Distribution and concentration of cyclosporin in human blood

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SUMMARY In patients receiving cyclosporin to minimise graft versus host disease after allogeneic bone marrow transplantation, whole blood cyclosporin concentration was roughly twice the serum concentration when blood was separated at 37°C. In turn, blood separation at 37°C resulted in a doubling of serum cyclosporin concentration compared with separation at room temperature. In vitro studies showed that the latter phenomenon was due to a temperature dependent partitioning of cyclosporin between plasma and red cells, such that increased cyclosporin was taken up from the serum into red cells at room temperature. Increasing delay in separation of patient blood (at either temperature) resulted in a gradually increasing cyclosporin serum concentration. Further in vitro studies showed that a distribution equilibrium between blood components was reached within 30 min incubation. Red cell uptake of cyclosporin was saturable at an incubation concentration of $>4 \mu\text{g/ml}$, while plasma and mononuclear cells showed a linear uptake to $7 \mu\text{g/ml}$. The cellular cyclosporin content of a mononuclear cell was roughly 1000 times greater than that of an erythrocyte. For clinical monitoring we recommend the measurement of cyclosporin concentration either in whole blood or in serum separated at 37°C without delay after venepuncture.

Cyclosporin, with its unique ability to inhibit T cell activity,^{1,2} has become an important drug in human organ transplantation. It is effective in reducing the incidence of solid organ graft rejection^{3,4} as well as bone marrow graft rejection.⁵ It is as effective as methotrexate in minimising acute graft versus host disease after marrow transplantation.⁶ Unfortunately, it is associated with nephrotoxicity,⁷⁻⁹ hepatotoxicity,^{10,11} and central nervous system toxicity.¹² Despite much effort aimed at measuring drug concentrations in serum and whole blood, relatively little information is available on either toxic or therapeutic concentrations. We report on the concentration and distribution of the drug in whole blood and blood components in man and on a number of factors that influence the concentration of cyclosporin in these components.

Material and methods

PATIENTS STUDIED

All patients were receiving cyclosporin immunosuppression to minimise graft versus host disease after allogeneic bone marrow transplantation as treat-

ment for severe aplastic anaemia or haematological malignancy. The protocol for marrow transplantation has been described previously.¹³ Blood samples were collected from patients receiving cyclosporin immediately before a regular 12 hourly dose of cyclosporin (trough concentration).

For measurement of cyclosporin serum concentration blood was allowed to clot at either room temperature or 37°C. The clotted blood was centrifuged at 1000 g for 10 min at the respective temperatures. All serum samples were stored at -20°C before radioimmunoassay.¹⁴ In our laboratory the inter-assay variation was 8.4% and the intra-assay variation 5.6%.¹⁵ To evaluate the effect of the temperature at which the blood was separated on subsequent cyclosporin serum concentration, aliquots of clotted blood were allowed to stand for 1 h at room temperature or 37°C before separation. To investigate the effect of delayed blood separation, aliquots of clotted blood were allowed to stand at room temperature or 37°C for 1, 4, 7, or 22 h intervals before separation. For measurement of whole blood cyclosporin concentration, EDTA blood was collected and stored frozen at -20°C until assayed. On the day of assay the sample was thawed, brought to room temperature, and mixed thoroughly. No intact

red cells were detectable by light microscopy after this procedure. Cyclosporin concentration was then measured by radioimmunoassay.¹⁴ Again, this was standardised such that a cyclosporin concentration as low as 30 ng/ml could be measured. The inter-assay variation was 8.9% and the intra-assay variation 5.8%.

IN VITRO STUDIES

Cyclosporin pure substance was dissolved in ethanol and Tween 80 and diluted with 0.9% sodium chloride solution to give concentrations in the range 0.625 µg/ml to 8 µg/ml. Fresh blood (anticoagulated with EDTA) from normal individuals was used. The desired amount of cyclosporin was added to whole blood at 37°C and incubated with gentle mixing for 1 h. Isolation of plasma, the mononuclear cell fraction, and the red cell/granulocyte fraction was performed on a Ficoll gradient. Two millilitres of incubated blood was diluted 1/3 with phosphate buffered saline (PBS) and the diluted blood layered carefully on to 4 ml Ficoll-Paque (Pharmacia, Sydney); the samples were then centrifuged at 400 g for 35 min. The top layer of diluted plasma was drawn off and retained for cyclosporin assay. The mononuclear cell layer was then removed, leaving the red cell pellet. Both the mononuclear layer and the red cell pellet were resuspended separately in PBS and centrifuged at 1000 g for 10 min. After two further washes, the cells were lysed to recover the cyclosporin. The mononuclear cells were resuspended in 50:50 ethanol-PBS and lysed by rapid freeze-thawing in liquid nitrogen and hot water. After centrifugation, the supernatant was retained for cyclosporin assay. The red cells were resuspended in 50:50 distilled water-ethanol and vortexed. The lysed cells were centrifuged and the supernatant was retained for cyclosporin assay.

