Biochemical Pharmacology, Vol. 43, No. 9, pp. 1997–2006, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

TRANSPORT AND METABOLISM OF CYCLOSPORINE IN ISOLATED RAT HEPATOCYTES

THE EFFECTS OF LIPIDS

THOMAYANT PRUEKSARITANONT, MASAHIRO KOIKE,* BETTY-ANN HOENER and LESLIE Z. BENET†
Department of Pharmacy, University of California, San Francisco, CA, U.S.A.

(*Received* 3 *October* 1991; *accepted* 9 *January* 1992)

Abstract—The effects of lipids on the uptake and metabolism of cyclosporine (CyA) were investigated in isolated rat hepatocytes. In the absence of lipids, CyA was rapidly taken up (reaching apparent steady state within 5 min) and highly associated with the cells (more than 80%). The CyA uptake was concentration independent over the concentration range studied (0.6 to 11.2 μg/mL). Metabolism, however, was relatively slow and saturable. Except for cholesterol (at concentrations up to 15.5 mM), all lipids tested [oleic acid; low density lipoproteins (LDL); and high density lipoproteins (HDL)] reduced CyA cell uptake as well as its metabolism in a concentration-dependent manner. The effects of LDL were much more pronounced when compared to those of HDL and oleic acid. At an LDL concentration of 1 μM, drug uptake, indicated by the cell-associated concentration at steady state, was about 49% of the control value, while CyA metabolism was inhibited completely. Drug uptake of about 82 and 91% and CyA disappearance of 75 and 68% of the relevant control values were observed with HDL and oleic acid at concentrations of 10 μM and 0.7 mM, respectively. Apparently, lipids decreased CyA metabolism by reducing the concentration of CyA available for transport into the cells. These findings further support the suggestion of an important role for plasma lipids in the disposition of CyA.

Cyclosporine (CyA‡), a cyclic undecapeptide, is used widely as a powerful immunosuppressant in organ transplantations. It has been shown to be metabolized extensively (more than 90%) in the liver of humans and animals [1–3]. Due to its lipophilicity, it binds mostly to plasma lipoproteins (up to 80% of the bound drug) and the binding primarily occurs with low density lipoproteins (LDL) and high density lipoproteins (HDL) [4–6]. Thus, changes in lipoprotein patterns have been thought to play an important role in CyA distribution and clearance.

Only a few studies have focused on the effects of lipids on CyA disposition with, unfortunately, inconclusive or contradictory results. In dogs, concomitant infusion of a fat emulsion resulted in a nonsignificant (28%) increase in area under the plasma concentration–time curve (AUC), using a nonspecific radioimmunoassay (RIA) [7]. Significantly smaller serum volume of distribution and clearance were found in Zucker hyperlipidemic rats as compared to Zucker lean or Sprague–Dawley rat models, also using a nonspecific RIA method [8]. Using specific HPLC assays, a negative relationship between plasma CyA clearance and lipoprotein concentrations was reported in uremic patients [9], whereas high fat meals have been found to increase the volume of distribution and clearance of CyA obtained from both plasma and blood measurements after intravenous administration of CyA to healthy volunteers [10, 11]. If increased lipoprotein levels result from high fat meals, the findings described above appear to contradict the expected effects of increased plasma protein binding on the distribution and elimination of a low extraction ratio drug such as CyA. As an explanation for these apparently anomalous results, Gupta *et al.* [11] suggested that a complex of CyA–lipoproteins, especially LDL, may enhance uptake into cells, possibly via LDL receptors, leading to increased intracellular drug–protein complex concentrations which are then cleaved by lipases making greater amounts of CyA available for P450-mediated metabolism. Little direct information, however, is available on the effect of plasma lipids on drug transport into and metabolism within intact cells.

In this paper, CyA uptake and metabolism were investigated in the presence of plasma lipids: cholesterol, oleic acid, LDL and HDL. We chose isolated rat hepatocytes as a model system which allowed simultaneous examination of drug uptake and metabolism processes in the presence of various additives, without the complexities commonly encountered *in vivo*. Rats have been used extensively in previous disposition studies of both CyA and lipoproteins, with a number of findings shown to be similar between rats and humans [2, 12–14].

* Present address: Shionogi Research Laboratory, 3-1-1, Futaba-cho, Toyonakashi, 561, Japan.
† Corresponding author: Leslie Z. Benet, Ph.D., Department of Pharmacy, University of California, San Francisco, CA 94143-0446. Tel. (415) 476-3853; FAX (415) 476-0688.

‡ Abbreviations: CyA, cyclosporine; CyC, dihydrocyclosporin C; LDL, low density lipoproteins; HDL, high density lipoproteins; VLDL, very low density lipoproteins; AUC, area under concentration–time curve; RIA, radioimmunoassay; BSA, bovine serum albumin; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid; and DMSO, dimethyl sulfoxide.

METHODS

Chemicals. Collagenase type IV, bovine serum albumin (BSA), essentially fatty-acid free BSA, ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid (EGTA), *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), oleic acid, cholesterol, and both LDL and HDL derived from human plasma were obtained from Sigma (St. Louis, MO). CyA, its metabolites: AM9, AM1, AM1c and AM4N, previously known as M-1, M-17, M-18 and M-21, respectively [15], and dihydrocyclosporin C (CyC) were a gift from Sandoz Ltd. (Basel, Switzerland). Other chemicals and solvents were from Fisher Scientific (Santa Clara, CA) and were either analytical or HPLC grade.

Cell preparation. Male Sprague–Dawley rats (250–300 g) from Bantin and Kingman (San Leandro, CA) with free access to standard rat chow and water were used. Liver perfusion was performed as described by Seglen [16] with slight modification. In brief, the liver was perfused *in situ* with HEPES buffers via the hepatic portal vein, with the superior vena cava serving as an outlet for the perfusate. The basic composition of the HEPES buffers was 0.83% NaCl, 0.05% KCl and 0.24% HEPES, adjusted to pH 7.45. Each liver was perfused with buffer A, the HEPES buffer containing 0.019% EGTA, for 8–10 min, followed by buffer B, the HEPES buffer with 0.1% collagenase, 0.075% $CaCl_2$ and 1.5% BSA for a further 5–8 min. Thereafter, the liver was excised and dispersed in buffer C (0.8% NaCl, 0.035% KCl, 0.016% $MgSO_4 \cdot 7H_2O$, 0.018% $CaCl_2 \cdot 2H_2O$ and 0.24% HEPES, pH 7.5, supplemented with 0.18% glucose). This cell suspension was then filtered through cotton gauze, washed twice with buffer C by slow centrifugation (50 *g*), and redispersed in the incubation medium, buffer C containing 1% fatty-acid free BSA. The inclusion of albumin in the incubation medium was found to help maintain cell viability during the incubation period. The cell yield as counted in a Burker Chamber, was 400–600 × 10^6 cells/liver. Cell viability, as assessed by Trypan Blue exclusion and LDH release tests [17], was greater than 90%. The cell supension was stored on ice before incubation, which always occurred within 1 hr of preparation.

