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Sensitivity of baboon lymphocytes to cyclosporin A and FK 506: relative resistance of alloactivated cells to CyA

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Abstract The interspecies differences in CyA pharmacokinetics necessitate the establishment of optimal immunosuppressive doses in the baboon, especially as its use as host for preclinical xenografts is anticipated. We assessed the immunosuppressive effects of CyA and FK 506 on lymphocytes from chacma baboons, using human cells for comparison. At concentrations up to 100 μmol/l, neither drug was toxic to lymphocytes. FK 506 inhibited baboon and human lymphocyte proliferation and IL-2 synthesis equally. In contrast, approximately four times higher doses of CyA were needed to inhibit baboon lymphocytes responding to alloantigens. This may explain the inadequate immunosuppression of baboon graft recipients treated with clinically acceptable doses of CyA. We propose that CyA whole blood target levels of ± 1500 ng/ml should be used in this species and we provide evidence that chacma baboons are able to tolerate such doses without nephrotoxicity.

Key words Baboon lymphocytes, cyclosporin A, FK 506

Introduction

The immunosuppressive effects of cyclosporin A (CyA) have been complicated by variable pharmacokinetics observed between species [28, 34], within species and within individuals [7, 30]. In experimental models and clinical practise this has necessitated individualizing doses based on careful monitoring of blood levels [27]. While CyA is now established as the principal immunosuppressant in clinical organ transplantation [15], its immunomodulating effects in chacma baboon *(Papio ursinus orientalis)* hosts with renal [26, 34] and heart allografts [26] and concordant xenografts [3] have been disappointing. This may be due to poor absorption, relative resistance of target lymphocytes or rapid metabolism and/or excretion of the drug. Saturating tissue stores [34] and administering CyA in alcohol and Intralipid [24] has resulted in blood levels defined as clinically immunosuppressive. Nonetheless, baboon renal and orthotopic heart allograft outcome using CyA has consistently remained significantly below that obtained with other immunosuppressive strategies such as total lymphoid irradiation [22] or CyA with 15-deoxyspergualin [26]. Our experiments with concordant vervet monkey *(Cercopithecus aethops)* to chacma baboon liver xenotransplantation have necessitated careful assessment of the immunoinhibitory effects of FK 506 and CyA in the host species. Also, an increased demand for the use of baboons as recipients of discordant donor organs is anticipated as rapid progress in this relatively new field is made and preclinical experimentation, using size-suitable primate hosts, becomes necessary. A clearer understanding of how to use current immunosuppressive drugs in this species to optimum effect has, therefore, become essential.

This paper compares the sensitivity of baboon and human target cells to the action of FK 506 and CyA and, in particular, investigates whether the target cell in baboons is more resistant to the effects of CyA [5] as a possible explanation for the disappointing immunosuppressive action of this drug in baboon organ transplantation.

Materials and methods

Drugs

CyA (Sandoz, Basel, Switzerland) and FK 506 (Fujisawa Pharmaceutical, Osaka, Japan) were dissolved in ethanol at 1 mmol/l and stored at –20 °C. All further dilutions were in RPMI 1640 medium and prepared just prior to setting up cell cultures. Samples (18 µl) of vehicle control (0.001 % ethanol in RPMI) or drug dilutions to ensure final concentrations of 1×10^{-4}– 1×10^{-12} mol/l were placed in microtitre wells before addition of cells.

Isolation of cells

Heparinized peripheral blood from normal human volunteers and baboons was centrifuged at 200 *g* for 10 min. Mononuclear cells (MNC) collected from the buffy coats were further isolated by flotation on a Ficoll-Hypaque gradient, density 1.078. When required, T cells were purified by elution off nylon wool [11]. T-cell purity was established as greater than 93 % [35] using a crossreactive anti-CD2 monoclonal antibody (OKT 11, Ortho Diagnostics). Contaminating red cells were lysed with buffered NH_4Cl (155 mmol/l). Erythrocyte and platelet-free preparations were used in all assays.

