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Distribution and Protein Binding of FK506, a Potent Immunosuppressive Macrolide Lactone, in Human Blood and Its Uptake by Erythrocytes

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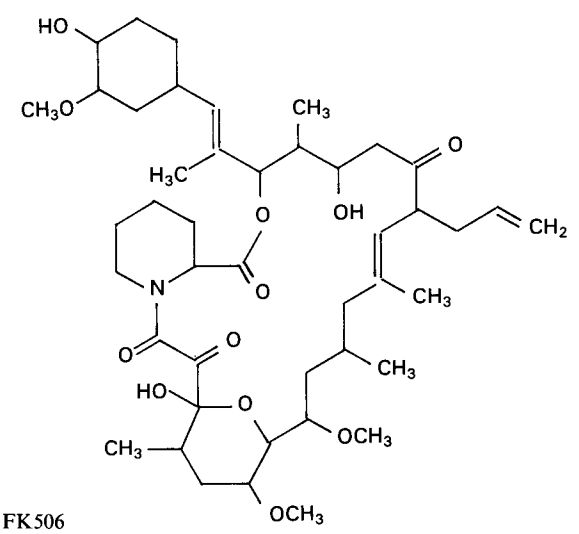
Abstract—The distribution of FK506 in the blood was estimated in-vitro. At a drug level of 5 ng mL^{-1}, FK506 mainly distributed in erythrocytes (95–98%) in dog, monkey and human blood, and its distribution was affected by drug concentration, temperature, and haematocrit values. In erythrocytes most of FK506 was distributed in cytoplasmic components and was bound strongly to a protein having a molecular weight of 10–11 kDa. The molecular weight of this protein agrees with FK506-binding protein found in various cells. Greater than 98·8% of FK506 was bound to the plasma proteins in all species studied. FK506 bound to various plasma proteins such as lipoproteins, globulins, α_1-acid glycoprotein and albumin.

FK506 is a macrolide lactone isolated from *Streptomyces tsukubaensis* and has strong immunosuppressive activities in-vitro and in-vivo (Thomson 1990; Goto et al 1991). FK506 is undergoing clinical trials as an immunosuppressant for organ transplantation and autoimmune diseases (Fung et al 1991; Japanese FK506 study group 1991a).



FK506

After intravenous injection or oral dosing of FK506 to rats, the blood/plasma concentration ratio varies with FK506 concentration. When the FK506 concentration in the whole blood is over 100 ng mL^{-1}, the ratio is less than 1, and conversely, when the FK506 concentration is less than 100 ng mL^{-1} the ratio is more than 1 (Iwasaki et al 1991). This suggests that FK506 has a higher affinity for blood cells than for plasma, and that its distribution between the plasma and blood cells is affected by its concentration.

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Cyclosporin A, which is similar to FK506 in its mechanism of immunosuppressive action and poor solubility in water, is essentially taken up by erythrocytes (58%) with the fractions present in leucocytes and plasma being 9 and 33%, respectively (Lemaire & Tillement 1982). In plasma, the protein binding of cyclosporin A is 90–95% and more than two-thirds of plasma-bound drug is distributed among lipoprotein fractions because of its lipophilicity (Lemaire & Tillement 1982; Sgoutas et al 1986). In erythrocytes cyclosporin A binds to a protein (cyclophilin) which has a molecular mass of about 16 kDa.

The FK506-binding protein is distinct from cyclophilin, and is widely distributed in different types of cells (Siekierka et al 1990).

In this study we investigated the distribution and plasma protein binding of FK506 in blood, the effect of several factors on its blood/plasma concentration ratio, and the nature of FK506 in erythrocytes.

Materials and Methods

Compounds

FK506 was biosynthesized in Fujisawa Pharmaceutical Co. Ltd (Osaka, Japan). [^{3}H]Dihydro-FK506 was prepared at Amersham International (Buckinghamshire, UK) by catalytic reduction of FK506 with $^3H_2$ gas.

Plasma protein binding

Freshly heparinized blood was collected from male Sprague-Dawley rats, male beagle dogs, male cynomolgus monkeys and healthy male volunteers. Plasma was obtained by centrifugation at 1500 *g* for 10 min. For determining the binding of FK506 with total plasma proteins, FK506 was incubated in plasma for 30 min at 37°C. Bound and free FK506 were separated by centrifugation of plasma (Centrifree MPS-3, Amicon Corporation, Beverley, MA, USA) at room temperature for 15 min at 1500 *g*. The extent of non-specific binding of FK506 to the membrane of Centrifree was checked; when FK506 was dissolved in protein-

free plasma ultrafiltrate, adsorption to the membrane was negligible.

