J. Clin. Chem. Clin. Biochem.
Vol. 25, 1987, pp. 71–76

Excretion of Urinary N-Acetyl-β-*D*-glucosaminidase Isoenzymes after Renal Transplantation in the Rat

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(Received December 20, 1985/September 25, 1986)

Summary: The urinary excretion of N-acetyl-β-*D*-glucosaminidase isoenzymes A and B following kidney transplantation was studied in rats. High enzymuria with permanent marked isoenzyme B excretion occurred from the immediate post-operative period to the irreversible rejection episode. Isoenzyme B could represent as much as 10–40% of total N-acetyl-β-*D*-glucosaminidase activity and it reflected the intensity of tubular lesions as observed by histological examination of allograft specimens. Thus, N-acetyl-β-*D*-glucosaminidase B isoenzyme determination may reinforce the diagnostic value of total (A + B) urinary N-acetyl-β-*D*-glucosaminidase activity determination during the various complications which can occur after transplantation.

Introduction

Measurement of the catalytic activity of urinary enzymes has attained clinical significance during recent years. Increased urinary N-acetyl-β-*D*-glucosaminidase activity has been well documented as an indication of renal tubular disorders in man and animal models (1, 2).

We investigated whether substitution of a differential measurement of urinary N-acetyl-β-*D*-glucosaminidase isoenzymes, instead of the commonly used total enzyme activity, could improve early monitoring of the human renal transplant function (3). For this purpose, an original method of measurement was used for monitoring the B isoenzyme excretion in addition to the N-acetyl-β-*D*-glucosaminidase (A + B) total enzymuria (4).

Urinary N-acetyl-β-*D*-glucosaminidase activity has been widely used in rat models to investigate the degree of renal lesions in many pathological processes (5–7). We decided therefore to choose the rat renal transplantation model in order to correlate the excretion of urinary N-acetyl-β-*D*-glucosaminidase isoenzymes A and B with the graft functional recovery.

Urinary enzymes originate from the kidney. N-Acetyl-β-*D*-glucosaminidase A and B isoenzymes are present in roughly equivalent amounts in human renal cortex (8), but isoenzyme B is present in smaller relative proportion in rat renal cortex (9). In the absence of typical electrophoresis isoenzymatic profiles for rat renal N-acetyl-β-*D*-glucosaminidases (10), a chromatographic method is required to monitor their urinary excretion.

The present results contribute to the interpretation of enzymuria data in human transplantation. The specific measurements of N-acetyl-β-*D*-glucosaminidase isoenzyme B activity indicates the intensity of proximal tubular lesions during the various complications that can occur after transplantation.

Materials and Methods

Animals and collection of urine samples

Renal transplantations were carried out according to the method of *Lee* (11). Male adult rats of 200–350 g body weight were used. Experiments were performed with 19 allograft recipients; 11 August-Lewis, 6 Sprague Dawley-Lewis and 2 Wistar-Wistar.

Four non-transplanted animals, 2 Wistar and 2 Lewis, were used as controls for 3 to 9 consecutive days.

The rats were housed individually in wire mesh metabolism cages. Water and food were available ad libitum. Immediate efficient diuresis occurred during a period of 5–13 days after surgery and before the irreversible rejection episode. The urine was collected in a fraction collector from the day of transplantation until discharge. The collecting vessels contained 0.1 ml of 2 g/l sodium azide. The 6-hour collections of rat urine were centrifuged at 3000 min^{-1} for 10 min and the supernatants tested after freezing and storage at −20 °C.

The collection of urine began always at 1100 hours for control rats, and transplanted rats were housed in metabolism cages after surgery between 1100 and 1300 hours (day J = 0).

Only 4 August-Lewis and 3 Sprague Dawley-Lewis allografts could be analysed due to technical failure in the others. The diagnosis of acute rejection was principally based on the observation of graft enlargement, and renal biopsy for 4 anuric sacrificed rats.

No irreversible rejection episode occurred during a three week observation period following functional recovery of the graft in a Wistar-Wistar allograft recipient.

