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Constitutive and acquired resistance to calcineurin inhibitors in renal transplantation: role of P-glycoprotein-170

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Abstract The present study examines whether resistance to Cyclosporin A (CyA) and Tacrolimus (FK506) develops in T cells from individual patients and the role of P-glycoprotein 170 (P-gp) in mediating drug resistance. IC_{50}s were established for CyA and FK506 in cell cultures from 46 renal allograft recipients. P-gp expression and functional activity were determined by flow cytometry. Mean ID_{50} for CyA was 29 μg/li (range 2.5–100) and for FK506 1.2 μg/li (range 0.085–5.5). The sensitivities to the two drugs were correlated ($P = 0.0001$). There was variation in the ratio of the ID_{50}s depending on the drug used for treatment ($P = 0.02$). There was no difference in P-gp expression and functional activity in patients with sensitive or resistant cells. The data indicate an association between the sensitivities to CyA and FK506 and evidence of selective resistance to whichever drug was used. P-gp drug transport does not explain this variation.

Key words Cyclosporin A · FK506 · Lymphocyte sensitivity · P-glycoprotein-170

Introduction

Renal transplantation is the treatment of choice for patients with end-stage renal failure. Kidney graft survival is approaching 90% at 1 year, due largely to improvements in anti-rejection treatment. Cyclosporin A (CyA) has formed the basis of transplant drug regimes since the early 1980s, however, its use has been limited by breakthrough rejection, nephrotoxicity and other adverse events. More recently, tacrolimus (also known as FK506, Tac and Prograf) has been introduced. It is now widely used to salvage grafts where there is an episode of ongoing acute rejection despite treatment with high dose corticosteroids and anti-lymphocyte preparations and apparent adequate levels of CyA [8]. It is also increasingly used as a first line agent with comparable patient- and graft survival to CyA and lower early rejection episodes [13]. Adverse effects have been observed with both drugs, particularly nephrotoxicity, hypertension, and development of diabetes mellitus [13].

The choice of immunosuppressive drugs for particular patients is largely an empirical clinical decision. Immunosuppressive protocols vary between centres with typical regimens which include CyA, azathioprine and prednisolone ('triple therapy'); CyA and prednisolone; and CyA monotherapy. Mycophenolate mofotil has largely replaced azathioprine in many US centres. Uncertainties in prescribing persist since it is not clear which drug is the most suitable for an individual patient, nor is the optimal dose clear that is required to prevent rejection and reduce the side effects.

In view of the efficacy of tacrolimus in salvaging grafts with apparent CyA failure, it would be beneficial to be able to predict those patients most at risk of CyA failure due to relative drug resistance. There is a supposition that this breakthrough-rejection represents CyA failure, despite apparently therapeutic blood levels of this drug. However, cyclosporine failure has not been demonstrated experimentally and remains a clinical observation. It would also be of value to assess the development of relative drug resistance occurring during

treatment. Previous reports have examined in vitro lymphocyte responsiveness to cyclosporine, prednisolone and azathioprine in preoperative recipients in mixed lymphocyte cultures, and also the pretransplant sensitivity to steroids of mitogen-stimulated recipient lymphocytes[4, 10]. In this study, we sought to compare the effects of both CyA and FK506 on lymphocyte responsiveness in vitro using the mitogen, Concanavalin A (Con A). This system is reproducible, and the maximum stimulus allows the pharmacological effects of both drugs to be defined.

The mechanisms of action of CyA and FK506 have been described qualitatively, but the mechanisms of quantitative inter- and intra-patient variation in their immunosuppressive action are poorly described, and previous work has concentrated on the absorption and elimination of the drugs, rather than their potency at lymphocyte level. Similarities in lymphocyte responsiveness could be expected since, although chemically unrelated, FK506 and CyA exert nearly identical biological effects in cells by inhibiting the same subset of early calcium associated events involved in lymphokine expression [17].

Alternatively, differences in sensitivities could be anticipated if, for example, the affinity of each drug for its respective binding protein were significantly different, or plasma levels of the binding protein, as demonstrated with FK506, increased rapidly, leading to reduced efficacy [18]. However, both CyA and FK506 are substrates for P-glycoprotein 170 (P-gp), the product of the MDR1 gene in man [20, 16]. P-gp acts as an efflux pump that extrudes hydrophobic drugs out of cells, reducing their intracellular concentration and subsequently their efficacy. This has been extensively studied in association with cancer chemotherapy with the introduction of specific P-gp inhibitors, such as PSC 833, in clinical trials [2]. P-gp is known to be expressed on a variety of solid human tissues, the cells of which perform specific secretory functions, and normal lymphocytes have been shown to express P-gp [14]. At present it is unclear what role P-gp may have in solid organ transplantation.

