Erythrocyte Uptake and Protein Binding of Cyclosporin A (CyA) in Human Blood: Factors Affecting CyA Concentration in Erythrocytes

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To further the understanding of the complexity of cyclosporin A (CyA) pharmacokinetics, we conducted an erythrocyte uptake and efflux study, and a protein binding study in human blood. The uptake study showed that the transport of CyA from the extracellular fraction to erythrocytes was retarded by increased human serum albumin (HSA) and lipid levels in this fraction. In addition, the concentration of CyA in erythrocytes increased with increases in CyA concentration in blood and reductions in hematocrit. The efflux study showed that the transport of CyA from erythrocytes to the extracellular fraction was essentially enhanced by increases of HSA and lipid levels in that fraction, but that these effects were relatively small. There were two affinity binding sites for CyA in ghost-free erythrocyte hemolysate, but not in the plasma fraction. The affinity binding constants for these binding sites were reduced by elevations in temperature, and under physiological conditions, 37°C, almost all the CyA in erythrocytes was bound to a CyA binding protein, namely, cyclophilin. These findings suggest that CyA distribution in blood is of two different types which are present in the erythrocyte and plasma fractions, respectively. Monitoring of blood biochemistry various showed that the concentration of CyA in erythrocytes had an interlocking relationship with these physiological factors, which were related to patient disease state, i.e., hematocrit, lipids, albumin, and total protein; the concentration of CyA in erythrocytes could be predicted from these physiological factors.

Keywords cyclosporin A; erythrocyte hemolysate; protein binding; blood distribution; immunosuppressant

Introduction

Cyclosporin A (CyA) is a powerful immunosuppressive agent that is used extensively in the clinical setting in liver, kidney, heart, and bone marrow transplantation. Since the pharmacokinetic profiles of CyA are highly variable both between and within patients, routine monitoring of CyA is advocated for its optimal use following transplantation. However, in clinical practice, it is often difficult to distinguish between the clinical signs of its major adverse effect, nephrotoxicity, and lack of therapeutic efficacy. Hence, an optimal drug protocol has not yet been established.

In a previous study, we found that the cell-to-plasma distribution ratio was increased when nephrotoxicity or hepatotoxicity was exhibited. We therefore suggested that changes in the cell-to-plasma distribution of CyA could serve as indicators to predict tissue toxicity. Further, based on the assumption that two different CyA binding sites are present in human blood, in the plasma and erythrocyte fractions, respectively, we derived a mathematical model to account for the distribution of CyA in human blood, and we simulated the effects of hematocrit and temperature on this distribution. However, the detailed mechanism responsible for this distribution and its clinical relevance remains unclear.

The present in vitro study was designed to confirm the characteristics of CyA erythrocyte uptake and/or efflux and protein binding in both erythrocyte hemolysate and plasma. In addition, using clinical monitoring data from transplant populations, we examined the relationship between erythrocyte CyA concentration and physiological factors to clarify CyA distribution in relation to the patients' disease state.

Materials and Methods

Chemicals CyA was kindly supplied by Sandoz Ltd., Basle, Switzerland. Human serum albumin (HSA, fatty acid free, lot 119F-5303) was obtained from Sigma Co. (St. Louis, MO, U.S.A.). Silicon oil (SH550, d = 1.07) was purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). All other chemicals were of analytical grade and were obtained commercially.