Cytotoxicity assay

Baboon and human MNC (1×10^7) were labelled with 3.7 MBq $^{51}CrNa_2CrO_4$ in RPMI (100 µl) for 1 h at 37 °C with gentle mixing. The cells were washed four times with RPMI to remove excess radioactivity and samples (5×10^4 cells/172 µl RPMI) dispensed in quadruplicate in microtitre plates containing FK 506 or CyA (18 µl; final drug concentration 10^{-4}–10^{-12} mol/l). Cells were incubated for 4 h at 37 °C, centrifuged at 100 *g* for 5 min and isotope released into supernatants measured in a gamma counter. Background isotope release (blank) was established using labelled cells incubated without drug and total isotope release (TR) was accomplished using detergent-induced cell lysis. Cytotoxicity (%) was calculated according to the formula:

[(Test c/min – blank c/min) ÷ (TR c/min – blank c/min)] ×100.

Lectin-induced mitogenesis

Quadruplicate cultures of MNC (1×10^5/90 µl) in final medium (FM), which consisted of RPMI 1640 supplement with 10 %–15 % decomplemented, pooled, male human AB (HAB) serum, 20 mmol/l HEPES (pH 7.3), 2 mmol/l L-glutamine and antibiotics (100 mg/l cefotaxime and 50 mg/l amikacin), were incubated with an equal volume of concanavalin A (con A; Pharmacia Fine Chemicals, Upsala; 55 µg/ml) for 72 h. Tritiated thymidine (18.5 kBq) was added 16 h before harvesting and results were expressed as an inhibition constant (IC_{50}), namely, the dose causing 50 % inhibition of proliferation.

Mixed leucocyte cultures (MLC)

One-way MLCs were performed as previously described [35] in FM containing 10 %–15 % HAB serum or heat-inactivated plasma autologous to responding cells. In order to obtain maximum stimulation, stimulator cells were pooled from normal humans ($n = 20$) or baboons ($n = 20$), irradiated (20 Gy) and cryopreserved until required. Briefly, human or baboon MNCs (1×10^5) were incubated in a total volume of 180 µl FM with an equal number of irradiated autologous or pooled stimulator MNC in the presence of increasing concentrations of FK 506 or CyA. Cultures were incubated at 37 °C in a humidified atmosphere of 5.5 % CO_2 for 6 days, and 18.5 kBq (methyl-^{3}H)thymidine (Amersham Life Sciences) was added 16 h before harvesting. All tests were performed in quadruplicate and percentage of inhibition of MLC was calculated according to the formula:

$$\left[100 - \frac{[\text{Test (c/min)} - \text{Bkgd (c/min)}]}{[\text{Maximum stimulation (c/min)} - \text{Bkgd (c/min)}]}\right] \times 100$$

where background (Bkgd) equalled MLC with vehicle control and autologous, irradiated stimulator cells, and maximum stimulation equalled MLC with vehicle control and pooled, irradiated stimulator cells. Results were expressed as IC_{50}.

Interleukin-2 synthesis and estimation

IL-2 synthesis in human and baboon MNC (5×10^6) was induced with phorbol 12-myristate 13-acetate (85 nmol/l, Sigma Chemical) and calcium ionophore A23187 (2.4 µmol/l, Sigma Chemical) in FM (1 ml) for 24 h at 37 °C. Duplicate samples of the supernates were assessed for IL-2 using a radioimmunoassay kit (Interleukin-2 [^{125}I] assay system, Code RPA 531, Amersham, UK).