Since current drug usage assumes all patients have equivalent sensitivities to CyA or FK506 and have no scientific explanation for breakthrough rejection despite therapeutic blood levels of drug, we have investigated individual patients' sensitivities to the drugs in terms of the effects on their target cells, the T-lymphocytes and the role of P-gp in effecting this. Assessing whether lymphocyte responses in vitro could predict if individuals demonstrate variability in the sensitivity of their responses to these drugs, may allow future planning of rational anti-rejection drug prescribing.

Subjects and methods

Drugs and reagents

CyA (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in a 9:1 ethanol/tween (Sigma Chemicals, Poole, UK) which was subsequently diluted with an equal volume of distilled water to give a 10 mg/ml stock solution which was stored frozen in aliquots. An FK506 (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) solution of 0.1 mg/ml was prepared and stored in a similar manner. PSC 833 (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in ethanol and subsequently diluted with an equal volume of sterile distilled water to give a 10 mg/ml stock solution which was stored at 40 °C. Tissue culture medium was RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% foetal calf serum (Gibco-BRL). Lymphocyte separation medium was Lymphoprep (Nycomed Pharma, Oslo, Norway). Round-bottomed 96-well tissue culture plates were purchased from Costar Inc (Badhoevedorp, The Netherlands). Con A was from Sigma Chemicals. ^{3}H thymidine (thy) was purchased from Amersham Radiochemicals (Amersham, UK). Rhodamine-123 (R-123) (Sigma, St. Louis, MO, USA), a fluorescent dye known to be transported by P-gp, was dissolved in Phosphate Buffered Saline (PBS) to give a stock solution of 1 mM which was stored in the dark at 4 °C. 12-well tissue culture plates were purchased from Costar Inc (Badhoevedorp, The Netherlands).

Antibodies

Monoclonal antibodies to external epitopes of P-gp were used. These were Mouse anti-Human 4E3-fluorescein isothiocyanate (-FITC) conjugated, IgG2a isotype, (Signet Labs. Inc, Glasgow, UK) and Mouse anti-Human MRK16, IgG2a isotype, (Kamiya Biomedical Company, Seattle, WA, USA). Mouse IgG 2a isotype control antibody was purchased from Sigma, St. Louis, MO, USA. Goat anti-Mouse IgG-FITC was used as secondary antibody (I.D. Labs. Inc, Glasgow, UK). CD4-phycoerythrin (PE) and CD8-PE were used to characterise cell subpopulations (Becton Dickinson, San Jose, CA, USA)

Cell line

The Adriamycin drug-resistant breast carcinoma cell line MCF-7/ADR was maintained in house and used as a positive control in P-gp experiments.

Patients

46 patients with renal transplants were selected at random from those treated at the Walsgrave Hospital Renal Unit. The majority of these patients were on "triple therapy": CyA or FK506 with azathioprine and prednisolone; 3 were not receiving prednisolone. Ages ranged from 23 to 73 years (mean 40 years) with 31 male and 15 female. Samples were taken 1–77 months post transplant (mean 19 months). Blood was taken in each patient in the morning and prior to ingestion of next dose of Cy A or FK506. The patients were entered into the study, which was approved by the local research ethics committee, after giving informed consent.

Preparation of peripheral blood mononuclear cells (PBMC)

Fresh venous blood collected into EDTA-tubes was mixed with an equal volume of culture medium and layered onto Lymphoprep and centrifuged 30 min at 1500 RPM. The interface layer was separated, washed, and cryopreserved in culture medium with 10% dimethylsulphoxide.

PBMC culture and incorporation of ^{3}H thy

PBMC aliquots from 32 patients were thawed rapidly, washed twice with culture medium to free them of dimethylsulphoxide, and a viable count was performed. If viability was below 90% (which was very rare) the cells were discarded. The cell concentration was adjusted to 10^6/ml, and the suspension was placed in 200 °µl aliquots into the wells of tissue culture plates. Six wells were reserved as negative controls. To a further 6 positive controls was added Con A alone to a final concentration of 5 µg/ml: to the remaining wells, Con A plus graded concentrations of the two drugs were added, in triplicate. Final concentrations of CyA were 1, 3, 10, 30, 100 and 300 µg/l and of FK506 were 0.1, 1, 3, 10, and 30 µg/l.

PBMC from a further 14 patients were treated in the same way, except that 2 µmolar PSC 833 was added along with either CyA (n = 9) or FK506 (n = 5). Controls with PSC 833 only were also established. After 48 h culture, 1 μC_i/ml ^{3}H thy was added to each well, the culture was continued overnight to allow incorporation, and then the cells were harvested to glass fibre pads, washed, and the incorporated ^{3}H counted. The concentration of drug required to inhibit 50% of ^{3}H thy incorporation (ID_{50} value) was established for all patients for both drugs by interpolation from graphs of incorporation against drug concentration.