Erythrocyte Uptake and Efflux Study These experiments were performed according to the method of Hanano et al. Human whole blood was obtained from nonmedicated normal volunteers. Erythrocytes were separated by centrifugation at 1000 × g for 10 min and washed three times with 0.9% saline at 4°C. Using these washed packed erythrocytes, we prepared 5, 10 and 20% erythrocyte suspensions with several incubation media, as shown in Table I. The incubation media were prepared with an isotonic buffer (pH 7.4) and included 20 mm Tris, 131 mm NaCl, 5.2 mm KCl, 0.9 mm MgSO4, 1.12 mm CaCl2, 3 mm Na2HPO4, and 5 mm glucose. Standard solutions of CyA were prepared by dissolving CyA in incubation buffer containing 0.01% of dimethyl sulfoxide and 0.2% HSA at 4 different final concentrations, from 50 to 300 μM. For the uptake experiments, after the erythrocyte suspension had been incubated for 5 min at 37°C, the uptake reaction was initiated by adding CyA standard solution. To 1 ml of erythrocyte suspension, 10 μl of CyA standard solutions was added, to final concentrations ranging from 0.5 to 3 μM. At an appropriate time, the transport reaction was stopped by placing samples (0.9 ml) into 1.5-ml plastic microcentrifuge tubes containing 0.2 ml of silicon oil and then centrifuging the tubes in a microcentrifuge at 37°C (9000 × g for 30 s). After centrifugation, the lower erythrocytes in the microcentrifuge tube were pipetted and diluted 10-fold with distilled water, after which the CyA concentration was measured. For the efflux experiments, CyA-entrapped erythrocytes were prepared by incubating 10% erythrocyte suspension in medium I at a final CyA concentration of 1 μM at 37°C for 30 min; the erythrocytes were then washed three times with 0.9% saline at 37°C. After these erythrocytes were incubated for 5 min at 37°C, the efflux reaction was initiated by adding CyA standard solution to the samples (1 ml) and centrifuging the samples at 9000 × g for 30 s. The upper plasma was removed and the concentration of CyA was measured.

Table I. Incubation Media Used in Uptake and Efflux Experiments

<table>
<thead>
<tr>
<th>Medium</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Isotonic incubation buffer (pH 7.4)</td>
</tr>
<tr>
<td>II</td>
<td>Isotonic incubation buffer (pH 7.4) including 0.4% HSA</td>
</tr>
<tr>
<td>III</td>
<td>Isotonic incubation buffer (pH 7.4) including 0.8% HSA</td>
</tr>
<tr>
<td>IV</td>
<td>Diluted plasma obtained before food intake</td>
</tr>
<tr>
<td>V</td>
<td>Diluted plasma obtained 90 min after food intake</td>
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a) See text. b) Plasma samples were obtained from one nonmedicated volunteer; the concentration of ALB in these samples was adjusted to 0.4% by diluting with the isotonic incubation buffer (pH 7.4).

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was initiated by diluting the cells 10-fold with the incubation media listed in Table 1, and the mixtures were incubated at 37°C. At an appropriate time, the transport reaction was stopped by the same procedure used for the uptake experiments above. All measurements of CyA in erythrocytes were expressed as the content within packed erythrocyte volume.

**Protein Binding Study in Erythrocyte Hemolysate and Plasma** In this study, we used 10-fold diluted samples to enhance the accuracy of measurement of unbound CyA. Ghost-free erythrocyte hemolysate was prepared from the washed packed erythrocytes by freezing and thawing after the addition of nine volumes of distilled water, and by ultracentrifugation (25000 × g at 4°C) for 1 h. The CyA standard solutions used in this study were prepared as described in the uptake experiments to make 10 different final CyA concentrations, from 4.16 × 10⁻¹⁴ to 4.16 μM. Four-tenth ml of erythrocyte hemolysate or 10-fold diluted plasma was mixed with 0.1 ml of CyA standard solution in a 1.5-ml microcentrifuge tube to give final concentrations of 8.31 × 10⁻⁵ to 8.31 × 10⁻¹ μM. The mixture was equilibrated at specified temperatures (21, 30 and 37°C) for 1 h. To separate unbound CyA from protein-bound CyA in the hemolysate, we used the method of Agarwal et al.³⁰ with some modifications, adding 0.1 ml of charcoal suspension (containing 25 mM Tris, 5 mM Tween 20, 10 g fine charcoal and 2 g bovine serum albumin per liter). The mixture was then vigorously vortexed and equilibrated for 10 min at the same specified temperature. After centrifugation (9000 × g, 30 s) at the same specified temperature, the bound CyA concentration (C_b) in the supernatant was measured. Total CyA concentration (C_t) was equal to added CyA concentration, and the unbound CyA concentration (C_u) was calculated in accordance with the following relationship: C_u = C_t/F - C_b, where F is the dilution factor = 1.2.