T-cell uptake of ^{3}H-CyA

A modification of the technique described by Sanghvi et al. [31] was used. ^{3}H-dihydrocyclosporin A (24 ng/ml, 4.4 TBq/mmol; Sandimmun kit, Sandoz, Basel, Switzerland) was dispensed at three concentrations (9.6 pg, 48 pg and 480 pg) in triplicate into microtitre wells. In initial experiments ethanol was evaporated, but later experiments utilized ^{3}H-dihydrocyclosporin A (480 pg/well) in ethanol to a final concentration of 10 % (v/v) which permitted increased absorption. Human or baboon T cells (2×10^5/180 µl RPMI containing 1 % BSA) were added, incubated for 1 h at 37 °C in a humidified CO_2 atmosphere and harvested onto a glass microfibre filter. The radioactivity incorporated was determined by scintillation counting.

Statistical methods

The significance of the difference between IC_{50} values of FK 506 and CyA using responder cells from human and baboon sources was calculated using the Mann-Whitney U-test, selected because of the relatively small number of experiments that disallowed parametric assumptions.

In vivo studies

One baboon received an orthotopic liver allotransplant and another a liver from a vervet monkey donor. Animals were immunosuppressed as previously reported [8], except that the target CyA whole blood trough level was raised to 1500 ng/ml. In the postoperative period, liver and kidney function tests were regularly determined and CyA and its metabolites were assessed on whole blood by the TDX system (Abbott, North Chicago, Ill., USA). Percutaneous liver biopsies were taken weekly for the first 2 months and processed for light microscopy and immunohistochemistry. Autopsy was performed upon death or sacrifice and renal biopsies were taken for histological examination.

Table 1 Toxicity of CyA and FK 506 to human and baboon mononuclear cells

CyA (mol/l)	^{51}Cr release (%)		FK 506 (mol/l)	^{51}Cr release (%)	
	Baboon	Human		Baboon	Human
10^{-4}	0	0	10^{-4}	0	0.2
10^{-5}	0	0	10^{-5}	0	0.2
10^{-6}	0	1.2	10^{-6}	0	0.8
10^{-7}	0	0	10^{-7}	0.1	1.0
10^{-8}	0	0	10^{-8}	0	0.9
10^{-9}	0	1.0	10^{-9}	0	0.8
10^{-10}	0	0.6	10^{-10}	1.1	1.9
10^{-11}	0.1	0.5	10^{-11}	1.2	1.2
10^{-12}	0.1	0.7	10^{-12}	0.5	0.1

Table 2 Inhibition of human and baboon mixed leucocyte cultures (MLC) with FK 506 and CyA

Drug	Cells[a]	Serum[b]	n	$IC_{50} \pm SE$ (nmol/l)	P
FK 506	Human	Human	7	0.6 ± 0.3	NS
FK 506	Baboon	Baboon	4	0.7 ± 0.3	NS
FK 506	Baboon	Human	3	0.6 ± 0.7	NS
CyA	Human	Human	18	11.3 ± 1.4	
CyA	Baboon	Baboon	9	49.9 ± 12.0	0.006
CyA	Baboon	Human	7	40.1 ± 11.0	NS

[a] Human and baboon lymphocytes were stimulated with irradiated, pooled baboon mononuclear cells
[b] Source of serum used to sustain MLC

Results

Drug cytotoxicity

The susceptibility of baboon lymphocytes to cytotoxic effects of the two drugs under investigation was examined. As shown in Table 1, no significant toxicity to cells from either species was noted, with ^{51}Cr release lower than 2 % over all drug concentrations.

Drug effects on human and baboon MLC

Experiments using human and baboon responder cells stimulated with irradiated, pooled baboon cells are shown in Table 2. The inhibitory effect of FK 506 remained similar for the two species while that of CyA showed a distinct differential effect with baboon cells requiring about four times higher concentrations of CyA than human cells to achieve an equal inhibition of proliferation ($P = 0.006$). In initial experiments MLCs were performed using decomplemented autologous plasma to achieve optimal stimulation and control for possible serum inhibitors of MLC. The possibility that sequestration by serum components differed between the two species and was responsible for the apparent insensitivity of baboon cells to CyA was also considered. The IC_{50} of FK 506 and CyA on baboon MLCs was unaffected by alterations in serum source, indicating equivalent quenching effects of serum from both humans and baboons (Table 2; P = NS). A requirement for higher doses of CyA was not invariable with all cell isolates from the baboons studied and IC_{50} from 5/16 experiments fell within the human cell range. This resulted in the large SE for baboon cells shown in Table 2.

