Transpl Int (1994) 7 [Suppl 1]: S97–S101

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The diagnostic value of GM-CSF and IL-6 determinations in patients after renal transplantation

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Abstract Cytokines are released by graft-infiltrating cells during cellular rejection. We studied the release of GMCSF and IL-6 and their prognostic significance in predicting rejection. Sequential measurements were made in serum and urine samples with an IL-6 specific cell line and a GMCSF ELISA. Biopsy tissue was snap frozen and examined with immunohistochemical methods. The IL-6 values for normal controls (CTR) and stable transplant patients (PTS) were 5–10 pg/ml in serum and 0–2.5 pg/ml in urine. In 51 biopsy-proven rejections (AR), serum IL-6 values at least doubled in 15 (sensitivity 29%, specificity 87%; 19 ± 7 vs. 7 ± 2 pg/ml; P = ns). In urine an increase was observed in 29 of 36 AR (sensitivity 80%, specificity 75%; 92 ± 34 vs. 5 ± 1 pg/ml; $P < 0.05$). After treatment, IL-6 decreased in urine in 26/29 PTS to 7 ± 2 pg/ml ($P < 0.05$). In three PTS, rejection persisted, as did their elevated IL-6 urine values. In PTS with urinary tract infections, IL-6 increased in the serum of 13/19 and in the urine of 10/12. GMCSF in serum was not influenced by rejection; however, urine values increased in 22/33 AR (sensitivity 67%, specificity 96%; 22 ± 5 vs. 4.8 ± 0.3 pg/ml; $P < 0.05$). These values decreased (5 ± 0.3; $P < 0.05$) after treatment. During infection, increased urinary GMCSF levels were observed in 2/9 PTS. Further analysis revealed a better correlation between elevated cytokine levels and rejection episodes in the early posttransplant period. In kidneys with acute rejection, IL-6 was found in the interstitium of all PTS tested. CTR tissue was negative. In PTS GMCSF was found in arterioles and in infiltrate; however, control tissue also showed some staining. Cytokine labeling in tissue could not be correlated with serum or urine values. We concluded: (1) serum IL-6 and GMCSF are of no value in rejection; (2) in urine, they reflect rejection, especially in the early posttransplant period; however, infection confounds the results; (3) IL-6 staining in tissue may be helpful, but requires more study.

Key words Allograft rejection
GM-CSF · IL-6
Renal transplantation

Introduction

The migration of inflammatory cells into the parenchyma of transplanted kidneys is an important feature of transplant rejection. These cells, when activated, release a series of cytokines that bear the messages for mediating the ensuing inflammatory reaction associated with rejection. Interleukin-6 (IL-6) is a pleiotropic cytokine with a molecular weight of 26 kDa [1]. IL-6 is a particularly important mediator of inflammation. Granulocyte-macrophage colony stimulating factor (GM-CSF), which was originally described in the differentiation of haematopoietic cells, is one of the most important activators of mature granulocytes and macrophages [2]. Thus, GM-CSF serves a pivotal role in mediating immunological responses.

Many cytokines, including IL-1, IL-2, IL-6, TNF-alpha, interferon-gamma and GM-CSF, are known to mediate not only the initial inflammatory response, but also to maintain the process of rejection [3]. Recent studies have shown that after renal transplantation, the expression of IL-6 is increased in tissue, and its circulating level in blood, as well as its elimination in the urine, is increased [4–6]. The purpose of the present study was to evaluate the value of cytokine determinations in blood and urine in diagnosing transplant rejection. We selected IL-6 and GM-CSF as possible candidates for that role because of their participation in the process.

Methods

Patients and immunosuppression

A total of 63 patients were monitored after renal transplantation. Twenty-one normal persons served as normal controls. The immunosuppressive therapy given to the patients consisted of methylprednisolone and cyclosporin-A. Methylprednisolone 250 mg was given on the day of transplantation. Thereafter, the dose was reduced to 60 mg per day for the following week and to 24 mg per day after the 1st month. The steroid dose was subsequently reduced in a step-wise fashion, until the goal of 4 mg per day was attained. Cyclosporin-A was adjusted daily according to blood levels (monoclonal antibody trough levels: 100–150 ng/ml).

Fifty-one rejection episodes occurred in the transplanted patients, all of which were histologically documented by biopsy. When rejection occurred, the patients were given methylprednisolone 250 mg daily for 3 days, followed by 40 mg per day, with a subsequent reduction if the rejection was contained. Steroid-resistant rejection was additionally treated with anti-thymocyte globulin (Fresenius AG, Bad Homburg, Germany) 4–6 mg/kg per day for 10 days. Samples of morning urine and serum were obtained routinely thrice weekly in all patients. The serum and urine were centrifuged at 600 and 1000 g, respectively, for 10 min. All specimens were stored at −20 °C until assayed.