P-gp expression

The percentage of total cells expressing surface P-gp was determined by immunofluorescence techniques in those patients that were defined as either drug sensitive or drug resistant, following the PBMC cultures. Briefly, cells were washed twice in ice-cold PBS. 50 µl aliquots (5×10^5 cells) were incubated with either 40 µl of the undiluted 4E3-FITC Mab (5 µg/assay) or 50 µl of 1:10 dilution of MRK16 Mab (2.5 µg/assay), as per manufacturers instructions, for 30 min on ice. After incubation, the cells were washed thoroughly in PBS and the secondary antibody (50 µl of 1:25 dilution of Goat anti-Mouse) was added in the case of MRK16, and incubated for 15 min on ice. Further washes in ice-cold PBS were performed, and the samples were resuspended in PBS and kept at 40 °C in the dark until analysis.

Control isotype antibodies at the same concentration as the test antibodies were treated in the same way.

Rhodamine efflux

Aliquots containing approximately 5 × 105 cells loaded with R-123 by incubating for 30 min at 37 °C with R-123 (final concentration 2 µmolar). After loading, the cells were washed and resuspended in complete RPMI-1640 in the presence or absence of PSC 833 (final concentration 2µmolar) and incubated for a further 4 h. At time points 0 and 4 h a sample was taken for study, and the cells washed and kept in ice-cold PBS in the dark until analysis. In some cases the samples were then incubated with anti-CD4-Phycoerythrin (PE) and CD8-PE Mab to examine which subsets of T cells are actively pumping the dye. Samples were analysed on a FACStar flow cytometer (Becton Dickinson), equipped with a 488 nm argon-ion laser and standard optical setup. 10000 events were analysed with Consort 30 software (Becton Dickinson) and subsequently analysed with DAKO Flowmate II for Windows software. The fluorescences were measured on a four-decade logarithmic scale. Manual gating via scattergram on Forward Scatter (cell size) versus Side Scatter (cell granularity) identified the cell population of interest. P-gp staining results are expressed as mean fluorescence intensity (MFI), and the percentage of cells actively effluxing R-123 is estimated from histograms obtained for each sample, see Figure 4.

Drug blood levels

Pre-dose (trough) CyA and FK506 levels were measured in whole blood using a system from Abbott (Maidenhead, UK).

Statistics

Tests of significance were carried out using the "Astute" software package (University of Leeds, UK) and "Excel" (Microsoft).

Results

Range of sensitivities of patient PBMC to CyA and FK506

Incorporation of ^{3}H thymidine into Con A stimulated patient PBMC was progressively inhibited by increasing concentrations of both immunosuppressive drugs. The inhibitory effects of the two drugs varied from patient to patient, both in the nature of the response and in the level of sensitivity to the drugs. Figure 1 shows examples of the response of Con A-stimulated PBMC from 4 renal transplant patients. Two (2 and 21), representative of the majority of the patients, showed essentially complete inhibition with both drugs. The other two (3 & 4), representative of 5 patients, showed a rather different response. With these, even at the highest concentrations of drug used, inhibition was incomplete, only just exceeding 50%.

The ID_{50}s are presented for 32 patients in Table 1. Where there were sufficient PBMC for duplicate assays of the same sample, performed on separate occasions, the ID_{50}s generated agreed within a 2-fold range, and the mean values where duplicate assays were done are quoted in the table. In the cases of some patients repeat samples were taken and the ID_{50} values obtained for these repeat samples are quoted separately. The values of the ID_{50}s for both drugs varied widely from patient to patient, with a 50 fold range. However, when the correlation between the two sets of ID_{50} values was analysed by the Spearman Rank method it was found to be significant (P = 0.0001). Figure 2 a shows the regression of the ID_{50} for FK506 against that for CyA. The ratio

