analysis was performed using SPSS 9.0 for windows and the Student t test and ANOVA for repeated measures. $P < 0.05$ was considered to be statistically significant.

Results

Histopathological changes and differences of daily serum BUN and Creatinine

The data of the daily serum creatinine and BUN levels from day 0 (before) and after renal transplantation (days 1–9) are shown in Fig. 1. We found no significant differences between the group of isografts and negative controls. However, there were significant differences between the group of allografts and negative controls. There was also no significant difference between levels found on the first and second days.

On day 1, the histopathological examination (Table 1) showed the allograft kidney to be similar to the negative control. On day 2 it showed only scattered lymphocyte infiltration in the allograft (Fig. 2) similar to borderline renal rejection as defined by Banff classification. On days 3 and 4, the picture progressed to active acute rejection. After day 5 there was diffuse infiltration of the mononuclear cells. On days 7–9, the grafts showed diffused infarction and necrosis. The histopathological examination of the isograft kidneys from days 0–9 demonstrated that they were similar each day without mononuclear cell infiltration and also similar to those of the negative controls.

Serial Th1/Th2 cytokines and IL-17 mRNA expression

To evaluate the serial changes of Th1/Th2 cytokines pattern associated with acute renal allograft rejection, RT-PCR was used to analyze mRNAs expression in the allograft, the isograft and the negative controls from postoperative days 1–9. mRNA expressions of IL-2, IFN-γ, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17 and IL-18 were detectable, but there was no IL-4 or IL-5 expression in the allografts (Table 2). All of them were absent in both isografts and negative controls. The IFN-γ, IL-6 and IL-8 expressed on the day 3. This indicates that both Th1/Th2 cytokines mRNA were transcripted during acute rejection.

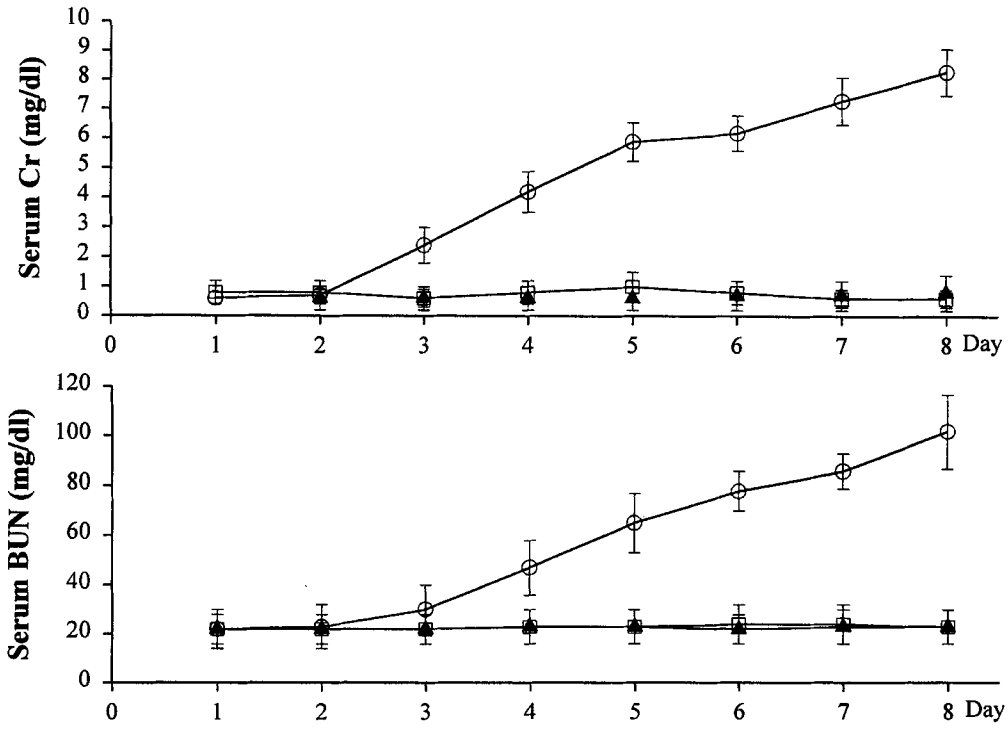

Fig. 1 The serum creatinine and blood urea nitrogen level from post-transplant day 1 to day 8. ○, the allograft group; □, the isograft group; ▲, the sham operation group as negative control. There were no significant differences between groups of isografts and negative controls. However, there were significant differences between groups of allografts and negative controls

