Indicators of Acute Renal-Transplant Rejection in Patients Treated with Cyclosporine

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We evaluated the ability of three enzymes—N-acetyl-β-d-glucosaminidase (NAG; EC 3.2.1.30), alanine aminopeptidase (AAP; microsomal aminopeptidase, EC 3.4.11.2), and γ-glutamyltransferase (GGT; EC 2.3.2.2)—and adenosine deaminase binding protein (ABP) in urine to predict or confirm renal-transplant rejection in patients treated with cyclosporine. We measured the enzymes daily during the early post-transplant hospital stay of 104 renal-transplant recipients (72 men and 32 women). We also measured ABP in 32 of these patients. We analyzed the data by calculating the activity ratio of each day’s test value to the previous day’s result and optimized the sensitivity (SN) and specificity (SP) to determine the optimal ratio for each test. The results indicate that cyclosporine treatment reduces the optimal sensitivity and specificity of these tests. Three comparable tests (ABP, GGT, and AAP) yield the best optimal values (SN = 0.77, 0.69, 0.77; and SP = 0.71, 0.74, 0.63, respectively), and the NAG test yields the lowest combination of sensitivity and specificity (SN = 0.62, SP = 0.66). All four tests were less sensitive and specific than the plasma creatinine test (optimal day-to-day difference = 5 mg/L). However, the ABP and AAP tests gave indications of rejection at least 24 h before clinical diagnosis for 50% of the patients experiencing rejection, while early plasma creatinine increases of 5 mg/L occurred in only 19% of this group.

During the past two decades, the practice of using enzyme excretion in the urine of renal-transplant patients as an indicator of transplant rejection has caused considerable controversy (1–18). Although results of studies of patients being treated with the immunosuppressant drugs azathioprine and prednisone generally indicate high sensitivity and specificity for these tests (1–9, 11–13, 15–17), few studies have been conducted to examine patients who receive cyclosporine (10, 18). The purpose of this study was to evaluate the use of these tests when cyclosporine is an immunosuppressant, this agent being used in 99% of organ transplants today (19). The use of cyclosporine has made the diagnosis of acute rejection more difficult in view of (a) its nephrotoxicity (20, 21) and (b) its ability to mask the clinical features of acute rejection (22, 23). In addition, we evaluated the proximal tubular antigen adenosine deaminase binding protein (ABP) when reagents for its assay became available because reports suggested that it was a useful test for detecting renal-transplant rejection (24, 25).

Materials and Methods

Patients and Samples

The study population comprised 104 patients, 72 men (mean age 39.9 years, range 18 to 68 years) and 32 women (mean age 37.6 years, range 17 to 62 years). Of these patients, 82 had received kidneys from cadavers, 19 from related donors, and three from nonrelated donors. All were treated with cyclosporine (8–14 mg/kg of body weight) and prednisone (1.5 mg/kg) immediately after the transplant operation. Twenty-three patients also received azathioprine (0.5–1.0 mg/kg). The patients were studied from one to a maximum of 28 days post-transplant. We divided the patients into three groups: (I) 68 without diagnosis of rejection during the hospital stay (nonreject group), (II) 26 with features of acute rejection (reject group), and (III) 10 with renal-transplant dysfunction secondary to various other causes, including cyclosporine toxicity, urinary infection and leakage, and wound infection.

The diagnosis of acute rejection was based on clinical and laboratory criteria. The clinical criteria included at least one of the following: (a) urine output <500 mL/24 h, (b) weight gain >1 kg/24 h, (c) fever without infection, and (d) enlargement and tenderness of the graft. The laboratory criteria included an increase in plasma creatinine of >5 mg/L per day and at least two of the following: (a) normal cyclosporine concentrations, (b) delayed and decreased perfusion by renal scintigraphy, (c) dampened diastolic flow with a resistive index of >0.72 measured by Doppler ultrasound, (d) a fine-needle aspirate biopsy showing immune activation (corrected increment >3.5), and (e) a core-needle biopsy showing the histological features of acute cellular rejection. The results of the urinary protein analyses were not available to the clinician at the time of diagnosis.

