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Serum hippuric acid concentration in renal allograft rejection, ureter obstruction, and tubular necrosis

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Abstract Plasma from 35 renal allograft recipients (21 males and 14 females) was sampled daily and analyzed for hippuric acid (HA) by high-performance liquid chromatography (HPLC) and serum creatinine. Twelve of these patients experienced an acute renal allograft rejection or a ureter obstruction as proven by clinical signs and biopsy, as well as by radiography or ultrasound, respectively. Two patients suffered from tubular necrosis followed by rejection during the postoperative period. Mean serum HA increased by 39.9 μmol/l from baseline (range 20.4–115.5 μmol/l) in patients with acute rejection 3 days after an initial increase that was observed 24 h before the mean serum creatinine increased by 107.1 μmol/l (range 21–193 μmol/l). In cases of ureter obstruction, HA rose by 1.6 μmol/l (range 1–8.2 μmol/l), significantly less than elevations due to rejection. The increase in creatinine, however, amounted to 65.3 μmol/l (range 22–140 μmol/l) and was not different from the change in rejecting patients. Successful antirejection treatment coincided with a decrease in serum HA starting 24 h earlier than the decrease in the serum creatinine concentration. Of special interest was the observation of a parallel decrease in HA with creatinine concentration in patients with tubular necrosis after allotransplantation; HA increased in cases of an additional rejection. Our data suggest that HA, which is excreted by tubular secretion and glomerular filtration, could be a sensitive and early marker of acute allograft rejection. Furthermore, it seems to discriminate between acute renal allograft rejection and ureter obstruction. It might, therefore, be of value in the diagnosis of rejection complicating tubular necrosis after transplantation.

Key words Kidney transplantation, rejection, serum hippuric acid · Ureter obstruction, serum hippuric acid · Rejection, kidney, serum hippuric acid · Hippuric acid in serum, rejection, kidney

Introduction

During the last 30 years of renal transplantation, great progress has been made in the diagnosis of renal rejection. Many immunological methods have been proposed, but just a few seem to be applicable in clinical practice.

Acute allograft rejection is clinically expected when patients develop temperature, a painful transplanted organ, edema, and high blood pressure. A significant increase in serum creatinine (>20%) suggests acute allograft rejection and requires further evaluation or exclusion of other causes such as ureter obstruction.

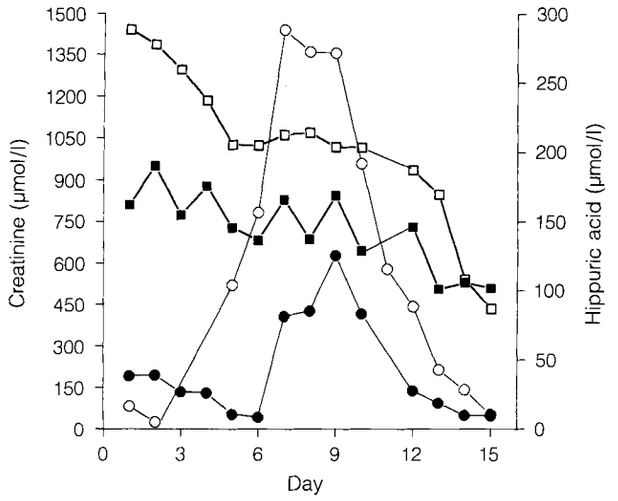

Fig. 1 Serum hippuric acid (◕) and creatinine (◪) concentrations after allotransplantation. Each symbol represents one patient. The increase in HA was due to rejection during tubular necrosis post-transplantation

Hippuric acid (HA), an organic acid with a molecular weight of 176 Da, is bound to protein and excreted by the proximal renal tubules through an organic acid transport carrier system; at higher concentrations and saturation of protein binding sites, free HA undergoes glomerular filtration. Up to 90% of HA is produced in the liver and up to 10% in the kidney itself. Endogenous production is approximately 0.14 mmol/day. Bergström et al. measured an HA clearance of 26.8 ± 16 ml/min in patients with advanced renal failure who had creatinine clearances of 6.5 ± 2 ml/min. No correlation between serum HA, creatinine, and urea was documented [12]. The urinary excretion rate for HA in severely uremic patients was the same as in healthy subjects, in contrast to creatinine, which is retained in spite of tubular secretion [12]. The precursor benzoic acid could not be detected in plasma ultrafiltrate, which might be due to complete conjugation with glycine to HA.

The aim of this study was to evaluate measurements of serum HA concentrations in order to provide diagnostic and differential diagnostic information on acute allograft dysfunction.