**Data Analysis** Analysis of the binding data was performed by a nonlinear least squares method using the following equation, where the C_u in binding protein solution is defined as:

\[
C_u = B_1 K_1 C_t (1 + K_1 C_t) + B_2 K_2 C_t (1 + K_2 C_t)
\]

This equation assumes one specific binding site and one nonspecific binding site. K_1 and K_2 are the affinity constants (μM⁻¹) for the first and second class of binding sites, respectively, and B_1 and B_2 are the respective binding capacities (μmol/ml). The binding parameters were estimated from the concentration data C_b and C_t by a damping Gauss-Newton method, using MULTI.¹¹¹ implemented on a PC-9801 Vm2 microcomputer. The initial parameters for the analysis were determined by drawing Rosenthal plots. Convergence was defined as a relative change of less than 10⁻³ in the residual sum of squares. The measured concentration values were weighted as to be numerically equal.

**Relationship between CyA Concentration in Erythrocytes and Blood Constituents in Transplant Patients** Eighty-five heparinized whole blood samples obtained from 15 transplant patients (13 with renal transplants and 2 with bone marrow transplants), between 14 and 56 years and undergoing immunosuppressive therapy with CyA, were used in this study. Whole blood samples were withdrawn at trough level in each patient. After the whole blood samples were equilibrated at 37°C for 3 h,²¹² erythrocytes were separated with silicon oil according to the method described in the uptake experiment, and the concentrations of CyA in erythrocytes and whole blood were measured. Biochemical findings [transaminase activity (GOT, GPT), total bilirubin (T-BIL), serum creatinine (CRE), blood urea nitrogen (BUN), albumin (ALB), total protein (TP), total cholesterol (CHO), triglyceride (TG), and hematocrit (HCT)] corresponding to the CyA measurements were obtained from the SHINE computer on-line system of Shiga University of Medical Science.

**Assay Method** CyA concentration in whole blood, erythrocytes, and erythrocyte hemolysate was measured by a monoclonal fluorescence polarization immunoassay method employing the TDx operation system (Abbott Laboratories Diagnostic Division, North Chicago, IL, U.S.A.) according to the Abbott assay manual.¹³ Caliberation curves were generated from the net polarization values obtained by adding a known amount of CyA to CyA-free samples.

**Statistical Method** A regression was considered to be significant if the p-value for a correlation coefficient was less than or equal to the 0.05 level of significance.

**Results**

**Effects of CyA Concentration in Medium on CyA Erythrocyte Uptake** Figure 1 shows the effects of CyA concentration in 10% erythrocyte suspensions on the erythrocyte uptake of CyA. When the concentration of HSA in the medium was fixed at 0.4% using medium II (Table I), the concentration of CyA in erythrocytes after the addition of the CyA standards at final concentrations between 0.5 and 3 μM increased in a CyA concentration-dependent manner (Fig. 1a). The erythrocyte concentration of CyA at 60 s after the addition of CyA standards to the 10% erythrocyte suspensions increased nonlinearly with the rise in CyA concentration in the suspensions (Fig. 1b).

These results suggest that the concentration of CyA in erythrocytes is regulated by a concentration gradient in the extracellular fraction, and that the uptake of CyA in the erythrocyte fraction is a saturable process.

