Selection of an Optimal Assay Method for Monitoring Cyclosporine Therapy

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CLINICAL use of cyclosporine (CyA) as an immunosuppressive agent requires monitoring drug levels in the blood to guide therapy and avoid toxicity. The original methods used to monitor therapy measured either parent drug by high performance liquid chromatography (HPLC) or a combination of CyA and metabolites by polyclonal radioimmunoassay (RIA). For routine monitoring of CyA levels, these methods have generally been replaced by newer RIA and fluorescent polarization immunoassay (FPIA) techniques. HPLC has been replaced by an RIA method using a monoclonal antibody specific for the parent compound.[1-4] Nonselective immunoassay of CyA and metabolites may now be performed by monoclonal RIA[1] or polyclonal FPIA methods,[5] and numerous comparisons of these techniques have been reported.[6-12] The selection of a method by transplant centers has been based on prior experience, assay convenience, and information concerning metabolite bioactivity. Controversy exists, however, over an optimum method and therapeutic range for monitoring treatment.[13] The nonselective immunoassays continue to be used in many centers,[14] even though parent drug monitoring by specific methods has been recommended.[13] Continued routine use of nonselective methods might be acceptable if CyA and metabolites were measured in relation to their potential contribution to therapy. In this study we correlated nonselective immunoassay results with specific measurement of CyA and primary metabolites. We evaluate the crossreactivity of purified metabolites with the nonselective immunoassays, and discuss our results in relation to current information concerning the biologic activity of metabolites.

MATERIALS AND METHODS

CyA and Metabolites

Purified CyA was obtained from Sandoz Pharmaceuticals (East Hanover, NJ). CyA metabolites AM1 (M17), AM9 (M1), AM19 (M8), AM 4N (M21), AM1c (M18), and AM1A (M203-218) were isolated from human urine by a preparative chromatographic procedure developed in our laboratory. Briefly, prefiltered urine was loaded onto a 40 μm octadecyl silica (Bondesil, Analytichem International, Harbor City, CA) adsorption column (125 × 4.6 mm inside diameter [ID]). The column was washed with acetonitrile/0.5 mol/L acetic acid, 40:60 (vol/vol). The metabolites were then eluted from the column with 100% acetonitrile, dried under air, and reconstituted in 1.0 mL acetonitrile/water, 50:50 (vol/vol). The concentrated extract was then chromatographed on a 5 μm, C8 (Zorbax, Dupont Instruments, Wilmington, DE) silica preparative HPLC column (250 × 21.2 mm ID) with acetonitrile/water, 50:50 (vol/vol), as the mobile phase. Fractions containing the purified CyA metabolites were collected with a fraction collector. The identity, concentration, and purity of the isolated metabolites were evaluated by analytical HPLC. Analyses were performed on both cyanopropyl (Zorbax, Dupont Instruments) and C8 (Supelcosil, Supelco Inc, Supelco Park, Bellefonte, PA) silica HPLC columns (250 × 4.6 mm ID) using photo diode-array detection. All metabolites were quantified based on peak area at 215 nm. Identification of the various peaks was performed by comparing retention times with those of structurally identified metabolites provided by Sandoz Pharmaceuticals (Basel, Switzerland) and Syva Company (Mountain View, CA); photo diode-array detection was used to confirm the purity of isolated metabolites.

Immunoassay and HPLC Methods

RIA specific (RIA-SP) and nonselective (RIA-NS) measurements with monoclonal antibodies[1] were performed with the Sandimmune kit (Sandoz Ltd, Basel, Switzerland) according to manufacturer's instructions. CyA-free whole blood was used to prepare CyA standards and quality control samples, and analytical grade ethanol was used for sample pretreatment. Whole blood samples were analyzed in duplicate, and radioactive counts were measured with a liquid scintillation counter (model LS 5000TD, Beckman Instruments Inc, Fullerton, CA) using an external quench correction. The FPIA method was performed with a TDX analyzer (Abbott Diagnostic Division, Abbott Park, IL) using manufacturer's reagents, controls, and assay protocol. Whole blood standards used with the monoclonal RIA were coanalyzed with the FPIA method in order to cross-validate calibration curves against those obtained by immunoassay. Whole blood concentrations of CyA, M1, M17, and M18 were determined by an HPLC procedure previously reported.[15] HPLC and immunoassay measurements were compared using whole blood samples obtained from renal allograft recipients. Patients received oral CyA in combination with methylprednisolone and azathioprine therapy as previously reported.[15] All samples were obtained during the first 3 months posttransplant and represent trough samples.