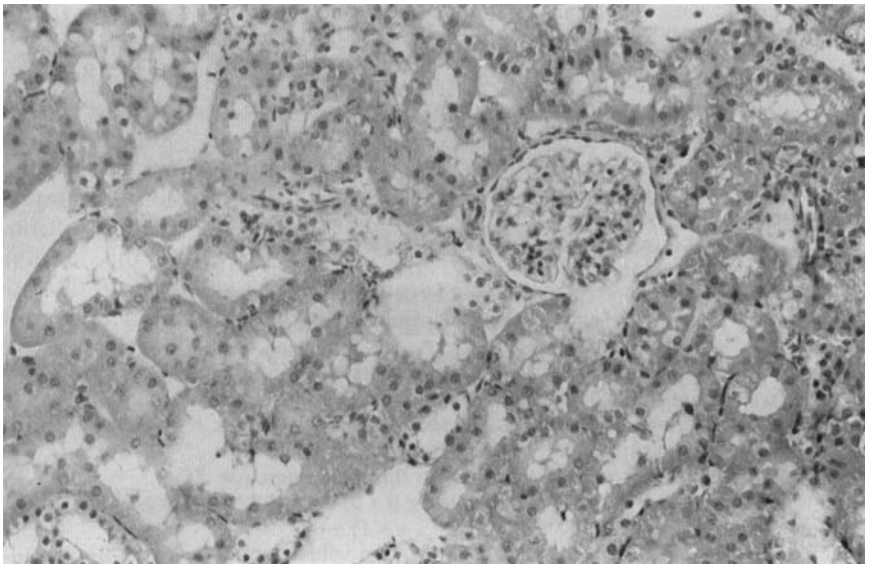

Fig. 2 The allograft kidney on post-transplant day 2 showing scattered lymphocyte infiltration similar to the borderline change of renal allograft rejection defined by the Banff criteria. Hematoxylin and eosin. × 200

A careful look at the serial changes of IL-17 (Fig. 3) shows that IL-17 mRNA appeared on the allograft early on postoperative day 2, peaked on day 5 and then declined until it was almost undetectable at day 9. IL-17 was induced from the mononuclear cells stimulated by the lipopolysaccharide, but the expression was lower than that on postoperative day 2. The expression of IL-17 in the allograft on postoperative day 3 showed statistical significance compared with the expression of IL-17 on postoperative days 5 and 6. This means that IL-17 is involved early during the course of acute rejection and may act as a trigger, rise and disappear quickly again. Another cytokine, IFN-γ, is usually considered as an early trigger during acute rejection, but it appeared on postoperative day 3, later than IL-17. From the above results only IL-17 mRNA expression correlated with serial quantitative changes starting from post-transplant day 2.

Table 1 Semiquantitative analysis for Hematoxylin-and-eosin stain of isograft and allograft renal tissues. *N* Normal, *D* day, *MNCs* mononuclear cells, *infil.* infiltrating

	Interstitium	Tubules		Glomerulus	
	Infil. round cells	Tubulitis	Focal atrophy	Mesangial cell	Sclerosis
Isograft					
D1	(–)	(–)	(–)	N.	(–)
D5	(–)	(–)	(–)	N.	(–)
Allograft					
D1	(–)	(–)	(–)	N.	(–)
D2	Occassional	0%	(–)	N.	(–)
D3	50%	40%	10% (+)	N.	(–)
D5	100%	100%	10% (+)	N.	(–)
D6	100%	100%	30% (+)	N.	(–)
D7	100%	60%	40% (+)	N.	(–)
D9	100%	50%	50% (+)	N.	(–)

Table 2 Expression of Th1/Th2 cytokines mRNA on allografts and isografts renal tissues. + Detectable, – undetectable, $n = 6$

	Th1 cytokines			Th2 cytokines	
	Allograft	Isograft		Allograft	Isograft
IL-2	+	–	IL-4	–	–
IL-12	+	–	IL-5	–	–
IL-18	+	–	IL-10	+	–
IFN-γ	+	–	IL-6	+	–
IL-15	+	–	IL-13	+	–

IL-17 expression and histopathological changes

The serial expression of IL-17 antigen in rat renal allografts, isografts, and sham group samples are shown in Table 3. In the isograft- and sham operation groups, there was no detectable IL-17 on renal tissue (Fig. 4a). In the allograft group, the abnormal expression of IL-17 antigen in the infiltrating MNCs was detectable on day 2 (Fig. 4b), corresponding to the borderline rejection according to the Banff classification, and it increased progressively until day 5. After day 5, the IL-17 antigen expression decreased. In comparison to day 1, there was a significant increase in IL-17 antigen expression from days 2–5. The infiltrating cells that expressed IL-17 antigen were also stained positive with anti-CD3 mAb.