Additionally the binding of FK506 to specific proteins was studied. Human albumin (Cohn Fraction V, Sigma Chemical Co., St Louis, MO, USA), human globulin (Cohn Fraction II, III, Sigma Chemical Co.) and human α_1-acid glycoprotein (Cohn Fraction VI, Sigma Chemical Co.) were dissolved in protein-free plasma ultrafiltrate to prepare physiological concentrations of protein solutions. Human very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) were obtained by ultracentrifugation according to the method of Havel et al (1955). The protein content of each lipoprotein fraction was determined by the method of Lowry et al (1951) and diluted to physiological concentration with protein-free plasma ultrafiltrate. Centrifugal conditions of the separation of free from bound drug were adjusted to make the volume of filtrate within 20% of the sample volume.

The concentration of FK506 in the plasma and filtrate was determined by extraction of FK506 from the sample into dichloromethane and by competitive enzyme immunoassay using anti-FK506 mouse monoclonal antibody (Tamura et al 1987); binding of FK506 to plasma protein was then calculated.

Distribution in plasma lipoproteins

FK506 was incubated in human plasma for 30 min at 20°C. Lipoproteins were separated by ultracentrifugation according to the method of Havel et al (1955).

The VLDL ($d < 1.019$), LDL ($1.019 < d < 1.063$), HDL ($1.063 < d < 1.21$) and remaining lipoprotein deficient fraction (LPDF) were separated and purity was checked by agarose and cellulose acetate electrophoresis. The concentration of FK506 in each fraction was determined by enzyme immunoassay as described above.

Determination of plasma binding protein

Plasma binding protein of FK506 was examined by HPLC gel filtration using TSK-GEL G3000SW column (7·5 × 300 mm, Tosoh Corporation, Tokyo, Japan).

Human plasma containing 5 ng mL^{-1} [^{3}H]dihydro-FK506 was incubated for 30 min at 37°C. Plasma samples were injected onto the column equilibrated with buffer containing 100 mM Na_2SO_4, 20 mM NaH_2PO_4, pH 6·8, 5 mM 2-mercaptoethanol and with or without 0·05 ng mL^{-1} [^{3}H]dihydro-FK506. The proteins were eluted with the equilibrating buffer at a flow rate of 1 mL min^{-1}. Fractions (250 μL) were collected and radioactivity was counted. Protein was monitored by measurement of the absorbance at 280 nm.

Distribution in whole blood

The blood containing various concentrations of FK506 was incubated for 1 h at 37°C. After the incubation, the whole blood was separated into fractions containing plasma, leucocytes and erythrocytes by centrifugation in a discontinuous density gradient with Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). To the incubated blood was added the same volume of the diluting medium (310 mOsm, 20 mM Tris-Hanks solution, pH 7·4, d = 1·009 g mL^{-1}). The diluted sample (1 mL) was carefully layered on 6 mL Percoll discontinuous density solution (from bottom to top; 1·095, 1·090, 1·080 and 1·065 g mL^{-1}). These samples were then centrifuged at 400 *g* for 30 min at 20°C. Fractions I to V were collected from the top to bottom of the tube. Fraction I, the top layer, and fraction V, the bottom phase, contained mainly plasma and erythrocytes, respectively. Intermediate fractions II, III and IV containing mainly leucocytes were collected from the interface between two different densities.

The numbers of erythrocytes, leucocytes, mononucleocytes and granulocytes were counted by conventional methods. The concentration of FK506 in the whole blood and isolated fraction was determined by enzyme immunoassay described above.

Effect of temperature and haematocrit on blood/plasma concentration ratio

Human blood was incubated with FK506 at various temperatures for 30 min and the plasma was separated by centrifugation at the same temperature. Human blood was diluted with plasma obtained from the same volunteer to obtain haematocrits of 45, 36, 27 and 18%, and incubated with FK506 at 25 and 37°C for 30 min; the plasma was separated at the same temperature.

The concentrations of FK506 in the whole blood and plasma were determined.

