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CLINICAL INVESTIGATION

Glutathione transferase in the urine: A marker for post-transplant tubular lesions

LARS BÄCKMAN, EEVA-LIISA APPELKVIST, OLLE RINGDÉN, and GUSTAV DALLNER

Departments of Pathology, Transplantation Surgery and Clinical Immunology, Karolinska Institutet, Huddinge Hospital, Stockholm, Sweden

Glutathione transferase in the urine: A marker for post-transplant tubular lesions. Basic glutathione transferase released from the proximal tubular epithelium in the kidney was monitored in the urine of 69 recipients of renal allografts. The enzyme was isolated from human liver and the urinary analysis performed with radioimmunoassay. Patients receiving cyclosporine A without toxicity or rejection did not excrete this enzyme in their urine; whereas the urine of patients with cyclosporine A-induced nephrotoxicity contained significant amounts of the transferase ($P < 0.001$). Patients with allograft rejection also showed increased urinary concentrations of the basic glutathione transferase, but had significantly lower values than patients with cyclosporine induced nephrotoxicity ($P < 0.001$). During aminoglycoside and co-trimoxazole treatment, the urinary concentration of this transferase also increased. Patients with renal infarction showed a sudden increase in urinary transferase to very high levels. The results indicate that quantitative analysis of the basic glutathione transferase in urine is useful for monitoring renal tubular lesions present in various complications following transplantation, such as cyclosporine and antibiotic induced nephrotoxicity and renal infarction.

The release of cyctoplasmic enzymes as a result of an increased permeability of the plasma membrane during various pathological conditions is established in diagnosing many disorders. Determination of a protein as such or of enzyme activity in serum and plasma is used in diagnosing heart and liver diseases [1, 2]. In case of the kidney this approach is less developed. However, the idea of analyzing proteins in the urine to diagnose renal diseases is not new [3]. Theoretically, an increased permeability of renal epithelial cells may lead to release of cytoplasmic proteins into the urine.

Glutathione transferases (GSH-transferases) play an important role in conjugation reactions and are present at high levels in the liver and kidney. Three major forms—basic, neutral and acidic—have been identified and isolated from human tissues, and it is probable that each major form consists of subgroups of isoenzymes [4, 5].

The basic GSH-transferase (mol wt 51,000), formerly known as ligandin, was reported to be localized in the proximal tubular epithelium in the kidney [6]. Earlier investigations have detected the enzyme in urine, which is normally free from GSH-transferases, as a result of leakage from damaged tubular cells and not as a result of increased glomerular filtration of the blood enzymes [7]. Zalneraitis, Arias and Cho observed GSH-transferase in the perfusates from cadaveric kidneys collected for transplantation showing acute tubular necrosis [8].

Cyclosporine A (CsA), known to be nephrotoxic, is reported to affect the renal proximal tubular epithelium [9, 10], raising the possibility that the appearance of basic GSH-transferase in urine may be an indicator of CsA-induced nephrotoxicity.

We have developed a radioimmunoassay (RIA) using human liver basic GSH-transferase as antigen, and utilized this procedure to evaluate the usefulness of analyzing basic GSH-transferase levels in urine to diagnose tubular disorders as well as to discriminate between CsA-induced nephrotoxicity and rejection in renal allograft recipients. The results indicate that this approach will be useful for monitoring renal tubular lesions present in various complications following transplantation.

Methods

Sixty-nine recipients of renal allografts (28 females and 41 males) with a median age of 40 years (range 6 to 69) were studied. These patients had received grafts from a total of 14 living and 55 cadaveric donors. Eight of the patients received their second renal allograft and 16 underwent a combined pancreatic and renal transplantation [11]. CsA and prednisolone was used in 48 patients; azathioprine (AZA) and prednisolone in 14 patients; and CsA, AZA and prednisolone were administered together in 7 patients [12, 13]. Forty-two patients were followed daily from transplantation until discharge from the hospital, and twice weekly for a total of three months. The patients were examined upon admittance to the hospital to confirm the diagnoses of deterioriation in renal function. In order to diagnose the different renal transplant deteriorations, the practical approaches applied daily in clinical transplantation were employed, such as morphological and radiological examinations, clinical chemical parameters, and clinical response of renal function to a given treatment.