Expression of IL-17 in human renal graft with borderline subclinical rejection

Five stable post-renal transplant patients with increasing serum Cr levels were found during regular follow-up in the outpatient clinic. Renal biopsy was performed, and borderline subclinical rejection with less then 25% lymphocyte infiltration around the tubules was noted in renal biopsy specimens. The patient characteristics, changes of serum BUN and Cr and immunosuppressive regimens are listed on Table 4.

IL-17 protein expression was found in the renal graft biopsy tissues that showed borderline subclinical rejection (Fig. 5a) but not in normal controls (Fig. 5b). The IL-17 expression as detected by the immunohistochemical method could be readily identified scattered around in the borderline-rejected renal graft. However, it was undetectable both in the graft biopsy tissue without any evidence of rejection and in the nephrectomy sample with normal pathological appearance.

Expression of IL-17 mRNA on the MNCs of human urinary sediment

Five patients suffering from borderline subclinical rejection, as was proven with renal biopsy samples, revealed a 100% detectable rate of IL-17 mRNA expression in the MNCs of their urinary sediment (Fig. 6a). In contrast, in the 20 urinary specimens taken either from patients who suffered from acute urinary tract infection or from renal transplant recipients with normal graft function and without evidence of rejection, as was proven with the biopsy samples, IL-17 mRNA was 100% undetectable (Fig. 6b) by the *in-situ* hybridization method. The sediment cells also demonstrated the expression of CD3 by the immunohistochemical method.

Discussion

Our previous study on human renal graft biopsy samples showed that both Th1 and Th2 cytokines were involved in the acute rejection, but only Th2 cytokines were expressed during chronic rejection [3]. However, which cytokines appear earlier and trigger the reaction is still unknown.

IL-17 is a newly found cytokine which is produced by $CD4^+$T cells. Aarvak *et al* had found that thirty-three $CD4^+\alpha\beta^+$ T cell clones were developed from the synovial membrane and the synovial fluid of rheumatoid ar-