Incubations. The cell suspension was diluted to a final concentration of 5 × 10^6 cells/mL and preincubated at 37° in a shaking water bath for 15–20 min. CyA (1 mg/mL in DMSO) was added to the cell suspension (about 10 mL) to obtain a final concentration of 0.6 to 11.2 μg/mL. For the studies of lipid effects, two different concentrations of each lipid were used while CyA concentration was fixed at 1.2 μg/mL (1 μM). These lipid concentrations were: cholesterol, 0.77 and 15.5 mM; oleic acid, 0.7 and 3.5 mM; LDL, 0.1 and 1 μM; and HDL, 1 and 10 μM. Oleic acid was always preincubated with the cell suspension before the addition of CyA. Other lipids were either added directly to the cell suspension during the preincubation period or were preincubated with CyA before they were added to the cell suspension. Cholesterol was suspended in the incubation medium and sonicated for about 1 hr before use. In all experiments, DMSO was adjusted to a final concentration of 1.5% (v/v). Cell viability was decreased to about 80–85% at the end of incubation (4 hr) and was not affected by the addition of DMSO, cholesterol, HDL, LDL, and oleic acid at the low concentration (0.7 mM). In the presence of oleic acid at 3.5 mM, viability decreased more rapidly, reaching about 50% after 4 hr. This deterioration was probably due to the acidity of oleic acid.

For uptake studies, samples (0.3 mL) were taken at 10, 40, and 80 sec, and 2, 3, 5 and 10 min after the addition of CyA and each sample was centrifuged immediately at 4000 *g* for 5 sec. The supernatants were separated as soon as possible and 1 mL of a precipitating reagent (20% acetonitrile, 30% methanol and 5% zinc sulfate in water) was added to the remaining cell pellets. No attempt was made to correct for adhering water since a preliminary study showed that cell-associated CyA concentrations were only about 10% higher than those obtained from the cell pellets washed with ice-cold saline. The samples were vortexed and stored frozen until analyses, always within 1 week.

Experiments were also carried out to determine the binding of CyA to nonviable cells. Cell suspensions were frozen and then thawed prior to incubation with CyA.

For metabolism studies, samples (0.7 mL) were taken, in duplicate, at 0, 1, 2, 3 (in some studies), and 4 hr and heated in a 100° water bath for 2–3 min to terminate the reaction. Due to the rigorous early sampling time schedule for the uptake studies, the zero time metabolism sample was actually taken 2–3 min after CyA was added. From a pilot study, no loss of CyA or formation of the metabolites was observed during the first 5 min. No significant loss of CyA and its metabolites was observed during heating.

All experiments were performed using at least three separate hepatocyte preparations. Since considerable inter-liver variability was observed, a control experiment was carried out for each cell preparation and the effects of lipids were compared to those of the respective controls.

HPLC analysis. CyA and its metabolite concentrations were determined by modifications of our previously described HPLC procedure [18] so as to achieve higher recoveries of metabolites and better resolution of all species. Each sample was spiked with CyC, the internal standard, to a final concentration of 400 ng/mL and the proteins were precipitated by 3 mL (for the metabolism study) and 2 mL (for the uptake study) of the precipitating reagent. After centrifugation, the supernatant was separated and loaded onto a C18 Bond Elut column (Analytichem International, Harbor City, CA). The column was washed with 1 mL of 25% acetonitrile in water and eluted with 1 mL of ethanol. The eluent was evaporated to dryness under a stream of nitrogen and the residue was reconstituted with 130 μL of the mobile phase (a 52:5:63 mixture of acetonitrile:tetrahydrofuran:diluted phosphoric acid, 0.05%, pH 3). An aliquot, 50–100 μL, was then injected onto a C18-ultrasphere reversed phase column (5 μm, 250 × 4.6 mm, Beckman, Deerfield,

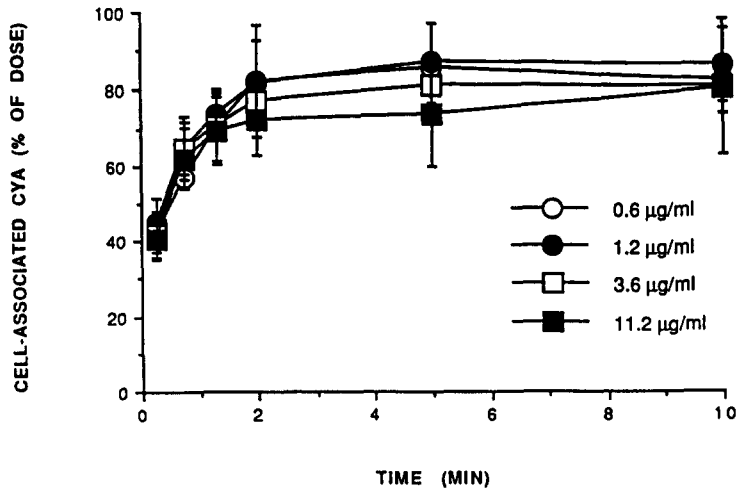

Fig. 1. Time course of CyA uptake by isolated rat hepatocytes at different initial CyA concentrations. Values are means ± SD (N = 4).

IL) maintained at 70° and the column eluent was monitored at 214 nm. At a flow rate of 1 mL/min, the retention times of CyA, CyC, AM9, AM1, AM1c, and AM4N were 46, 36, 14.6, 13.6, 16, and 24.5 min, respectively. The extraction recovery of CyA was 80%, which was approximately the same as that obtained previously [18], while the recoveries of AM9 and AM1 were about 4-fold and AM4N was about 2.5-fold higher than those found using the earlier method [18]. Since insufficient quantities of the metabolites were available for construction of standard curves, the metabolite concentrations were estimated assuming that their extinction coefficients and extraction recoveries were identical to those of CyA as proposed by previous workers [19, 20]. These assumptions are reasonable considering the close similarity of the chemical structures and the consistent high extraction recoveries obtained via the modified extraction procedure. In addition, Wallenmacq *et al.* [21] found a comparable response using UV and radioactive measurements for CyA and the four metabolites considered here. The intra-day and inter-day coefficients of variation of the CyA assay ranged between 3 and 8%.