Twenty-eight patients with stable renal function, referred to as the stable group, were studied. They had serum creatinine levels below 200 μmol/liter and were free from rejection, clinically significant CsA-induced nephrotoxicity, hypertension and infection. This limit was used since CsA treated patients in general have higher serum creatinine levels. In this group 23 patients received CsA, 2 patients AZA, and 3 received CsA, AZA and prednisolone.

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Acute tubular necrosis, rejection, nephrotoxicity and renal infarction

Acute tubular necrosis (ATN) was presumed to be present in patients requiring dialysis due to uremia after the renal transplantation ($N = 9$), if no other reason for the impairment could be established. In 33 patients improvement in renal function was immediate. Samples from the first post-operative day were used for comparisons. Theoretically, it is possible that patients with a mild form of ATN not requiring dialysis were placed in the group with an immediate onset in renal function here. An absolute diagnosis of ATN in the post-transplant period would require a biopsy, which was, however, not possible.

A biopsy from the graft was taken in 32 of the patients with increased serum creatinine. In those cases where the morphological changes were compatible with rejection ($N = 21$) or if there was strong clinical suspicion, anti-rejection therapy was given ($N = 38$). If the morphological examination revealed no signs of acute rejection in patients treated with CsA ($N = 11$), nephrotoxicity was assumed and the dosage of CsA reduced. In cases where no biopsy was taken, the diagnosis of CsA nephrotoxicity was made if the graft function improved after reduction of the CsA dose ($N = 16$). Anti-rejection treatment consisted of 0.5 g methylprednisolone intravenously (i.v.) for one day, followed by 0.25 g for at least three additional days (range 3 to 8 days). Eighteen patients treated with CsA, 14 treated with AZA and 6 patients treated with both CsA and AZA suffered rejection episodes. Renal allograft infarction ($N = 3$) was diagnosed by renal angiogram. The infarctions were total in two cases and partial in one. One patient developed a venous thrombosis in the allograft, diagnosed by renal angiogram. One patient suffered from hydronephrosis due to allograft uretary stenosis diagnosed by ultrasound examination. In cases of rejection, CsA nephrotoxicity, infarction and hydronephrosis, the values on the day of diagnosis were used for comparison.

Antibiotic treatment

Six patients with infections were treated with netilmicin (2 mg/kg body wt/dose) in combination with co-trimoxazole. The dose interval was based on renal function and on serum levels of netilmicin. Co-trimoxazole was given either orally or intravenously at a dose of 640 mg trimetoprim and 3400 mg sulphametoxazole per day with precaution to the renal function. Two patients were treated with netilmicin or co-trimoxazole alone. The maximal concentration of GSH-transferase during the treatment, together with the concomitant serum creatinine level, were compared with the values obtained immediately before treatment.

Protein purification and iodination

Basic GSH-transferase from a human liver was purified according to Warholm et al [5] with certain modifications. The 105,000 g supernatant fraction was filtered through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) equilibrated with 100 mM Tris-HCl buffer, pH 7.8, containing 1 mM EDTA. The fractions containing GSH-transferase activity were applied to a DEAE-cellulose column. The protein was further purified by affinity chromatography on S-hexylglutathione coupled to epoxy-activated Sepharose 6B. The active fractions were desalted on a Sephadex G-25 column and concentrated in an Amincon ultrafiltration cell. GSH-transferase activity was measured spectrophotometrically at 340 nm with 1-chloro 2,4-dinitrobenzene as substrate. The purified enzyme was labeled with 1 mCi ^{125}I (Amersham International, Arlington Heights, Illinois, USA) [14], followed by removal of unbound ^{125}I on a Sephadex G-25 column. The labeled protein was purified on a affinity column before being used as tracer in the assay.

Antibody production

Rabbits were injected in the foot pads with 0.3 ml Freunds complete adjuvant (FCA; Gibco, London, UK), enlarging the lymph nodes in the knee joints. The purified protein (50 to 70 μg) in FCA was then injected into these lymph nodes. This procedure was repeated several times. Blood was tapped and antiserum prepared. The specificity of the antiserum was confirmed using the 105,000 g supernatant fraction and human neutral and acidic GSH-transferases as antigens for Ouchterlony gel diffusion [15].