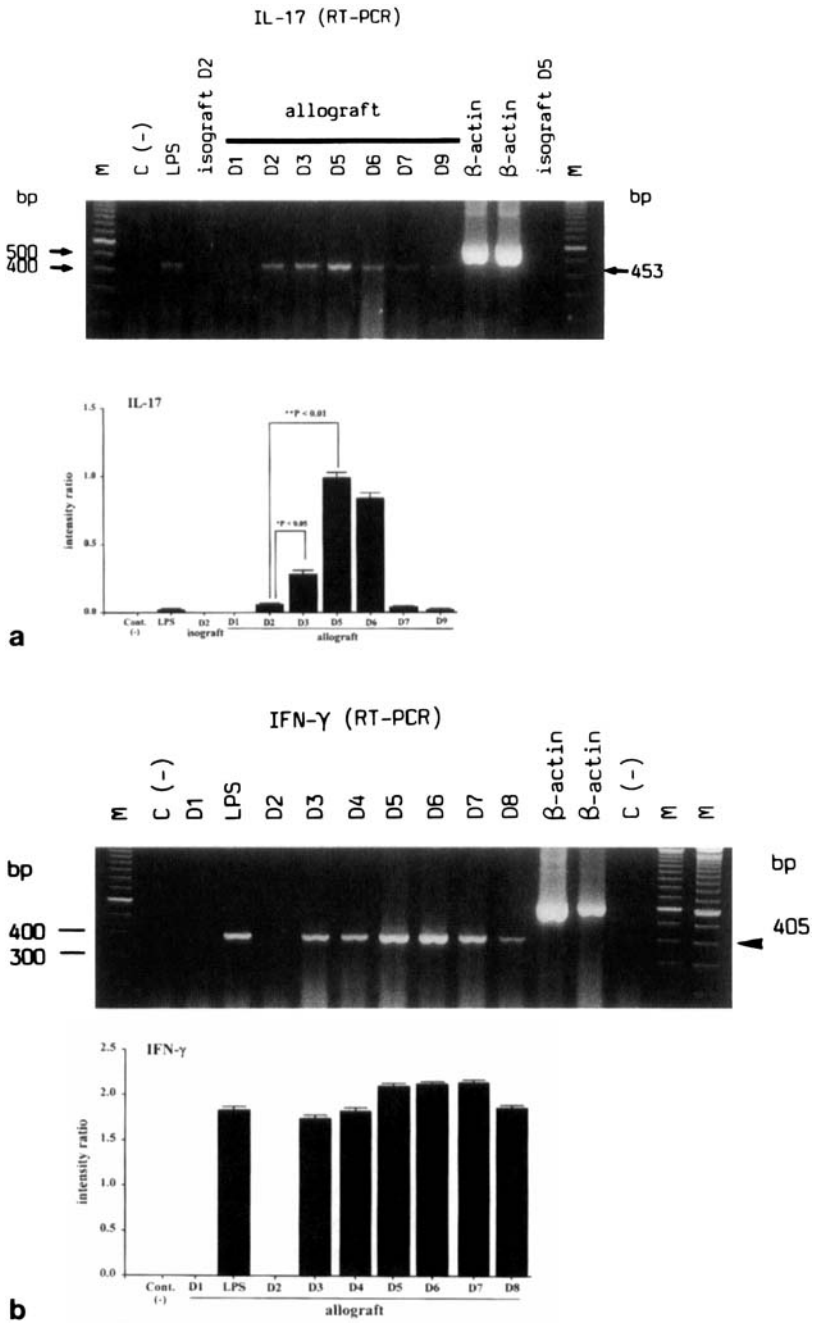

Fig. 3 The IL-17 (**a**) and IFN-γ (**b**) mRNA expression in the isograft and serial change in the allograft done by RT-PCR. Cont.(Controls); LPS(lipopolysaccharide); The IL-17 did not express in the isograft. In the allograft group, IL-17 expressed early on the postoperative day 2, and peaked on day 5. The expression of day 3 compared with days 5 and 6 showed statistical significance. Then it declined quickly on days 7 to 9. However, IFN-γ expression started from day 3

thritis patients. Three Th1 clones and two Th0 clones produced IL-17, but none of the sixteen Th2 clones did. Six Th2 clones were further cultured under the conditions to switch to Th1 phenotype, then two of them produced IL-17. It is consided that some cells of the Th1/Th0 phenotype produced IL-17 rather than cells of Th2 phenotype [1]. In mice, IL-17 was originally cloned by Rouvier et al and named cytotoxic T lymphocyte-associated antigen-8 (CTLA-8) [23]. The activated $CD4^+$T cells appear to be strong producers of mIL-17. However, the $\alpha\beta TCR^+CD4^-CD8^-$ T cells are also thought to be responsible for IL-17 production [11]. In humans, however, the $CD4^+CD45RO^+$ population of T cells has been demonstrated to be the major source of IL-17 [9, 35]. IL-17 can induce activation of the transcription factor NF-κB in a variety of cell types including mouse fibroblasts [36] and human macrophages [10]. The expression of cytokines that are known to contain NF-κB recognition sites in their promoters is thought to be regulated by IL-17. These cytokines include IL-6, IL-8 and the