**Effects of HSA Concentration in Medium on CyA Erythrocyte Uptake and Efflux** Figure 2 shows the erythrocyte uptake and efflux of CyA in the absence of HSA (medium I) or in its presence (medium II, III) at a final CyA concentration of 1 μM. When the values of HCT in the erythrocyte suspensions were fixed at 10%, and the erythrocyte concentration of CyA at 60 s after the addition of CyA standards to the erythrocyte suspensions was reduced by HSA in a concentration-dependent manner (Fig. 2a). On the other hand, the efflux of CyA from erythrocytes was enhanced as HSA concentration in the medium increased (Fig. 2b), while the effects of changes in HSA

**Fig. 1. Time Course of CyA Erythrocyte Uptake at Different CyA Concentrations**

(a) After 5-min preincubation of 10% erythrocyte suspensions prepared using medium I at 37°C, uptake was initiated by the addition of CyA standards at final concentrations of 0.5 to 3 μM, at 37°C and pH 7.4. Key: ○, 0.5 μM; △, 1.0 μM; ■, 2.0 μM; ●, 3.0 μM. (b) Relationship between CyA concentration in erythrocytes and CyA concentration in erythrocyte suspension using medium I. Each point represents the mean ± S.D. of 3 experiments.
Fig. 2. Effects of HSA on CyA Erythrocyte Uptake and Efflux

a) After 5-min preincubation of 10% erythrocyte suspensions prepared using incubation media I, II, and III and 37°C, uptake was initiated by the addition of CyA standards at a final concentration of 1.0μM at 37°C and pH 7.4. b) After 5-min preincubation of CyA-entrapped erythrocytes at 37°C, efflux was initiated by the addition of nine volumes of incubation media I, II, and III, at 37°C and pH 7.4. Key: —○—, HA-free; —●—, 0.4%; —▲—, 0.8%. Each point represents the mean ± S.D. of 3 experiments.

Fig. 3. Effects of Food Intake on CyA Erythrocyte Uptake and Efflux

a) After 5-min preincubation of 10% erythrocyte suspensions prepared using media IV and V at 37°C, uptake was initiated by the addition of CyA standards at a final concentration of 1.0μM at 37°C and pH 7.4. b) After 5-min preincubation of CyA-entrapped erythrocytes at 37°C, efflux was initiated by the addition of nine volumes of incubation media IV and V, at 37°C and pH 7.4. Key: —○—, medium I; —●—, medium II; —▲—, medium IV; —▲—, medium V. Each point represents the mean of 3 determinations.

concentration on the transfer of CyA from erythrocytes to extracellular fraction were relatively small in comparison with the case in medium I (HSA free).

Effects of Food Intake on CyA Erythrocyte Uptake and Efflux Figure 3 shows the effects of food intake on the erythrocyte uptake and efflux of CyA at a final CyA concentration of 1μM. Where the values of HCT were fixed at 10%, in media IV and V, the concentration of ALB in plasma was adjusted to 0.4% with the incubation buffer and, therefore, the concentration of other plasma components (globulins, lipoproteins, and so on) was also about one-tenth of that in normal human plasma. The concentration of lipids, TG + CHO, in media IV and V was 0.491 and 0.811nm (mean of 3 determinations), respectively, and we recognized that the elevation of these lipid levels in plasma was due to food intake. As shown in Fig. 3a, the initial rate of CyA uptake into erythrocytes was markedly retarded in the order, medium I, medium IV, and medium V. On the other hand, CyA efflux from the erythrocytes was enhanced in the order medium V, medium IV, and medium I (Fig. 3b). However, the effects of changes in plasma components, aside from ALB, due to food intake on the transfer of CyA from erythrocytes to extracellular fraction were relatively small in comparison with the case in medium I (HSA free).

Effect of HCT on CyA Erythrocyte Uptake Figure 4 shows the effect of HCT of the erythrocyte suspensions on the erythrocyte uptake of CyA using media II and IV at a final CyA concentration of 1μM. As shown in Fig. 4a, the concentration of CyA in erythrocytes in medium II decreased with a rise in HCT, and the concentration of CyA in erythrocytes in medium IV also decreased with a rise in HCT (Fig. 4b). However, the retardant effect of HCT in medium IV was less than that in medium II.