Statistical analysis. The results were evaluated by analysis of variance using Statview™ 512+ (BrainPower, Inc., Calabasas, CA).

RESULTS

Characteristics of CyA uptake. Figure 1 depicts the time courses of cell-associated CyA, expressed as percentages of initial concentrations over an initial concentration range of 0.6 to 11.2 μg/mL. CyA was taken up rapidly by the hepatocytes, reaching an apparent steady state within 5 min. CyA was highly associated with the cells with 86 ± 9% of the initial CyA concentration found in the cell portion after a 5-min incubation. Since no correction for water entrapment was made in the present study, the cell-associated CyA estimates may be slightly higher than the actual values. Over the concentration range of 0.6 to 11.2 μg/mL, CyA uptake was found to be concentration independent (Fig. 1), consistent with its passive transport property as reported in an earlier rat study [22]. Adsorption to nonviable (frozen and thawed) hepatocytes averaged 60% (range 58–64%) of the initial CyA concentration after 10 min.

Characteristics of CyA metabolism. Unlike the uptake process, CyA metabolism was relatively slow and exhibited concentration dependence. Over the 4-hr incubation period, about 30% of CyA disappeared at initial concentrations of 3.6 μg/mL or less, while about 16% of CyA was lost at the 11.2 μg/mL concentration. Saturable metabolism was also reported in isolated rabbit hepatocytes over the CyA concentration range 1 to 2.5 μg/mL, although a much faster metabolism rate was found [3]. With regard to metabolite formation, AM9 was a major component, followed by AM1 at all sampling times (Fig. 2), in agreement with a previous *in vivo* observation in rats [2]. The formation of all four metabolites appeared to exhibit nonlinearity with time (Fig. 2) and with concentration (Fig. 3). Because of very low concentrations of AM4N and difficulty in separating AM1c (secondary metabolite) from endogenous substances in some experiments, these two minor metabolites were not measured in subsequent experiments.

Effects of lipids on CyA uptake. Figure 4 shows uptake patterns of CyA in the presence of cholesterol, oleic acid, LDL, and HDL. All lipids tested, with the exception of cholesterol (Fig. 4a) and oleic acid during the first 80 sec (Fig. 4b), appeared to inhibit CyA uptake in a concentration-dependent manner. In the presence of 15.5 mM cholesterol, cell-associated CyA concentrations during the first 40 sec were significantly ($P < 0.02$) higher (up to 1.8-fold) when cholesterol was preincubated with CyA as compared to the control or when cholesterol was first incubated with the cell suspension (Fig. 4a).

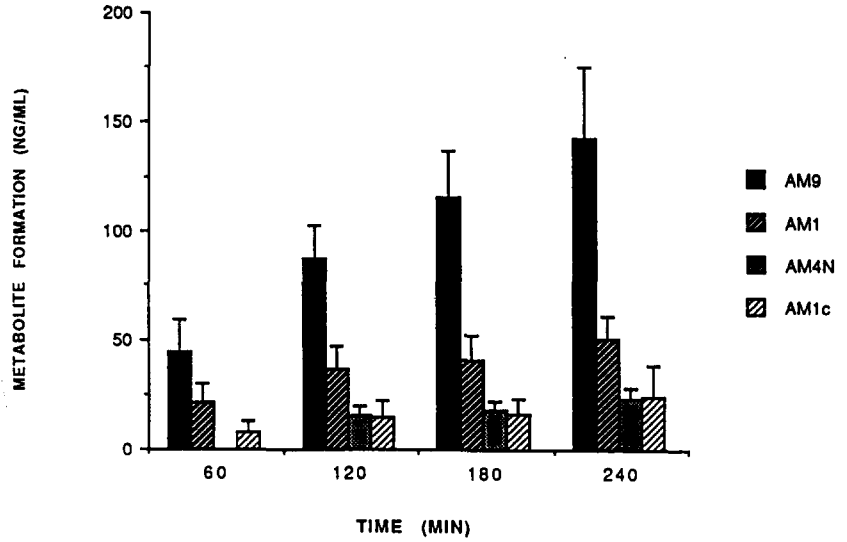

Fig. 2. Formation of measurable CyA metabolites as a function of time at an initial CyA concentration of 1.2 μg/mL. Values are means ± SD (N = 6).

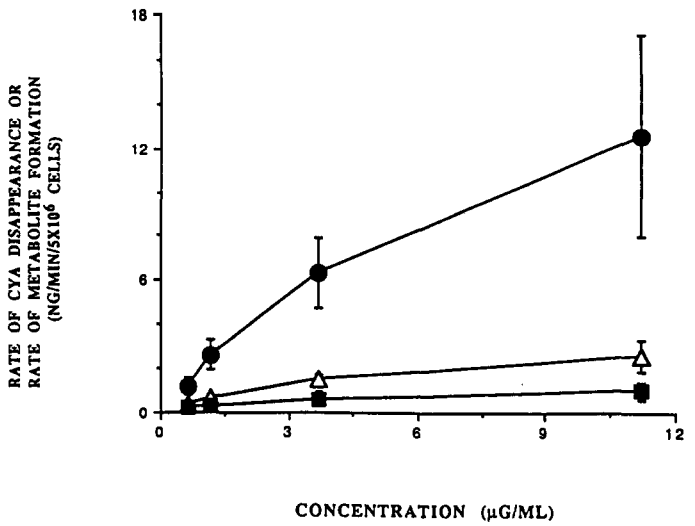

Fig. 3. Relationship between concentration of CyA and rate of CyA disappearance (●), formation of AM9 (△) and formation of AM1 (■). Values are means ± SD (N = 4).

Nevertheless, CyA cell association at equilibrium in the presence of cholesterol at both concentrations used was not different from the control.