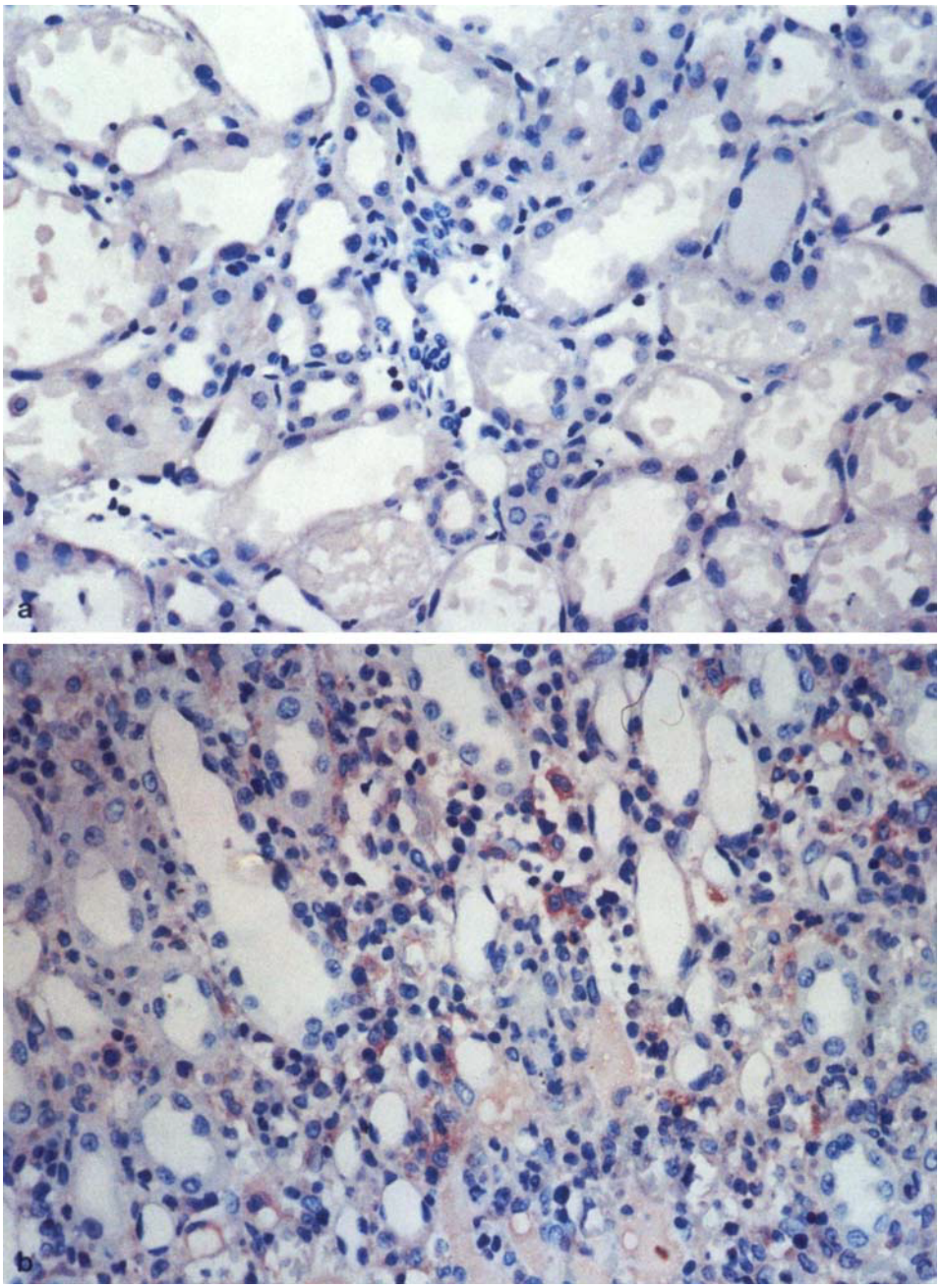

Fig. 4 **a** The immunohistochemical study using anti-IL-17 monoclonal antibody showing no detection of any IL-17 antigen in the kidney of the sham operation group. Hematoxylin. × 200. **b** The immunohistochemical study using anti-IL-17 monoclonal antibody showing in the allograft on post-transplanted second day some abnormal expression of IL-17 antigen in the infiltrating mononuclear cells. Hematoxylin. × 200

granulocyte colony stimulating factor (GCSF) [9, 35, 36]. hIL-17 can also induce stromal cells including epithelial cells, endothelial cells, and fibroblasts to secrete inflammatory and hematopoietic cytokines [9]. In vitro, IL-17 also was demonstrated to induce primary human proximal tubular epithelial cells, a type of cell regulating local interstitial inflammatory response, to secrete higher levels of IL-6, IL-8, and monocyte chemoattractant protein-1, but not the chemokine RANTES [12]. In addition, IL-17 may induce the secretion of IL-1β and TNF-α by human macrophages [10] and enhance the surface expression of the intracellular adhesion molecule-1 (ICAM-1) in human fibroblasts [35]. But IL-17 can specifically and dose-dependently augment IFN-γ-induced ICAM-1 expression on keratinocytes at both the mRNA and at the protein levels. IL-17 alone did not affect ICAM-1 or enhance TNF-α-induced ICAM-1. It may both directly and in synergism with IFN-γ and/or TNF-α stimulate synthesis and release of IL-8 by keratinocytes [2]. From the above evidence it seems

Table 3 Semiquantitative analysis for histochemical stain of IL-17 antigen on renal tissues. *D* Day

	D-1	D-2	D-3	D-4	D-5	D-6	D-9
Allograft (Gr. A) ($n = 6$)	0	$10 \pm 2^{a,b}$	30 ± 3^{b}	35 ± 4	65 ± 5	62 ± 6	23 ± 3
Isograft (Gr. B) ($n = 6$)	0	0	0	0	0	0	0
Controls (Gr. C) ($n = 6$)	0	0	0	0	0	0	0

[a] total score of positive IL-17 Ag all histological evaluation was done by two "blind" observers
[b] comparosin between Day 2 and Day 3 (paired t test $P < 0.05$)

Table 4 The patients characteristis, change of serum *BUN* and *Cr* and immunosuppressive regimens. *Anti-HPN* Anti-hypertensive drugs, *ALP* allopurinol, *(–)* absent, *IS* immunosuppressive

Case	Previous		At biopsy					
	BUN (mg/dl)	Cr (mg/dl)	BUN (mg/dl)	Cr (mg/dl)	IS regimens	Other medications	WBC (/mm^3)	Local pain of allograft
1	18	1.2	26	1.9	CsA + Pred + MMF	Anti-HPN + ALP	7300	(–)
2	19	0.9	31	1.5	FK + Pred + MMF	(–)	6300	(–)
3	23	0.8	31	1.3	CsA + Pred + Aza	Anti-HPN + ALP	5700	(–)
4	34	2.1	44	3.0	CsA + Pred + Aza	Anti-HPN + ALP	4300	(–)
5	36	2.4	45	3.2	CsA + Pred + Cellcept	Anti-HPN + ALP	5800	(–)

that IL-17 is closely correlated with early cytokines such as IFN-γ and TNF-α.