Daily urine samples (first-voiding morning samples and some 24-h samples) were collected during the patients’ stay in the hospital after the renal transplantation. We measured three urinary enzymes: N-acetyl-β-d-glucosaminidase (NAG; EC 3.2.1.30), alanine aminopeptidase (AAP; microsomal aminopeptidase, EC 3.4.11.2), and γ-glutamyltransferase (GGT; EC 2.3.2.2). During the study, we also began measuring a proximal tubular antigen, ABP (defined by the specificity of the URO-4 (previously S27) antibody (26, 27)). NAG samples were untreated and stored at −20 °C until analyzed (28); AAP and GGT samples were treated with 1 mL of glycerol per 10 mL of urine and stored at −20 °C (28); and to ABP samples we added, per liter, 20 mmol of Tris base, 1 mmol of benzamidine HCl (cat. no. 6506; Sigma Chemical Co., St. Louis, MO 63178), and 10 mmol of Na₂EDTA, and then stored them at 4 °C.

Assays

We assayed AAP by the optimized method of Jung and Scholz (29), which we modified for analysis on the Cobas-Bio centrifugal analyzer (Roche Diagnostics Systems, Nutley, NJ). We incubated 50 μL of each sample with buffer for 15 min at 37 °C, then started the reaction by adding the
substrate. The final assay mixture contained 2 mmol/L L-alanine p-nitroanilide substrate in 50 mmol/L Tris buffer (total volume 327 μL). After a 60-s delay, we measured the absorbance at 405 nm, then made 10 more readings at 30-s intervals. We calculated the results (U/L; micromoles of p-nitroanilide formed per minute per liter of sample) by using an absorptivity of 9.9 × 10³ L mol⁻¹ cm⁻¹. The Cobas-Bio settings were as follows: (1) units, U/L; (2) calculation factor, 505; (3–6) standards and limit, 0; (7) temperature, 37.0°C; (8) type of analysis, 3; (9) wavelength, 405 nm; (10) sample volume, 50 μL; (11) diluent volume, 47 μL; (12) reagent volume, 205 μL; (13) incubation time, 900 s; (14) start reagent vol, 5 μL; (15) time of first reading, 60.0 s; (16) time interval, 30 s; (17) number of readings, 11; (18) blanking mode, 0; and (19) printout mode, 1. The first reagent (setting 12) was Tris HCl (74.2 mmol/L, pH 7.8, at 37°C); the start reagent (setting 14) was L-alanine-p-nitroanilide HCl (Sigma, cat. no. A-7540), 0.131 mol/L in 0.236 mol/L Tris HCl, pH 7.8, at 37°C.

We assayed GGT on the Cobas-Bio by modifying the method of Szasz (30) and using S.V.R. GGT Test Reagents (Behring, La Jolla, California). We diluted the 15.0-mL reagent vial, which contained the substrates γ-glutamyl-p-nitroanilide and glycylglycine, with 13.5 mL of de-ionized water to compensate for a dilution the Cobas-Bio makes. We mixed the diluted sample with the reagent at 30°C. After a 180-s delay, we measured the absorbance at 405 nm, then made 14 more readings at 10-s intervals. We calculated results (U/L; micromoles of p-nitroanilide formed per minute per liter of sample) by using an absorptivity of 9.9 × 10³ L mol⁻¹ cm⁻¹. The Cobas-Bio settings were as follows: (1) units, U/L; (2) calculation factor, 5050; (3–6) standards and limit, 0; (7) temperature, 30.0°C; (8) type of analysis, 2; (9) wavelength, 405 nm; (10) sample volume, 5 μL; (11) diluent volume, 20 μL; (12) reagent volume, 180 μL; (13) incubation time, 0 s; (14) start reagent vol, 0 s; (15) time of first reading, 160 s; (16) time interval, 10 s; (17) number of readings, 15; (18) blanking mode, 0; and (19) printout mode, 2.

We assayed NAG by modifying the method of Leachback and Walker (31). We incubated the sample (50 μL) with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma, cat. no. M-2133), 0.263 mmol/L, and human serum albumin, 0.1 g/L in 0.05 mol/L sodium citrate buffer, at pH 5.0 (total volume, 1 mL) for 15 min at 37°C. We stopped the reaction by adding 5 mL of 0.2 mol/L glycine buffer, pH 10.65. We measured the fluorescence of the product, 4-methylumbelliferone, with a fluorometer spectrophotometer (Model LS-2B; Perkin-Elmer, Beaconsfield, Bucks., U.K.) with λₑₓ = 366 nm and λₑₘᵢₓ = 450 nm and compared the fluorescence with a standard curve.