Distribution in erythrocytes

Human blood was incubated with [^{3}H]dihydro-FK506 at a concentration of 100 ng mL^{-1} blood for 30 min at 37°C, and then separated into plasma, blood cell membrane and cytosol. The erythrocytes were washed three times in physiological saline solution, lysed with 20 vol 10 mM Tris-HCl (pH 7·4), containing 5 mM 2-mercaptoethanol, and kept on ice for about 1·5 h. Haemolysate was centrifuged at 20 000 *g* for 30 min at 4°C. The pellet (blood cell membrane) was washed 4 times by the same method. The radioactivity in each fraction was counted using a Packard LSC-1500 liquid scintillation counter (Downers Grove, IL, USA).

Binding of [^{3}H]dihydro-FK506 to cytoplasmic components was assessed by HPLC gel filtration using a TSK-GEL G3000SW column (7·5 × 300 mm, Tosoh Corporation). One hundred microlitres of the cytosol was injected and eluted with buffer containing 100 mM Na_2SO_4, 20 mM NaH_2PO_4, pH 6·8, and 5 mM 2-mercaptoethanol at a flow rate of 1 mL min^{-1}. Fractions (250 μL) were collected and radioactivity was counted.

Results

Protein binding and distribution in plasma

FK506 was highly bound to plasma proteins, at a drug level of 5 or 50 ng mL^{-1} (Table 1), and to human serum, indicating that heparin had no effect on binding of FK506.

α_1-Acid glycoprotein and HDL exhibited the highest binding rates among the proteins studied, but for all specific plasma proteins the binding rates were lower than that of total plasma proteins.

Distribution of FK506 in plasma lipoproteins is shown in Table 2. Contamination by other lipoproteins of each lipoprotein fraction collected by ultracentrifugation was negligible and little albumin was detected in the lipoprotein fractions (data not shown).

Table 1. Binding of FK506 to plasma protein.

Protein	Protein concn (mg mL^{-1})	Protein binding (%) Concn added (ng mL^{-1}) 5	50
Rat plasma	—	>99·1	99·4±0·1
Dog plasma	—	>98·8	99·1±0·1
Monkey plasma	—	>98·8	99·0±0·1
Human plasma	—	⩾98·8	98·9±0·1
Human serum	—	⩾98·5	98·8±0·1
Human albumin	40	—	80·5±0·5
Human globulin	40	—	93·8±0·1
Human α_1-acid glycoprotein	1	—	96·6±0·4
Human VLDL	0·88	—	88·0±1·3
Human LDL	2·0	—	94·6±0·6
Human HDL	2·0	—	96·3±0·4

Values represent means±s.e.m. of three individuals.

The binding of FK506 to the various plasma proteins after fractionation by HPLC gel filtration is shown in Fig. 1. Plasma proteins were eluted from the column in three main peaks, I (VLDL, LDL), II (HDL, globulin), and III (albumin, α_1-acid glycoproteins). In the absence of FK506 equilibration, the major part of the radioactivity was eluted in a broad peak after fraction number 60 as free drug. When the column was equilibrated with FK506, radioactivity was eluted in at least four peaks, indicating that FK506 was bound to various proteins. These results show good agreement with data shown in the distribution in plasma lipoprotein and the binding of the specific protein. Furthermore, a radioactive peak was observed in fraction number 41 which eluted behind protein peak III. This radioactive peak was also found in the absence of FK506 equilibration as a small peak. The position of this peak corresponds to a molecular weight of 10–11 kDa, similar to that of the FK506 binding protein described by Siekierka et al (1989).

Distribution in whole blood

Distribution of FK506 in the whole blood was studied at a drug level of 5 ng mL^{-1} and is shown in Table 3.

More than 95% of FK506 was distributed in the erythrocyte fraction of dog, monkey and human blood, while less than 2% of FK506 was detected in plasma of these species. In rat blood, about 50, 35 and 15% of FK506 were detected in mononucleocytes, erythrocytes and plasma, respectively.

Distribution of FK506 in human blood was investigated as a function of drug concentration (Table 4). The percentages in the plasma differed only marginally at concentrations between 5 and 50 ng mL^{-1} of blood but sharply increased at 500 ng mL^{-1}. These changes in human blood were much smaller than in rats at concentrations between 5 and 500 ng mL^{-1} (Iwasaki et al 1991).

Table 2. Distribution of FK506 in plasma lipoproteins of human blood.