Assay of N-acetyl-β-*D*-glucosaminidase activity

Urine enzyme catalytic activity was measured by minor modification of a method previously described (4). Urine samples (0.1 ml), 10 or 20-fold diluted, were incubated with 0.5 ml of 2.5 mmol 4-methylumbelliferyl-β-*D*-N-acetylglucosaminide (Sigma).

Enzyme activity is expressed as nanomoles of 4-methylumbelliferone released per hour per mg of urinary creatinine. In addition, results were also expressed in nmol · h^{-1} per 6-hour collections of rat urine.

Separation of N-acetyl-β-*D*-glucosaminidase isoenzymes by chromatography on DEAE Trisacryl M columns

Isoenzymes were separated by ion exchange chromatography on DEAE Trisacryl M (I. B. F.) equilibrated with sodium phosphate buffer (0.01 mol/l, pH 7) which retains isoenzyme A (4).

Urine samples (0.5 ml) from the acrophase of the circadian rhythm in urinary activity were added to 2 ml of phosphate buffer and placed on a 1 ml DEAE Trisacryl M column. Isoenzyme B is completely excluded by the exchanger and isoenzyme A is eluted with 0.25 mol/l NaCl.

N-Acetyl-β-*D*-glucosaminidase activity was determined on each eluted fraction (2 ml) with 0.25 ml or 0.1 ml aliquots of eluate containing B and A isoenzyme activities. Aliquots were incubated for 30 min (isoenzyme B activity) and 15 min (isoenzyme A activity) at 37 °C in an incubation medium containing 2 mmol 4-methylumbelliferyl-β-*D*-N-acetylglucosaminide. The relative activity of each isoenzyme fraction was derived by calculating the corresponding individual areas, results being expressed as fractions of the total area.

All three assays for total, A and B N-acetyl-β-*D*-glucosaminidase isoenzymes are combined in table 1.

Tab. 1. Determination of total N-acetyl-β-*D*-glucosaminidase activity and relative activity of each urinary isoenzyme.

Total N-acetyl-β-*D*-glucosaminidase activity	Urine samples (6-hour collections) 10 or 20-fold diluted
Chromatography on DEAE Trisacryl M of equilibrated urine samples	
Isoenzyme B	activity excluded by the exchanger
Isoenzyme A	activity retained by the exchanger and eluted with 0.25 mol/l NaCl

Statistical analyses were carried out using the nonparametric *Mann-Whitney* tests.

Results

For the four non-transplanted animals used as controls, N-acetyl-β-*D*-glucosaminidase activity varied considerably during a single 24-hour cycle and from one day to another. Excretion of enzyme was elevated during the first days following housing in the metabolism cage (early stage), then excretion of urinary N-acetyl-β-*D*-glucosaminidase fell and remained low (late stage). For the four non-treated controls, mean values (nmol · h^{-1} per 6-hour collections) between early stage (3–5 first days) and late stage (> 5 days) were statistically significant; 809 ± 1030 (n = 37) and 37 ± 70 (n = 27), $p < 0.001$. Figure 1 provides an example of daily N-acetyl-β-*D*-glucosaminidase fluctuations in a Lewis control rat. This graphical representation shows that N-acetyl-β-*D*-glucosaminidase activity fluctuated similarly when it is expressed as the catalytic activity per milligram of creatinine in an aliquot of 6-hour collections. In spite of a low baseline, the late stage shows the same daily fluctuations as early stage.

N-acetyl-β-*D*-glucosaminidase isoenzyme profiles determined on day 3 and 9 showed similar results. In spite of the different enzymuria of the two urine samples, no significant differences in the amplitude of peak activity corresponding to isoenzyme B was observed. The major from excreted was isoenzyme A, whereas isoenzyme B accounted for at most 5% of the total urinary activity (fig. 1; *insets* I and II).

This study demonstrates, despite marked individual variations in enzymuria, a similar pattern in renal transplant rats with a functioning allograft.