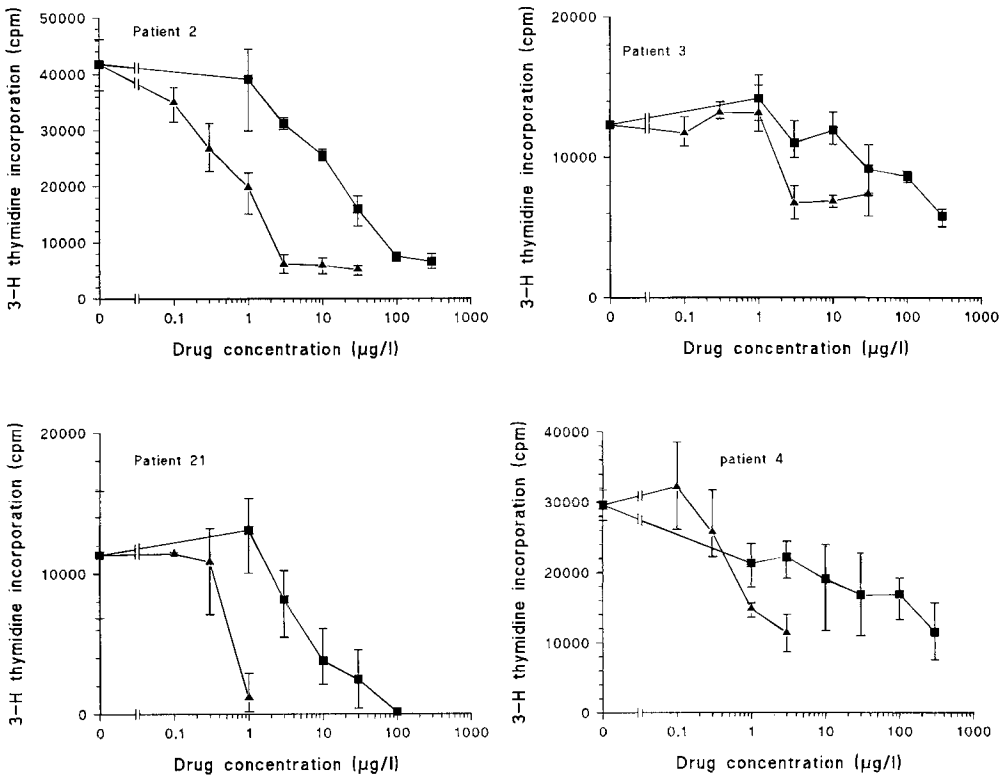


Fig. 1 Inhibition of incorporation of ^{3}H thymidine into PBMC from renal transplant patients by CyA and FK506. *Squares* CyA, *triangles* FK506

of the mean values for the ID_{50} for CyA and FK506 was 24.

This correlation conceals some considerable variation between patients in the ratio of the two ID_{50}s, from less than 10 (e.g. patients 4, 8 and 21) to 100 or more (patients 3, 28 and 35). This indicates that individual patients are relatively resistant to one or the other drug. To test whether this resistance was selective, i.e. patients' PBMC were relatively resistant to the drug used for treatment, the ratio of the ID_{50}s was compared for the patients treated with either CyA or FK506. Figure 2b shows that patients' PBMC were relatively resistant to the drug used for treatment. The median value for the ratio of ID_{50} for CyA to ID_{50} for FK 506 for patients treated with CyA was 47, and for patients treated with FK 506 was 24; *P* value for the comparison by Mann-Whitney U test was 0.02.

Drug ID_{50} values and blood drug levels

Since both CyA and FK506 have significant toxic side effects, an aim of therapy must be to use the minimal dose of drug consistent with preventing rejection. As can be seen from Table 1, the ratio of mean blood level to ID_{50} was about 30 for CyA and about 49 for FK506. Again comparing means conceals wide individual variations. For example, for patients treated with CyA, the ratio may be less than 3 (patients 3, 30 & 35) or more than 60 (18, 27 and 38): likewise for FK506, 18 and 22 respectively have low and high ratios (18 had been transferred from FK506 to CyA).

If the optimum dose of immunosuppression were being given, one might expect that the sensitivity to the drug should correlate with the blood level: but analysis by the Spearman Rank method shows no correlation whatever.

ID_{50} values do not correlate with clinical parameters

Comparing ID_{50} for either drug between patients who did or did not suffer acute rejection, no difference was found. However, the number of patients rejecting was very low (7). Likewise, there was no correlation between sensitivities and blood creatinine levels, nature of therapy (triple therapy with prednisolone against therapy without prednisolone) or time since transplant.

Range of sensitivities of patient PBMC to CyA and FK506 in presence of P-gp inhibitor PSC 833

In order to determine whether P-gp function could influence the sensitivity of PBMC to the immunosuppressive drugs, the effect of the inhibitor PSC 833 on ID50s

Table 1 ID_{50s} and trough blood levels for CyA and FK506

Patient number	ID_{50} for CyA	CyA blood levels[a]	Ratio blood level : ID_{50} (CyA)[a]	ID_{50} for FK506	FK506 blood levels[a]	Ratio blood level : ID_{50} (FK506)	Ratio CyA : FK ID50s
1	28.5 (2)[b]	nd[c]		nd	nd		
2	14	128	9	0.65			21.5
2 (rep)[d]	45 (2)	166	3.7	0.65 (2)			69
3	85 (2)	234	2.8	0.8			106
4	100 (2)	170	25	5.5			18
4 (rep)	30	nd		5			6
6	20	131	6.5	0.5			40
7	32	200	6.3	0.6			53
8	16			2.3	13.5	5.9	7
9	23 (2)			0.7 (2)	17.4	25	33
9 (rep)	28	nd		0.37			76
11	30 (2)	155	5.2	0.4 (2)			75
12	21 (2)	118	5.6	0.39 (2)			54
13	40 (2)	nd		0.68 (2)			59
14	9	nd		0.27			33
17	30	nd		0.8			38
17 (rep)	15			0.6	11.8	19.7	25
18	60			4	8.6	2.2	15
18 (rep)	2.2	227	103	0.17 (2)			13
19	5	117	23	0.15			33
20	12	176	15	0.3			40
21	4.6	210	46	0.6			7.7
22	1.5			0.085	24.6	290	18
23	17			1.4	11.1	8	12
24	30			1.5	15.8	10.5	20
25	14			0.45	11.3	25	31
26	28	nd		2			14
26 (rep)	12			0.35	9.5	27	34
26 (rep)	13			0.55	11.2	20	24
26 (rep)	11.5 (2)	296	26	0.44			26
27	15	179	12	0.2			75
27 (rep)	22.5 (2)	1000[e]	44.4	nd			
28	27.5 (2)	169	61	0.24 (2)			115
29	9.5	nd		0.16			59
30	100	247	2.5	10			10
30 (rep)	50	457	9	0.55 (2)			91
31	30	137	4.5	0.5			60
34	20 (2)			0.4 (2)	14.1	35	50
35	100	171	1.7	0.5			200
36	40	131	3.3	1.2			33
37	34 (2)	nd		0.5 (2)			68
38	2.5	159	64	nd			
Mean	29	230	30	1.2	14	49	
SD	25	190		1.9	5		
Median	25	170		0.55	12		