Concn added (ng mL^{-1})	Distribution of FK506 (%) VLDL	LDL	HDL	LPDF
5	3·5±0·2	10·9±0·3	29·0±1·4	56·6±1·4
50	6·2±0·6	11·8±0·8	31·6±1·5	50·3±2·7

Values represent means±s.e.m. of three individuals.

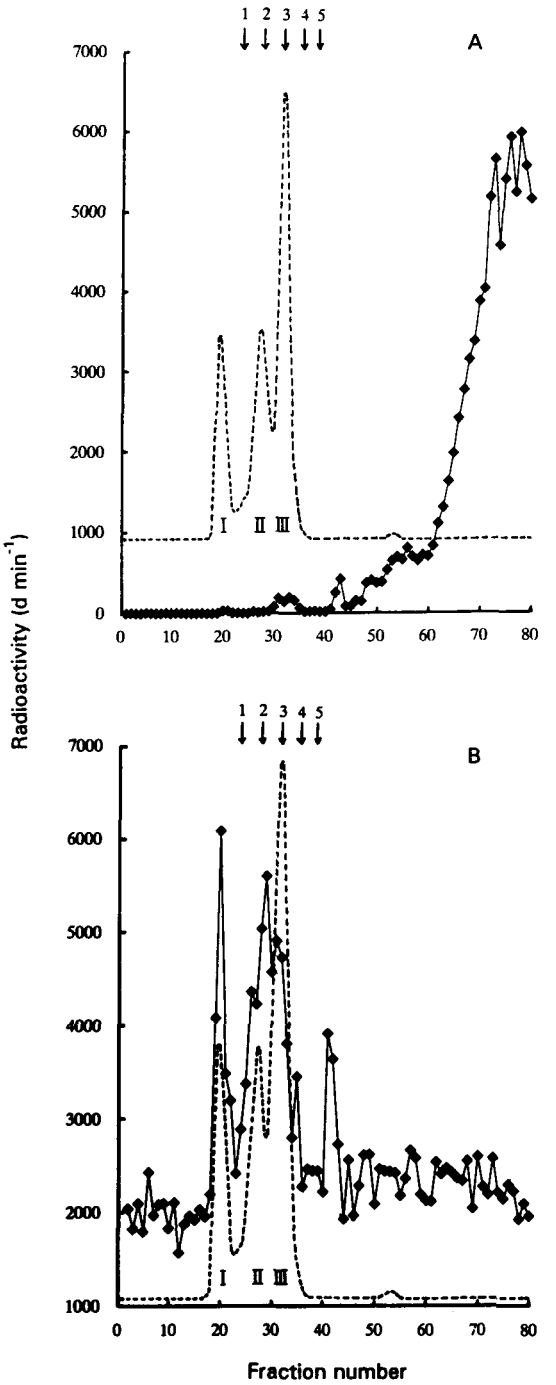

FIG. 1. Elution profile of human plasma containing [^{3}H]dihydro-FK506. A. Human plasma containing 50 ng mL^{-1} [^{3}H]dihydro-FK506. B. Human plasma containing 5 ng mL^{-1} [^{3}H]dihydro-FK506, after equilibration with FK506. ◆ Radioactivity; - - - - absorbance at 280 nm. The arrows mark the eluting positions of the protein markers as follows: 1, 290 kDa; 2, 142 kDa; 3, 67 kDa; 4, 32 kDa; 5, 12·4 kDa.

The effect of temperature of whole blood incubation and plasma separation on distribution of FK506 in human blood is shown in Table 5.

The effect of the haematocrit value on distribution of FK506 in human blood is shown in Table 6.

Table 3. Distribution of FK506 in rat, dog, monkey and human blood at a concentration of 5 ng mL^{-1}.

	Distribution (% of drug mass in each fraction)			
	Rat	Dog	Monkey	Human
Fraction I	14·92±1·06 (P)	0·86±0·43 (P)	1·30±0·76 (P)	1·89±0·34 (P)
II	46·39±1·85 (M)	3·31±1·70 (M)	1·41±0·71 (M)	n.d. (M)
III	5·24±3·08 (M+G)	n.d. (G)	n.d. (M)	n.d. (G)
IV	n.d. (M+G)	n.d. (G)	69·11±10·07 (E+G)	n.d. (G)
V	33·44±3·64 (E)	95·82±2·12 (E)	28·18±11·36 (E)	98·11±0·34 (E)

Values represent means±s.e.m. of three individuals. n.d.: not detected. In parenthesis: P, plasma; M, mononucleocytes consisting of monocytes and leucocytes; G, granulocytes; E, erythrocytes.