Efficient diuresis was observed on the 24-hour postoperative period, and polyuria occurred during the first days following transplantation. Diuresis was then sufficient for enzyme tests until the irreversible rejection episode.

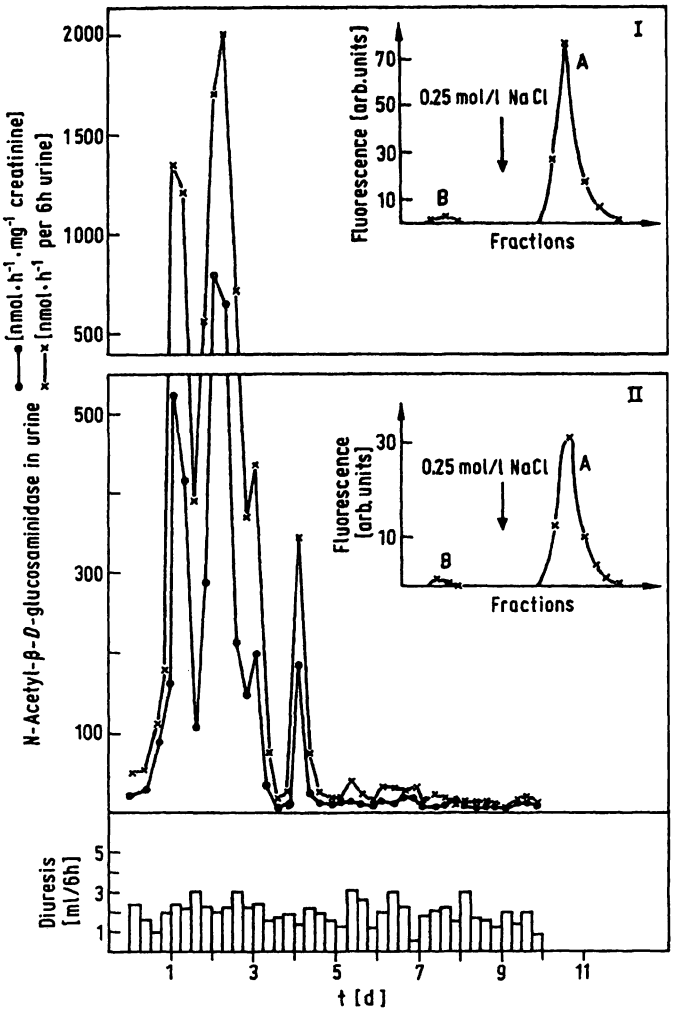

Fig. 1. Urinary N-acetyl-β-*D*-glucosaminidase activity during 10 consecutive days in Lewis rat control.
The collection of urine began at 1100 hours (day J = 0). N-Acetyl-β-*D*-glucosaminidase activity is expressed as nmol · h^{-1} per mg of creatinine (●—●), and as nmol · h^{-1} per 6-hour urine collections (x—x).
N-Acetyl-β-*D*-glucosaminidase isoenzymes A and B were separated by DEAE Trisacryl M chromatography according to conditions described in the text from urine samples on day 3 (inset I) and 9 (inset II).