Besides inducing proinflammatory cytokine production, IL-17 also augments mature T cell proliferation induced by suboptimal concentration of PHA stimulation (data not published). All of the findings suggest that IL-17 might play a role as early initiator of the T cell-dependent inflammatory reaction as previously stated [9].

Recently, a soluble mIL-17R:Fc fusion protein as an IL-17 antagonist has been found, which markedly inhibited T cell proliferation *in-vitro*, and also significantly prolonged nonvascularized and vascularized rat cardiac allograft survival. Further findings suggested that IL-17 plays a role during allogenic T cell proliferation that may be mediated partly via a maturation-inducing effect on dendritic cells [3]. Besides, IL-17 expression has been studied in 40 transplant biopsies by using RT-PCR with the conclusion that IL-17 transcripts were solely, but not reliably, observed in the rejection samples [33]. This result might be limited to the different period of acute rejection that was studied. Using the immunostaining method, expression of IL-17 in kidney biopsies was observed in patients suffering from graft rejection, but not in pretransplant biopsies or in normal kidneys. Biopsy samples from rejected grafts were further analyzed for IL-17 mRNA with RT-PCR. The expression of IL-17 was also found in cultured and activated graft-infiltrating T cells [12]. This evidence shows that IL-17 was involved in the alloimmune response, and it might be during an early period.

To verify our hypothesis that IL-17 appears early in the acute rejection process, we performed heterotopic rat renal transplantation and also evaluated different cytokines including Th1 and Th2 in the grafts. From the data of RT-PCR, the Th1 cytokines such as IFN-γ, IL-2 and IL-12 were all expressed, while among Th2 cytokines, IL-6, IL-10, and IL-13 were expressed but not IL-4 and IL-5. These results demonstrated that both Th1/Th2 cytokines were expressed during acute rejection, but the reason behind the differential expression of Th2 cytokines is presently not known. Using serial rat renal tissue specimens to evaluate the correlation between IL-17 expression and the degree of rejection, we found that only IL-17 protein and mRNA expression were inducible early on the infiltrating MNCs of renal allograft even during borderline subclinical rejection on postoperative day 2. This led to expression of IL-6 and IL-8 on day 3. Together with IFN-γ, the progression of the rejection response was more pronounced, and the IL-17 Ag expression peaked on days 5 and 6, the expression of IL-17 then disappeared, before the rats died on days 8 and 9. There was no correlation between the severity of acute rejection as defined by the Banff criteria, and the expression levels of any other Th1/Th2 cytokines. Our results are unique in that only IL-17 mRNA and protein could be detected within kidneys that were undergoing acute allograft rejection and were correlated with the severity of rejection by Banff criteria.

The Banff criteria for borderline change or a liklihood of acute rejection are foci of mild tubulitis with 1–4 MNCs on tubular cross sections and at least 10–25% inflamed parenchyma, without presence of internal arteritis. From a retrospective study, 78% of the patients' biopsy samples with "Borderline" change and elevated serum creatinine were treated as acute rejection [31]. When routine or sequential protocols in renal biopsies carried out early after kidney transplantation, 30% of non-symptomatic patients showed subclinical