[a] Patients were treated with CyA or FK506: hence a blank in one column indicates that the patient was treated with the other drug. Blood levels were determined at the time of PBMC sampling

[b] Indicates mean of 2 determinations: otherwise single determinations

[c] nd = not done

[d] Indicates determination with a repeat sample (i.e. taken on a separate occasion)

[e] Minimum value

for the two drugs was determined. The ID_{50}s are presented in Table 2. PSC 833 did not significantly affect the ID_{50s} in either CyA- of FK506 treated cells. Control experiments confirmed that PSC 833 did not have direct immunosuppressive effects at this concentration. The ID_{50} for CyA alone was 31.8 μg/l (SD 16.1 range 16–80) and the ID_{50} for CyA plus PSC 833 at 2 μmolar was 28.9 μg/l (SD 20.9 range 15–78). This was not statistically significant, as tested by the paired *t*-Test ($P = 0.62$). The ID_{50} for FK506 alone was 0.56 μg/l (SD 0.71 range 0.044–1.75) and the ID50 for FK506 plus PSC 833 was 0.572 μg/l (SD 0.67 range 0.04–1.4: $P = 0.32$). The con-

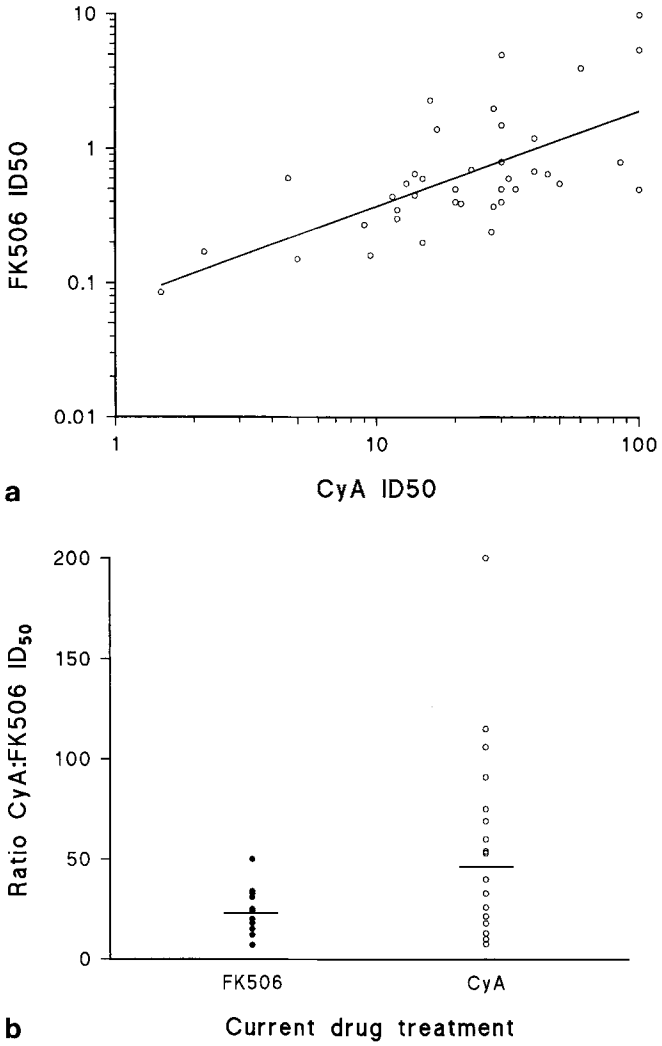


Fig. 2 **a** Correlation of values of ID_{50} for FK506 and CyA for all patients. **b** Ratios of ID_{50}s for the two drugs in patients treated with either one or other drug

centration of PSC 833 used (2μmolar) is equivalent to the concentration used to inhibit R-123 efflux. This concentration in molar terms is approximately 8 fold higher than the highest dose of CyA used.

