Diagnostic Significance of Some Urinary Enzymes for Detecting Acute Rejection Crises in Renal-Transplant Recipients: Alanine Aminopeptidase, Alkaline Phosphatase, γ-Glutamyltransferase, N-Acetyl-β-D-glucosaminidase, and Lysozyme

Klaus Jung,1 Jesus Diego,1 Volker Strobel,2 Dietmar Scholz,1 and Gottfried Schreiber3

We compared the diagnostic validity of five urinary enzymes—alanine aminopeptidase (EC 3.4.11.2), alkaline phosphatase (EC 3.1.3.1), γ-glutamyltransferase (EC 2.3.2.2), N-acetyl-β-D-glucosaminidase (EC 3.2.1.30), and lysozyme (EC 3.2.1.17)—as indicators of acute rejection crises in renal-transplant recipients. In 82 patients (group A), the excretion of each of these five enzymes was measured daily from transplantation until discharge from hospital. In another 69 patients (group B), enzyme determinations were made when the patient came for regular checkups (about every four to eight weeks). We used an “activity ratio” (the activity measured at a particular time compared with the activity on the preceding determination) value of 1.5 as the decision point. In group A, use of this discrimination point for alanine aminopeptidase, γ-glutamyltransferase, and N-acetyl-β-D-glucosaminidase yielded a specificity and sensitivity of about 90%. In group B, only alanine aminopeptidase had a greater diagnostic sensitivity than creatinine alone. Evidently, measurement of alanine aminopeptidase can be a helpful indicator of acute rejection crises, when interpreted in combination with other available relevant clinical, biochemical, and immunological data.

Currently there is no consensus as to the superiority of any of several enzymes measured in urine as an indicator of acute rejection crises in renal-transplant recipients (1–6). Numerous methodological as well as clinical factors account for these contradictory views (7): general uncertainty in detecting rejection crises, use of different criteria for detecting rejection crises, insufficient number of patients, comparison of results from noncomparable groups of patients, determination of different enzymes, determination of identical enzymes by different methods, selection of different discrimination points for denoting a significant increase of enzyme excretion, and insufficient consideration of diagnostic criteria (e.g., sensitivity, specificity, efficiency) in evaluating the diagnostic significance of enzyme excretion. The most important differences affecting these factors have been variations in urinary enzyme excretion affected by nonrenal sources and by changes in renal blood flow during imminent rejection (1, 2). We previously demonstrated the method of “activity ratios” (7), which takes these factors into account as much as possible and minimizes their effects on measurements of urinary enzymes. Using our previous recommendations, we compare the diagnostic significance of five enzymes often used as indicators of acute rejection crises in renal transplant recipients. Having a uniform standard for comparing changes in the activities of urinary enzyme measurements, we applied more general diagnostic criteria (e.g., sensitivity, specificity) to resolve contradictory views on their diagnostic utility. Here we report our investigations with alanine aminopeptidase (AAP), alkaline phosphatase (AP), γ-glutamyltransferase (GGT), N-acetyl-β-D-glucosaminidase (NAG), and lysozyme (LYS).

Materials, Methods, and Patients

Apparatus. We used photometer PCP 6121 and analyzer ACP 5040, and pipettes (all from Eppendorf Gerätebau, Netheler & Hinz, Hamburg, F.R.G.), a refrigerated centrifuge (Model K 28; VEB Zentrifugenbau, Leipzig, D.D.R.), and 25 cm × 1 cm (i.d.) chromatographic columns.