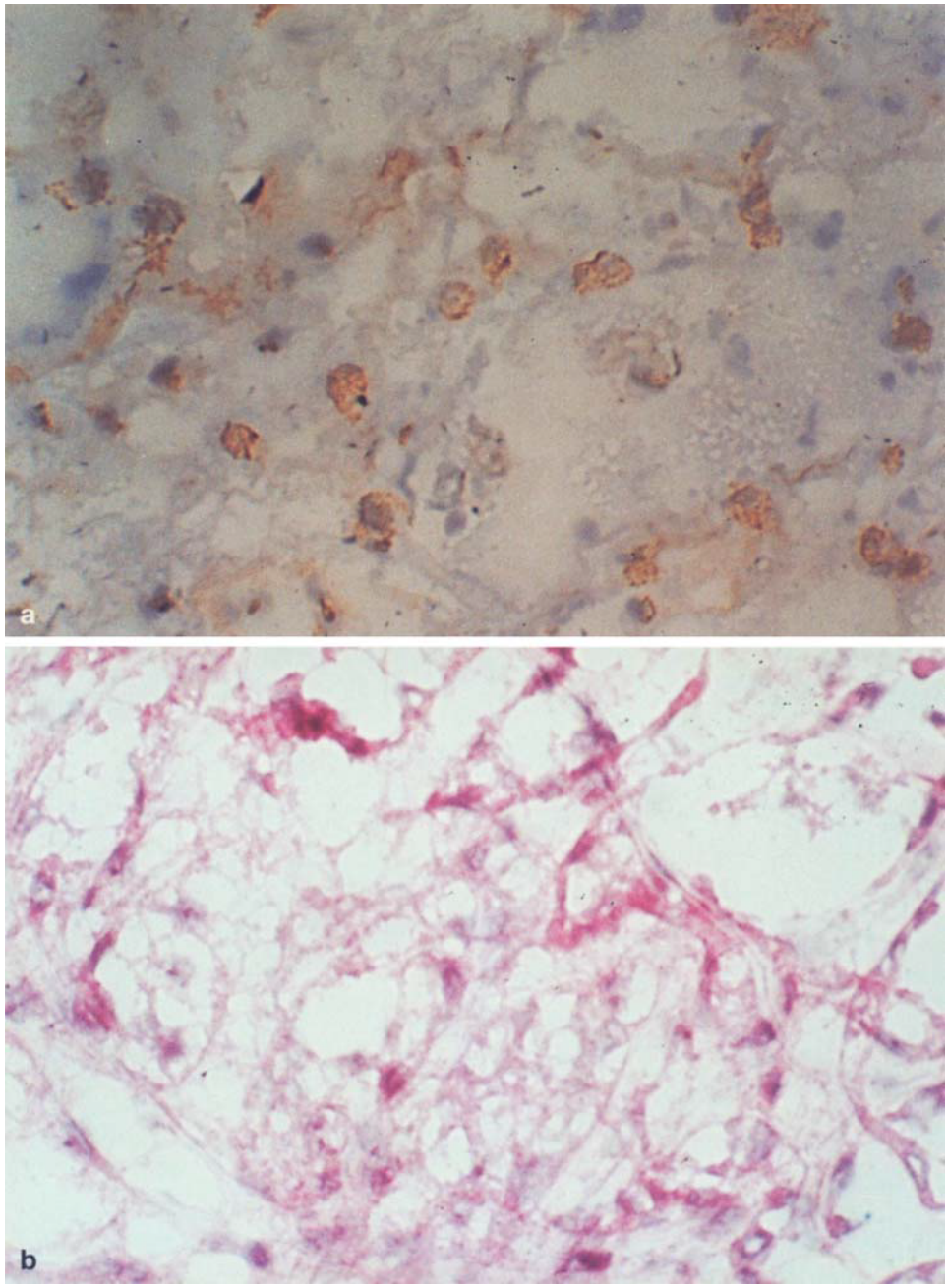

Fig. 5 **a** Immunohistochemical study of IL-17 antigen expression in a human renal biopsy sample with borderline subclinical rejection. The expression of IL-17 antigen is found scattered around the graft. Hematoxylin. × 200. **b** Immunohistochemical study of IL-17 expression in a normal human renal sample not showing any expression of IL-17 antigen. Hematoxylin. × 200

rejection that could lead to renal dysfunction [26, 25]. Added immunosuppressive treatment in the course of this borderline change resulted in a significant decrease in early and late acute rejection episodes [27, 28], whether the borderline change is associated with clinically elevated serum creatinine or subclinical rejection. In contrast, other investigators found that 72% of the patients with borderline infiltrates who did not undergo additional anti-rejection therapy did not progress to acute rejection for over 40 days of follow-up [17]. The controversy might be due to the duration of follow up. Possibly, the category of "Borderline Change" needs a subdivision.

Clinically, the difference between "Borderline Change" and acute rejection as defined by the Banff criteria is that there are more severe tubulitis and interstitial infiltration in acute rejction, and some acute rejections are even associated with arteritis. However, molecular analyses have shown that there is an increased expression of perforin gene during subclinical acute rejection [15]. In addition, the infiltrating cells of acute rejection are predominantly Th1 and secret more IFN-γ [8].