Materials and methods

Patients

Thirty-five recipients of renal allografts (21 males and 14 females) with a mean age of 39.5 years (range 22–62 years) were studied. Two patients received second transplants and two patients third transplants. The causes of chronic renal disease included chronic glomerulo- and pyelonephritis, polycystic kidneys, diabetic nephropathy, Alport syndrome, reflux nephropathy, and dysplastic kidneys. Twenty-nine patients were treated with chronic hemodialysis and 6 patients with chronic ambulatory peritoneal dialysis (CAPD).

Immunosuppressive treatment

The immunosuppressive treatment included prednisone, azathioprine, and cyclosporin A. The prednisone dose was 0.5 mg/kg body weight during the first 3 weeks after transplantation, followed by 40 mg/day until the beginning of the 9th week. Azathioprine was given according to body weight, 25–75 mg/day, and adapted to the number of leukocytes in the peripheral blood. The cyclosporin A dosage amounted to 5 mg/kg body weight per day, divided into two doses, for maintenance of a plasma through level of 300–500 µg/l within the first 3 months after transplantation (polyclonal assay). Acute renal allografting rejection episodes were treated with additional prednisone boluses, 1 g/day (patients < 40 kg body weight received 0.5 g/day), five times (on days 1, 2, 3, 5, and 7), eventually followed by antilymphocyte globulin (ATG) infusions (3 mg/kg per day) for 10–14 days or OKT3 orthoclone infusions (5 mg/day) for 10 days in cases of persisting rejection. Cytomegalovirus (CMV)-negative patients receiving a CMV-positive organ were treated prophylactically with Cytotect, 2 ml/kg per day, on days 1, 2, 4, and 14 and 4, 8, and 12 weeks after transplantation to prevent CMV disease. One patient had to be treated with plasmapheresis over a period of 10 days because of a hemolytic-uremic syndrome. Prophylactic treatment with ATG was given to 16 patients who experienced prolonged warm or cold ischemia times. The diagnosis of acute rejection was based on clinical signs, renal functional deterioration, transplant biopsy, and therapeutic success. Increments in serum HA and creatinine were estimated as the difference between an initial increase of more than 20% continuing until day 3 of observation.

Blood samples were taken frequently after transplantation up until day 30 or until discharge before day 30. After discharge, blood samples were obtained during visits to the outpatient clinic. One milliliter serum was ultrafiltrated through a Centrifree micropartitions system (Amicon 10, cut-off 10000 Da) and then stored at −30 °C until analysis.

High-performance liquid chromatography (HPLC) analysis

Analyses were performed with a Biorad Model 700 HPLC gradient system, including two model 1350 Soft-Start pumps, high pressure dynamic mixer, variable wavelength ultraviolet detection model 1305A, and an As-100 HPLC automatic sampling system. One hundred microliters of the ultrafiltrate was injected into a Riosil C18 HL column 250*4.6 mm, packed with 5-µm particles, in conjunction with an Octyl guard column, ODS-10, 30*46 mm (Micro-Guard Refill Cartridges, Bio-Rad Laboratories, Richmond, Va., USA) packed with 10-µm particles. The elution was done using 100% 0.05 M ammonium formate (PH = 4) to 60% methanol within 45 min and a flow rate of 0.9 ml/min, followed by a 45-min long column wash program to eliminate column contaminations. Loop volume was 100 µl with an overfill volume of 10 µl.

Quantification

HA standard solutions were added in concentrations from 10 to 400 µmol/l to ultrafiltrated serum samples from healthy students as well as from uremic patients. The resulting calibration standard

curve ($r = 0.99$, $P < 0.0001$) was used for further quantification procedures. Continuous registration of ultraviolet absorbance at a wavelength of 254 nm was a concentration-dependent measurement. The area integration curve at known rentention time was compared with data obtained from standard solution in order to quantify HA concentration.

Materials

For standardization, HA (benzoylaminoacetic acid), sodium salt, was obtained from Sigma Chemical (St. Louis, Mo., USA). Formic acid was delivered by Fluca Chemie (Buchs, Switzerland), as well as ammonium hydroxide in water and methanol for HPLC, $H_2O < 0.05\%$, Kp 65°.

Reproducibility

Running ten HPLC chromatograms with the same ultrafiltrated pooled serum sample with an average serum HA concentration of 10 µmol/l, we observed a coefficient of variation of 1.5%. For routine measurements, a test mixture was included after every 15 samples in order to obtain long-term analysis reproducibility.

Drug interference

Immunosuppressive therapy with azathioprine, prednisone, and cyclosporine A, cotrimoxazole for *Pneumocystis carinii* pneumonia prophylaxis, was excluded to influence the HPLC chromatogram and the HA quantification procedure. Adding these substances to normal and uremic serum samples before ultrafiltration at concentrations comparable to those observed in patients did not result in the observation of new peaks nor in a change of the retention time of the HA acid peak.