Radioimmunoassay

After titration, the antiserum was used at a dilution of 1:175,000. The standard curve, constructed according to the Spline function [16], consisted of 12 duplicate samples of the purified protein in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 10 mM EDTA, 1.0% bovine serum albumin and 0.1% sodium azide. The concentrations ranged from 1.0 to 1,000 ng/ml. Urine was collected and stored at room temperature without addition of protease inhibitors. This storage did not influence the enzyme levels measured with RIA. Samples were centrifuged and frozen at −20°C. Duplicate samples consisting of 100 μl urine were mixed with 100 μl (20,000 dpm) tracer and 100 μl diluted antiserum. The mixture was shaken at room temperature for 20 hours. Two hundred fifty microliters (1 mg) of goat-antirabbit secondary antibody (Immunobead reagent, Bio-Rad Laboratories, Richmond, California, USA) was then added and incubated for two hours at 37°C. Each sample was washed with 2 ml of the RIA buffer. The samples were centrifuged at 1500 g for 10 minutes and decanted. The radioactivity in the pellet was counted and the enzyme amount calculated from the standard curve.

Analysis of creatinine

Serum creatinine (S_{Cr}), normal value <115 μmol/liter, was analyzed using the kinetic Jaffe method. The accuracy of this method has been evaluated using isotope dilution mass spectrometry [17].

Statistics

For comparisons between the different groups the Mann-Whitney U-test was used. Students *t*-test, chi-square analysis and Fishers exact test were also used when appropriate. The linear correlation coefficient was calculated. GSH-transferase concentrations of less than 1 ng/ml were arbitrarily assigned the value 0.5 ng/ml for the statistical analyses.

Table 1. Purification of basic glutathione transferase from human liver

Step	Volume *ml*	Protein concentration *mg/ml*	Total activity *μmol/min*	Specific activity *μmol/min/mg protein*	Purification factor	Yield of activity %
Cytosolic fraction	100	13.9	5400	3.9	1	100
Sephadex G-25	140	9.2	5230	4.1	1.1	96.9
DEAE-cellulose	175	1.5	4060	15.5	4.0	75.2
Affinity chromatography + sephadex G-25 concentration	86	0.11	2511	265.4	68.1	46.5
Amincon cell	10	0.91	2340	257.1	65.9	43.3

Results

Protein purification

This study required the isolation of a highly purified human basic GSH-transferase to be used as antigen for the antibody production and as tracer and standards in the RIA. The gel filtration and ion exchange chromatography resulted in a limited purification. Successful isolation required the use of affinity chromatography. The purification factor with this step included was about 70 (Table 1) and the yield was 40 to 50% of the cytosolic activity. The purity of the isolated enzyme was investigated by sodium dodecylsulphate (SDS)-gel electrophoresis (Fig. 1). Coomassie Blue staining showed a single and well-defined band with an apparent molecular weight of 26,000 for the two subunits. No contaminating bands were visible.

Antiserum and tissue localization

The anti-serum had a titer of 1:175,000. Single precipitations were obtained upon immunodiffusion with the 105,000 g supernatant fraction or the purified GSH-transferase. No precipitations were obtained when human neutral or acidic transferases were tested.

Immunohistochemical staining of formalin fixed non-pathological renal biopsies with use of the antiserum, and the horseradish peroxidase reaction showed an intense positive reaction in the proximal tubular epithelium (Fig. 2). Staining of biopsies from patients with CsA nephrotoxicity did not reveal any alteration in the distribution of the transferase.