Reagents: 4-Nitrophenyl phosphate and Tris were obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G.; alanine-4-nitroanilide and diethanolamine (distilled before use) from E. Merck GmbH, Darmstadt, F.R.G.; Sephadex G50 (medium) from Pharmacia Fine Chemicals AB, Uppsala, Sweden; 4-nitrophenyl-N-acetyl-β-D-glucosaminide and the test combinations for GGT activity from Lachema, Brno, Czechoslovakia; and Micrococcus luteus from

1 Department of Experimental Organ Transplantation, University Hospital Charité, Humboldt University Berlin, Leninallee 49, DDR-1017 Berlin, D.D.R.
2 Kidney Transplantation Center, and 3 Central Laboratory at the City Hospital Friedrichshain, Leninallee 49, DDR-1017 Berlin, D.D.R.

Received February 3, 1986; accepted June 5, 1986.
Ferak, West Berlin. Other chemicals, obtained from various suppliers, were of analytical grade.

Procedures: We measured AAP (8), AP (9), GGT (10), and NAG (11) activities as described previously (12). The reaction mixtures for the methods used contained, per liter, 2 mmol of alanine-4-nitroanilide and 50 mmol of Tris HCl (pH 7.80) for AAP; 10 mmol of 4-nitrophenyl phosphate, 0.5 mmol of MgCl₂, and 1 mol of diethanolamine (pH 9.80) for AP; 4 mmol of γ-glutamyl-4-nitroanilide, 101 mmol of NaCl, and 101 mmol of glycylglycine (pH 8.20) for GGT; and 2 mmol of 4-nitrophenyl-N-acetyl-β-D-glucosaminide and 100 mmol of citrate buffer (pH 4.40) for NAG. We determined lysozyme by an improved turbidimetric method, monitoring the decrease in absorbance at 365 nm during 90 s (13); final concentrations in this reaction mixture were, per liter, 70 mmol of phosphate buffer (pH 6.2) and 250 mg of Micrococcus luteus. Creatinine was measured by a kinetic procedure (14).

Patients and samples: We investigated two groups of recipients of cadaver renal allografts.

Group A (early period after transplantation) comprised 82 recipients (24 women, 58 men; mean age 37.4 years, range 11 to 54 years) studied after transplantation until their discharge from hospital. Forty of these recipients did not show any rejection crises (average stay in hospital, 31 days); 42 recipients had altogether 47 rejection crises: three patients had two rejections each; and one patient had three rejections. The mean stay in hospital of these 42 patients was 46 days. Kidney transplants of five patients had been removed in consequence of irreversible rejections.

On weekdays, urinary enzyme excretion was determined daily in urines collected between 05.00 and 09.00 hours. On weekends, the samples were stored at 4 °C and analyzed on the subsequent Mondays.

Rejection episodes were diagnosed by clinicians on the basis of an increase of serum creatinine, diminished sodium concentration in urine, the results of immunological monitoring, and clinical signs of rejection (fever, diminished urine volume, increase of graft size, hypertension). Patients with other causes of renal damage (e.g., medication with nephrotoxic drugs) were excluded from this study.

Immunosuppression treatment consisted of azathioprine, 2–4 mg/kg of body weight daily, and prednisolone, 4 mg/kg on the day of transplantation, 3 mg/kg on the first day after transplantation, 2 mg/kg on the second, and 1.9 mg/kg on the third. Between the fourth and the 12th days prednisolone was decreased by 0.1 mg/kg daily; from the 13th day onward it was decreased biweekly by 5 mg, until the daily dose was about 15 mg.

Group B comprised 69 patients (30 women, 39 men; mean age 30.3 years, range six to 54 years) studied in the late period after transplantation (mean time after transplantation 42.1 months, range nine to 140 months). We selected these patients because in this group we changed treatment, administering prednisolone only every other day (15 to 25 mg). Enzyme determinations were made when the patient came to the outpatient department for regular checkups (about every four to eight weeks). Before the treatment was changed, we measured the urinary enzyme excretion two or three times. In all, we made 951 determinations for patients in this group. In the eight patients who experienced rejection crises we discontinued the alternate-day treatment with prednisolone.