Determination of creatinine

Creatinine was measured kinetically using the Jaffe method (Vitalab Eclipse photometer, Merck; coefficient of variation 3.1%).

Data analysis and statistics

All data were expressed as mean ± SD. Confidence interval analysis was used for comparison [6]. A P value lower than 0.05 was considered significant.

Results

Of the 35 transplant recipients, 6 were diagnosed by transplant biopsy as suffering from acute cellular rejection and 6 were documented by radiography and/or ultrasound as exhibiting ureter obstruction. Another two patients investigated showed impaired excretory function with high serum creatinine but only slightly elevated HA concentrations. Rejection was accompanied by an early and marked rise in HA (Fig. 1). Antirejection therapy and surgery, respectively, were successful in all but one case.

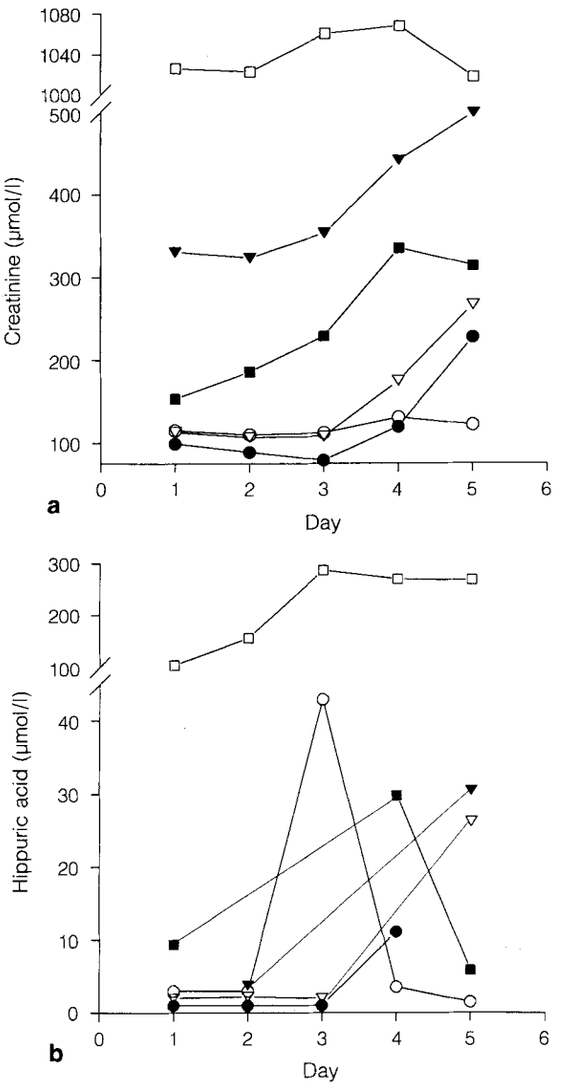

Fig. 2 **a** Creatinine and **b** hippuric acid in acute allograft rejection

In this particular case, the transplanted organ had to be removed because of necrosis. Comparison of serum HA and creatinine measurements revealed a rise in HA together with creatinine in cases of acute transplant rejection. Elevations were characterized as follows (Table 1, Fig. 2): mean creatinine increase 107.1 µmol/l, CI 31.3–183 µmol/l, $P < 0.05$; mean HA increase 39.9 µmol/l, CI 3.1–76.7 µmol/l, $P < 0.05$. In contrast, ureter obstruction was accompanied by a much lower increase in HA, which often occurred earlier than the increment in creatinine: mean increase in HA 1.6 µmol/ml, CI 1.4–4.6 µmol/ml, P = NS; mean increase in creatinine 65.3 µmol/ml, CI 24.4–106, $P < 0.05$. Decrements in serum HA concentrations preceded those of the creatinine concentration after successful treatment (Fig. 1).

Table 1 Serum concentration of hippuric acid and creatinine before and during allograft rejection and ureteric obstruction respectively. First measurement indicates baseline value. Second measurement indicates top value. Numbers in parenthesis indicate confidence interval. n.d, < 3 μmol/l (detection limit); for calculation assumed to be 1 μmol/l

Acute allograft rejection			Ureter obstruction		
Patient no.	Creatinine (μmol/l)	Hippuric acid (μmol/l)	Patient no.	Creatinine (μmol/l)	Hippuric acid (μmol/l)
1	110– 131	n.d– 43	7	80–124	n.d–n.d
2	108– 267	n.d– 26.3	8	137–176	n.d–9.2
3	79– 120	n.d– 11.2	9	126–186	n.d–n.d
4	324– 517	3.8– 30.7	10	152–237	n.d–n.d
5	1023–1069	156.5–272	11	99–121	n.d–n.d
6	153– 336	9.4– 29.9	12	129–269	5.8–5.8
Mean change (range)			Mean change (range)		
	107.1 (21–193)	39.9 (20.4–115.5)		65.3 (22–140)	1.6 (1–8.2)

In contrast in all six cases of ureter obstruction that were confirmed by radiography and/or ultrasound, serum HA did not change and was most often below the detection limit. Increases in serum creatinine were comparable to those observed during rejection. Successful antirejection treatment coincidenced with a decrease in HA, sometimes earlier than the decrease in serum creatinine. In one patient, the HA did not decrease during intensive antirejection treatment and the organ had to be removed due to necrosis (data not shown).