CyA Protein Binding in Erythrocyte Hemolysate and Plasma The Rosenthal plot analysis for the membrane free erythrocyte hemolysate and 10-fold diluted plasma at 3 different storage temperatures is shown in Fig. 5. This analysis for the erythrocyte hemolysate indicates the presence of more than one site at lower storage temperatures (Fig. 5a). However, the low affinity site apparently disappeared with rises in the storage temperature. In contrast, although the Rosenthal plot analysis in 10-fold diluted plasma also showed temperature-dependent distribution, there appeared to be no affinity binding site in plasma, since the slope calculated from the binding data was equal to zero. The respective binding parameters of CyA in erythrocyte hemolysate at 3 different storage temperatures are shown in Table II. The affinity constants at high affinity binding sites (aside from those at 37°C) were 3- to 7-fold those at low affinity binding sites, while the capacity constants at low affinity sites were 2-fold those at
high affinity sites. The affinity constants at low affinity sites decreased with a rise in temperature; we were unable to estimate the affinity constant at a low affinity site at 37°C.

**Relationship between Erythrocyte CyA Concentration and Blood Biochemistry Variables in Transplant Patients**

A total of 85 samples from 15 transplant patients were analyzed. There were significant correlations between the concentration of CyA in erythrocytes and TG + CHO (sum of the value of measurements, mm), ALB, TP, and HCT in the bivariate analysis (Table III). Of these blood biochemistry variables representing changes in blood components due to a disease state, HCT was found to show the highest degree of correlation with the concentration of CyA in erythrocytes in both bivariate analysis and multiple linear regression analysis. The blood biochemistry variables representing hepatic and renal functions (GOT, GPT, T-BIL, CRE, and BUN) did not correlate with the concentration of CyA in erythrocytes. In Fig. 6, scatter diagrams of the relationships between the erythrocyte concentration of CyA and HCT, TP, ALB, and TG + CHO as a function of HCT are shown. Where the erythrocyte concentration of CyA was normalized by the measurements in whole blood to minimum plot-scattering due to CyA concentration in each whole blood sample. In clinical practice, although it is difficult to draw a definite boundary line between anemic and nonanemic patients, we found that lipid levels, TP and ALB in samples with HCT of less than 30% decreased with advanced anemia (Figs. 6b, c), while the erythrocyte concentration of CyA in these samples increased with decreased HCT (Fig. 6a). When we divided samples into two classes according to HCT, namely, an anemic group (HCT < 30%) and a nonanemic group (HCT ≥ 30%), the indices of hepatotoxicity were 31.8% (27 per total samples) and 17.9% (7 per total samples), respectively (Table IV). The chi-square test showed a correlation between HCT changes and hepatic function ($\chi^2 = 14.601, p < 0.01$).

**Discussion**

CyA, which is an extremely lipophilic substance with an octanol water partition coefficient of 991,14 is widely distributed in blood components. In plasma, about 98% is bound to proteins, 85–90% to lipoproteins, and 5–15%
Fig. 6. Relationship between Erythrocyte CyA Concentration and Blood Constituents in Transplant Patients Receiving Immunosuppressive Therapy with CyA

(a) The concentration of CyA in erythrocytes was normalized by the concentration of CyA in whole blood, and is given as a cell-to-blood concentration ratio. (b) Plasma lipid level is given as the sum of TG and CHO in plasma.