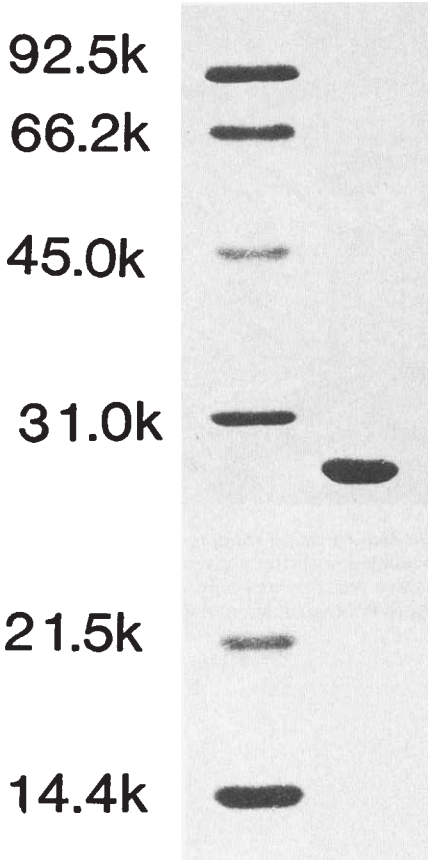

Fig. 1. *Appearance of purified glutathione transferase on a polyacrylamide gel.* SDS-PAGE was performed in a 1.5 mm thick 10% polyacrylamide gel. Twenty μg of the purified protein and the standards were electrophoresed at 30 mA in 0.05 M Tris-HCl, 0.38 M glycine, 0.1% SDS, pH 8.3, in a vertical slab gel unit and stained with Coomassie Blue. On the left side are the references used: lysozyme (mol wt 14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200) and phosphorylase B (92,500). On the right side are the subunits of the purified human liver basic GSH-transferase, overlapping each other with a molecular weight of approximatively 26,000.

Stable renal function and ATN

Patients who belonged to the stable group had a S_{Cr} level of 133 ± 8 μmol/liter (mean ± SEM; Fig. 3). They demonstrated a somewhat impaired renal function in comparison to a healthy population (S_{Cr} <115 μmol/liter). The level of basic GSH-transferase was 0.7 ± 0.4 ng/ml (mean ± SEM) in the patients with stable renal function. About 80% (22 of 28) had a level less than 1.0 ng/ml. Of all measurements on patients with stable renal function (N = 298) 12% showed values greater than 1.0 ng/ml.

During ATN the S_{Cr} level was higher ($P < 0.001$) than in patients with an immediate onset of renal function, the mean values being 1041 ± 71 and 461 ± 40 μmol/liter (±SEM), respectively. The urinary GSH-transferase concentration was also significantly higher in the ATN group ($p < 0.05$) in comparison with patients demonstrating an immediate onset in renal function (19.8 ± 8.1 and 7.1 ± 2.0 ng/ml, mean ± SEM, respectively).

Renal infarction and hydronephrosis

In the three cases of renal infarction the urinary GSH-transferase concentration was 134 ± 90 ng/ml (mean ± SEM) and the mean S_{Cr} level was 420 ± 142 μmol/liter (± SEM). The patient with a venous thrombosis in the renal allograft had a