Metabolite Crossreactivity Studies

Known quantities of individual metabolites were reconstituted with HPLC-grade methanol and diluted with whole blood. Further serial dilutions were performed with blood to attain metabolite concentrations ranging from 25 to 3000 ng/mL. Dilutions were performed with CyA-free whole blood or with blood containing

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CyA to produce metabolite-dilution series containing either 0, 160, or 320 ng/mL levels of CyA. All samples were analyzed by the RIA-NS and FPIA methods.

Assay for Immunosuppressive Activity

Stock concentrations of CyA or a metabolite were prepared in absolute ethanol. Serial dilutions were prepared and aliquoted into 24-well tissue culture plates and then evaporated under a laminar flow hood. Jurkat cells were then added at 5×10^5 cells/mL in 1 mL of RPMI-1640 supplemented with 10% fetal bovine serum. The cells were incubated at 37°C for 60 minutes prior to stimulating them with phytohemagglutinin (PHA)-M (1:40 dilution) and phorbolmyristate acetate (PMA) (1 ng/mL). Supernatants were harvested 20 hours later and assayed for interleukin-2 (IL-2) using the IL-2-dependent murine HT-2 cell line as previously described.[16] This line does not respond to human IL-1, IL-4, or IL-6, nor does it proliferate in response to the concentrations of PHA and PMA used in these experiments.

RESULTS

Analytical results with the nonselective immunoassays were compared with specific measurements of CyA and the primary metabolites that predominate in blood. Parent drug monitoring performed with the HPLC and RIA-SP methods show statistical agreement of results for renal transplant patients (Fig 1). Both nonselective immunoassays, however, overestimated the actual concentration of parent drug (Figs 2 and 3) such that CyA accounted for only 29% and 36% of the measured concentration by RIA-NS and FPIA methods, respectively. When compared with specific measurements of CyA and primary metabolites, the closest agreement was observed between results with the RIA-NS method and HPLC measurement of CyA and its primary metabolites (Fig 4). For clinical samples with RIA-NS concentrations below 1000 ng/mL, 96% of the RIA-NS concentration could be empirically accounted for by the combined levels of CyA, AM1, and AM9.

Cross-reactivity of metabolites with the nonselective immunoassays was determined by dose-response curves

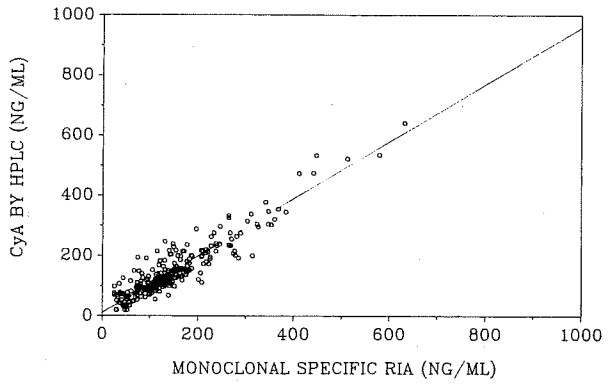

Fig 1. Comparison of CyA concentrations as measured in whole blood (n = 277) by monoclonal-specific RIA and HPLC. Linear regression analysis resulted in a regression line equation of Y = 0.95X + 10 and an *r* value of 0.954.