In the initial experimental system, pooled baboon MNC were used as MLC stimulators. This meant that human cells were xenostimulated while those of baboons were allostimulated. As the stimulating antigen may influence the effect of CyA, we compared the IC_{50} of this drug on allo- and xenostimulated human and baboon responder cells. CyA inhibition of human responder cells gave identical IC_{50} values with both species of stimulating antigen (Fig. 1). Similarly, the dose of CyA necessary to inhibit baboon responder cells was not significantly altered by allo- or xenoantigen stimulation ($P = 0.12$, $n = 9$; data not shown).

Inhibition of lectin-induced mitogenesis

The inhibitory effect of increasing concentrations of FK 506 and CyA on con A-induced cytoproliferation was assessed in cells from four and ten baboons, respectively, using human MNCs for purpose of comparison. No significant difference in the inhibition of baboon and human cells by FK 506 was observed (Fig. 2 A). Mean ± SE IC_{50} were 0.13 ± 0.09 nmol/l and 0.17 ± 0.04 nmol/l for baboon and human cells, respectively. Values for CyA inhibition are shown in Fig. 2 B. There was a consistent requirement for marginally higher doses of CyA to inhibit baboon lymphocyte mitogenesis (mean ± SE IC_{50} 8 ± 2 nmol/l on seven human experiments and 14 ± 2 nmol/l for ten baboon experiments, $P = 0.04$), but the magnitude was much lower than that found to be necessary for inhibition of baboon MLC. The increased potency of FK 506 (50 to 70-fold) was confirmed in both species.

Inhibition of IL-2 synthesis

FK 506 inhibited IL-2 synthesis equally in both species (IC_{50} of 0.3 ± 0.1 nmol/l for human and 0.2 ± 0.1 nmol/l for baboon cells; $n = 3$). The differential effect of CyA on human and baboon MLC was not reproduced using synthesis of IL-2 as an endpoint (Fig. 3).

^{3}H-CyA Uptake

To determine whether the differential effect of CyA in baboon and human MLCs was due to differences in cell uptake, T cells from two normal humans and two normal

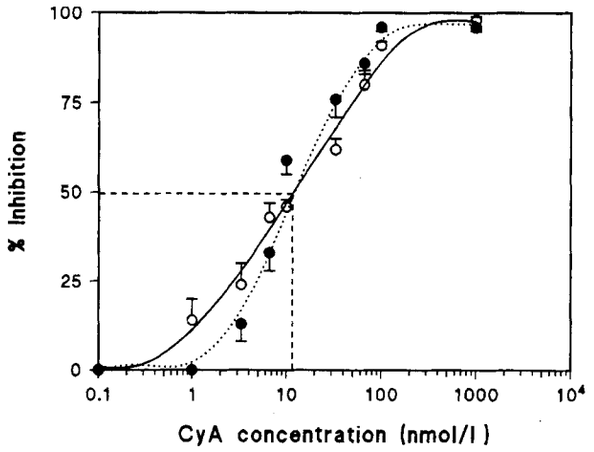

Fig. 1 CyA inhibition of human peripheral blood lymphocytes stimulated with pooled allogeneic (o—o) and xenogeneic (•···•) cells. CyA IC_{50} values were identical (12.5 nmol/l). Mean data from five experiments

baboons, from which contaminating platelets and erythrocytes had been removed, were incubated with radiolabelled CyA. Figure 4 shows no significant difference in CyA uptake between T cells of the two species at any of the three concentrations used. Subsequent experiments using liquid ^{3}H-dihydrocyclosporin A confirmed these findings with 41 %±1 % uptake of isotope in human (n = 4) and 42 %± 0.6 % in baboon (n = 6) purified T cells.