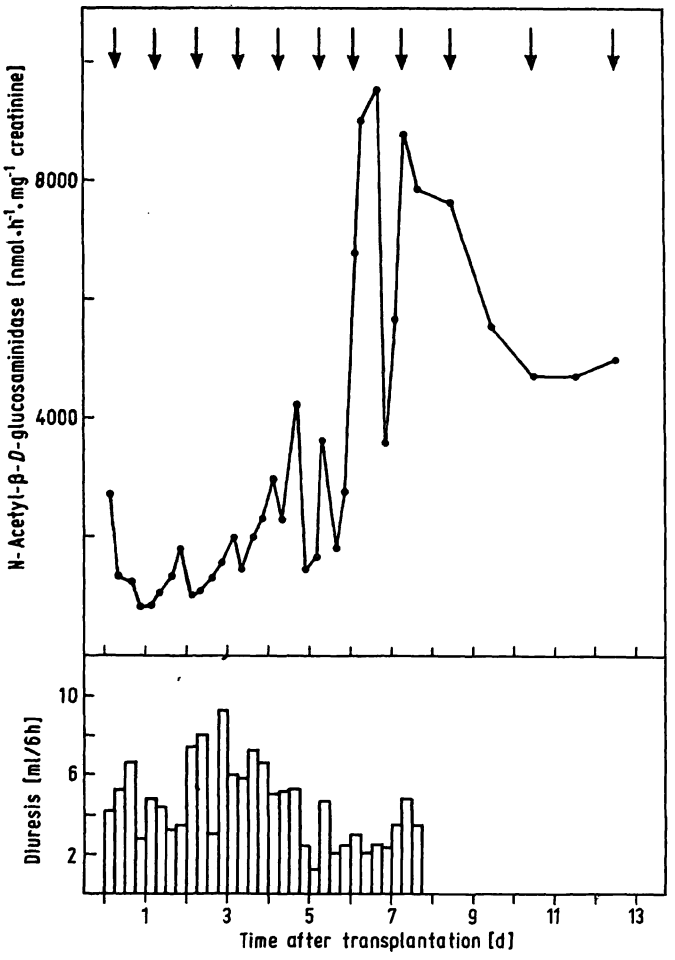

Fig. 2. Urinary N-acetyl-β-*D*-glucosaminidase activity in a Sprague Dawley-Lewis allograft during the 13 days before irreversible rejection episode.
Immediate diuresis occurred after surgery and collection of urine began at 12 hours (day J = 0). N-Acetyl-β-*D*-glucosaminidase activity is expressed as nmol · h^{-1} per mg of creatinine (●—●) for 6-hour urine collections during the 8 days following transplantation and subsequent 24-hour urines.
Arrows indicate urinary samples used for isoenzymatic profiles by chromatography on DEAE Trisacryl M.

The values of the urinary output of N-acetyl-β-*D*-glucosaminidase in renal transplant rats with functioning allograft are shown in table 2.

Figure 2 provides an example of N-acetyl-β-*D*-glucosaminidase excretion in a representative case of Sprague Dawley-Lewis allograft. This rat died on day 14 following transplantation, so a large number of experimental data were available. Excretion rates expressed as nmol · h^{-1} per 6-hour collections remained very high without significant day-to-day variations (tab. 2) from the post-operative period to the irreversible rejection episode (2838 ± 694, n = 31). On the other hand, N-acetyl-β-*D*-glucosaminidase activity, expressed per milligram of creatinine, persistently increased during the follow-up after transplantation (fig. 2). N-Acetyl-β-*D*-glucosaminidase activity varied considerably during a single 24-hour cycle as observed in controls.

The main feature of this enzymuria was permanent marked isoenzyme B excretion. Isoenzyme profiles were determined in samples indicated by an arrow in figure 2, and isoenzyme B represented as much as 10—40% of the enzymuria (fig. 3 b).

A positive correlation (r = 0.75, n = 10) between A and B isoenzyme activities was observed (fig. 3 a).

Histologic analysis showed mixed features of tubular lesions and rejection. There was no changes of glomeruli and vessels. Interstitial tissue changes consisted of oedema and inflammatory cells in the infiltrates.

Tab. 2. Urinary output of N-acetyl-β-*D*-glucosaminidase in renal transplant rats with functioning allograft.
N-Acetyl-β-*D*-glucosaminidase activity is expressed as nmol · h^{-1} for 6-hour urine collections during the 8 days following transplantation and subsequent 24-hour urines.
– no diuresis
NS not significant: enzyme activity of the urine sample did not account for > 10 nmol · h^{-1} per ml.
A/L August-Lewis allograft, SD/L Sprague Dawley-Lewis allograft, W/W Wistar-Wistar allograft.
SD/L* Sprague Dawley-Lewis allograft corresponding to fig. 2.