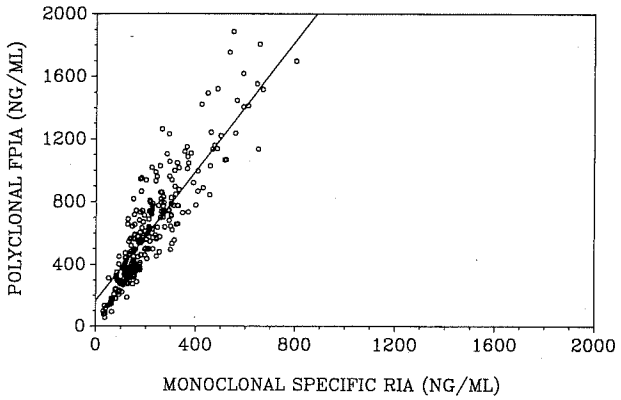

Fig 2. Correlation of monoclonal-specific RIA and FPIA results for whole blood samples (n = 280) from renal transplant recipients. Linear regression analysis resulted in a regression line equation of Y = 2.07X + 168 and an *r* value of 0.891.

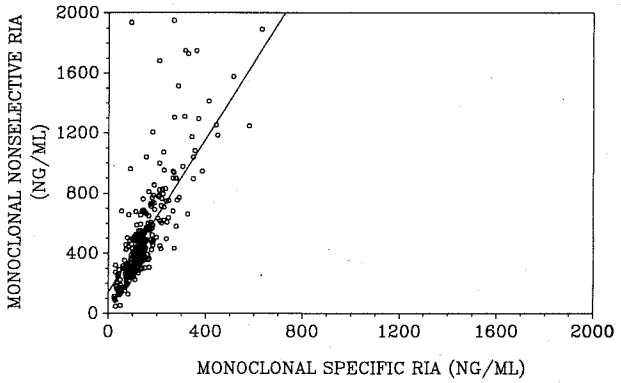

Fig 3. Correlation of monoclonal-specific and nonselective RIA results for whole blood samples (n = 280) from renal transplant recipients. Linear regression analysis resulted in a regression line equation of Y = 2.56X + 141 and an *r* value of 0.815.

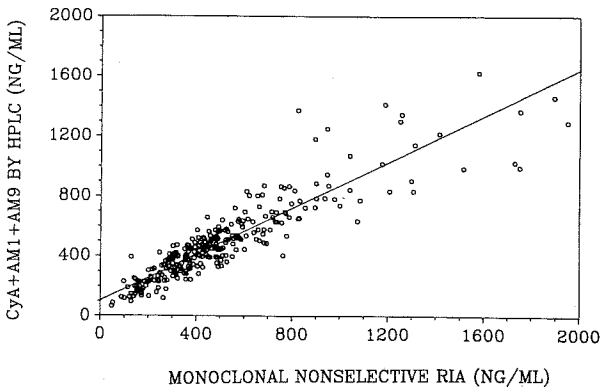

Fig 4. Correlation of monoclonal nonselective RIA versus CyA and primary metabolite concentrations in whole blood samples (n = 277) from renal transplant recipients. Linear regression analysis resulted in a regression line equation of Y = 0.77X + 102 and an *r* value of 0.920.

for the individual purified metabolites. The degree of crossreactivity varied significantly between nonselective immunoassays, depending on both metabolite structure and concentration. Reactivity of the CyA in the FPIA method (Fig 5) followed the order AM1 > CyA > AM1A > AM1c > AM4N > AM9 > AM19, resulting in overestimation of the actual AM1 concentration and significant underestimation of AM9 and AM19 concentrations. In addition, AM1c, AM4N, and AM1A produced nonlinear dose-response curves, indicating decreased crossreactivity at the higher concentrations. The RIA-NS method (Fig 6) resulted in a completely different set of metabolite dose-response curves. Crossreactivity of AM9, AM19, and AM1 were close to 100%, while AM1c and AM1A concentrations were significantly overestimated and AM4N was underestimated by the assay. A nonlinear dose response was observed for AM9, AM19, and AM1c, but, in this assay, the crossreactivity increased with the higher metabolite concentration. The presence of CyA in blood appeared to reduce the crossreactivity of metabolites in both nonselective immunoassays, as demonstrated with AM1 data in Fig 7. Recovery of AM1 decreased significantly in the FPIA (24% decrease) and RIA-NS (21% decrease) assays in the presence of CyA (320 ng/mL).