In this study, values for creatinine and HA before an increment were compared with data obtained on day 3 or 4 after an initial rise. During that time period, there were often no clinical signs of rejection, in spite of later confirmation by clinical signs and/or transplant biopsy.

Discussion

Frequent monitoring of serum HA and creatinine in 35 renal allograft recipients resulted in the identification of six rejection episodes and six ureter obstructions. Two patients suffered from ischemic renal failure postoperatively complicated by acute rejection. A significant increase in serum HA was observed during acute rejections in all of our patients, whereas the serum creatinine increased later. In contrast, ureter obstruction was accompanied by minor increments in serum HA. These findings indicate that HA seems to be an early and sensitive indicator of allograft rejection; it also discriminates from ureter obstruction. If serum creatinine and HA is already in the normal range, the extent of increases in HA is likely to be of great value in cases where single point measurements are taken at the time of suspected rejection and obstruction, respectively, thereby avoiding continuous monitoring.

Of special interest was the HA level in two cases of ischemic tubular necrosis. Superimposed rejection was accompanied by an early rise in HA (Fig. 1), whereas the serum creatinine concentration was stable and elevated.

Although small numbers of patients have been evaluated up until now, the consistency of our observation showing no false-positive or false-negative data seems promising. Allograft rejection has been associated with an increase in neopterin in serum and urine [7, 9]. Plasma and urine neopterin measurements have shown up to 64% false-positive results in transplanted patients with viral infections [9]. The acute phase reactant C-reactive protein has been said to detect early renal allograft rejection with a high sensitivity [5]. Some 4.7% false-positive and 11.1% false-negative results in 38 transplanted patients have been reported [5]. An increase in the number of macrophages in renal rejection has been used for diagnosis with fine needle biopsy [2]. Monoclonal antibody WT14, showing a consistent, intensive binding to interstitial macrophages, was used to differentiate between cyclosporin A nephrotoxicity and acute interstitial rejection [2]. HLA-DR expression from renal tubules cells coincidenced with the degree of renal rejection [1]. Morphometric analysis with antilymphocytic antibodies showed a close relationship between infiltration and graft function in the 279 human renal allograft biopsies tested [8]. The usefulness of all of these techniques is limited, however, because they involve invasive procedures or have a low level of accuracy.

The rapid decrease in elevated HA levels during acute cellular rejection in cases of successful therapy is another clinically useful finding that might eliminate prolonged, additional immunosupression in order to salvage allografts with irreversible or nonimmunological damage.

The reason why HA reflects transplant function is most probably due to its metabolism and renal excretion. Benzoic acid originates from the oxidative breakdown of phenylalanine by intestinal bacterias as well as from fruits, vegetables, and food preservation. B-oxidation of phenyl fatty acids is also said to produce benzoic acid. High serum HA concentrations are found after contact with toluene and xylene in the enviroment [3]. About 35% is protein-bound [10], accounting in part for the impaired protein binding of phenytoin and theophylline [11]. HA was found to inhibit the glucose utilization in human red blood cells, blood platelets, kidney cortex, and striated muscle [4]. In 1975, Richet et al. showed that HA interferes with para-aminohippuric acid and urate transport in the cortical renal tubules, suggesting that there is compe-

tition to transport various organic substances. High HA serum concentrations are found in acute and chronic renal failure. HA has a five times higher clearance than creatinine in patients with an impaired creatinine clearance below 15 ml/min [12], while the endogenous production of 0.14 mmol/l seems to be constant.

The interstitial cellular infiltration in cases of allograft rejection seems to be linked to impaired HA excretion by tubular secretion earlier and to a higher degree than creatinine excretion, thereby inducing the early elevation of serum HA concentration. There were no false-positive results, even in patients with viral infections like CMV or herpes simplex virus.

Early detection of acute rejection episodes is important in view of early therapeutic interventions. The diagnosis has to be correct in order to avoid unnecessary and dangerous treatments. HA seems to be a reliable marker for the early detection of acute renal allograft rejection and offers specific and valuable information. The preliminary observation of a low HA concentration associated with tubular necrosis, but of a rise in cases of complicating rejection, requires further study.

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