Calculations: Results for AAP, AP, GGT, and NAG were expressed in units (U = μmol × min⁻¹) of enzyme activity excreted per gram of urinary creatinine (7). Values for LYS were calculated as milligrams per gram of urinary creatinine. We calculated the "activity ratio for every day" by comparing the activity measured on a particular day to the activity measured on the preceding day. In group A, an activity ratio above or below a certain value was defined as a truly positive or a falsely negative test result, respectively, if it was found in patients with rejection episodes during the period between the day that rejection was diagnosed and the four days preceding this diagnosis. In group B, the corresponding test result was defined by considering the activity measured on the day of the diagnosis of rejection and the activity measured during the latest checkup before rejection. A ratio value below or above a certain discrimination point determined in patients without rejections was considered as a truly negative or falsely positive test result, respectively.

Results

Figure 1 shows the behavior of the enzymes AAP, GGT, and NAG during the four days before the rejection episode. A tendency toward increased excretion of these enzymes was apparent, but excretions of AP and LYS (not presented) showed no marked change. As already stated in a previous paper (12), these excretion rates varied greatly among individuals. For example, on the day of rejection the coefficients of variation of activity per gram of creatinine excreted were between 69% (for AAP, the smallest CV) and 122% (for AP, the largest).

The extent of the changed enzymuria during a rejection period was not related to the outcome of the rejection. We could not observe differences both in the rate of the enzyme excretions and the calculated activity ratios between patients who had reversible or irreversible rejection crises. However, the changed enzymuria during a rejection period depended somewhat on the functioning of the allograft preceding the rejection crisis. We drew this conclusion by comparing the enzymuria in patients with similar course of reversible rejection crises (without the removal of allograft), but differing serum creatinine values (Figure 2). In patients with serum creatinine <800 μmol/L (Figure 2A), the increase in enzymuria was less pronounced than in patients with creatinine >800 μmol/L (Figure 2B).

This general behavior of these five enzymes during rejec-

![Graph](image-url)
tion episodes is not sufficiently related to specific outcome to permit assessment of the diagnostic power of enzyme measurements for the detection of acute rejection crises.

Because the interindividual excretion rates varied so greatly, we applied the "activity ratio" procedure (7) to evaluate the diagnostic validity of the activities of the enzymes. In this procedure, the baseline value for every patient is used as a decision criterion to compare the activity ratio measured at a particular time with the activity determined at the preceding measurement. In group A, i.e., renal-transplant recipients during their stay in hospital after transplantation, daily values were available for our study. In group B, the preceding value was about four to eight weeks older than the current excretion rate determined.

In group A we evaluated the influence of the discrimination point of the activity ratio on the diagnostic criteria of sensitivity and specificity, calculating their values at different decision points. Figure 3, representing the results for the activity ratios 1.2, 1.5, and 1.7, clearly demonstrates that sensitivity and specificity depended on the discrimination point. Sensitivity considerably declined when a ratio greater than 1.5 (intermediate points of lines) was selected. Therefore, we chose the activity ratio of 1.5 as a decision criterion. Under such conditions, AAP, GGT, and NAG were the three most efficient enzymes in group A. This discrimination point guarantees a balance between sensitivity and specificity, which is a sign of efficiency not essentially influenced by prevalence.

The mean value for the highest activity ratios of AAP, the most sensitive enzyme, was 2.61 (SD 0.63) during the four days preceding the rejection, but only 1.96 (SD 0.75) on the day of the clinical diagnosis of rejection (p <0.001). This underlines the fact that increased AAP excretion precedes the clinical diagnosis of rejection; in our study, this "forewarning" period averaged 1.9 days.

To improve sensitivity and specificity, we combined the three most useful measures: AAP, GGT, and NAG (Table 1). For group A, the occurrence of a simultaneous increase in two of these enzymes improved the specificity but decreased the sensitivity, in comparison with an increase in only one enzyme.