Table IV. Sample Frequency of Anemia and Hepatotoxicity

<table>
<thead>
<tr>
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<th>Anemia</th>
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<tbody>
<tr>
<td></td>
<td>(HCT &lt; 30%)</td>
</tr>
<tr>
<td>Hepatotoxicitya</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>27 (31.8)</td>
</tr>
<tr>
<td></td>
<td>19 (22.4)</td>
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- a) % sample frequency against total number of samples. b) Hepatotoxicity was judged by transaminase activity in plasma.

to other proteins. In whole blood, about 60% of the total is bound to erythrocytes. Theoretically, the pharmacological effects of a drug are more closely related to its unbound concentration in plasma than to its total concentration. However, in the case of CyA, the unbound fraction in plasma is very small because of its extremely lipophilic nature, and it exhibits marked within and between variability at various therapeutic phases. Further, direct measurements of CyA unbound concentration in plasma cannot easily be performed in routine monitoring. Recently, Legg and Rowland demonstrated that estimation of erythrocyte CyA concentration could be used to predict the unbound CyA concentration. On the basis of this quantitative chemical observation, we examined the way in which changes in physiological factors in blood affect the concentration of CyA in erythrocytes. The results described in this report indirectly show aspects of changes in the unbound concentration of CyA in blood under several physiological conditions.

The in vitro uptake study (Figs. 1a, 2a, 3a and 4) showed that the concentration of CyA in erythrocytes was affected by CyA concentration in blood samples, HSA concentration, lipid concentration in plasma and HCT. The concentration of CyA in erythrocytes increased as its concentration in the blood sample increased, whereas its concentration in erythrocytes decreased as HSA, lipids and HCT value increased. In addition, the extent of suppression of erythrocyte uptake produced by diluted plasma (media IV and V, Table I) was greater than that produced by 0.4% HSA solution (medium II, Table I) (Figs. 2a, 3a). These findings suggest that the fractions involving lipids, lipoproteins or globulins aside from ALB contributed more to the cell-to-plasma distribution of CyA than the ALB fraction. Gupta et al. reported that, in normal subjects, elevations in plasma lipid levels after food intake enhanced the total body clearance, as estimated from CyA measurements in whole blood or plasma. One reason for this increased clearance of CyA after food intake is that transport of the bounded CyA from plasma into erythrocytes is retarded by increased lipids in plasma (here, chylomicrons), and that the hepatic uptake of CyA may increase.

The results of the in vitro efflux study, shown in Figs. 2b and 3b, indicate that the transfer of CyA from erythrocytes to the extracellular fraction was not as subject to the influence of HSA, other proteins, or lipids in plasma as that from the extracellular fraction to erythrocytes. These results suggest the possibility that CyA transferred into erythrocytes may, in part, remain in the erythrocytes if the elimination of the drug from the blood circulation proceeds. To reveal the mechanism underlying the differences between erythrocyte-to-plasma and plasma-to-erythrocyte transport of CyA, it is necessary to characterize the interaction between the erythrocyte membrane and CyA.

From the Rosenthal plots shown in Fig. 5a, the presence of two classes of binding sites for CyA in erythrocyte hemolysate was expected. In addition, the affinity constants in both binding sites were reduced as the incubation temperature increased, and the low affinity site apparently disappeared with rise in temperatures. These results suggest that erythrocyte hemolysate has essentially two binding sites for CyA, however, most of the CyA in the erythrocyte hemolysate was bound to the first binding site at the physiological body temperature (37°C). In previous reports, the existence of CyA binding proteins in tissue cells, such as calf-thymus, pig-spleen, human-erythrocyte, has been recognized. At present, although it is not yet clarified whether or not these CyA binding proteins are the same, they are called cyclophilins. Using a charcoal separation method, Agarwal et al. described that the cyclophilin in erythrocytes has a temperature-dependent binding property for CyA with a monobinding aspect between 4°C and 37°C. Moreover, they confirmed that erythrocyte cyclophilin has a molecular mass of about 16000, and is distinct from hemoglobin, carbonic anhydrase, calmodulin and cytochrome b,

The conflict in relation to the number of binding sites between our results and theirs may be due to the difference in CyA concentration range used in the protein binding study: namely the concentration range they used was far higher than that we used (at final concentrations, 3.9—31.2 μM versus 8.31 × 10⁻³—8.31 × 10⁻¹ μM), and no multibinding aspect was detected. Although we have no evidential data to decide the binding
species for CyA at present, we speculate that the first affinity binding site may be cyclophilin, whereas the secondary site, which has relatively lower affinity but higher capacity binding than the first, may be a cyclophilin-like substance or other erythrocyte proteins in relation to the glycolysis pathway or maintenance of membrane structure.