To evaluate the effect of the temperature at which the blood was separated, diluted blood was separated on Ficoll at either room temperature or 37°C. To evaluate the effect of an increase in cyclosporin concentration during incubation, increasing concentrations were added to the incubation tube. Con-

Table 2 *Effect on serum cyclosporin concentration of reheating blood to 37°C after initial cooling to room temperature*

	Blood reheated to 37°C after cooling to room temperature*	Blood separated at 37°C without cooling
Trough serum cyclosporin concentration (ng/ml) (n = 26)	405 ± 369	401 ± 375

*Values represent mean ± 1SD. Difference is not significant.

centrations used were 0.062, 0.125, 0.25, 0.5, 1, 2, 3, 4, 6, 7, and 8 µg cyclosporin/ml blood. To evaluate the effects of the duration of incubation, the incubation period was varied: blood was incubated with cyclosporin for 30, 60, 90, or 120 min before separation on Ficoll.

STATISTICAL ANALYSIS

This was performed using the two tailed Student's t test.

Results

EFFECT OF TEMPERATURE AT WHICH BLOOD WAS SEPARATED

Trough serum cyclosporin concentration was significantly increased when blood from patients receiving cyclosporin was separated at 37°C compared with separation at room temperature (Table 1). When patient blood was reheated to 37°C before serum separation, after it had initially been allowed to cool to room temperature, the cyclosporin serum concentration was the same as that obtained in parallel samples that were separated at 37°C without cooling (Table 2). These findings were further investigated using blood from normal subjects incubated with cyclosporin in vitro (Fig. 1). The proportion of cyclosporin in plasma was lower, and the proportion of cyclosporin in red cells higher, at room temperature compared with 37°C. Thus, partitioning of cyclosporin between plasma and red cells was temperature dependent, and it appeared that some cyclosporin was taken up from the serum into red cells at room temperature.

EFFECT OF DELAYING BLOOD SEPARATION

With increasing delay in separation of patient blood after venesection there was a gradual increase in the cyclosporin serum concentration, regardless of whether the blood was separated at room temperature or at 37°C (Fig. 2).

Table 1 *Effect of temperature of blood separation on serum cyclosporin concentration*

	Temperature at which blood was separated	
	37°C	Room temperature
Trough serum cyclosporin concentration (ng/ml) (n = 12)	283 ± 155	104 ± 96

Values represent mean ± 1SD. Difference is significant (p < 0.00001).

Fig. 1 Effect of temperature at which blood was separated on distribution of cyclosporin in blood components. Blood from normal subjects incubated with cyclosporin *in vitro* was used ($n = 11$). Values represent mean $\pm$ 1SD. The difference between the two temperatures was significant for each component ($p < 0.001$).

SATURABILITY OF BLOOD COMPONENTS WITH CYCLOSPORIN

The saturability of the three blood components (plasma, red cells, and mononuclear cells) is shown in Fig. 3. Red blood cells showed an apparent linear uptake up to an incubation concentration of 4 $\mu\text{g/ml}$, after which their uptake appeared to be satur-

Fig. 2 Effect on serum cyclosporin concentration of delay in separation of blood (at either room temperature or 37°C). Blood from patients receiving cyclosporin was used ($n = 5$). Values represent mean $\pm$ 1SD. Differences between 1 h and 7 h values and 1 h and 22 h values were significant ($p < 0.05$ for room temperature values and $p < 0.1$ for 37°C values).

Fig. 3 Effect on concentration of cyclosporin in blood components of increasing concentrations of cyclosporin during incubation. Blood from normal subjects incubated with cyclosporin *in vitro* was used ($n = 4$). Values represent mean $\pm$ 1SD.

able. Both the plasma and the mononuclear cell components showed an apparent linear uptake up to 7 $\mu\text{g/ml}$. Plasma cyclosporin concentration increased more rapidly than red cell cyclosporin concentration with increasing concentrations of cyclosporin during incubation.

Fig. 4 Cyclosporin content of mononuclear cells and erythrocytes at increasing incubation concentrations of cyclosporin. Blood from normal subjects incubated with cyclosporin *in vitro* was used ($n = 4$). Values represent mean $\pm$ 1SD.

Fig. 5 Effect on cyclosporin content of blood components of increasing duration of incubation of blood with cyclosporin. Blood from normal subjects was used ($n = 3$). Values represent mean $\pm$ 1SD. Differences for different durations of incubation not significant.

CELLULAR CYCLOSPORIN CONTENT

Mononuclear cells had a cyclosporin content roughly 1000 times greater than that of erythrocytes at all concentrations of cyclosporin (Fig. 4).

EFFECT OF DURATION OF INCUBATION

There was no change in the distribution of cyclosporin between blood components when cyclosporin and blood were incubated for increasing periods of time (30–120 min) (Fig. 5). A distribution equilibrium had thus been reached within 30 min of incubation.