Days after transplantation	Renal transplant rats							
	A/L	A/L	A/L	A/L	SD/L	SD/L*	SD/L	W/W
J = 0	–	–	–	–	4073	2730	4133	382
	–	–	948	4120	4920	2862	3485	1165
	–	1821	297	1690	4160	2114	3694	486
	–	955	150	2640	3680	728	3194	712
J = 1	576	836	NS	2880	3510	1104	2556	406
	375	840	NS	5280	6624	1936	4649	616
	495	805	–	1660	3822	2145	3261	260
	390	667	NS	3460	1924	2625	2755	48
J = 2	158	–	NS	5270	3000	3108	941	756
	1124	876	NS	5740	4680	5103	477	598
	1029	1090	NS	4320	4092	1800	347	NS
	382	–	1100	7200	2706	5612	114	NS
J = 3	378	–	227	6630	3782	4800	–	118
	610	1560	144	3300	4560	5452	564	20
	543	–	552	4610	3420	5329	1012	21
	1128	765	1190	5100	10045	4224	676	–
J = 4	1205	550	NS	3770	3100	3213	245	107
	881	–	NS	3510	1456	4368	133	28
	351		30	1920	1056	3869	–	29
	898		225	–	648	2064		NS
J = 5	1604		NS	3890	608	1140		NS
	1281		–	2660	286	2726		–
	205		94	3220	1862	2184		–
	578		462	2800	1742	3216		753
J = 6	894		282	2040	384	4920		1534
	1149		–	1500	624	3128		74
	2801		165	–	816	3600		71
	4752		1904		1360	1488		46
J = 7	1584		–		1452	3010		1015
	–		–		3552	4224		101
			190		1764	3570		NS
			–		1550	3600		42
J = 8			595		3648			29
			–		3618			65
			–		3950	11600		24
					2464			54
J = 9					–	10920		
J = 10						7440		
J = 11						8680		
J = 12						14800		

The most prominent morphological damage affected tubular cells with flattening of tubular epithelium and loss of brush border and lumen enlargement but without cast.

Mean excretion levels of N-acetyl-β-*D*-glucosaminidase activity (nmol · h^{-1} per 6-hour collections) for the 7 transplantation cases in which efficient diuresis occurred (tab. 2), are significantly different from the early stage of rat controls:

2821 ± 2332 (n = 147) versus 809 ± 1030 (n = 37), $p < 0.001$. On the other hand, correlation between A and B isoenzyme activities for the 7 allografts cases varied from r = 0.59 to r = 0.87, and histologic analysis showed predominant tubular lesions.

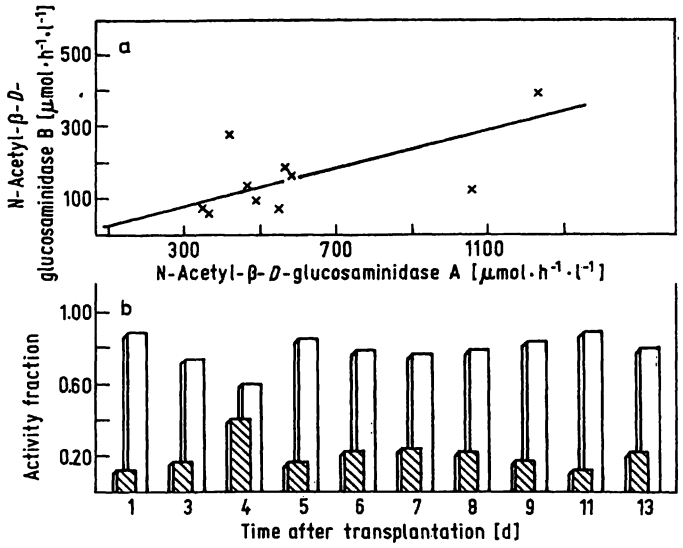

Fig. 3. Urinary N-acetyl-β-*D*-glucosaminidase isoenzyme excretion following a Sprague Dawley-Lewis allograft.
N-Acetyl-β-*D*-glucosaminidase isoenzymes (A, white columns; B, hatched columns) were separated from urine samples (indicated by arrow on fig. 2) by DEAE Trisacryl M chromatography according to conditions described in the text.
a) correlation between isoenzyme A and B activities ($r = 0.75$, $n = 10$).
b) Isoenzyme activities expressed as fraction of the total N-acetyl-β-*D*-glucosaminidase activity.