In vivo studies

The xenotransplanted baboon survived 27 days and the allograft recipient 32 days. The cause of death was a biliary leak and haemorrhage from the aortic anastomosis, respectively. The mean CyA level was 1492±931 ng/ml whole blood, and liver biopsies showed less histological evidence of rejection than previous studies on animals that had received less CyA [8]. Mean values for blood urea nitrogen and serum creatinine were 54.5±18.5 mg/dl and 0.75±0.14 mg/dl, respectively, indicating no impairment of renal function. Renal biopsies taken at autopsy failed to show histological evidence of CyA toxicity.

Discussion

Neither CyA nor FK 506 has been shown to be toxic in vitro to lymphocytes of other species [12], and this was also true of baboon and human MNC exposed to increasing concentrations of either drug (Table 1). The inhibition of a proliferative response of baboon cells by FK 506 closely paralleled that of human cells, regardless

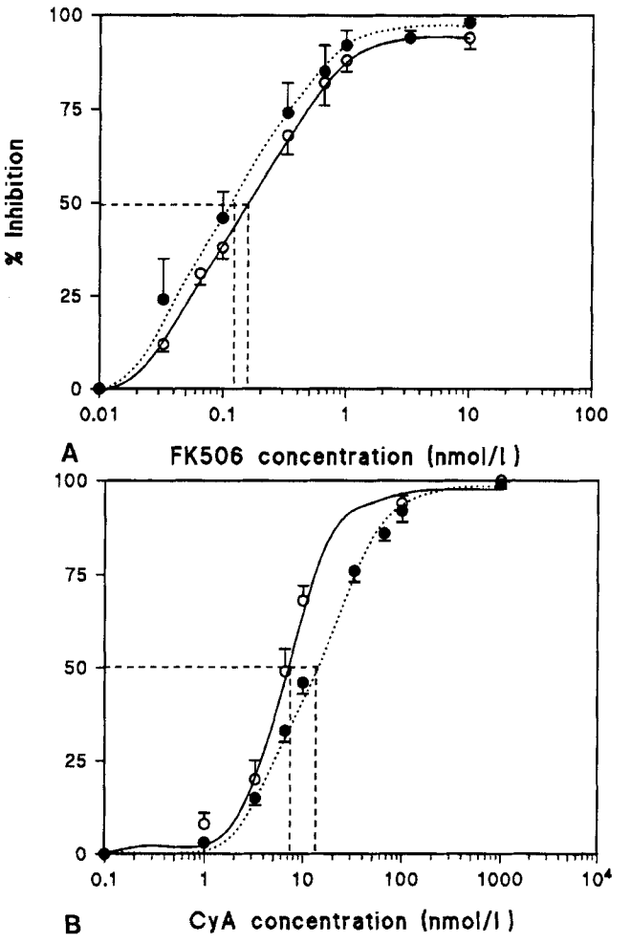

Fig. 2 **A** Similar inhibition of con A-induced proliferation of human (o—o) and baboon (•···•) mononuclear cells was obtained with FK 506 (mean data from four experiments; IC_{50} 0.13 ± 0.09 nmol/l for baboon and 0.17 ± 0.05 nmol/l for human cells, respectively). **B** CyA inhibition of con A-induced proliferation of human (o—o) and baboon (•···•) mononuclear cells. Data points respresent mean ± SE of seven and ten experiments, respectively

of whether these were stimulated with allo- or xenoantigens or the plant lectin con A and IC_{50} values for both species were similar to the published range for humans (0.2–0.5 nmol/l) [5, 10, 19, 33]. These observations are in agreement with those of Metcalfe et al. [19] but not with the results of Eiras and coworkers [5], who reported that baboon cells were 13 times less sensitive to FK 506 than human cells in MLC. A significant discrepancy in CyA IC_{50} values between human and baboon cells was observed when an MLC system was used (Table 2). The mean IC_{50} we obtained (11.3 nmol/l) for human cells was in the lower range of the 10–100 nmol/l reported by Sigal and Dumont [33] but consistent, with a SE of 1.4 nmol/l. We confirmed the findings of Eiras et al. [5] that, on average, four times more CyA was required to inhibit

