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Interaction of cyclosporin A with human lipoproteins*

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The ultracentrifugal fractionation of human serum after previous incubation with cyclosporin A showed that, in healthy fasting individuals, 8% of cyclosporin A was found in the very low density lipoproteins (VLDL), 31% in the low density lipoproteins (LDL), 46% in the high density lipoproteins (HDL) and 15% in the non-lipoprotein protein fraction. In non-fasted, healthy and in non-fasted, lipaemic individuals, 7 and 6% of cyclosporin A was found in chylomicrons, 9 and 13% in VLDL, 28 and 30% in LDL, 39 and 37% in HDL, and 12 and 13% in the non-lipoprotein protein fraction, respectively. In patients receiving cyclosporin A the distribution varied from 12 to 19% in VLDL, 21 to 28% in LDL, 33 to 43% in HDL, and 13 to 20% in non-lipoprotein proteins. The interaction between cyclosporin A and isolated normal human lipoproteins was also studied by ultrafiltration. All lipoproteins exhibited a non-saturable, low affinity, high capacity uptake for cyclosporin A. Analysis of the uptake by phospholipid vesicles showed a similar uptake, suggesting that cyclosporin A dissolves in the lipophilic portion of the lipoprotein molecule rather than being associated with specific binding sites.

In a report by Lemaire & Tillement (1982) the distribution of cyclosporin A (CsA) among plasma proteins, leucocytes and erythrocytes was studied in-vitro. More than two-thirds of the plasma-bound drug was distributed among the lipoprotein fraction and less than one-third associated with the fraction containing non-lipoprotein proteins. Mraz et al (1983) have presented evidence for the exclusive association of CsA with lipoproteins in plasma from renal transplant patients.

We have examined the distribution of CsA in human serum lipoprotein classes from serum incubated with CsA in-vitro and compared it with distribution of the drug in serum or plasma lipoproteins from transplant patients receiving the drug. Furthermore, in an effort to understand better the relationship between lipoprotein and CsA, binding of CsA to isolated human serum lipoproteins was determined by ultrafiltration.

MATERIALS AND METHODS

Blood samples from healthy individuals in the fasting state or from non-fasted patients with moderate hyperlipidaemia were collected into plain evacuated tubes. Lipidaemia was defined as fasting triglyceride levels above 2·5 mg ml^{-1} and/or cholesterol above 2·5 mg ml^{-1}. Fresh, heparinized blood was obtained from patients receiving daily oral doses of CsA (12–17 mg kg^{-1}) after organ transplantation. Normolipidaemic patients who had fasted 8–12 h were sampled 12 h before the next dose.

Serum or plasma was obtained by centrifugation at 1200*g* for 10 min at room temperature (20 °C). All work was carried out at room temperature, to eliminate temperature-dependent changes of CsA distribution among blood components in-vitro (Wenk et al 1983; Dieperink 1983).

Human serum lipoproteins were prepared by ultracentrifugation according to Havel et al (1955). The VLDL, LDL and HDL obtained gave a single band on agarose electrophoresis.

[^{3}H]Dihydrocyclosporin A, ([^{3}H]CsA), (608 Ci mol^{-1}), synthesized at the Pharmaceutical Department of Sandoz, Ltd (Basel, Switzerland), had a radiochemical purity of approximately 98% confirmed by thin layer chromatography on silica gel G plates with appropriate solvent systems ($CHCl_3$–MeOH, 93 : 7). [^{3}H]CsA mixed with non-radioactive drug at different concentrations in ethanol was added to human serum (not to exceed 50 μmol ethanol $litre^{-1}$ serum) and the mixture swirled gently in a water bath at 23 °C. After 1 h incubation, samples were withdrawn and centrifuged to separate serum lipoproteins as described by Havel et al (1955). Repeated determinations of the same pool after addition of [^{3}H]CsA (200 ng ml^{-1} serum) gave a coefficient of variation 18·2% for VLDL, 5·4% for LDL, 2·9% for HDL and 2·8% for the non-lipoprotein protein fraction, respectively.

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Levels of CsA in whole plasma and plasma lipoproteins isolated by ultracentrifugation in several patients undergoing chronic therapy with CsA were determined by radioimmunoassay (RIA) using reagents supplied by Sandoz. Determinations were in triplicate using 20 μl plasma, diluted 1/50 with 0·05 M Tris buffer, pH 8·5 (Donatsch et al 1981). The interassay variation was ±5%, and the assay covered the range 60–4000 ng ml^{-1}. RIA was also used to determine CsA levels in serum lipoproteins from serum or whole blood which was spiked with different amounts of drug and incubated for 1 h at 23 °C before ultracentrifugation and lipoprotein separation.