In the presence of oleic acid, cell-associated CyA concentrations were not affected significantly during the first 10–80 sec of incubation, but decreased thereafter. At an oleic concentration of 3.5 mM, CyA cell association gradually declined reaching a plateau of 67 ± 12% of the control value after 10 min (Fig. 4b). In contrast, at the 0.7 mM oleic acid concentration, the cell-associated CyA concentration at equilibrium was only slightly lower than that of the control.

CyA uptake values in the presence of LDL (0.1 and 1 μM) and HDL (10 μM) as depicted in Fig. 4c and 4d, respectively, were less than those of the controls at all sampling times, independent of the order of lipid addition (data not shown). These results are in agreement with the preferential binding of CyA to plasma lipoproteins [4–6, 23, 24]. The effect of LDL was much more pronounced than that of HDL. At equilibrium, cell-associated CyA was found to be 84 ± 9 and 49 ± 15% of the control with 0.1 and 1 μM LDL, respectively, and 82 ± 10% of the control with 10 μM HDL.

Effects of lipids on CyA metabolism. The

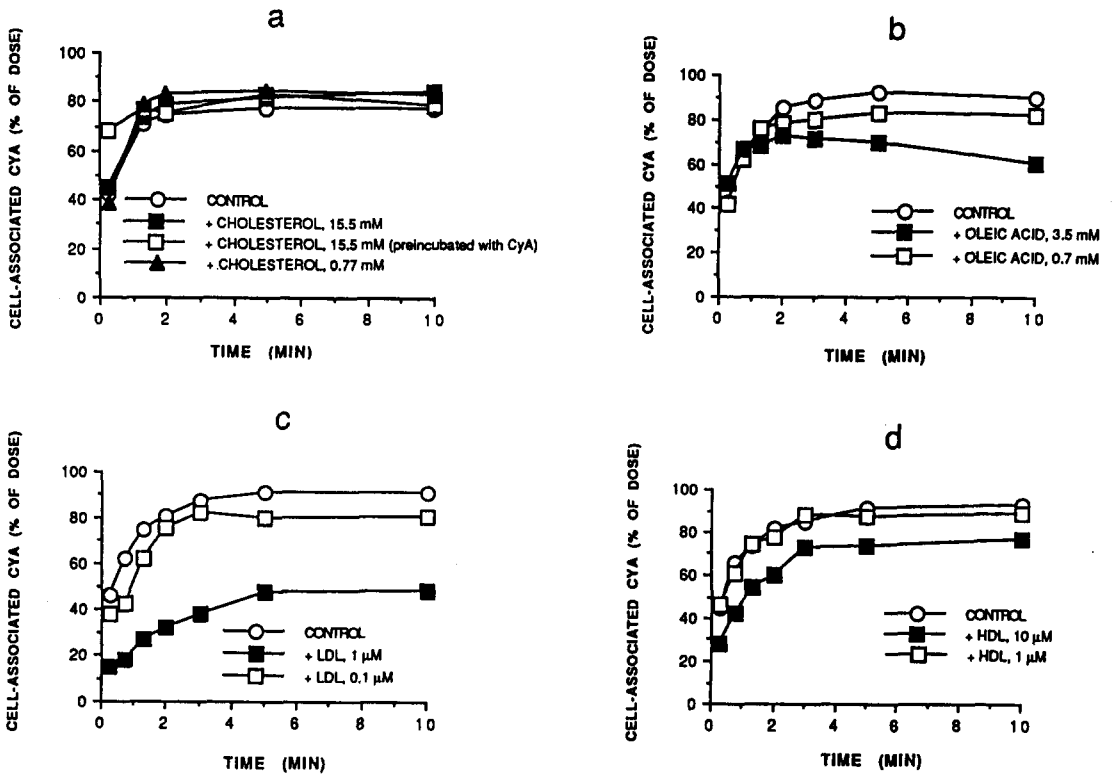

Fig. 4. Uptake of CyA by isolated rat hepatocytes at the initial CyA concentration of 1.2 μg/mL in the presence of cholesterol (a), oleic acid (b), LDL (c), and HDL (d). Each value is the mean of at least three experiments.

concentration–time profiles of CyA disappearance and AM9 and AM1 formation in the presence of lipids are shown in Figs. 5–7, respectively. The patterns of CyA disappearance during the 4-hr incubation period in the presence of lipids appeared to be the inverse of those for AM9 and AM1 formation. Cholesterol, in the concentration range studied, did not affect CyA metabolism significantly although slightly less drug disappearance and metabolite formation were found at the end of incubation with cholesterol at 15.5 mM (Figs. 5a, 6a, and 7a). In contrast, both oleic acid at 3.5 mM and LDL at 1 μM completely inhibited the drug metabolism. No significant loss of CyA was found (Fig. 5, b and c), and no metabolites were detected (Figs. 6, b and c, 7, b and c). A significant decrease in CyA metabolism was also observed at the lower concentrations of oleic acid and LDL used. HDL at a concentration of 10 μM, but not at 1 μM, markedly affected CyA metabolism (Figs. 5d, 6d, and 7d, and Table 1). The order of lipid addition did not appear to alter the drug metabolism pattern (data not shown).

Relationship between CyA uptake and metabolism. Table 1 summarizes the effects of lipids on: CyA cell association at steady state (column 3), cell association corrected for adsorption to nonviable hepatocytes (column 4), the disappearance of CyA (column 5) and the formation of AM9 at 4 hr (column 6). The positive relationship between CyA uptake and metabolism in the presence of lipids (column 4 vs column 5 or 6) was apparent ($r^2 > 0.90$). Cholesterol in the concentration range studied and HDL at 1 μM affected neither drug uptake nor drug metabolism. HDL at 10 μM decreased the concentration of CyA associated with the cells to 82% of the control and the drug metabolism to 75% of the control. LDL at 0.1 μM diminished the drug uptake, the drug disappearance and AM9 formation to 84, 85 and 69% of the control values, respectively. In the presence of oleic acid at 0.7 mM, CyA metabolism was decreased significantly (62–68%), and the uptake was also lower, although statistically insignificant, than the control (91%). No metabolism was observed when the values of CyA associated with the cells fell below about 67% of the control, as in the case of 3.5 mM oleic acid and 1 μM LDL.