P-gp expression and function

PBMC from 15 patients were tested for analysis of P-gp expression by immunofluorescence staining and for function by R-123 dye efflux. These patients were selected on the basis of their ID50 s to CyA and FK506 into high or low sensitivity groups (see Table 3). Overall there was not significant staining above isotype control as assessed by flow cytometry for any patient when either 4E3 or MRK16 antibodies were used (data not shown). PBMC from all the patients exhibited a degree of R-123 dye efflux. By flow cytometry, in the order of half the lymphocytes as defined by forward and side scatter pumped out the dye over a period of 4 h This efflux appeared invariably to be P-gp dependent since it was inhibited by PSC 833 (see Fig. 3). There was no dif-

Table 2 Effect of addition of PSC 833 to ID_{50s} for CyA and FK506

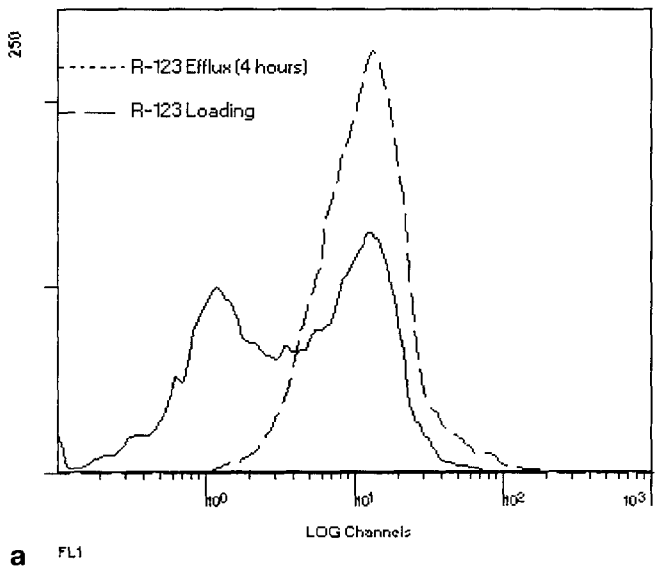
Patient number	ID_{50} for CyA	ID_{50} for CyA plus PSC 833	ID_{50} for FK506	ID_{50} for FK506 plus PSC 833
39	25	15.5		
40	18	14		
7	38	20		
41	23	28		
42	68	47		
43	16	20		
44	48	23		
45	58	78		
46	21	15		
47			0.044	0.04
48			0.058	0.05
49			0.12	0.17
29			1.2	1.2
50			1.7	1.4
Mean	35	28.9	0.62	0.57
SD	19	21	0.77	0.67

Paired t-Test for FK506: FK506/PSC 833 $P = 0.45$
Paired t-Test for CyA: CyA/PSC 833 (2 μmolar) $P = 0.24$

Table 3 Individual drug sensitivity and R-123 efflux

Patient number	ID50 for CyA	ID50 for FK506	Percentage of cells effluxing R-123
Low resistance group			
22	1.5	0.085	49
18	2.2	0.17	35
38	2.5		31
21	4.6	0.6	37
19	5	0.15	22
14	9	0.27	37
29	9.5	0.16	26
26	11.5	0.440	28
Mean	5.7	0.28	33.1
SD	3.79	0.19	8.4
High resistance group			
2	45	0.65	48
3	85	0.8	30
13	40	0.68	41
36	40	1.2	32
30	100	10	27
4	100	5.5	25
35	100	0.5	64
Mean	73	2.8	38.1
SD	30	3.7	14

t-Test comparing percentage of cells effluxing in high and low resistance groups; $P = 0.42$