Separate series of experiments investigating binding of CsA to isolated lipoprotein fractions were performed by ultrafiltration. Binding assays were performed in duplicate in polystyrene tubes containing [^{3}H]CsA in appropriate concentrations ranging from 1×10^{-10} to 5×10^{-7} M in a final reaction volume of 1 ml. Specificity in binding was determined with and without a 500-fold excess of unlabelled CsA.

Ultrafiltration with Amicon Centrifree devices (Amicon Corp. Danvers, MA), which are designed to separate free from bound ligands in plasma, was as described by Vlahos et al (1982). To make a direct comparison of the interaction of CsA with different lipoproteins, its uptake was expressed as B/F × P; B, F and P being the molar concentrations of bound [^{3}H]CsA, unbound [^{3}H]CsA and lipoprotein, respectively.

Protein concentrations were determined by the method of Lowry et al (1951). The concentration of lipoproteins was then calculated on the assumption that the protein part of the lipoprotein (apoprotein) occupies 4·3% of the chylomicron, 8·3% (w/w) of the VLDL, 22·7% (w/w) of the LDL, and 58·1% (w/w) of the HDL molecules (Burley 1971). The average molecular weights of VLDL, LDL and HDL were estimated to be $5{\cdot}2 \times 10^6$, $2{\cdot}3 \times 10^6$ and $2{\cdot}5 \times 10^5$, respectively (Burley 1971).

Cholesterol and triglycerides were determined with an Abbott ABA 100 instrument, according to Allain et al (1974) and Bucolo & David (1973), respectively.

Liposomes were prepared using the method of Bangham et al (1974). The starting material contained 65 μmol each of egg yolk phosphatidyl choline, and cholesterol. Vesicles (3·2 μmol total lipid) were incubated for 30 min in 1 ml phosphate-buffered saline (PBS) containing concentrations of [^{3}H]CsA ranging from $1{\cdot}2 \times 10^{-10}$ to $2{\cdot}4 \times 10^{-7}$ M. Specificity in binding was determined using a 500-fold excess of unlabelled CsA.

To measure partition of [^{3}H]CsA between an organic solvent and an aqueous phase the following systems were used: (1) chloroform vs PBS, pH 7·4 and (2) hexanes (b.p. 68–70 °C) vs PBS. The labelled drug was dissolved in 10 ml of the organic solvent (pre-equilibrated with PBS), 10 ml of PBS (pre-equilibrated with the organic solvent) added and the system shaken for 5 min; the phases were allowed to separate and the concentration of labelled material in each phase was determined by counting. For comparison, the same experiment was performed with another hydrophobic molecule, [1,2,6,7,^{3}H]-progesterone (100 Ci $mmol^{-1}$) purchased from New England Nuclear Corp. Boston, MA.

The measurement of radioactivity was in a LKB-Wallac liquid scintillation counter (model 1210 Ultrabeta) with Maxifluor as scintillation cocktail. Counting efficiency, as determined by automatic external standard, was approximately 35% and did not change when either buffer or protein solution was assayed.

The data are expressed as mean and standard deviation (s.d.). Significance of differences was assessed by Student's paired *t*-test.

RESULTS

In preliminary experiments it was established that [^{3}H]CsA rapidly bound to blood components, an equilibrium being established during the first 30 min of incubation (Atkinson et al 1983; Niederberger et al 1983) that was temperature-dependent with serum proteins binding less CsA at lower temperatures than they did at higher temperatures (Dieperink 1983). Hence, we routinely allowed all samples to remain at room temperature for 2 h before centrifugation at the same temperature.

Table 1 shows the distribution of [^{3}H]CsA among serum components from healthy fasted and non-fasted individuals as well as from non-fasted lipidaemic patients. Serum [^{3}H]CsA concentration was 100 ng ml^{-1}. The values varied within the same category (see s.d. in Table 1). Significant ($P < 0{\cdot}01$) differences in the [^{3}H]CsA uptake by HDL were observed among samples from fasted and non-fasted individuals. In samples from non-fasted healthy and lipidaemic individuals, CsA binding to chylomicrons caused lower binding to other serum proteins.

Table 2 shows the distribution of [^{3}H]CsA among lipoproteins in serum from fasted individuals as a function of serum drug levels. The results appeared

Table 1. Distribution of [^{3}H]CsA in human serum lipoprotein fractions. The distribution in each fraction was calculated as the amount of radioactivity present in the fraction divided by the total amount of radioactivity present in all the fractions.