Using "Nephroscreen" Adenosine Deaminase Binding Protein enzyme immunoassay kits (Cambridge Research Laboratory, Cambridge, MA), we assayed ABP by the method of Thompson et al. (32). Some samples had values below the detection limit for this assay, 0.2 arb. unit/0.1 mL (one arbitrary unit is defined by the manufacturer as the amount of ABP present in 100 μL of sample that increases the absorbance by one absorbance unit, 1.000 A); for those samples we reported ABP values of 0.2 arb. unit/0.1 mL.

Data Analysis

Urinary creatinine concentrations were used to correct for renal flow, and plasma creatinine concentrations were used as an indicator of renal function. We expressed the protein test results as a ratio of enzymatic activity or ABP concentration to that of urinary creatinine, because Jung et al. (33) demonstrated that this method had the "highest diagnostic potential." All data for urine samples with urine creatinine concentrations <200 mg/L were deleted before we analyzed the protein test data. Forty-nine patients in the nonreject group and 22 patients in the reject group had at least one urinary creatinine value of <200 mg/L or missing.

We calculated the pooled intraperson two-way correlations for GGT, AAP, ABP, NAG, and plasma creatinine for each patient in the reject and nonreject groups. To obtain these pooled correlations, we first removed outliers (34) and then computed, separately for each individual, the two-way correlation between two tests (e.g., for NAG and GGT) on the basis of daily values. We then pooled these correlations across individuals (35) and applied the inverse hyperbolic tangent transformation of Fisher (36) to provide statistics for hypothesis tests of nonzero correlations in the nonreject and reject groups and for tests of nonequality of correlations between the nonreject and reject groups.

We calculated activity ratios as the ratio of each day's protein test value to the previous day's value. If a sample was missing, we used the previous available value, provided it had been obtained within the preceding three days. The data were evaluated at ratios from 1.5 to 3.5 at intervals of 0.1. At each ratio we defined a true positive as an activity ratio equal to or greater than the cut point and occurring within four days of the earliest clinically diagnosed rejection. We defined true negative as all activity ratios less than the value under consideration in a patient without clinically diagnosed rejection. At each ratio cut point for each test, we determined the sensitivity (SN, the number of true positives divided by the number of patients with clinically diagnosed rejection) and specificity (SP, the number of true negatives divided by the number of patients without clinically diagnosed rejection). We evaluated plasma creatinine data similarly, except that, instead of ratios, we used differences between each day's plasma creatinine and the previous day's value (going back a maximum of three days if values were missing).

The optimum ratio (or difference) for each test was defined as the ratio cut point (or difference cut point) giving the lowest value for D, where

\[ D = \sqrt{(1 - SN)^2 + (1 - SP)^2}. \]

For a given test, D is the euclidean distance from the point with coordinates (SP, SN) to the point (1,1) that represents perfect specificity and sensitivity. Such optimization gives equal weight to—and simultaneously maximizes—the sensitivity and specificity.

Results

As is commonly observed among transplant recipients, the background concentrations of enzyme and ABP excretion in the study patients were higher than for people with normal renal function, and these values varied considerably from one patient to another (11, 17). We also found a large variation in the degree to which test values fluctuated among the patients who were not clinically diagnosed as rejecting the transplants; some patients had relatively stable background values, and others had fluctuating ones. Approximately 84% of the nonreject group experienced minor increases in plasma creatinine at some point during
their hospital stay, and 57% had at least one blood cyclosporine measurement above normal. Both patients whose data are shown in Figure 1A and B experienced minor increases in their blood cyclosporine concentrations. The patient in Figure 1B experienced a plasma creatinine increase of 4 mg/L on day 5 and again on day 10, with an additional 1 mg/L increase on days 11 and 12, increases less than that associated with clinically diagnosed rejection. The patient in Figure 1C experienced clinically diagnosed rejection on day 10. We observed an average within-person correlation (r) near −0.3 between plasma creatinine and blood cyclosporine values in both the nonreject and reject groups. We found no significant pooled correlations, however, between enzyme or ABP values and blood cyclosporine values.