DISCUSSION

Isolated hepatocytes have been used successfully in investigations of drug transport and metabolism for over a decade [25, 26], and proved to be a useful tool for the study of CyA disposition. The relatively fast uptake process of CyA as compared to its metabolism allows each process to be evaluated independently. The high cell association of CyA is consistent with the drug's previously reported

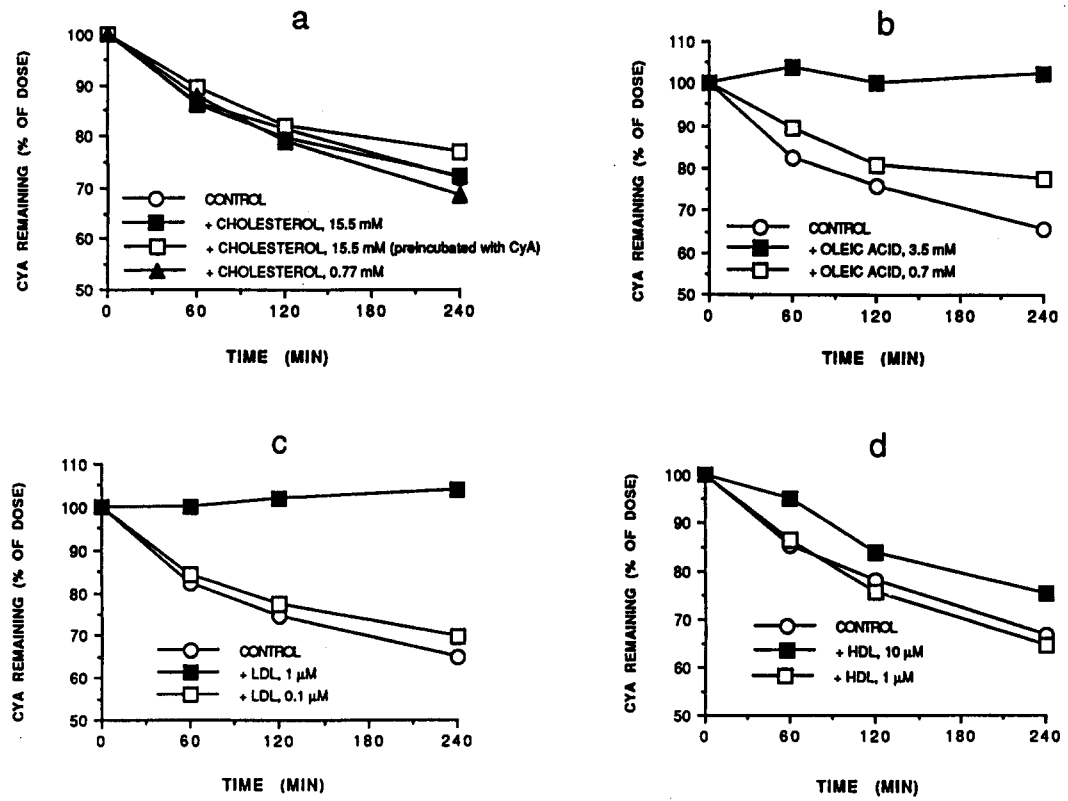

Fig. 5. Time course of CyA disappearance at the initial CyA concentration of 1.2 μg/mL in the presence of cholesterol (a), oleic acid (b), LDL (c), and HDL (d). Each value is the mean of at least three experiments.

pronounced tissue binding and high affinity to cyclophilin, an intracellular protein [2, 3]. The apparently slow metabolism observed, even in the absence of lipids, suggests that the intrinsic clearance of CyA is low, in agreement with the observation *in vivo* [2]. Since CyA was metabolized much faster in rabbits [3], there appears to be species differences in CyA metabolism. The saturable kinetics of CyA metabolism observed in this study was also consistent with the earlier study in rabbits [3]. Although the concentrations of CyA tested here were limited (four concentrations over an 18-fold range), it is possible to obtain preliminary estimates of V_{max} and K_m from initial formation rates of AM9 and AM1 and the disappearance of CyA. The K_m values of the initial formation rate of AM9 and AM1, and the disappearance of CyA were approximately 6.5, 4.7, and 12.8 μg/mL and the V_{max} values were 4.2, 1.5, and 26 ng/min/5 × 10^6 cells, respectively. Assuming negligible binding of CyA to albumin [4–6], the CyA intrinsic clearance, estimated by dividing the V_{max} of CyA disappearance by its relevant K_m, was 2 μL/min/5 × 10^6 cells. The intrinsic formation clearances of AM9 and AM1 were 0.65 and 0.32 μL/min/5 × 10^6 cells, accounting for 32.5 and 16% of the CyA disappearance, respectively. If the estimates of metabolite concentrations, assuming approximate equivalence to the CyA extinction coefficient are reasonable, then only about half of the CyA dose can be accounted for as identified primary metabolites. Similar results were found in rats [2], where a significant portion of unidentified polar metabolites (up to 36% of total radioactivity) was reported. Most recently, a sulfate conjugate of CyA was identified at high concentrations in human blood and bile [27].

To investigate the effects of plasma lipids on CyA disposition, LDL and HDL were chosen for study, since they represent major plasma lipoproteins that bind to CyA [4–6]. Oleic acid is one of the fatty acids commonly found in dietary fats [28]. It probably also represents very low density lipoproteins (VLDL) to some extent, as VLDL synthesis has been reported to be stimulated by incubating oleic acid (0.25 to 1 mM) with rat hepatocytes [29, 30]. Cholesterol is present in plasma in the form of lipoproteins (about 60% in LDL and 30% in HDL) [31]. The higher concentrations of lipids used in the present study, 1 μM LDL, 10 μM HDL and 0.7 mM oleic acid, are comparable to the levels normally found in human plasma [28, 31, 32]. Lower concentrations of LDL and HDL were also tested since they probably reflect the concentrations in the extracellular fluid of most organs [33]. The low and high concentrations of cholesterol utilized represent the range of the free and total cholesterol in human plasma [32]. As shown in Table 1, all lipids tested, with the exception of cholesterol, decreased the drug uptake and