Cytokine assays

To measure IL-6 we used a bioassay that relied on the IL-6 dependent cell line B9. After sterile filtration and heat treatment, B9 cells were placed in 96 wells, 5000 in each well. Serum specimens at a concentration of 10% and urine samples at concentrations of 2.5 and 1.25% were added in triplicate to the wells and the samples were incubated. The results were determined with densitometry via the MTT test. Because of the intra-individual variability of the test, all samples from a single patient were determined in the same batch. The specificity of the method was verified by the addition of a monoclonal antibody against IL-6. GM-CSF levels in serum and urine were measured by a sandwich ELISA method (R&D Systems, Minneapolis, USA).

Immunohistochemistry

Renal tissue from patients undergoing rejection and normal control renal tissue were compared. The latter was obtained from kidneys excised because of hypernephroma. Tissue was stored in liquid nitrogen at −70 °C. After preparation of frozen sections and fixation with acetone, the APAAP stain was with monoclonal antibodies against IL- and GM-CSF (Genzyme, Cambridge, USA). We used an antibody against CD45 (Serotec, Oxford, England) as a positive control. Our negative control was an antibody against mouse IgG (Dako Diagnostika GmbH, Hamburg, Germany).

Results

The IL-6 concentrations of 21 normal persons and 45 patients with stable graft function ranged from 5–10 pg/ml in serum and 0–5 pg/ml in urine. In serum (Table 1 a; Fig. 1), we identified a 100% increase in IL-6 titers in 15 of 51 patients developing histologically documented rejection (sensitivity 29%, specificity 87%). The mean value was 19 ± 7 pg/ml vs. 7 ± 2 pg/ml (not significant; n.s.) compared to baseline values 5–10 days before rejection was evident. In urine (Table 1 b) the results were more promising. Of 36 patients with documented rejection, 29 developed a 100% increase in IL-6 values (sensitivity 80%, specificity 88%). The mean value was 85 ± 44 pg/ml in patients with rejection, compared to 5 ± 1 pg/ml 5–10 days before rejection. Successful treatment of rejection resulted in a decrease in the urine values to normal (7 ± 2 pg/ml). In three patients, the rejection persisted, as did their elevated IL-6 values. Further analysis revealed that elevated urinary IL-6 concentrations showed a better correlation with rejection episodes in the initial phase after transplantation (Table 1 b, Fig. 2). In 88% (23 of 26) of rejection episodes in the first 2 months after transplantation, elevated IL-6 levels were found (4.6 ± 1.2 pg/ml vs. 93 ± 34 pg/ml; $P < 0.05$), whereas in only 60% (6 of 10) of rejection episodes after this period was an increase in IL-6 concentration evident

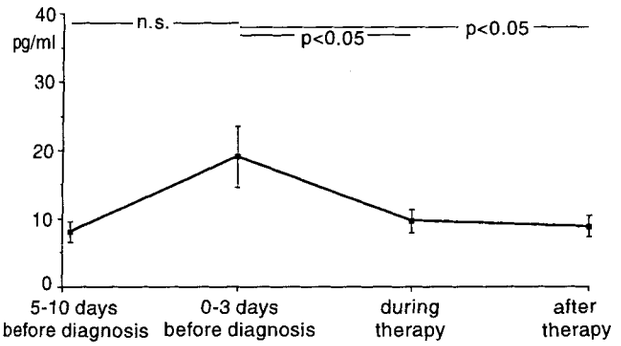

Fig. 1 Serum interleukin-6 (IL-6) levels (±SEM) of 45 patiens with acute rejection ($n = 51$) after renal transplantation

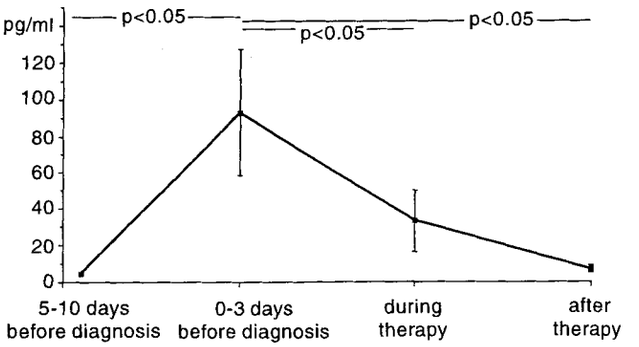

Fig. 2 Urine IL-6 levels (±SEM) of 18 patients with acute rejection ($n = 26$) in the initial phase (< 2 months) after renal transplantation