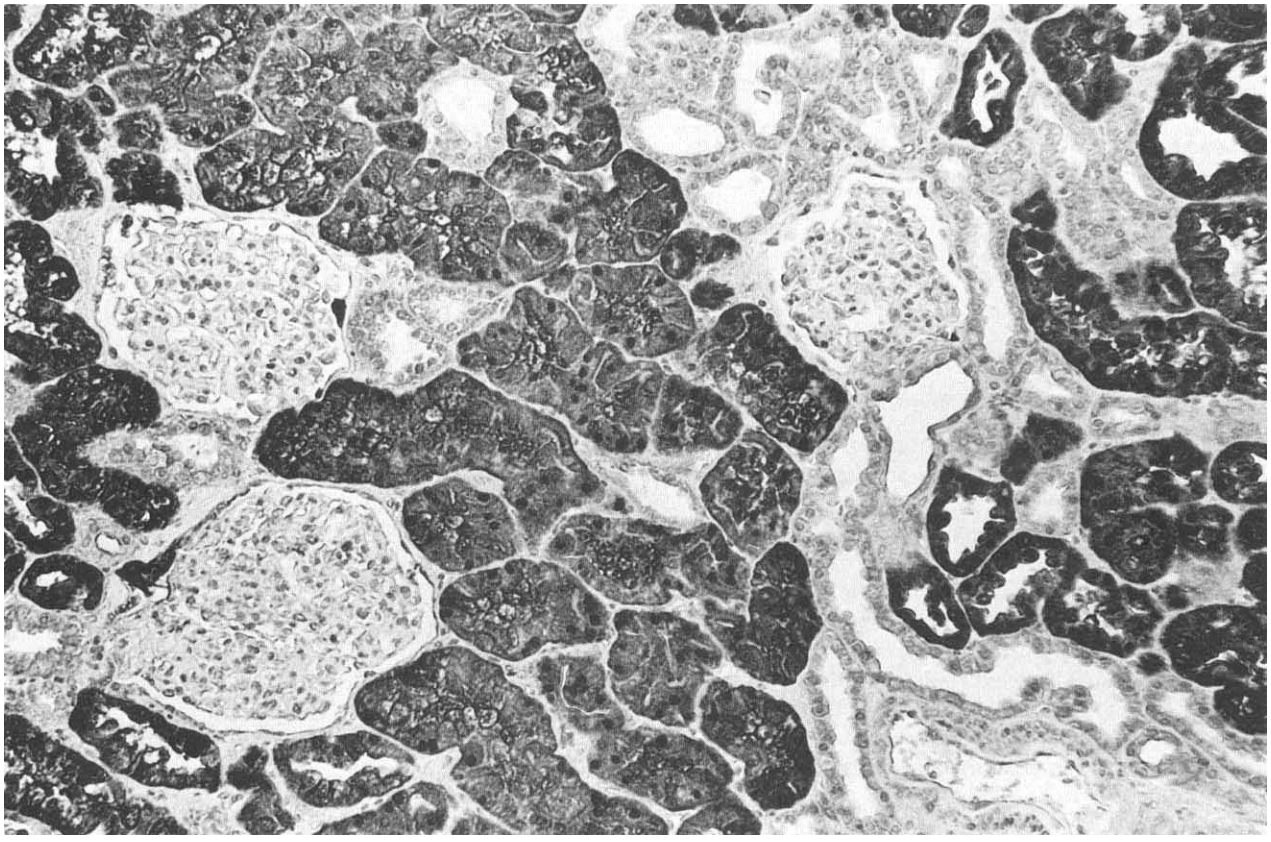

Fig. 2. *Immunohistochemical staining of a human kidney biopsy with rabbit anti-human basic glutathione transferase serum.* The formalin-fixed slices were incubated with the antiserum in a dilution of 1:200. The antigen-antibody complex was visualized by the horseradish peroxidase technique. Positive reaction was only visible in the proximal tubular epithelium. Staining after incubation with antigen absorbed or nonimmune serum was negative. Magnification ×400.

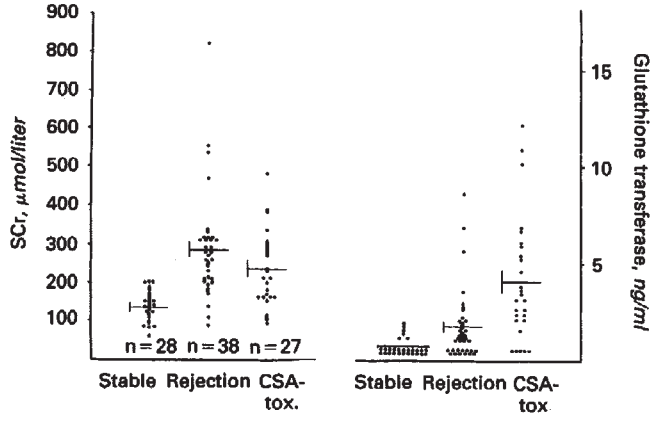

Fig. 3. *Serum creatinine and urinary GSH-transferase concentrations in renal transplant recipients.* The criteria for stable, rejection and cyclosporine induced nephrotoxicity are given in **Methods**, *N* = number of patients. The horizontal line is the mean value, while the vertical bar indicates SEM.

transferase concentration of greater than 1,000 ng/ml. In these patients the increased urinary concentration was observed on the same day as the increased S_{Cr}. In the case of hydronephrosis there was no detectable GSH-transferase in the urine.

Rejection and CsA-induced nephrotoxicity

Patients with acute rejection had a S_{Cr} of 283 ± 21 μmol/liter (mean ± SEM; Fig. 3), significantly higher than for patients with stable renal function ($P < 0.001$). The urinary GSH-transferase concentration for the rejection group was 1.7 ± 0.3 ng/ml (mean ± SEM), also differing from the stable group ($P < 0.001$). In comparison with the ATN and "immediate onset" groups, those with rejection had significantly lower GSH-transferase concentrations in their urine ($P < 0.001$ and $P < 0.01$, respectively).