CyA and individual metabolite preparations were also tested for relative immunosuppressive activity using a human T cell line. These results are summarized in Table 1. The human T-cell leukemia line Jurkat was found to be more sensitive than human peripheral blood mononuclear cells (PMNC) to the inhibitory effects of CyA (data not shown). The IC_{50} for the effect of CyA on Jurkat cell IL-2 production was 5 to 10 ng/mL, compared with 100 ng/mL using normal PMNC.[17] The relative order to immunosuppressive activity, however, was the same as previous reports using normal lymphocytes.[18,19] As can be seen in Table 1, CyA was approximately 10-fold more potent than the next most active CyA metabolite, AM1. AM9 was slightly less active than AM1, with all of the other metabolites at least 10 to 20-fold less immunosuppressive than the primary metabolites, AM9 and AM1.

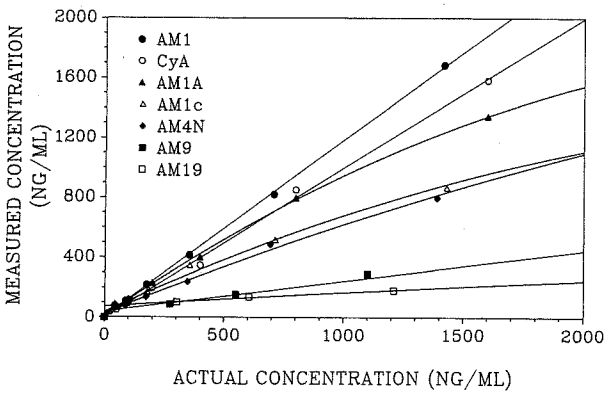

Fig 5. Crossreactivity of CyA metabolites with the nonselective polyclonal FPIA method. Dose-response curves were generated with dilutions of each metabolite in CyA-free blood. First or second order linear regression analysis was performed to curve-fit the data.

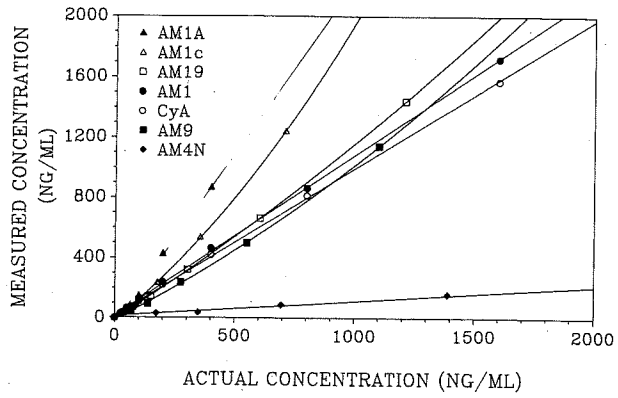

Fig 6. Crossreactivity of CyA metabolites with the nonselective monoclonal RIA method. Dose-response curves were generated with dilutions of each metabolite in CyA-free blood. First or second order linear regression analysis was performed to curve-fit the data.

DISCUSSION

Specific measurement of the therapeutic agent has been the standard of practice in therapeutic drug monitoring. Antibody crossreactivity with metabolites is routinely performed to validate this specificity. CyA monitoring by nonselective immunoassay is a unique practice in which metabolites contribute significantly to the drug measurement. In our assessment of blood from renal transplant recipients, the parent drug contributed only an average of 29% and 36% to the concentration measured by the RIA-NS and FPIA methods, respectively. Clinical correlations further indicated a greater metabolite contribution to the RIA-NS than to the FPIA measurement. Monitoring with the RIA-NS method correlated closest with the total concentration of CyA and its primary metabolites. How-