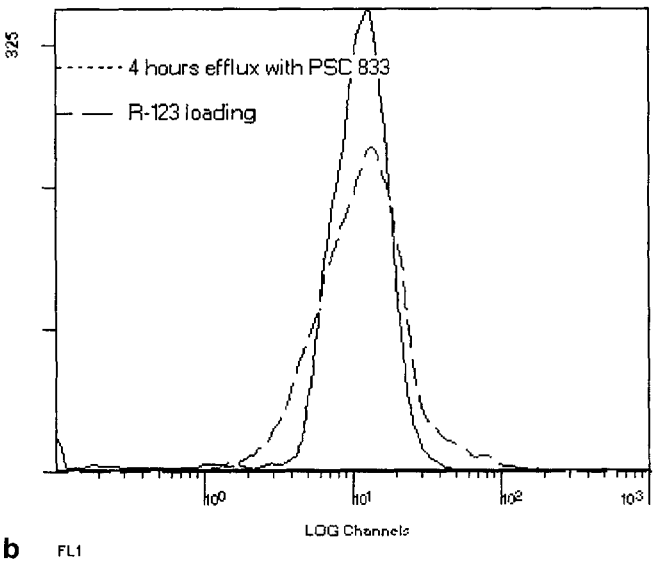
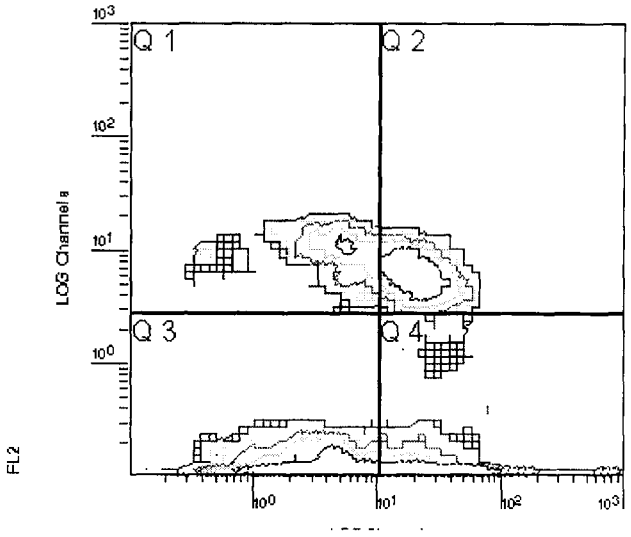


Fig. 3 **a** R-123 Efflux at time points 0 and 4 h. **b** Effect of PSC 833



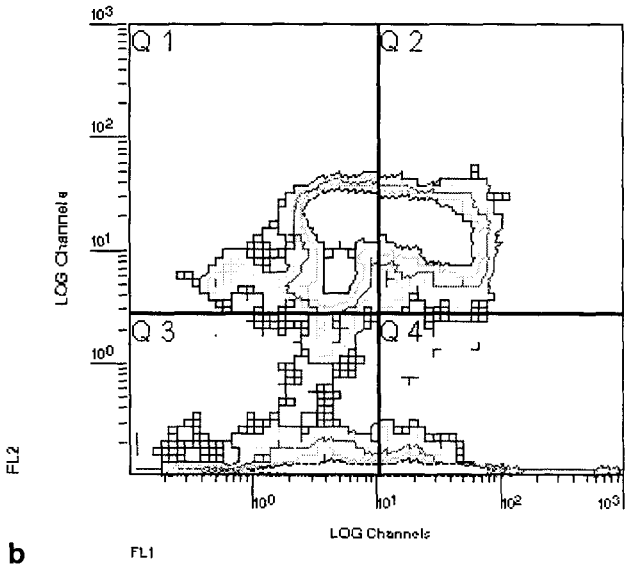


Fig. 4 **a** Contour graph showing double staining of R-123 pumping cells ('*x*'axis; FL1) with anti CD4-PE ('*y*'axis;FL2). **b** Contour graph showing double staining of R-123 pumping cells ('*x*'axis; FL1) with anti CD8-PE ('*y*'axis;FL2). Note increased number of cells in Q1 in anti-CD8 compared to anti-CD4 stained cells

ference between PBMC from the 2 groups in terms of mean percentage of cells actively effluxing the dye ($P = 0.4$). Following double staining with CD4 and CD8 lineage markers the majority of cells that are effluxing were found to be CD8 positive (see Fig. 4).

Discussion

The response to immunosuppressive drugs of PBMC activated by a T cell mitogen varied both quantitatively and qualitatively. The use of Con A to produce a maximal stimulus for lymphocyte proliferation allows the pharmacological effects of each drug to be defined unambiguously. The data presented above indicate that there is a wide variation in the sensitivity to the drugs. The two independent associations we have described, namely an association between CyA- and FK506 sensitivities between patients, and also selective resistance to the drug received by an individual patient, suggest that more than one mechanism may determine drug sensitivity at cellular level.

The interpatient correlation of ID_{50}s for the two drugs implies that there may be common mechanisms to account for differing sensitivities. Our stated hypo-

thesis that this could be through the intracellular drug concentration being modulated by multidrug resistance mechanisms has not been proven, although there are a number of reasons for it to be an attractive model. Both CyA and FK506 are substrates for at least one of the mechanisms implicated in multidrug resistance to cancer chemotherapy, the P-glycoprotein encoded by the MDR-1 locus [16], a membrane protein which pumps hydrophobic substances out of cells [20]. This is known to be expressed by subsets of lymphocytes [9] and Con A stimulated mouse spleen cells have been shown to become more sensitive to doxorubicin in the presence of P-gp inhibitors [15].

In our experiments with mononuclear cells from renal transplant patients there was no demonstrable effect on sensitivity to CyA or FK506 following the addition of P-gp inhibitor, PSC 833. The patients have received treatment with either drug as part of their immunosuppressive regime and it may be that MDR1 gene expression and P-gp function is down-regulated following prolonged treatment with the drugs. Indeed, it has been shown previously that no patient who had a transplant for more than 40 months expressed the MDR1 gene in his lymphocytes [5].