In patients with CsA-induced nephrotoxicity the S_{Cr} (235 ± 20 μmol/liter, mean ± SEM) was increased in comparison to the stable group ($P < 0.001$). However, patients with acute rejection had a higher S_{Cr} level than those with acute CsA nephrotoxicity ($P < 0.05$). The concentration of basic GSH-transferase in the urine in case of toxicity was 4.1 ± 0.6 ng/ml, (mean ± SEM), exceeding the values for the stable ($P < 0.001$) and rejection groups ($P < 0.001$). A urinary GSH-transferase value greater than 2 ng/ml was seen in 21 of 27 (78%) of the patients diagnosed to have CsA nephrotoxicity, and only in 8 of 38 (21%) of the patients with rejection ($P < 0.001$, chi-square analysis).

To determine whether CsA-induced nephrotoxicity was also present in the eight patients with high urinary GSH-transferase levels and rejection, those treated with AZA, or experiencing

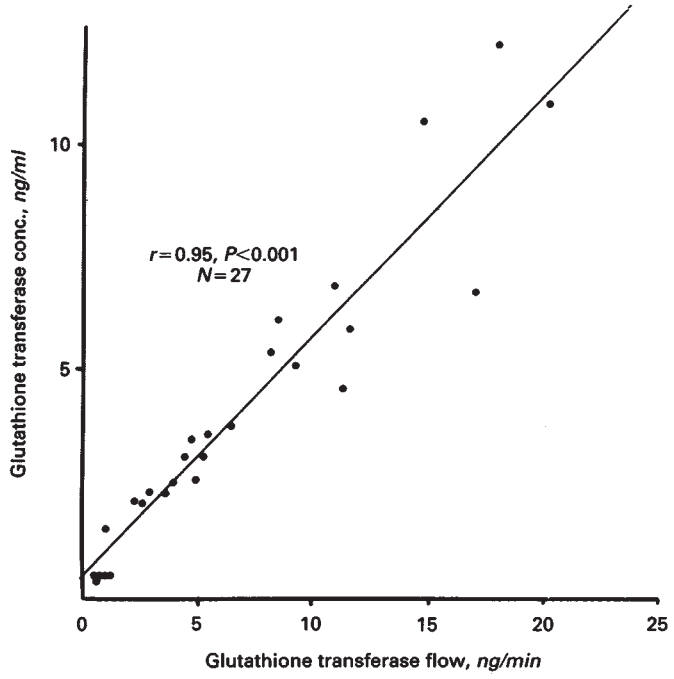

Fig. 4. *Correlation between urinary GSH-transferase concentration and the amount of enzyme excreted per minute in the urine during cyclosporine induced nephrotoxicity.*

re-rejection episodes or irreversible rejections were excluded. The three remaining patients with the highest GSH-transferase levels all developed nephrotoxicity within six months, while only two of the corresponding 12 patients in the group with urinary concentrations less than 2 ng/ml suffered a CsA nephrotoxic episode within six months ($P < 0.05$, Fishers' exact test).

During CsA-induced nephrotoxicity there was no linear correlation ($r = 0.16$, NS) between S_{Cr} and the GSH-transferase concentration in the urine. The correlation between urinary GSH-transferase concentration and the amount excreted per minute was linear in the patients with CsA nephrotoxicity ($r = 0.95$, $P < 0.001$; Fig. 4).

Of the patients with CsA nephrotoxicity 13 underwent daily monitoring of GSH-transferase. In nine (69%) of these patients the GSH-transferase concentration increased at the same day as did the S_{Cr}. In two patients the increase occurred one day before and in two patients two days before the increase in S_{Cr}. During episodes of nephrotoxicity 77% of the GSH-transferase values were increased during the five day period after the diagnosis was made. Of the 25 patients with acute rejection and urinary GSH-transferase concentrations greater than 1 ng/ml, 16 underwent daily monitoring and 81% of these demonstrated elevated levels within five days after diagnosis.

Antibiotic treatment

In 10 patients treated with netilmicin alone and/or co-trimoxazole, the GSH-transferase level in the urine increased from 0.7 ± 0.1 ng/ml (±SEM) before treatment to a maximum of 3.1 ± 0.8 ng/ml (mean ± SEM, $P < 0.001$; Table 2). The mean S_{Cr} level increased from 203 ± 42 to 279 ± 56 μmol/liter (± SEM, $P <$ 0.01) on the day when the maximal GSH-transferase was attained.