In contrast to these favorable results in group A we obtained markedly worse sensitivity and specificity for all the investigated enzymes in group B by using activity ratios and combinations of two enzymes (Table 1). Only the sensitivity of AAP exceeded the sensitivity of serum creatinine in detecting allograft rejection. When we used the combination of AAP and serum creatinine, and used as a decision value an increase in both of 50% over the preceding values, the specificity increased.

---

**Table 1. Diagnostic Sensitivity and Specificity of Urinary Excretion of AAP, GGT, and NAG in Detecting Acute Rejection Crises in Two Groups of Renal Transplant Recipients**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Group A</th>
<th>Group B</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP</td>
<td>0.93</td>
<td>0.75</td>
<td>0.88</td>
<td>0.60</td>
</tr>
<tr>
<td>GGT</td>
<td>0.86</td>
<td>0.37</td>
<td>0.90</td>
<td>0.76</td>
</tr>
<tr>
<td>NAG</td>
<td>0.82</td>
<td>0.50</td>
<td>0.86</td>
<td>0.32</td>
</tr>
<tr>
<td>Serum C</td>
<td>0.62</td>
<td>0.62</td>
<td>0.73</td>
<td>0.84</td>
</tr>
</tbody>
</table>

**Two-analyte combination, with either one positive**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Group A</th>
<th>Group B</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP + NAG</td>
<td>0.94</td>
<td>0.75</td>
<td>0.75</td>
<td>0.16</td>
</tr>
<tr>
<td>AAP + GGT</td>
<td>0.94</td>
<td>0.75</td>
<td>0.77</td>
<td>0.51</td>
</tr>
<tr>
<td>NAG + GGT</td>
<td>0.90</td>
<td>0.63</td>
<td>0.84</td>
<td>0.37</td>
</tr>
<tr>
<td>AAP + serum C</td>
<td>0.94</td>
<td>0.75</td>
<td>0.70</td>
<td>0.56</td>
</tr>
</tbody>
</table>

**Two-analyte combination, with both positive**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Group A</th>
<th>Group B</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP + NAG</td>
<td>0.78</td>
<td>0.50</td>
<td>0.96</td>
<td>0.88</td>
</tr>
<tr>
<td>AAP + GGT</td>
<td>0.87</td>
<td>0.38</td>
<td>0.93</td>
<td>0.84</td>
</tr>
<tr>
<td>NAG + GGT</td>
<td>0.72</td>
<td>0.30</td>
<td>0.98</td>
<td>0.54</td>
</tr>
<tr>
<td>AAP + serum C</td>
<td>0.57</td>
<td>0.62</td>
<td>0.96</td>
<td>0.98</td>
</tr>
</tbody>
</table>

*The test result was interpreted as positive when the enzyme activity ratio exceeded 1.5 (see Calculations) and the creatinine (C) in serum increased by 44 µmol/L.*
Discussion

The enzymes AAP, AP, and GGT are located in the brush-border membranes of the proximal renal tubule, whereas NAG is found in lysosomes of this part of the nephron. The activities of these enzymes in urine increase when the tubular cells are damaged, as in the transplant rejection, and thus release the enzymes into the ultrafiltrate. Injury to the tubular cells reduces the reabsorptive-digestive capacity (i.e., the catabolism of low-molecular-mass proteins such as lysozyme) of the tubules and causes increased urinary excretion of lysozyme. Thus the increased excretion rates of the components investigated here are caused by different pathobiochemical mechanisms and reflect general cell damage. For this reason, measurements of all urinary enzymes have only a limited specificity for detecting acute rejection crises. Causes of renal damage other than rejection (especially by medication with certain antibiotics), urinary infections, urinary leaks, and enzymes deriving from sources outside of the kidney (e.g., serum enzymes in case of proteinuria; enzymes from erythrocytes and leukocytes) also have to be considered when an increased enzyme output is measured.