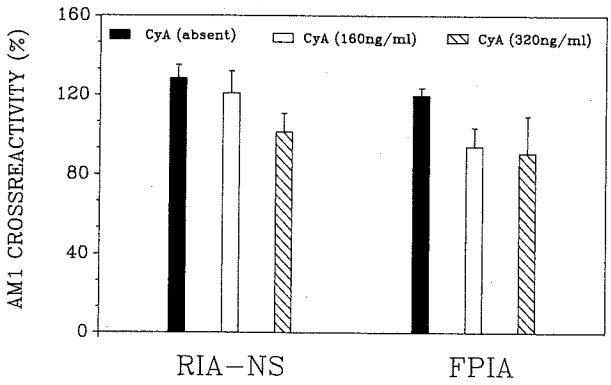

Fig 7. Effect of CyA concentration on the crossreactivity of M17 with the RIA-NS and FPIA methods. Data represents the mean percent (SD) of the actual M17 concentration measured in either assay.

Table 1. Relative Immunosuppressive Activity of CyA and Its Metabolites

Compound	IC_{50}* (ng/mL)
CyA	5-10
AM1	50-100
AM9	75-150
AM1c	1,000-1,300
AM19	1,800-2,000
1M1A	>2,000

*Concentration producing 50% inhibition of IL-2 production by Jurkat T cells stimulated with PHA and PMA.

ever, the contribution of individual metabolites to nonselective monitoring methods cannot be determined from these correlations.

Studies with purified metabolites reveal the complexity of metabolite crossreactivity with nonselective immunoassay methods. Consistent with the original report by Quesniaux et al,[1] we find that the monoclonal antibody used in the RIA-NS method does not discriminate effectively between CyA and many of the metabolites. For metabolites included in our evaluation, only AM4N showed a significantly lower binding affinity, suggesting antibody discrimination of an epitope involving amino acid number 4. Several of the metabolites (AM1A, AM1c, and AM1), all of which contain structural modifications of amino acid number 1, showed enhanced affinity for the antibody. Crossreactivity in the RIA-NS and FPIA assays was similar for M17 but differed significantly for the other metabolites. The FPIA assay data revealed a reduced antibody affinity for all of the evaluated metabolites except AM1. This may explain the clinical finding of lower concentrations by FPIA versus RIA-NS monitoring, since we have shown that AM9 is present in blood in significant quantities.[20] In addition, both the RIA-NS and FPIA methods produced nonlinear dose-response curves with several of the metabolites. This observation could be explained by differences in antibody-binding kinetics between the metabolite and the CyA used in calibrating the assay. Competitive interactions between mixtures of CyA and the metabolites have also been demonstrated for AM1. Crossreactivity may therefore depend not only on the concentration of metabolite, but also on the concentration of CyA and other metabolites in blood. Based on these crossreactivity data and the HPLC measurements of clinical samples, we conclude that M17 is the major CyA structure measured by either the RIA-NS or FPIA methods. The difference in clinical monitoring between the nonselective immunoassays may result from differences in crossreactivity with other metabolites present in blood, such as AM9, which is underestimated by the FPIA method. Therefore, discrepancies between the assays can occur when AM9 or more polar metabolites accumulate, as we have observed during episodes of nephrotoxicity.[15]

Although these studies clearly show that metabolites are the major contributors to therapeutic monitoring with nonselective immunoassay, they probably do not provide a corresponding therapeutic effect. As seen in Table 1, the major metabolites AM1 and AM9 have only 5% to 10% of the immunosuppressive activity of CyA. Although the trough blood levels of primary metabolites AM1 and AM9 exceed CyA by twofold to threefold, metabolites probably provide less than 20% of the total immunosuppressive effect with CyA therapy. Therefore, routine monitoring of the parent drug by specific methods would appear to provide the best index of the level of immunosuppression, based on our current knowledge. Measurement of specific metabolites may be needed if further studies reveal a significant in vivo contribution to immunosuppression or nephrotoxicity. Nephrotoxic activity of metabolites has not been demonstrated, although we have preliminary evidence to suggest a correlation between the accumulation of metabolites and the onset of nephrotoxicity.[15] However, toxic metabolites must be identified and clinical studies performed before routine monitoring of specific metabolites can be recommended. The use of nonselective immunoassays will only continue the confusion over method selection, therapeutic/toxic ranges, and comparison of clinical data among the transplant centers.

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