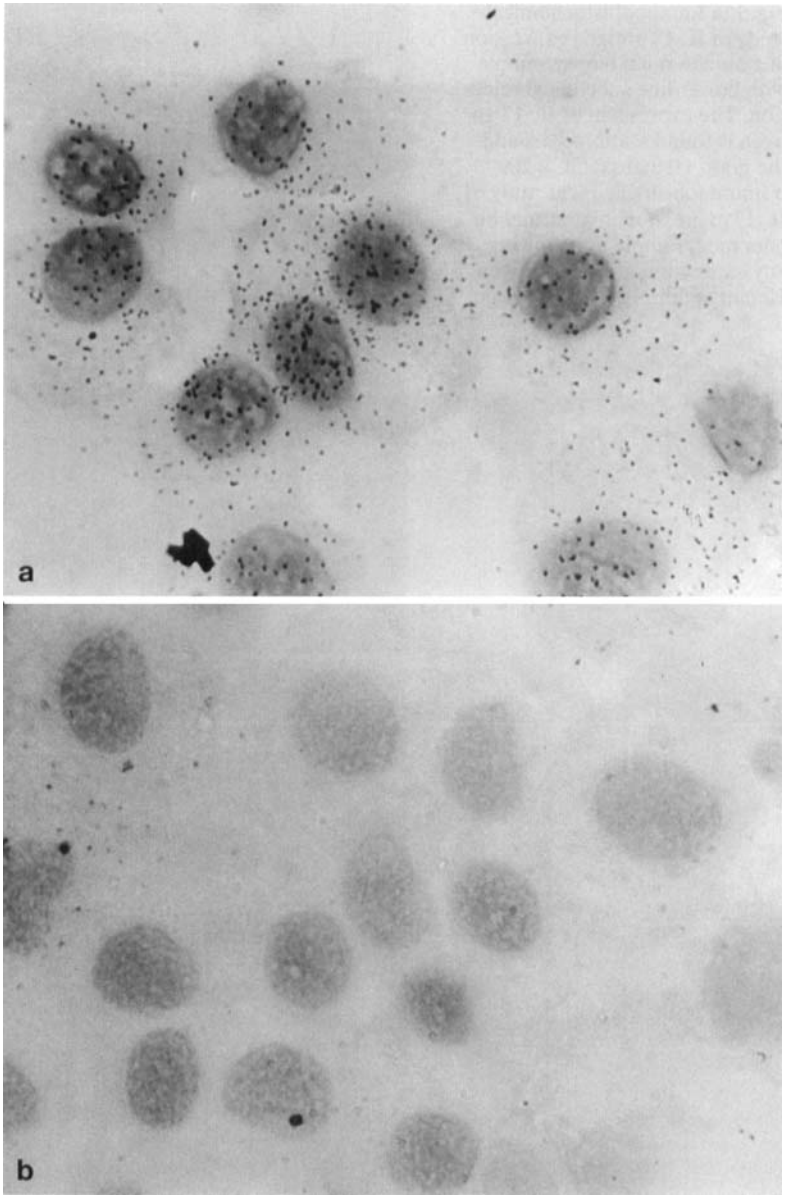

Fig. 6 **a** *In-situ* hybridization study using [^{3}H]-labeled IL-17 cDNA probe showing prominent expression of IL-17 mRNA in the mononuclear cells from the urinary sediment of a patient with borderline rejection. Eosin. × 800. **b** *In-situ* hybridization study using [^{3}H]-labeled IL-17 cDNA probe not showing any expression of IL-17 mRNA in the mononuclear cells from the urinary sediment of a patient with acute urinary tract infection or transplanted patient with normal renal histopathology. Eosin. × 800

Therefore, from the molecular point of view, borderline change may be different from acute rejection, or it may represent the early period of acute rejection, as was found by others [25]. In summary, the above findings indicate that the effects of IL-17 are involved in the early alloimmune responses. With regard to clinical application, IL-17 may serve as a useful parameter for warning against renal graft rejection.

In the present study, we also stained the IL-17 in the renal biopsy specimen. We found that even with only borderline subclinical rejection, IL-17 protein was expressed in the graft infiltrating MNCs. We did not have the same findings from the transplantation recipients or normal control patients with normal graft histopathology. These results suggest that IL-17 may serve as a useful index for an early diagnosis even in cases of borderline subclinical renal rejection.

Although some authors suggest that protocol- or sequential biopsy would be important to detect subclinical rejection during the first three months after transplantation [24], recipients in our country are reluctant to accept these examinations out of fear from graft loss in a situation of donor scarcity. Non-invasive methods being favoured, we have previously used urinary neopterin and β2-microglobulin to detect renal graft rejection early [14]. Other investigators have used urinary cytology or cytokine assay [5, 32]. Recently, urine flow immunocytometry is used to evaluate the graft function with

good results [22]. In this study we used in situ hybridization to detect IL-17 mRNA expression in the urinary sediment MNCs of different patients. IL-17 was detectable in patients with borderline subclinical rejecetion, but not in the patients who suffered from urinary tract infection or renal transplantation patients with normal graft tissue.

In summary, the above findings indicate that the effects of IL-17 are involved in the early alloimmune responses. With regard to clinical application, IL-17 may serve as a useful parameter for giving a warning of renal graft rejection.

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