Table 1a Elevated (> 100%) serum IL-6 levels

	Total (patients)	Positive	Percentage
Normal course	6 (6)	0	0%
Rejection episodes (< 2 months after Tx)	40 (34)	10	25%
Rejection episodes (> 2 months after Tx)	11 (11)	5	45%
Infection episodes	19 (12)	13	68%
False positive results	18 (63 patients) 3 × ATG therapy 13 × Infections 2 × unknown		
< 2 months after Tx: Sensitivity 25% Specificity 85%		> 2 months after Tx: Sensitivity 45% Specificity 89%	

Table 1b Elevated (> 100%) urinary IL-6 levels

	Total (patients)	Positive	Percentage
Normal course	6 (6)	1	16%
Rejection episodes (< 2 months after Tx)	26 (18)	23	88%
Rejection episodes (> 2 months after Tx)	10 (10)	6	60%
Infection episodes	12 (11)	10	83%
False positive results	15 (45 patients) 2 × ATG therapy 10 × Infections 3 × unknown		
< 2 months after Tx: Sensitivity 88% Specificity 90%		> 2 months after Tx: Sensitivity 60% Specificity 86%	

Table 2 Elevated (> 100%) urinary GM-CSF levels

	Total (patients)	Positive	Percentage
Rejection episodes (< 2 months after Tx)	23 (18)	20	87%
Rejection episodes (> 2 months after Tx)	10 (10)	2	20%
Infection episodes	9 (8)	2	22%
False positive results	5 (36 patients) 1 × ATG therapy 2 × Infections 2 × unknown		
< 2 months after Tx: Sensitivity 87% Specificity 96%		> 2 months after Tx: Sensitivity 20% Specificity 97%	

(69 ± 56 pg/ml vs. 7.7 ± 4.3 pg/ml; n.s.). The addition of the monoclonal antibody against IL-6 reduced the activity in plasma and urine in the B9 cell assay to zero in every instance.

Bacterial infection proved to be a major confounder. Of 19 patients developing such infections, 13 also displayed increased IL-6 serum titers (Table 1a) of greater than 100% (33 ± 9 vs. 11 ± 3 pg/ml in controls). Of 12 such infections in urine (Table 1b), 10 were associated with increased urinary titers (77 ± 25 pg/ml). After antimicrobial therapy, the serum values of infected patients decreased to 6 ± 1 pg/ml, while urine values decreased to 10 ± 5 pg/ml.

GM-CSF concentrations in serum were not increased in patients undergoing biopsy-proved rejection (2.1 ± 0.5 vs. 1.7 ± 0.4 pg/ml; n.s.). On the other hand, 100% increases in urine (Table 2) were observed in 22 of 33 rejection episodes. During the early posttransplant period (< 2 months after transplantation), elevated GM-CSF

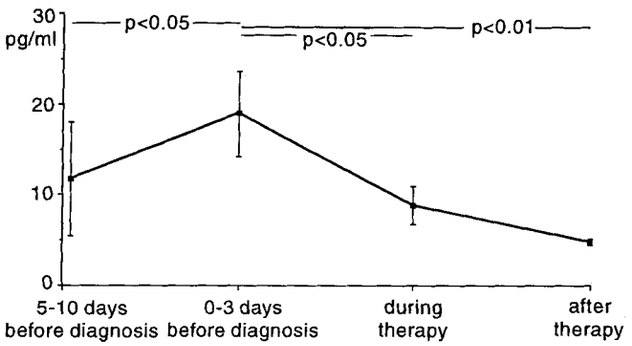

Fig. 3 Urine granulocyte-macrophage colony stimulating factor (GM-CSF) levels (±SEM) of 23 patients with acute rejection ($n = 26$) in the initial phase (< 2 months) after renal transplantation

levels were detected (specificity: 96%, sensitivity: 87%) in 20 out of 23 biopsy proved rejection episodes. In these patients, the urinary GM-CSF concentrations showed a significant increase (18.9 ± 4.7 vs. 11.7 ± 6.3; $P < 0.05$; Fig. 3). After successful antirejection therapy, these values decreased to normal baseline values (4.8 ± 0.3 pg/ml; $P < 0.01$). In patients with rejection episodes after the initial 2 months, only two out of ten patients had elevated GM-CSF levels (7.7 ± 2.5 vs. 6.5 ± 2.2; n.s.; Table 2). Two of nine patients with bacterial infections also demonstrated 100% increases in GM-CSF values in their urine (5.1 ± 1.0 vs. 3.8 ± 1.0; n.s.; Table 2).