Table 4. Distribution of FK506 in human blood as a function of drug levels in whole blood.

	Distribution (% of drug mass in each fraction)		
	Blood concn (ng mL^{-1})		
Fraction	5	50	500
I Plasma	1·89±0·34	1·06±0·14	17·94±1·31
II Mononucleocytes	n.d.	2·81±0·25	5·77±0·80
III Granulocytes	n.d.	0·63±0·08	1·57±0·31
IV Granulocytes	n.d.	1·15±0·35	1·14±0·42
V Erythrocytes	98·11±0·34	94·35±0·10	73·58±2·07

Values represent means±s.e.m. of three individuals. n.d.: not detected.

Distribution of dihydro-FK506 in erythrocytes

Subcellular distribution of [^{3}H]dihydro-FK506 was investigated at a drug concentration of 100 ng mL^{-1}; 94% of the [^{3}H]dihydro-FK506 was distributed in the cytoplasmic compartment and only 6 and 0·05% were distributed in the plasma and blood cell membrane, respectively.

Binding of [^{3}H]dihydro-FK506 to cytoplasmic components was assessed by HPLC gel filtration. A single peak of radioactivity was observed eluting at a position corresponding to a molecular weight of 10–11 kDa (Fig. 2). The molecular weight of this binding protein was in good agreement with that of the binding protein in plasma eluted in fraction number 41 and the FK506-binding protein of Siekierka et al (1989). When the complex of [^{3}H]dihydro-FK506 and the binding protein was incubated with a 200-fold molar excess of unlabelled-FK506, [^{3}H]dihydro-FK506 was displaced by unlabelled-FK506 and radioactivity was detected in the late-eluting free fraction; incubation with cyclosporin A had no effect on the complex. These findings suggest that this binding protein is specific for FK506.

Table 5. Effect of temperature of whole blood incubation and plasma separation on distribution of FK506 in human blood.

Concn added (ng mL^{-1})	Incubation and separation temperature (°C)	Plasma concn (ng mL^{-1})
20	1 (on ice)	0·25±0·03[a] (0·63)
20	10	0·28±0·03 (0·70)
20	20	0·27±0·02 (0·68)
20	25	0·24±0·05 (0·60)
20	30	0·38±0·06 (0·95)
20	37	0·52±0·10 (1·30)
200	1 (on ice)	8·9±1·6 (2·23)
200	10	5·2±1·3 (1·30)
200	20	7·3±1·1 (1·83)
200	25	8·7±0·7 (2·18)
200	30	14±1 (3·50)
200	37	22±4 (5·50)

[a] Values represent means±s.e.m. of three healthy volunteers. Values in parentheses are % of drug mass in plasma fraction. Mean (s.e.m.) of haematocrit values of these volunteers was 0·50 (0·02). Whole blood containing FK506 was incubated at temperatures indicated in the table for 30 min and plasma was separated at the same temperature.

Table 6. Effect of haematocrit on distribution of FK506 in human blood.

		Plasma concn (ng mL^{-1})			
		Incubation and separation temperature			
Concn added (ng mL^{-1})	Haematocrit (%)	25°C		37°C	
20	44·7±0·9[a]	0·19±0·01	(0·53)	0·61±0·12	(1·69)
20	35·7±0·7	0·29±0·06	(0·93)	0·71±0·09	(2·28)
20	26·8±0·5	0·38±0·05	(1·39)	1·1±0·2	(4·03)
20	17·9±0·4	0·68±0·03	(2·79)	1·5±0·3	(6·16)
200	44·7±0·9	5·7±0·7	(1·58)	20±5	(5·53)
200	35·7±0·7	16±2	(5·14)	24±2	(7·72)
200	26·8±0·5	63±5	(23·06)	76±5	(27·82)
200	17·9±0·4	110±0	(45·16)	97±7	(39·82)

[a] Values represent means±s.e.m. of three healthy volunteers. Values in parentheses are % of drug mass in plasma fraction. Whole blood containing FK506 was incubated at temperatures indicated in the table for 30 min and plasma was separated at the same temperature.