The values for a 26-year-old male pancreatic and renal graft recipient who underwent netilmicin treatment due to septicemia are shown in Figure 5. The S_{Cr} level was only slightly elevated, while the GSH-transferase level in the urine increased continuously and considerably during the period.

Discussion

GSH-transferases are present in many different tissues and play a central role in the biotransformation of drugs and toxic chemicals. Their catalytic action produces glutathione conjugates, which are either excreted or hydrolyzed to cysteine derivates, and subsequently N-acetylated to yield mercapturic acids [18]. The basic form has been identified in the liver, kidney, adrenal gland, duodenum and gonads [19, 20]. The enzyme is present in high concentrations constituting 5% and 2% of the cytoplasmic protein content in the liver and proximal tubules of the kidney, respectively.

The basic isolated GSH-transferase demonstrated a high specific activity and was found to be pure; the antisera did not cross-react with the neutral or acidic forms of the enzyme.

There are two explanations for the appearance of GSH-transferase in urine: excretion from plasma by glomerular filtration or release from damaged cells in the proximal tubular epithelium. It has been reported that glomerular disorders do not increase the urinary excretion of GSH-transferase, making the first explanation unlikely [7].

In patients with ATN the urinary GSH-transferase concentration was higher than in patients with immediate onset in renal function after transplantation, which may be useful in differential diagnosis for example, low GSH-transferase concentrations in spite of high S_{Cr} values may indicate the absence of ATN or CsA nephrotoxicity. Urinary GSH-transferase excretion was massive during allograft infarction. This finding is useful for an early diagnosis of allograft infarction, since potentially-dangerous immunosuppressive treatment can then be stopped.

Two major complications often occur after renal transplantation in patients receiving CsA: acute rejection and CsA-induced nephrotoxicity. Monitoring of blood and plasma levels of CsA is of limited value in detecting toxicity, since there seems to be only a small difference between the therapeutic and toxic levels of CsA, and which sometimes appear to overlap [21]. Methods for distinguishing between these conditions are limited. The most effective are needle biopsy and fine needle aspiration biopsy [22–25].

CsA-induced nephrotoxicity in human renal transplant recipients was accompanied by a release of basic GSH-transferase in the urine, indicating a toxic effect of the drug on the proximal tubular system. CsA has several well-defined effects on peroxisomes and microsomes [26]. Experimental and clinical studies of CsA-induced toxicity have revealed histological changes in the proximal tubules [9, 10, 27]. However, we cannot exclude the possibility that CsA also affects other parts of the kidney.

The patients suffering acute rejection usually had a moderately elevated level of basic GSH-transferase in their urine, but the level was significantly below the level obtained during CsA nephrotoxicity. However, S_{Cr} levels were higher in patients with rejections than in those with CsA-induced nephrotoxicity. In some cases of rejection elevated urinary levels of transferase

Table 2. Effect of antibiotic treatment on glutathione transferase concentration in urine and serum creatinine level

Patient no.	Antibiotic treatment	Urinary GSH-transferase concentration *ng/ml*		Serum creatinine concentration *μmol/liter*	
		Before treatment	Maximal value during treatment	Before treatment	When GSH-transferase was maximal
1	Co-trimoxazole, p.o.	0.5	1.6	202	269
2	Co-trimoxazole, p.o.	0.5	1.3	69	85
3	Netilmicin	0.5	7.8	246	255
4	Netilmicin	1.1	1.6	196	279
5	Co-trimoxazole, i.v. + netilmicin	0.5	0.5	62	85
6	Co-trimoxazole, i.v. + netilmicin	0.5	2.1	185	325
7	Co-trimoxazole, i.v. + netilmicin	0.5	1.0	524	679
8	Co-trimoxazole, i.v. + netilmicin	0.5	5.1	273	285
9	Co-trimoxazole, i.v. + netilmicin	0.5	6.0	167	413
10	Co-trimoxazole, i.v. + netilmicin	1.4	3.6	105	113
	Mean ± SEM	0.7 ± 0.1	3.1 ± 0.8	203 ± 42	279 ± 56
	Significance[a]	$P < 0.01$		$P < 0.01$	

The maximal urinary GSH-transferase concentrations attained during the antibiotic treatment and serum creatinine values measured on the same day were used.

[a] Levels of significance were determined by the Mann-Whitney U-test.