WHOLE BLOOD CYCLOSPORIN CONCENTRATION

Estimation of cyclosporin concentration in whole

blood and in the corresponding serum separated at 37°C was performed in 20 marrow transplant recipients. Multiple samples, most of which were drawn on consecutive days from the day of transplant, were analysed from each patient (Table 3). The proportion of cyclosporin detected in serum compared with that detected in whole blood ranged from 44% to 72% (mean $\pm$ 1 SD, 59 $\pm$ 9%).

Discussion

Although cyclosporin is an effective immunosuppressive agent in human organ transplantation, it also has considerable toxicity. In marrow transplant recipients there have been few studies showing a correlation between trough serum cyclosporin concentrations and toxicity. Barrett¹⁶ showed a correlation between trough serum cyclosporin concentration and blood urea concentration, and Hows⁸ showed a correlation between trough serum cyclosporin concentration and serum creatinine values. In the latter study, however, patients with other causes of nephrotoxicity or potential nephrotoxicity (such as aminoglycoside antibiotic treatment) were included in the analysis and make interpretation of the data difficult. No studies have yet been reported correlating serum cyclosporin values and efficacy in preventing graft versus host disease. Difficulties in the techniques for measuring cyclosporin concentration in human serum or blood may be a reason for the lack of such studies. Lemaire¹⁷ and Hows¹⁸ have previously shown that the concentration of cyclosporin in human serum is increased roughly twofold if the blood is separated at 37°C rather than at room temperature before analysis. We have confirmed

Table 3 Cyclosporin content of serum separated at 37°C and of whole blood

Patient no	No of samples	Serum cyclosporin concentration (ng/ml)	Whole blood cyclosporin concentration (ng/ml)	Serum: whole blood cyclosporin concentration ratio
81	8	962 $\pm$ 506	1412 $\pm$ 783	0.68
84	12	183 $\pm$ 131	337 $\pm$ 220	0.61
85	13	347 $\pm$ 140	606 $\pm$ 270	0.57
95	12	592 $\pm$ 425	867 $\pm$ 494	0.68
97	9	345 $\pm$ 201	567 $\pm$ 192	0.61
100	7	172 $\pm$ 259	334 $\pm$ 410	0.51
101	19	220 $\pm$ 202	444 $\pm$ 470	0.50
102	19	162 $\pm$ 102	369 $\pm$ 199	0.44
105	29	307 $\pm$ 145	609 $\pm$ 172	0.50
107	41	284 $\pm$ 195	564 $\pm$ 287	0.50
108	42	168 $\pm$ 129	339 $\pm$ 181	0.50
109	31	215 $\pm$ 170	331 $\pm$ 211	0.65
110	40	263 $\pm$ 147	445 $\pm$ 135	0.59
111	66	297 $\pm$ 261	446 $\pm$ 273	0.67
112	32	308 $\pm$ 161	535 $\pm$ 244	0.58
113	29	385 $\pm$ 396	532 $\pm$ 509	0.72
114	41	461 $\pm$ 306	665 $\pm$ 329	0.69
115	21	284 $\pm$ 242	500 $\pm$ 260	0.59
116	24	436 $\pm$ 269	622 $\pm$ 248	0.70
117	14	207 $\pm$ 121	429 $\pm$ 185	0.48
Mean		330 $\pm$ 185	548 $\pm$ 245	0.59 $\pm$ 0.09

Values represent mean $\pm$ 1SD.

this in our study and have shown that this is due to uptake of cyclosporin by red cells at room temperature. We have also shown that increasing time before separation of the blood results in an increased serum cyclosporin concentration, probably due to release of cyclosporin from haemolysed red cells during the delay.¹⁹ In contrast to previous reported practice,¹⁹ however, we would recommend either that human blood be separated at 37°C without delay after venepuncture (rather than storing it for 2–3 h at room temperature) before separation and measurement of the serum cyclosporin concentration, or that whole blood cyclosporin concentration be measured. Separation at 37°C without delay minimises the uptake of cyclosporin by red cells, thus allowing a closer approximation to in vivo serum values. Such a policy would at least lessen confusion caused by two factors—the temperature dependence of serum cyclosporin concentration and the increased cyclosporin concentration due to delay in separation. If such practice is not possible, similar results can be obtained by rewarming the whole blood sample to 37°C before separation. Once separated, the serum can be stored at –20°C with impunity for several months until the time of assay.¹⁹ The measurement of whole blood cyclosporin concentrations would avoid all these difficulties, but, again, no correlation between whole blood concentrations, toxicity, and efficacy is available.

In vitro studies showed that the distribution of cyclosporin between the three major components of the blood (plasma, erythrocytes, and mononuclear cells) depends on the temperature at which the blood was separated, the concentration of cyclosporin present during the incubation, but not on the duration of incubation. Of the three components, only the erythrocytes appeared saturable in the concentration range tested and only then at a concentration higher than would normally occur in vivo. A mononuclear cell contained much more cyclosporin than an erythrocyte. Work is in progress to determine the cyclosporin content of the T cell, T cell subsets, and non-T cell components of mononuclear cells in order to determine if any of these parameters are useful for clinical monitoring.

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