Pooled within-person correlations of the protein tests with one another are statistically significant (r = 0.43 to 0.66, P = 0.0001) in the reject group, but the correlations between protein tests in the nonreject group are weaker (P < 0.0002), except for ABP with AAP and ABP with NAG. Although in the reject group the correlations of plasma creatinine values with all of the protein tests (except the ABP test) are statistically significant (P < 0.05), the correlation coefficients are low (NAG, r = 0.21; GGT, r = 0.19; AAP, r = 0.13; ABP, r = 0.02). Plasma creatinine values do not correlate with any of the protein test results in the nonreject group.

The relationship between sensitivity and specificity estimates for the ABP, GGT, AAP, and NAG tests at different activity ratios shows that ABP, GGT, and AAP give higher sensitivities and specificities than does NAG and that the receiver-operating characteristic (ROC) curves are flatter than those observed by Jung et al. (17) for patients who did not receive cyclosporine (Figure 2). The results for ABP, however, are less reliable than those for GGT, AAP, and NAG, because ABP values were available for only 13 of the 26 transplant recipients in the reject group and for 19 of the 68 patients in the nonreject group. The optimum activity ratios, obtained by minimizing D (Figure 2, Table 1), are higher and the sensitivities and specificities are lower than those reported as optimal in patients who did not receive cyclosporine (17). At each test’s optimum ratio, ABP and AAP give the highest sensitivity and GGT the highest specificity. Of the four tests, ABP gives the highest and NAG the lowest combination of both specificity and sensitivity. ABP still has the highest combination of sensitivity and specificity when the optimum ratios for the enzymes are recomputed based on the same subset of 32 transplant recipients for whom ABP data were available.

A similar analysis of increases in plasma creatinine alone, performed on the basis of the differences between the plasma creatinine on a given day and a previous day, gives a minimum D value of 0.18, with a sensitivity of 0.85 and a specificity of 0.91, at an optimal difference of 5 mg/L. Analyzing plasma creatinine alone, therefore, yields better sensitivity and specificity than any of the urine protein tests. This finding is in accord with current reliance on increases in plasma creatinine for the diagnosis of transplant rejection.

We evaluated sensitivities and specificities of the possible combinations of two and three tests. No combination gives a D < 0.41, which is in accord with the strong two-way correlations observed for the enzymes and ABP values. In addition, we computed the optimum activity ratios (or differences in the case of plasma creatinine) separately for

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**Fig. 1. Examples of plasma creatinine and protein test data for three renal-transplant recipients:** (A) patient in the nonreject group with minor increase in blood cyclosporine concentration, (B) patient in the nonreject group with minor increase in blood cyclosporine and subclinical increases in plasma creatinine, (C) patient in the reject group whose rejection was clinically diagnosed on day 10.
the 20 patients given, and the 74 patients not given, azathioprine. The analysis yields only slight differences for NAG, AAP, and plasma creatinine (differences explainable by sampling variation) and no differences for GGT and ABP.

The percentages of positive indications (an activity ratio equal to or greater than the optimal activity ratio, occurring within four days of the first clinically diagnosed acute rejection) for each test in the reject group that occurred before, on the same day as, or after the day of clinical diagnosis of rejection show that 63% to 70% of the positive indications occurred before the clinical diagnosis for three of the four protein tests (Table 2). In contrast, GGT gave only 39% positive indications before the date of clinical diagnosis and 44% after that date. ABP and AAP gave the highest percentage of positive indications before the date of diagnosis (70% and 65%, respectively), and resulted in positive indications of rejections for approximately 50% of the total reject group. Plasma creatinine increases of 5 mg/L or greater, however, gave only 23% of the positive indications (19% of reject group) before the date of clinical diagnosis, lower than any of the protein tests; 36% of the positive indications occurred on the same day and 41% on the day after diagnosis.