Despite a report in the literature [14] that P-gp is readily detected on the surface of normal lymphocytes, we found that none of the patients tested in this study had even moderate levels of P-gp detectable on the surface of their lymphocytes with the monoclonal antibodies 4E3 and MRK16. These antibodies were chosen since they recognise external epitopes on the cell surface and can be applied to unfixed cells in suspension [6]. The lack of MRK16 staining in our patients may reflect insufficient sensitivity to detect P-gp rather than non expression, since another group found little expression of P-gp on peripheral lymphocytes despite attempting to increase sensitivity by using biotinylated MRK16 [19]. Similarly it has been shown that P-gp mRNA can be detected, despite the absence of immunological expression of P-gp [11].

However, despite the lack of immunologically detectable P-gp, all the patients tested in this study exhibited P-gp functional activity as demonstrated by R-123 dye efflux. R-123 is a fluorescent dye that diffuses into the cell, and if not actively transported by an efflux pump, it is trapped in the cell and accumulates within mitochondria. Approximately 42% of total normal adult PBMC export the dye, and this decreases with age [14]. The dye efflux is secondary to P-gp activity since it is readily inhibited by specific inhibitors such as the cyclosporine-analogue PSC 833. Our patients exhibited similar levels of cells actively pumping, and there was no difference in pumping activity in those patients where the Con A stimulated lymphocytes were sensitive to CyA and FK506, compared to those where the cells were resistant to the drugs. This indicates that there is no gross difference between the drug-sensitive and drug insensitive PBMC in P-gp function. However, the majority of lymphocytes pumping were CD8 positive, and this is consistent with an earlier reports [14]. One could speculate that the responsive cells in the PBMC cultures stimulated with Con A are primarily CD4 positive, and we are therefore unlikely to see a vast difference in pumping activity between high- and low sensitivity groups, since the background level of pumping is relatively low. In summary, alternative mechanisms to explain variations in lymphocyte sensitivity need to be considered.

Both drugs act by binding to intracellular receptors of the immunophilin type [1]. The differences in drug response could be accounted for in differences in the amounts of these proteins in cells from different donors. Downregulation of cyclophilin levels in T lymphocytes from CyA-treated patients, and likewise of FK506 Binding Protein (FKBP) in FK506-treated patients, is one possible explanation for the selective drug resistance we observed. There is some evidence that both cyclophilins and FKBP proteins are regulated genes, for example varying in their expression with differentiation status [3, 12], but there is as yet no evidence that this may influence the response of cells to CyA or FK506.

The ratio of mean sensitivities to the two drugs was 24, and this is in agreement with the commonly accepted value of about 30. In vitro inhibition of mitogen-induced lymphocyte proliferation by both CyA and FK506 has been performed on cells from healthy volunteers and uraemic dialysis patients [7]. These studies confirmed that FK506 is effective in concentrations approximately 30–100 times lower than that of CyA and that there is wide interindividual variability. Our study, an extension of the above, was performed on renal transplant patients cells, and confirms the wide variability in sensitivity. The wide individual variation we have described may be important when transferring patients from one drug to another, clinically. We have already observed that it is better to convert between CyA and FK506 by factoring the existing drug dose by 30, than to start the new drug at the standard dose in mg/kg per day (the "rule of 30", Higgins et al. to be published). If the actual relative sensitivities were known, the new drug doses could be predicted more precisely.

Furthermore, measurement of lymphocyte sensitivity may define drug failure (i.e. rejection occurring despite therapeutic blood levels) more scientifically. Even the current widespread clinical use of FK506 for "CyA failure" has no scientific validity. Patients with trough CyA blood levels of about 200 μg/l are transferred to FK506 at starting levels of about 15 μg/l, which means that if FK506 is about 30 times more potent that CyA, the same immunosuppressive result might have been achieved by increasing the CyA level to 450 μg/l. The advantage of FK506 could therefore solely be due to its

relative dose, and perhaps to a better therapeutic index (ratio of desired effect to adverse effects).

Comparison of the ID_{50}s and serum concentrations suggests that although in most cases there is a reasonable excess, some patients may be underdosed, i.e. trough blood concentration may be close to the ID_{50}, and others may be overdosed, i.e. the blood concentration may be many times the ID_{50}. The question arises as to whether this is of clinical significance. Underdosing implies potentially inadequate immunosuppression, whilst overdosing would exacerbate side effects. One difficulty in assessing the data is that the trough blood levels do not represent the peak or the area under the curve (AUC).

The clinical outcome after transplantation is ultimately determined by lymphocyte responsiveness. However, it is notable that more work has hitherto been performed on the mechanisms of lymphocyte activation and on the pharmacology of immunosuppressive drugs than on the interaction between drugs and lymphocytes in the patient. Our data indicate that several mechanisms may be of clinical importance in this area and that further work is required.

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