	Drug bound (%)[a]		
	Normal		Lipaemic
	Fasted	Non-fasted	Non-fasted
Fraction	n = 8	n = 7	n = 7
Chylomicrons	—	8·6 (3·1)	13·0 (1·7)
VLDL	8·4 (2·5)	6·7 (2·6)	5·9 (0·9)
LDL	31·0 (4·0)	28·4 (3·4)	30·3 (2·5)
HDL	45·7 (5·4)	38·9[c] (5·0)	36·9[c] (4·1)
Proteins[b]	14·7 (1·7)	11·2 (2·0)	12·3 (3·5)

[a] Mean of n experiments and s.d. in parentheses.
[b] Non-lipoprotein proteins remaining after the removal of lipoproteins by ultracentrifugation.
[c] Significantly different ($P < 0·01$) from the fasted.

Table 2. Distribution of [^{3}H]CsA in human serum lipoproteins as a function of plasma CsA level.[a]

	Drug concn (ng ml^{-1})		
	100	200	500
Fraction		Drug bound (%)	
VLDL	8·5	6·5	7·1
LDL	29·3	28·1	31·5
HDL	47·6	44·8	46·2
Protein[b]	12·1	9·3	11·0

[a] Mean of two independent experiments.
[b] Non-lipoprotein proteins remaining after the removal of lipoprotein by ultracentrifugation.

Table 3. Distribution of CsA in plasma from transplant patients undergoing CsA therapy.

		Drug (%)			
Range[a]	n	VLDL	LDL	HDL	Proteins[b]
100–300	6	19·1 ± 7[c]	28·2 ± 7·5	34·9 ± 5·8	15·4 ± 4·9
300–500	7	17·9 ± 5·6	22·3 ± 6·1	38·8 ± 8	15·2 ± 4·1
500–1000	6	16·6 ± 7·3	20·5 ± 11·5	33·3 ± 10·3	13·5 ± 5·8
1000–1400	3	12·3 ± 5·2	24·9 ± 7·3	43·1 ± 5·9	14·9 ± 4·4

[a] CsA in plasma, ng ml^{-1}.
[b] Non-lipoprotein protein remaining after the removal of lipoprotein by ultracentrifugation.
[c] Each value is the mean ± s.d. of n samples.

to be concentration-independent up to and including 500 ng ml^{-1} serum.

In other experiments (not shown), non-radioactive CsA was incubated in human serum or plasma, before the lipoproteins were separated by ultracentrifugation, and CsA was determined by RIA. The results were in agreement with those in Table 2, showing the equivalence in the in-vitro distribution of radioactive CsA. Other results (not shown) and those of Smith et al (1983) have indicated that, provided blood samples containing CsA are carefully handled after collection, they show an excellent correlation between serum and plasma CsA.

Table 3 shows the distribution of CsA, as determined by RIA, in plasma lipoproteins from 22 transplant patients being treated with CsA. The drug levels ranged from therapeutic to toxic. Distribution among plasma lipoproteins showed a wide inter-individual variation with no other characteristic pattern relating to CsA concentration in plasma except that its distribution among lipoproteins was always: HDL > LDL > VLDL and non-lipoprotein protein.

To substantiate the above observations, the binding of CsA to individual lipoproteins in-vitro was studied. Fig. 1 shows results from binding experiments of [^{3}H]CsA with isolated lipoproteins. Within

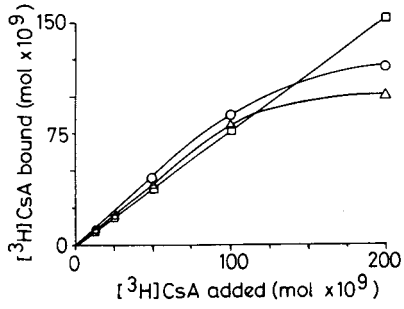

FIG. 1. Uptake of [^{3}H]CsA by isolated human serum lipoproteins: VLDL (○), LDL (△) and HDL (□) containing the indicated concentrations of [^{3}H]CsA. Each point represents the mean value of duplicate experiments. All lipoproteins at a concentration of 4 mg ml^{-1}.

a concentration range up to and including 100 pmol ml^{-1} serum, the drug interacted linearly with VLDL, LDL and HDL. At concentrations including 200 pmol ml^{-1}, the uptake of CsA by VLDL and LDL showed signs of saturation whereas uptake by HDL continued to be linear.