Discussion

The results of this study suggest that, when cyclosporine is used as an immunosuppressant, the sensitivity and specificity of tests (such as increases in ABP, GGT, AAP, and NAG in urine) to predict or confirm renal-transplant rejection are reduced. When activity ratios are optimized by minimizing the parameter D, we find sensitivities of ABP, GGT, and AAP ranging from 0.69 to 0.77 and specificities from 0.63 to 0.74; NAG yields the lowest combination of sensitivity and specificity (Table 1). These results indicate higher sensitivity for ABP and AAP than that indicated in the study of Kotanko et al. (18), who evaluated the use of several other enzymes in urine, including NAG, as indicators of transplant rejection. Although Kotanko et al. found a sensitivity of 1.00 and specificity of 0.85 in a small group of patients treated with azathioprine and prednisolone, only 67% of the rejections in a larger group of patients treated with cyclosporine were recognized correctly when a nonoptimized model of enzyme combinations was used. Our results for NAG agree with this finding and also with the findings of the smaller study of Loertscher et al. (10), in which 12 conventionally treated patients and 11 cyclosporine-treated patients were evaluated only for NAG. Significant increases in NAG accompanied only 67% of the rejection episodes in the cyclosporine-treated patients.

Our finding that AAP and GGT are more sensitive and specific than NAG agrees with the results of studies of patients treated with conventional immunosuppressants (13, 16, 17). These results also indicate that ABP is among the best urinary protein tests for detecting transplant rejections. The use of ABP as an indicator of rejection was evaluated with only a few patients, however, and final conclusions on the value of this test should be based on results for a larger study group.

Analyzing data in studies of this type has several problems, including three of particular importance: (a) which model to choose for data analysis, (b) how to handle periods of oliguria, and (c) whether to use samples with low urinary creatinine (dilute urine samples). Many studies do not include information on how these problems are handled, but the choices made are likely to influence results. Analyzing data is complicated by variations in the patterns of enzyme excretion among patients not experiencing clinical rejection. These variations make calculating background values difficult. We, therefore, chose the data analysis method of Jung et al. (17), which uses a ratio of the enzyme value on a particular day to that on the previous day...
However, the sensitivities calculated from such an analysis must be regarded as minimum values, because the analysis may miss steady increases in protein values that peak after several days but may not show a significant increase in one day (Figure 1C, GGT).

Transplant recipients have periods of oliguria, particularly during rejection episodes, when urine samples are frequently not available. In those situations, we chose to calculate ratios on the basis of the previous day's values or, if necessary, values up to three days previous. Undoubtedly, critical data are unavoidably lost during the oliguric period, both in this study and in the usual clinical setting.

We also found several urine samples with low creatinine concentrations. These low values can result in increases in the data when urine creatinine is used to correct for variations in flow; the artificial increases appear to have no relation to a physiological cause (37). Because we found spurious increases in protein results to be particularly apparent when urine creatinines were <200 mg/L, we deleted such data from our analysis of the protein tests.

Both the higher optimal activity ratios observed in this study and the changes in protein excretion of patients not experiencing a clinically diagnosed rejection may be related to characteristics of cyclosporine action. Rejections by transplant recipients treated with cyclosporine are less aggressive than rejections by recipients who are given conventional treatment, and the typical clinical symptoms of rejection are suppressed in the former (22, 23). In addition, cyclosporine itself is nephrotoxic (38); therefore, as the observed minor increases in serum creatinine and above-normal concentrations of cyclosporine suggest, the increases in urine protein measurements in the nonreject group may be related to subclinical rejections and cyclosporine toxicities (Figure 1A and B). These protein tests are known to be very sensitive to damage of the renal tubules (11, 39), which could account for the low specificity observed in studies of cyclosporine-treated transplant recipients. Low specificity causes a shift to higher ratio values when the activity ratio is optimized. The sensitivities of the tests at an activity ratio of 1.5 are 0.85 (ABP), 0.96 (GGT), 1.00 (AAP), and 0.88 (NAG), which generally agree with sensitivities found when patients are treated with conventional immunosuppressants (17). The specificities at the 1.5 ratio, however, are only 0.12 to 0.24 (Figure 2).

In summary, the results of this study indicate that the measurements of urinary ABP, GGT, AAP, and NAG in cyclosporine-treated patients appear to be less sensitive and less specific indicators of acute rejection at their optimal activity ratio for patients treated with cyclosporine than for patients treated with conventional immunosuppressants. For cyclosporine-treated patients, all four protein tests are less sensitive and specific than the plasma creatinine test alone. However, increases of 5 mg/L or greater in plasma creatinine detect only 19% of the total number of patients with clinically diagnosed rejection before the actual date of diagnosis, whereas ABP and AAP give early indication for 50% of the patients with rejection.

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