Apart from the pathobiochemical background, we compared the diagnostic power of these five enzymes and arrived at the following conclusions:

- The urinary enzymes AAP, GGT, and NAG can reliably be used to detect acute rejection crises in the early post-transplantation period via calculation of their activity ratios.
- The diagnostic sensitivity and specificity of all enzymes depend especially on the selected discrimination point of the activity ratio. We found that an activity ratio of 1.5 gave a good balance between the two diagnostic criteria.
- Combining the AAP, GGT, and NAG measurements improves their diagnostic power.
- For renal-transplant recipients who attend the outpatient department for regular checkups, measurement of AAP, but not of the other enzymes, can help evaluate the state of their transplants. This decreased sensitivity in long-term follow-up in comparison to the acute period after transplantation may be caused by the long interval between measurements for two checkups, because urinary enzyme excretion by renal-transplant recipients depends on the interval after transplantation (12).

All the enzymes mentioned in this paper have already been applied as indicators of acute rejection crises in renal-transplant recipients (2). However, different groups have reported discordant results on the usefulness of urinary enzyme measurements for this purpose (3–6). In a previous publication we compiled numerous factors that could be responsible for these contradictory findings (7). To settle this issue, we suggested comparative determinations that would take into account these methodological and clinical variations. Using the example of AAP, we demonstrated that the expression of enzyme excretion rates in terms of activity per amount of urinary creatinine (U/g creatinine) was better suited than the volume activity or time-related output for the diagnosis of rejection crises (7). Moreover, there have been few comparisons in terms of diagnostic sensitivity, specificity, and efficiency (1, 2).

According to published data (1), NAG and AAP presumably are the most valuable enzymes for detecting acute rejection crises. The present findings and a recent report (15) published after we analyzed our data clearly support this assumption. After our 1973 recommendation for the determination of AAP as an indicator of acute rejection crises (16), this enzyme was especially monitored in German laboratories (17, 18). All reports about AAP so far have underlined its clinical reliability. In 1977 Horpacey et al. (17) stressed the high sensitivity of AAP in comparison with other enzymes; however, they gave no further details regarding the criteria of evaluation.

The use of NAG as the diagnostic tool for monitoring acute rejection crises was introduced in 1970 (19). Both reliable and insufficient results regarding this enzyme have been published so far (3–6, 15), but in general, favorable reports predominate (1). Separation of NAG into its isoenzymes forms A, B, and M may further improve the diagnostic power of measurements of this enzyme (20). Measurements of only the major enzyme forms A and B, however, supplied no additional information over that obtained from measuring total NAG activity (21).

Our data show that AAP and NAG both meet the requirements as reliable indicators of acute rejection crises in the early period after transplantation. However, we prefer to measure AAP, not only because of its higher sensitivity and specificity, but also because of its easier assay. In 1980 we introduced an optimized kinetic assay of AAP (8) by which urinary AAP activity can be rapidly and precisely measured with automated analyzers. This procedure is more advantageous, methodologically, than the colorimetric determination of NAG, which is not automatable. This fact should especially be kept in mind for smaller laboratories that do not have a fluorimeter required for the preferred fluorimetric method of NAG determination (9).

In contrast to AAP and NAG, the urinary excretion of AP, GGT, and LYS have been monitored more rarely (2, 6). Our data reveal, in contrast to the findings of other authors (6), that GGT seems to be an appropriate analyte for detecting acute rejection crises, whereas AP and LYS are not.

In conclusion, certain urinary enzymes are reliable markers for detecting acute rejection crises in renal-transplant patients. In particular, the daily measurement of AAP in the early post-transplantation period can provide helpful information in the management of renal-transplant recipients. Other urinary enzymes also offer promising results, especially in combination with other available data on the excretory function of a transplant, such as creatinine clearance values (22). In no case, however, do we intend to replace other biochemical data (e.g., serum creatinine and urinary sodium concentration) by measurements of urinary enzymes, and recommend using all available chemical, biochemical, and immunological data when making the diagnosis of a rejection.

References