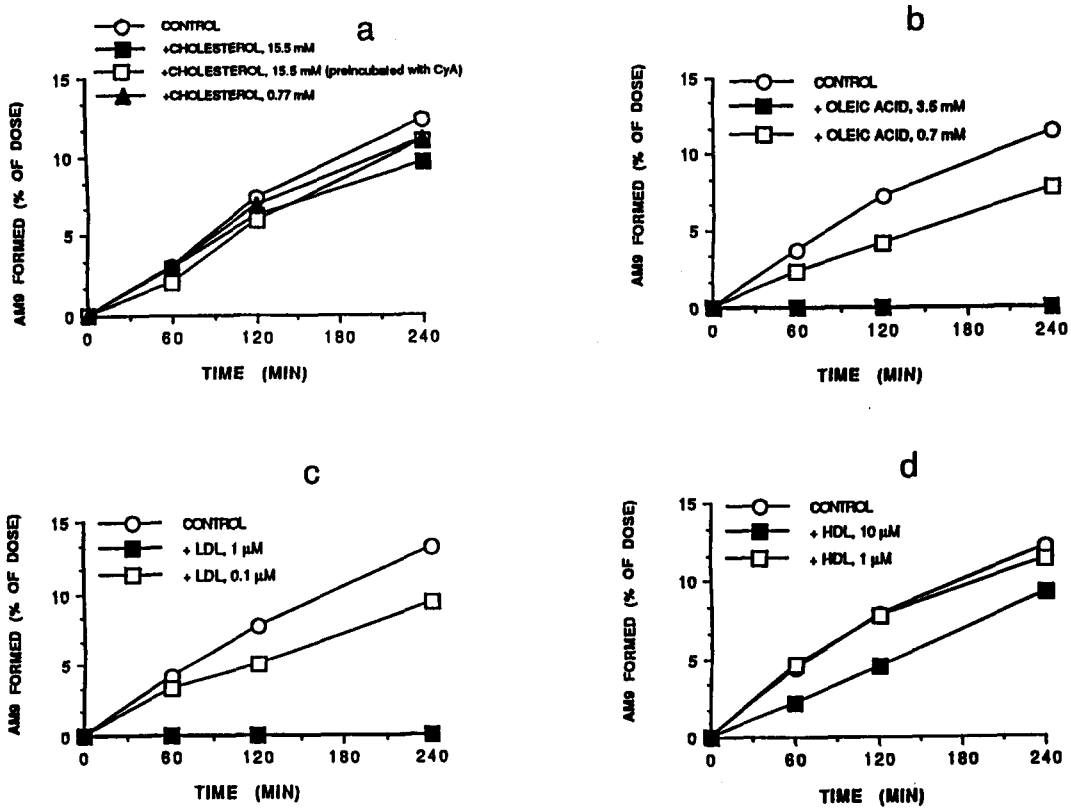

Fig. 6. Time course of AM9 formation at the initial CyA concentration of 1.2 μg/mL in the presence of cholesterol (a), oleic acid (b), LDL (c), and HDL (d). Each value is the mean of at least three experiments.

metabolism. LDL possessed the strongest effects as compared to other lipids at comparable molar concentrations. Since the effects of cholesterol on drug uptake (Fig. 4a, Table 1) and metabolism (Figs. 5a, 6a, and 7a, Table 1) were negligible, the strong binding of CyA to LDL probably results from lipoprotein structure which is not found with the major LDL component, cholesterol. Although VLDL was not directly tested in this study, the considerable drug uptake in the presence of 0.7 mM oleic acid may imply an insignificant effect of VLDL on the drug uptake.

Ideally, the measurements of the effects of lipids on CyA cell uptake and metabolism should have been made as a function of unbound CyA concentrations. Measurement of free CyA concentrations, however, is known to be difficult with results generally believed to be nonreproducible and unreliable with the commonly used methods such as equilibrium dialysis and ultrafiltration [34]. It is, nevertheless, apparent from this study that the decrease in drug metabolism by lipids tested is most likely due to the reduction in the free drug concentration available for the uptake as suggested by the similarity in the effects of lipids on the CyA uptake and metabolism (Table 1). In the case of oleic acid, the parallel decrease in uptake and metabolism also is explained by the loss of cell viability [35]. The values of CyA associated with the cells (column 3, Table 1) of about 49 and 67% of the controls found in the presence of LDL and oleic acid at high concentrations, respectively, when no metabolism occurred (Figs. 4, b and c, 5, b and c, 6, b and c, and Table 1) are probably explained as due to CyA adsorption to the cell membranes (60% of initial CyA concentration was associated with nonviable cells after a 10-min incubation). The lower CyA adsorption to the cells obtained in the presence of 1 μM LDL is consistent with the suggestion of preferentially binding of CyA to LDL.

The decrease in CyA uptake and metabolism in the presence of LDL found in this study is in contrast to the hypothesis proposed by Gupta and Benet [10] that a CyA–LDL complex may enhance CyA uptake into cells, thereby enhancing the metabolism of the drug. The negative finding in the present study was not likely to result from an inability of rat hepatocytes to take up human LDL, as used here, since Chao *et al.* [33] observed comparable metabolism rates of human and rat LDL in rat livers. Differences, however, may exist between LDL receptor expression in isolated cells used in the present study versus an *in vivo* system in humans as in the previous study [10]. In that study increases in the volume of distribution and clearance of CyA following high fat meals were accompanied by decreased levels of