IL-6-positive cells were identified with APAAP in every biopsy specimen showing rejection. IL-6-positive histiocytes within the interstitium were a hallmark. Positive cells were often observed within the interstitial infiltrate around the glomeruli. The degree of positive staining was variable; some biopsies had only a few positive cells, whereas in other biopsies, positive cells were present by the hundreds. Moreover, in some preparations, tubular cells showed IL-6-positive staining. The blood vessels and structural cells of the glomeruli were uniformly negative. GM-CSF-positive staining was identified even in normal tissue. Rejection increased the intensity of this staining, which was identified within glomeruli, interstitium and blood vessels. Interestingly, we were unable to find a correlation between the severity of rejection and the intensity of the staining in the biopsy specimens.

Discussion

The aim of this study was to evaluate the serum and urine concentration of marker cytokines, as well as their presence in renal tissue as indicators for acute renal transplant rejection. We chose IL-6 and GM-CSF because of their pivotal participation in the inflammatory process.

In serum, IL-6 concentration appears to increase with rejection [3, 4] the GM-CSF levels, however, were not different from baseline values. However, the IL-6 values in our patients showed such variability, that reliability could not be shown. For both cytokines, serum determinations failed to display sufficient reliability to recommend their clinical use.

Urine determinations showed considerably more promise than serum values. Both IL-6 and GM-CSF appeared reliable indicators of rejection. The increases were sufficiently prompt (1–2 days before clinical diagnosis was made) to be of clinical value. The values were decreased concomitantly with the suppression of the rejection episodes with medication. Our results and those of others [4] suggest that urine determinations of these cytokines may assist in the diagnosis and management of rejection.

Interestingly, the cytokine concentrations during the early posttransplant period are more sensitive diagnostic markers of rejection than in later rejection episodes. Our results suggest a central role for IL-6 and GM-CSF in the rejecton process, especially in the initial phase after renal transplantation. Thereafter, IL-6 and GM-CSF are poor diagnostic markers of rejection, although the presence of these cytokines has been demonstrated in biopsies [6, 7]. Probably due to a more sustained rejection process or to a different mechanism with involvement of other cytokines, less IL-6 and GM-CSF "spillover" in urine was found in these patients.

Bacterial infection proved to be the major confounder in our study. The participation of bacteria in cytokine release, the mediation of the "systemic inflammatory response syndrome" and similar cytokine-mediated manifestations is well recognized. This response in IL-6 and GM-CSF serum and urine levels was anticipated [5]. Unfortunately, it renders the use of these parameters as rejection indicators worthless if bacterial infection is included in the differential diagnosis. However, in many instances, bacterial infection can be ruled out, particulary, in urine. In such circumstances, cytokine determinations could conceivably be of value.

We also observed increased cytokine release after therapy with anti-thymocyte globulin. This observation confirmed earlier findings indicating that anti-thymocyte globulin results in cytokine release [5, 8, 9]. Mechanisms involved in this process may include T cell activation via surface receptors. It is probable that cytokine release is

responsible for the side-effects of anti-thymocyte globulin therapy.

We believe that the site of cytokine production in our patients was in the rejected graft itself and that the elevated serum and urine concentrations reflected a "spillover" of cytokines from the inflammatory process within the transplanted kidney. The immunohistochemical staining we observed supports this point of view. These observations confirmed those reported earlier [6, 7]. We saw no clear-cut correlations between histochemical staining and rejection severity or cytokine levels. A new finding was the GM-CSF staining we observed even in normal kidney. With rejection, this staining was much more prominent and also included the walls of blood vessels. Also of interest was the tubular staining with IL-6. Thus, rejection is not only an acute interstitial process, but may involve tubular and vascular structures of the transplanted organ as well. Additional studies will be necessary to delineate to what extent tubules and smooth muscle cells participate in this process. Involvement of tubular cells may not be too surprising, since tubular cells have been shown to exhibit immune-mediated signalling [10, 11]. The clinical relevance of these observations remains to be elucidated.

Our results suggest that cytokines may be of value in monitoring acute transplant rejection, especially in the early posttransplant period. However, inflammation from other causes, notably bacterial infection, is a serious confounding variable. If infection is unlikely, a cytokine profile may be a promising avenue of approach.

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