No irreversible rejection episode occurred during a three week observation period for a Wistar-Wistar allograft (tab. 2). In this case, no statistically significant difference occurred compared with the early stage of rat controls; 361 ± 419 ($n = 26$) versus 809 ± 1030 ($n = 37$) nmol · h^{-1}, $p < 0.07$.

Significant isoenzyme B excretion occurred only during the immediate post-operative period and on the 6th day following transplantation. Isoenzyme B activities in the other urine samples only accounted for a small proportion of enzymuria as among rat controls, and it was not possible to establish any positive correlation between A and B isoenzyme activities.

Discussion

This study documents a circadian rhythm in the urinary enzymuria in control and experimental animals, similar to that reported in clinically healthy human subjects (12, 13). On the other hand, *Grötsch* et al. described biorhythms in rats (14). This group reported a biorhythm with a dominant period of 7 days for urinary N-acetyl-β-*D*-glucosaminidase. Additionally, a marked decrease of the enzyme excretion after the early stage of urine sampling has not been reported. A possible explanation for these different findings is that data from this group are from long term experiments, and rats show good toleration of single-cage housing. Moreover, baselines of urinary excretion of N-acetyl-β-*D*-glucosaminidase are not comparable; *Grötsch* et al. employed the 4-nitrophenyl derivative of the monosaccharide as opposed to the methylumbelliferyl derivative employed here.

In control rats, enzymuria is constituted almost exclusively by the isoenzyme A. Although high N-acetyl-β-*D*-glucosaminidase activity in urine was observed during the first days following the housing of control rats in metabolism cage, it was not accompanied by a marked excretion of isoenzyme B. This finding suggests that a physiological process (stress) could lead to an increase in isoenzyme A excretion with low amounts of isoenzyme B. It has been pointed out that urinary lysosomal enzyme excretion in mice could be extensively modulated by both hormonal and genetic manipulations (15, 16). This hypothesis would therefore explain the high level in N-acetyl-β-D-glucosaminidase activity during the early stage, but we cannot offer any other evidence to support it.

As compared with control rats, high enzymuria with permanent marked isoenzyme B excretion occurred from the immediate post-operative period to the irreversible rejection episode.

Our study confirms the assumption (17) that the enzyme excretion reported in relation to creatinine offers the best diagnostic results.

The high level of isoenzyme B excretion following transplantation reflects the intensity of tubular lesions observed by histological examination of allograft specimens.

Present data confirm our previous clinical and experimental investigations (3, 10, 18). A positive correlation between isoenzyme A and B activities was observed. Thus a specific measurement of isoenzyme B activity did not seem to provide major information for the early diagnosis of rejection episodes after transplantation, if total N-acetyl-β-*D*-glucosaminidase activity is also regularly monitored. However, an increase in urinary excretion of isoenzyme B may reflect an elective tubular disorder and reinforce the diagnostic value of urinary N-acetyl-β-*D*-glucosaminidase catalytic activity during the various complications which can occur after transplantation.

In addition, although excretion of N-acetyl-β-*D*-glucosaminidase appears to be a sensitive indicator of renal cell integrity, the wide variability of normal values and biorhythms in rats make it difficult to discriminate between normal and pathological conditions of the kidney. The results presented clearly demonstrate that for pharmacological and toxicologi-

cal monitoring isoenzyme B excretion can be of greater diagnostic value than measurement of total urinary activity of N-acetyl-β-*D*-glucosaminidase.

Acknowledgement

We thank Dr. *H. Beaufils* for histological examination of allograft specimens.

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