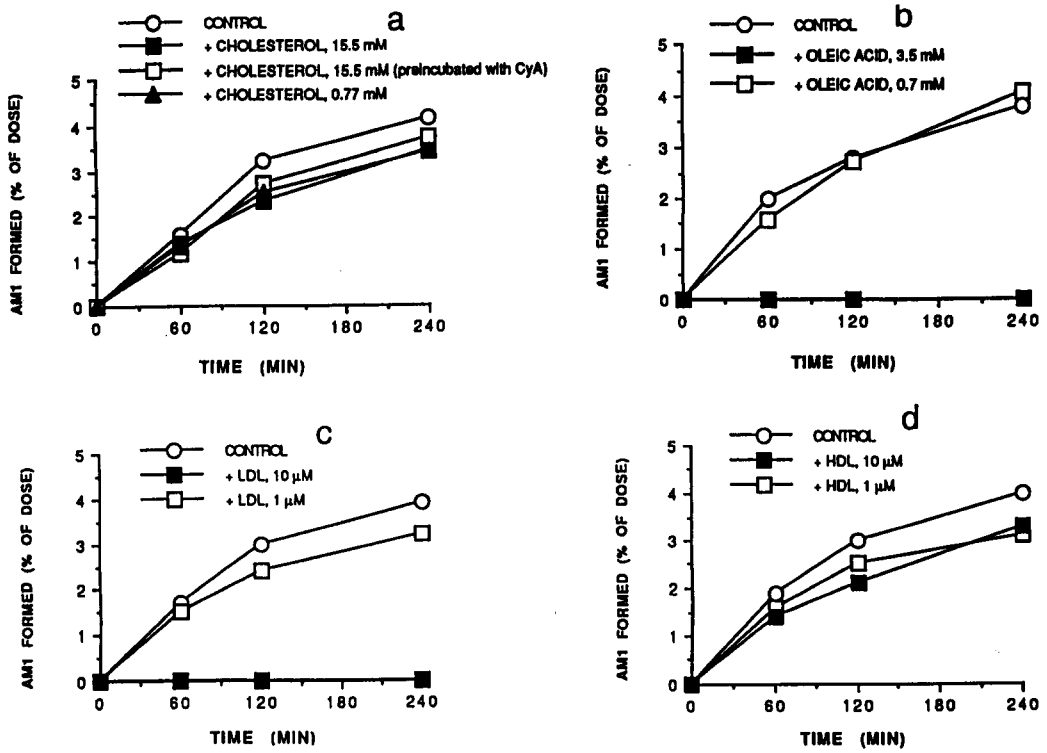

Fig. 7. Time course of AM1 formation at the initial CyA concentration of 1.2 μg/mL in the presence of cholesterol (a), oleic acid (b), LDL (c), and HDL (d). Each value is the mean of at least three experiments.

Table 1. Summary of lipid effects on CyA uptake and metabolism at a CyA concentration of 1 μM

Lipid	Concentration	Cell-associated CyA during 5–10 min (% of control)	Cell-associated CyA after subtraction of binding to nonviable cells (% of control)	CyA disappearance in 4 hr (% of control)	AM9 formation in 4 hr (% of control)
Cholesterol (4)*	0.77 mM	104.8 ± 6.3	101.1 ± 14.7	108.9 ± 9.2	100.2 ± 19.0
	15.5 mM	106.1 ± 8.3	108.8 ± 16.7	93.6 ± 16.0	91.0 ± 17.0
Oleic acid (4)	0.7 mM	91.3 ± 3.5	74.3 ± 10.4†	68.4 ± 10.9†	62.3 ± 7.1†
	3.5 mM	66.7 ± 11.6†	−2.3 ± 24.0†‡	0†	0†
LDL (3)	0.1 μM	84.3 ± 9.2†	76.2 ± 20.5†	85.4 ± 6.3†	69.2 ± 10.6†
	1 μM	48.6 ± 14.7†	−46.2 ± 35.5†‡	0†	0†
HDL (3)	1 μM	96.4 ± 5.7	91.4 ± 15.7	107.9 ± 12.1	91.1 ± 19.0
	10 μM	81.6 ± 9.5†	53.0 ± 19.5†	75.2 ± 10.7†	75.9 ± 4.5†

Values are means ± SD.

* Number of hepatocyte preparations used.

† Significantly different from the control experiment ($P < 0.05$). Control values are depicted in Fig. 1 for CyA at 1.2 μg/mL.

‡ Cell-associated CyA was less than that obtained from the binding of CyA to nonviable cell experiments, but the values are not significantly different from zero.

cholesterol and increased triglycerides [10, 11]. These apparently opposite findings may be rationalized as follows. A decrease in total cholesterol level would probably be a reflection of a decrease in plasma LDL since the majority of plasma cholesterol is in LDL [31]. As a consequence, more free drug was available to be taken up by the liver and probably other cells, resulting in greater drug distribution and metabolism. Although an increase in triglyceride levels was also obtained, its effect on the drug disposition was probably small. Triglycerides are mostly found in chylomicron (more than 80%) and

VLDL (50–60%) fractions [31]. Both chylomicrons and VLDL levels in plasma in healthy subjects [31] are relatively low and CyA bindings to these lipoproteins are low as compared to HDL and LDL [4–6]. This explanation is also supported by the negative correlation found between measured free fractions of CyA and serum cholesterol levels in healthy volunteers and renal transplant patients [36, 37]. No such correlation was observed with measured serum triglyceride levels in healthy subjects [36]. The results reported here are also consistent with the findings of Awni *et al.* [38] who noted lower CyA AUCs following oral drug dosing in a subgroup of renal transplant patients who exhibited lower LDL concentrations as compared to a subgroup with mean LDL concentrations about 50% higher. In this study significantly lower levels of cholesterol, HDL and triglycerides were also observed in the low LDL subgroup.

In summary, the present study demonstrates that plasma lipids inhibit CyA uptake into liver cells and consequently slows its rate of metabolism. These findings support the suggestion of the important role of drug binding to plasma lipids in CyA disposition. Further investigations using isolated liver perfusion are now under way to confirm the present results.

Acknowledgements—The authors wish to acknowledge Dr. Maria Almira Correia for her valuable suggestions and Ms. Susan Wong for excellent technical assistance. This work was supported by NIH Grant 26691.