Legg et al. derived physiochemically a mathematical model to account for the distribution property of CyA in renal transplant patients' plasma involving a partition rule between plasma water and lipids, and evidenced statistically that the distribution of CyA in plasma was not achieved by affinity bindings, but by a partition rule between lipoproteins and plasma water.24 Our observations, shown in Fig. 5b, are essentially in agreement with those of Legg et al., namely, no affinity binding aspect for CyA in plasma was recognized on the Rosenthal plot at three different storage temperatures. Generally, the binding phenomenon of drugs in plasma most often studied is the association of drugs with albumin, in which binding sites, often saturable process can be characterized. Since the erythrocyte uptake of CyA was suppressed by HSA as shown in Fig. 2, CyA also has an association with albumin. However, CyA distributes simply into the lipid moiety of lipoprotein molecules with a high partition ratio in plasma fraction.25 Therefore, an affinity binding of CyA in plasma may apparently be masked. Nevertheless these results, shown in Fig. 5, confirmed that the distribution of CyA in blood was regulated by two different types of distribution in the erythrocyte and plasma fractions.

In a previous study, we derived a mathematical equation to account for the binding relationships of CyA in human blood, and we predicted changes in the cell-to-plasma distribution of CyA involving the unbound CyA concentration in plasma,89 where the unbound fraction of CyA was accounted for by the affinities both in plasma and erythrocyte fraction, and the binding capacities in both fractions were decided by hematocrit and plasma lipids. The biochemical variables, HCT, TP, ALB, and TG + CHO reflect physiological changes in blood components due to patients' disease states (Fig. 6, Table IV). In the present in vitro studies, although we separately investigated the effects of several factors in blood on the erythrocyte uptake of CyA, we consider that these factors, acting together, influence the erythrocyte uptake of CyA in vivo. An increase in erythrocyte CyA concentration will be produced not only by the elevation of CyA concentration in whole blood, but also by a reduction in CyA binding species in plasma and/or in the erythrocyte fraction. Therefore, it is necessary to consider that blood components have multiple effects on the cell-to-plasma distribution of CyA.

As shown in Table IV, episodes of hepatotoxicity were accompanied by anemia in transplant populations. Indeed, it is well known that the synthesis of ALB, lipoproteins, or hematogenous functions are suppressed when hepatotoxicity is exhibited. Therefore, the main causes for elevation of CyA concentration in erythrocytes during hepatotoxicity are firstly, suppression of hepatic metabolism of the drug, and secondly, a decrease in blood constituents which regulate CyA distribution in blood. In addition, during hepatotoxicity, the secondary cause may enhance the CyA distribution to other blood cells, i.e., lymphocytes, or tissues. Hence, the concentration of CyA in erythrocytes could serve as an indicator to predict a shift of unbound CyA concentration and tissue toxicities.

In conclusion, the concentration of CyA in erythrocytes was affected by CyA concentration in the blood, plasma lipids, albumin and other proteins, and hematocrit. Further, the concentration of CyA in erythrocytes was inversely proportional to these blood chemistry variables. Namely, as the erythrocyte concentration of CyA is essentially regulated by two different types of distribution which exist in the plasma and erythrocyte fractions, the erythrocyte concentration of CyA is interlocked with the changes in these biochemical parameters when disease state-dependent changes occur in such parameters. Hence, changes in the erythrocyte concentration of CyA indirectly indicate changes in the unbound concentration of CyA. Accordingly, we are now studying the relationships between CyA measurements in erythrocytes or whole blood and these biochemical parameters to establish a drug protocol for CyA in transplant patients during episodes of toxicity.

References