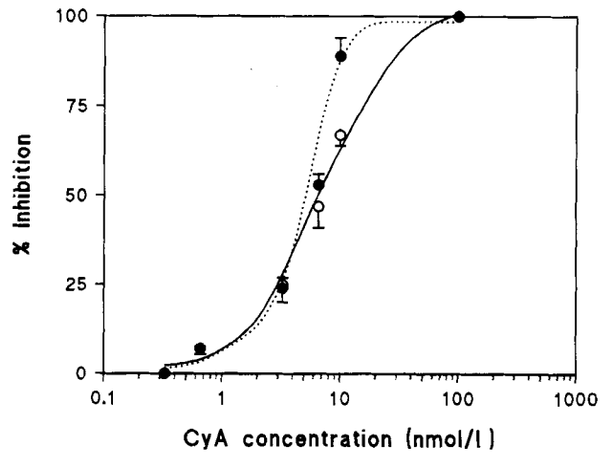

Fig. 3 CyA inhibition of IL-2 synthesis by human (o—o) and baboon (●···●) mononuclear cells. Although the shape of the curves was not the same, IC_{50} values were similar (5.1 and 5.8 nmol/l, respectively). Results of five different experiments expressed as mean ± SE

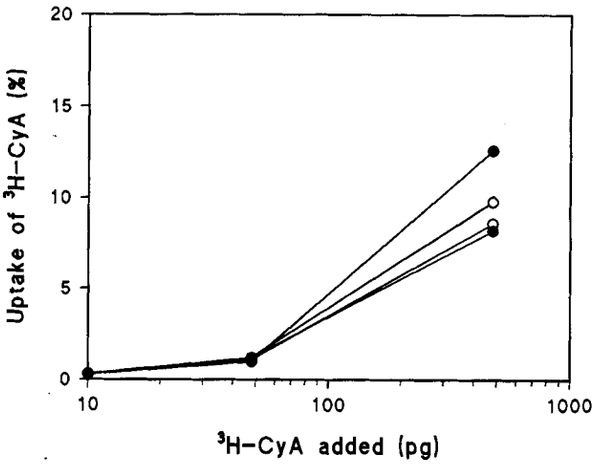

Fig. 4 No significant differences in uptake of ^{3}H-CyA into human (o—o) and baboon (●—●) T cells were noted

baboon than human MLC. Nevertheless, as has been demonstrated in other models [7, 30], intraspecies differences also occurred in the baboon, with requirements for higher doses of CyA not an invariable finding. Higher CyA requirements were not due to species differences in serum quenching effects (Table 2), nor was this related to whether responder cells were stimulated with allo- or xenoantigens. However, it appeared that the activating signal did influence CyA requirements. Thus, only marginal differences were noted between the IC_{50} values for baboon or human cells stimulated with con A, but discrepant MHC class II antigens, the main trigger for MLC responses [17], led to a significant differential effect.