REFERENCES

1. Maurer G, Metabolism of cyclosporine. *Transplant Proc* **17** (Suppl 1): 19–26, 1985.
2. Wagner O, Schreier E, Heitz F and Maurer G, Tissue distribution, disposition, and metabolism of cyclosporine in rats. *Drug Metab Dispos* **15**: 377–383, 1987.
3. Fabre G, Bertault-Peres P, Fabre I, Maurel P, Just S and Cano J-P, Metabolism of cyclosporin A. I. Study in freshly isolated rabbit hepatocytes. *Drug Metab Dispos* **15**: 384–390, 1987.
4. Gurecki J, Warty V and Sanghvi A, The transport of cyclosporine in association with plasma lipoproteins in heart and liver transplant patients. *Transplant Proc* **17**: 1997–2002, 1985.
5. Mraz W, Zink RA, Graf A, Preis D, Illner WD, Land W, Siebert W and Zottlein H, Distribution and transfer of cyclosporine among the various human lipoprotein classes. *Transplant Proc* **15** (Suppl 1): 2426–2429, 1983.
6. Sgoutas D, Macmahon W, Love A and Jerkunica I, Interaction of cyclosporin A with human lipoproteins. *J Pharm Pharmacol* **38**: 583–588, 1985.
7. Wassef R, Cohen Z and Langer B, In vivo interaction of cyclosporine and intralipid. *Transplantation* **41**: 266–268, 1986.
8. Brunner LJ, Vadiei K and Luke DR, Cyclosporine disposition in the hyperlipidemic rat model. *Res Commun Chem Pathol Pharmacol* **59**: 339–348, 1988.
9. Lithell H, Odlind B, Selinus I, Lindberg A, Lindstrom B and Frodin L, Is the plasma lipoprotein pattern of importance for treatment with cyclosporine? *Transplant Proc* **18**: 50–51, 1986.
10. Gupta SK and Benet LZ, High-fat meals increase the clearance of cyclosporine. *Pharm Res* **7**: 46–48, 1990.
11. Gupta SK, Manfro RC, Tomlanovich SJ, Gambertoglio JG, Garovoy MR and Benet LZ, Effect of food on the pharmacokinetics of cyclosporine in healthy subjects following oral and intravenous administration. *J Clin Pharmacol* **30**: 643–653, 1990.
12. Bouis P, Brouillard J-F, Fischer V, Donatsch P and Boelsterli UA, Effect of enzyme induction on Sandimmun^{R} (cyclosporin A) biotransformation and hepatotoxicity in cultured rat hepatocytes and *in vivo*. *Biochem Pharmacol* **39**: 257–266, 1990.
13. Havel RJ and Hamilton RL, Hepatocytic lipoprotein receptors and intracellular lipoprotein catabolism. *Hepatology* **8**: 1689–1704, 1988.
14. Griglio S, Lipoprotein metabolism in isolated and cultured hepatocytes. Part 2: Lipoprotein catabolism by liver cells. In: *Research in Isolated and Cultured Hepatocytes* (Eds. Guillouzo A and Guguen-Guillouzo C), pp. 135–154. John Libbey Eurotext, INSERM, Paris, 1986.
15. Consensus Document: Hawk's Cay Meeting on Therapeutic Drug Monitoring of Cyclosporine. *Transplant Proc* **22**: 1357–1361, 1990.
16. Seglen PO, Preparation of isolated rat liver cells. In: *Methods in Cell Biology* (Ed. Prescott D), Vol. 14, pp. 29–83. Academic Press, New York, 1975.
17. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 52, pp. 61–71. Academic Press, New York, 1978.
18. Gupta SK and Benet LZ, HPLC measurement of cyclosporine in blood, plasma and urine and simultaneous measurement of its four metabolites in blood. *J Liq Chromatogr* **12**: 1451–1462, 1989.
19. Lensmeyer GL, Wiebe DA and Carlson IH, Identification and analysis of nine metabolites of cyclosporine in whole blood by liquid chromatography. 1: Purification of analytical standards and optimization of the assay. *Clin Chem* **33**: 1841–1850, 1987.
20. Shah AK and Sawchuk RJ, Improved liquid chromatographic determination of cyclosporine and its metabolites in blood. *Clin Chem* **34**: 1467–1471, 1988.
21. Wallenmacq PE, Lhoest G and Dumont P, Isolation, purification and structure elucidation of cyclosporin A metabolites in rabbit and man. *Biomed Environ Mass Spectrom* **18**: 48–56, 1989.
22. Ziegler K, Polzin G and Frimmer M, Hepatocellular uptake of cyclosporin A by simple diffusion. *Biochim Biophys Acta* **938**: 44–50, 1988.
23. Rodl S and Khoshsorur G, Binding of cyclosporine A to human serum lipoproteins. *Transplant Proc* **22**: 287–288, 1990.
24. Urien S, Zini R, Lemaire M and Tillement JP, Assessment of cyclosporine A interactions with human plasma lipoproteins *in vitro* and *in vivo* in the rat. *J Pharmacol Exp Ther* **253**: 305–309, 1990.
25. Schwenk M, Transport systems of isolated hepatocytes: Studies on the transport of biliary compounds. *Arch Toxicol* **44**: 113–126, 1980.
26. Chenery RJ, The utility of hepatocytes in drug metabolism studies. In: *Progress in Drug Metabolism* (Ed. Gibson GG), Vol. II, pp. 217–265. Taylor & Francis, London, 1988.
27. Johansson A, Henricsson S and Moller E, A novel sulfate conjugate of cyclosporine occurring in high concentrations *in vivo*. *Transplantation* **49**: 619–622, 1990.
28. Hill JO, Peters JC, Swift LL, Yang D, Sharp T, Abumrad N and Greene HL, Changes in blood lipids during six days of overfeeding with medium or long chain triglycerides. *J Lipid Res* **31**: 407–416, 1990.
29. Åkesson B, Effect of different fatty acids on triacylglycerol secretion in isolated rat hepatocytes. *Lipids* **15**: 677–681, 1980.
30. Davis RA and Boogaerts JR, Intrahepatic assembly of

very low density lipoproteins: Effect of fatty acids on triacylglycerol and apoprotein synthesis. *J Biol Chem* **257**: 10908–10913, 1982.
31. Scanu AM, Plasma lipoproteins: An overview. In: *Biochemistry and Biology of Plasma Lipoproteins* (Eds. Scanu A and Spector A), pp. 1–10. Marcel Decker, New York, 1986.
32. Altman PL and Dittmer DS, *Biology Data Book*, Vol 3, p. 79. Federation of American Societies for Experimental Biology, Bethesda, MD, 1974.
33. Chao Y, Windler EE, Chen GC and Havel RJ, Hepatic catabolism of rat and human lipoproteins in rats treated with 17 α-ethinyl estradiol. *J Biol Chem* **254**: 11360–11366, 1979.
34. Legg B and Rowland M, Cyclosporin: Measurement of fraction unbound in plasma. *J Pharm Pharmacol* **39**: 599–603, 1987.
35. Danis M, Kauffman FC, Evans RK, Holtzclaw D, Reinke LA and Thurman RG, Inhibition of *p*-nitroanisole *O*-demethylation in perfused rat liver by oleate. *Biochem Pharmacol* **34**: 609–616, 1985.
36. Legg B, Gupta SK, Rowland M, Johnson RWG and Solomon LR, Cyclosporin: Pharmacokinetics and detailed studies of plasma and erythrocyte binding during intravenous and oral administration. *Eur J Clin Pharmacol* **34**: 451–460, 1988.
37. Lindholm A, Henricsson S and Gang P, The free fraction of cyclosporine in plasma: Clinical findings with a new method. *Transplant Proc* **20** (Suppl. 2): 377–381, 1988.
38. Awni WM, Heim-Duthoy K and Kasiske BL, Impact of lipoproteins on cyclosporine pharmacokinetics and biological activity in transplant patients. *Transplant Proc* **20**: 1193–1196, 1990.