Discussion

FK506 was highly bound to plasma protein and its rate of binding (>99%) was higher than that of cyclosporin A (90~95%). The distribution pattern of FK506 in lipoprotein was parallel with the extent of binding to the separated lipoproteins. Maximal plasma concentration of FK506 after intravenous or oral administration to kidney-transplant patients ranged from 0·4 to 48 ng mL^{-1} (Japanese FK506 study group 1991b). For complete characterization of pro-

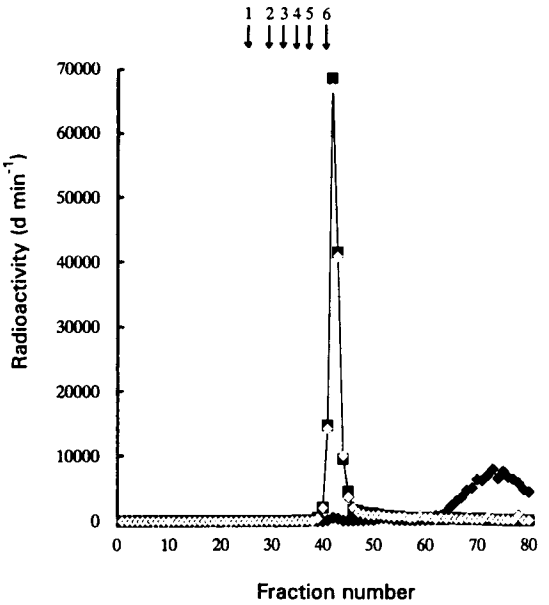

FIG. 2. HPLC gel filtration of human erythrocyte lysate containing [^{3}H]dihydro-FK506. ■ Erythrocyte lysate and [^{3}H]dihydro-FK506, ◆ erythrocyte lysate, [^{3}H]dihydro-FK506 and 200-fold molar excess of unlabelled FK506, ◇ erythrocyte lysate, [^{3}H]dihydro-FK506 and 200-fold molar excess of cyclosporin A. The arrows mark the eluting positions of the protein markers as follows: 1, 290 kDa; 2, 142 kDa; 3, 67 kDa; 4, 32 kDa; 5, haemoglobin; 6, 12·4 kDa.

tein binding of FK506, lower concentrations than those used in this study would have been desirable. However, FK506 is so highly bound to plasma protein that accurate determination of the binding characteristics at lower concentrations is not possible, even when using the very sensitive method in this study.

The amount of binding protein in erythrocytes seems to be less than in the plasma; consequently the role of FK506-specific binding protein in plasma protein binding would be of minor importance.

Maximal whole blood concentrations of FK506 after intravenous and oral administration to kidney-transplant patients ranged from 12 to 160 ng mL^{-1} (Japanese FK506 study group 1991b). In our experiment on distribution in whole blood, therapeutic concentrations (5–500 ng mL^{-1}) were studied. More than 95% of FK506 was distributed in erythrocytes of dog, monkey and human blood.

The distribution of FK506 in erythrocytes is much higher than in leucocytes, but as the number of erythrocytes in the whole blood is 300 to 800 times more than that of leucocytes, the distribution of FK506 per cell would be higher in leucocytes than in erythrocytes. This binding of FK506 to leucocytes may correlate with the biological effect in-vivo.

The blood/plasma ratio of FK506 was affected by FK506 concentration temperature of blood handling and haematocrit values, as was cyclosporin A (Lemaire & Tillement 1982; Niederberger et al 1983). The nonlinearity of distribution confuses blood level monitoring. It is difficult to decide whether levels should be assayed in whole blood or in plasma, because there is little information relating whole blood or plasma concentration to immunosuppression and toxicity. However, it has recently been reported that the whole blood concentration of FK506 correlated more closely with immunosuppression and toxicity than plasma concentration in kidney-transplanted patients (Japanese FK506 study group 1991b).

In this study, we have shown that FK506 in erythrocytes is mainly bound to a protein having a molecular weight of 10–11 kDa, not to haemoglobin which is a major protein in erythrocytes. Similarly, it has been reported that cyclosporin A binds to an intra-erythrocytic protein with a molecular weight of 16 kDa (Agarwal et al 1986; Foxwell et al 1988). The binding protein of FK506 is specific to FK506, indicating that binding proteins of FK506 and cyclosporin A are distinct from each other.

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