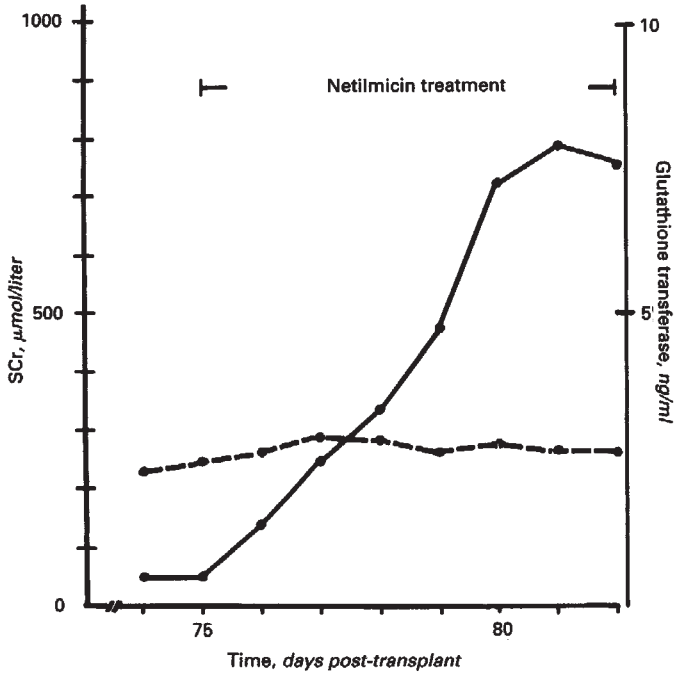

Fig. 5. *Effect of aminoglycoside treatment on urinary glutathione transferase concentration.* The values are for a 26-year-old male recipient of a pancreatic and renal allograft suffering from a Klebsiella enterobacterium septicemia diagnosed with a positive blood culture on day 75 after transplantation. Netilmicin was given intravenously at a dose of 100 mg/24 hour (2 mg/kg body wt). The serum concentration of netilmicin was 1.9 μg/ml immediately before administration on day 77 and 7.6 μg/ml 1 hour after administration. The patient was treated with azathioprine (AZA) and prednisolone, but the AZA treatment was discontinued due to the severe infection. (——) Urinary glutathione transferase concentration; (---) serum creatinine level.

were obtained, which is not surprising, since some degree of toxicity induced by CsA or other factors may also be present. In some case of nephrotoxicity the level of transferase in urine was not elevated, while in some cases of rejection it was elevated to the same level as in the toxicity group. We have no immediate explanation for these findings. Future studies regarding subgroups (that is, released isoenzymes not detected by our antibodies), other associated pathological conditions and the influence of other drugs, may provide some answers. It is possible that a severe rejection episode might give rise to tubular damage, followed by a pronounced excretion of basic GSH-transferase. On the other hand, in our material not even the patients with the most severe and irreversible rejections displayed such increased excretion. In most cases then, obviously, rejection episodes were not accompanied by any marked effects on the tubular cells containing the basic GSH-transferase.

The antibiotics co-trimoxazole and aminoglycosides are known to be nephrotoxic [28] and have been reported to potentiate CsA-induced nephrotoxicity [29, 30]. During netilmicin treatment no patient had extreme levels of the drug in his serum. The increase in GSH-transferase suggests that this enzyme is a more sensitive indicator of tubular damage than serum creatinine levels.

The results demonstrate that quantitation of basic GSH-transferase in urine is a useful method in detecting and monitoring disturbances of tubular function. The test developed was, in fact, specific for the detection of tubular damage and, therefore, useful for detecting different pathological conditions giving rise to tubular epithelial damage. This enzyme excretion was increased during several conditions, but the degree of excretion, its time course and the clinical circumstances may be of help in differentiating between various disturbances of the tubular system.

Epithelial cells in the nephron differ both in chemical and enzymatic composition [31, 32]. In the future isolation of proteins present at high concentrations in specific tubular cells and their use for quantitation in urine may therefore be of importance. There are good reasons to believe that establishment of a few assays for specific proteins in urine will provide improved tools for diagnosis of renal impairments, such as

those available for analysis of disturbances in liver and heart function.

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Reprint requests to L. Bäckman, Department of Pathology F-42, Huddinge Hospital, S-141 86 Huddinge, Sweden.

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