That sensitivity to CyA may be a function of the activating signal is not a new concept. Kay and Benzie [13, 14] showed that sensitivity of T cells to CyA inhibition is related to the type of stimulator used, while Little et al. [18] reported that more CyA was needed to inhibit stimulation with purified murine allogeneic B cells than either spleen cells or macrophages. Takaori and coworkers [36] have recently reported that lymphocytes isolated from dogs immunosuppressed with CyA showed inhibition of responses to con A, PHA and IL-2, but responses to alloantigen stimulation were essentially unaffected. This in vivo effect of CyA is similar to our in vitro observations with baboon lymphocytes. Why significantly higher doses of CyA should be required to inhibit allo- or xenoantigen but not mitogen-driven cytoproliferation in the baboon is not clear. Both responses rely on IL-2 availability and it is now accepted that FK 506 as well as CyA directly interfere with IL-2 gene transcription [6, 37], leading to inhibition of IL-2 synthesis. We were unable to demonstrate species differences in CyA inhibition of IL-2 synthesis, probably because cells were stimulated with phorbol myristate acetate (PMA) and Ca ionophore rather than allo- or xenoantigens. This was necessary as stimulation of primate cells with mitogen or antigen in the absence of phorbol ester results in very low levels of IL-2 production [2]. During the course of the IL-2 experiments we observed that baboon cells were maximally stimulated with one-fifth the concentration of PMA necessary for optimum IL-2 production by human cells (unpublished observation). Cell activation is thought to occur via two pathways, namely, the activation of protein kinase C (PKC) and the elevation of cytoplasmic Ca^{2+} [12]. As PMA acts directly on the PKC pathway and CyA is known to interfere with the Ca^{2+}-dependent pathway of cell activation [12], it may be that PKC is more readily activated by alloantigen in baboon cells than human cells, thus rendering them more resistant to the inhibitory effect of CyA. Why a similar differential effect was not observed with FK 506, which also inhibits Ca^{2+}-dependent activation signals, remains conjecture. However, it is worth noting that despite the remarkable similarities in the activity of CyA and FK 506, important differences do exist. For example, each has a distinct carrier protein (cyclophilin and FKBP) [32] and probably also distinct intracellular acceptor molecules [33]. In the presence of Ca^{2+} and chemical-inducing agents, mitochondria undergo a permeability transition that allows normally impermeant solutes to cross the inner membrane at significant reates [1]. CyA potently inhibits this process, yet FK 506 has no effect [33].

The apparent resistance of baboon lymphocyte proliferation to CyA was not due to impaired uptake of the drug, which was comparable in human and baboon lymphocytes (Fig. 4). This was not unexpected considering that cyclophilin is known to be both ubiquitous and phylogenetically highly conserved and that only a small

fraction of the cellular pool needs to be occupied by the drug to achieve maximum immunosuppression [33].

Since the MLC is an in vitro correlate of the rejection response [9], and as the in vitro concentration of CyA needed to inhibit lymphocyte proliferation has been shown to be closely comparable to serum levels required for in vivo effects [12], we predict whole blood CyA levels of approximately 1500 ng/ml would be more effective in the chacma baboon. The importance of antibody-mediated rejection in concordant xenograft transplantation is becoming more fully appreciated [29]. Since CyA also inhibits early B-cell activation via cell surface immunoglobulin receptors [21, 25], it is possible that increasing CyA blood levels would improve control of B-cell responses. The limiting factor in increasing CyA doses is the toxicity of the drug, the major manifestation being nephrotoxicity [16]. Sustained high-dose treatment has resulted in irreparable kidney damage in humans [20, 23], while rhesus monkeys have been shown to be highly resistant to organ toxicity [28]. In a previous study on orthotopic liver transplants in baboons [8], we showed that mean CyA blood levels of ± 700 ng/ml resulted in no evidence of nephrotoxicity and rejection was only partially controlled. These findings, in conjunction with the in vitro studies reported here, prompted us to monitor rejection and nephrotoxicity on two additional baboons given higher doses of CyA (mean whole blood trough level of ±1500 ng/ml). In terms of liver function tests and histology, rejection was shown to be better controlled in both animals compared with animals that had received lower doses of CyA [4, 8] and there was no evidence of nephrotoxicity. Therefore, like rhesus monkeys, chacma baboons tolerate higher doses of CyA than humans and require higher doses for equivalent immunosuppression.

In summary, we have shown that allo- or xenoactivated baboon lymphocytes are less sensitive than human cells to CyA, but not FK 506, inhibition. This may partly explain the disappointing results of CyA in the prolongation of allo- and xenograft survival in this species, and we suggest that whole blood levels of 1500 ng/ml may be more appropriate.

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