Fig. 2 shows the effect of varying the concentration of serum lipoproteins on the binding at a fixed concentration (200 ng ml^{-1}) of [^{3}H]CsA. The lipoprotein concentration varied from physiological to 20-fold dilutions with PBS. At 1 mg lipoprotein, 15% of [^{3}H]CsA was free in VLDL, 9% in LDL and 5% in HDL. Higher dilutions gave inconsistent results due to losses of free [^{3}H]CsA on the wall of the vessel. The effect of temperature on the binding of CsA to human serum lipoproteins was investigated at 20 and 37 °C. The results (not shown) demonstrated a tighter binding of CsA to all lipoproteins at 37 °C.

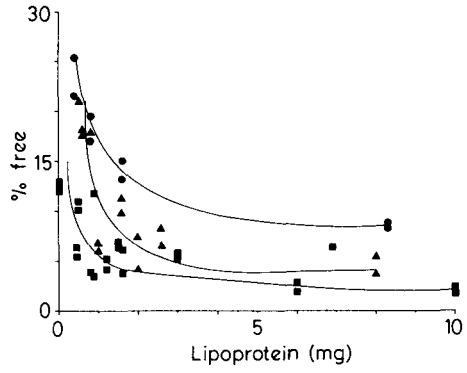

FIG. 2. Uptake of [^{3}H]CsA by isolated serum lipoproteins as a function of lipoprotein concentration: VLDL (●), LDL (▲) and HDL (■). Each point represents an experiment in the presence of 200 ng [^{3}H]CsA ml^{-1} serum. Lines represent the best fit.

In Fig. 3, results from the binding experiments with VLDL, LDL and HDL are plotted according to Scatchard (1949). The concentration of each lipoprotein was approximately 20 times less than that in normal serum (Nichols 1969). At high dilutions of lipoproteins, there was an excess of free [^{3}H]CsA which is a condition required for valid radioligand binding studies (Klotz 1982). Fig. 3 shows that for the

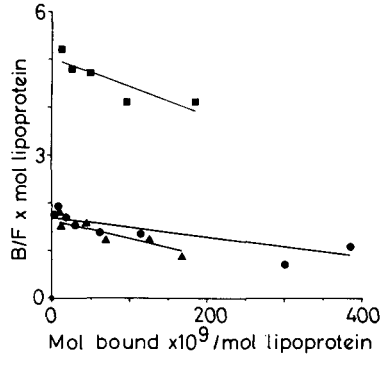

FIG. 3. Computer-generated least square plot of bound (B) over free (F) [^{3}H]CsA per mole of lipoprotein against bound [^{3}H]CsA per mole of lipoprotein: VLDL (●), LDL (▲) and HDL (■). Each point represents the average value of duplicate experiments.

range of drug tested the response B/F × P vs B/P gave practically straight lines which descended slowly to intercept at the X-axis, suggesting an apparent single kind of binding site of low affinity and high capacity for all lipoproteins. Excess of non-radioactive CsA failed to displace bound [^{3}H]CsA from lipoproteins suggesting its unspecific binding to all serum lipoproteins. Although theoretical considerations did not allow calculation of affinity and capacity constants, the binding data in Fig. 3 strongly suggested that [^{3}H]CsA exhibited higher affinity for HDL and less (and almost equal) for VLDL and for LDL.

Considering that CsA is a very hydrophobic molecule, it is possible that these studies were simply reflecting partitioning of CsA into the lipid component of the lipoproteins. To test this hypothesis, two sets of experiments were conducted. Fig. 4

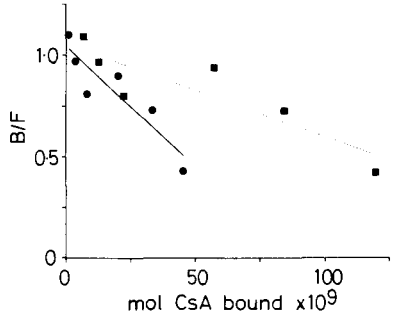

FIG. 4. Scatchard plots of the unspecific (■) and specific (●) uptake of [^{3}H]CsA by phospholipid vesicles. Each point represents the average of duplicate experiments. Computer-generated least square plots.

shows the results from the first experiment in which the uptake of [^{3}H]CsA by liposomes was measured. Analysis of the data showed binding, displaceable by excess of CsA, which exhibited a single binding site with an apparent dissociation constant of Kd = 0·8 × 10^{-7} M. In the second experiment, the partition of [^{3}H]CsA at different concentrations between organic solvents and PBS was examined. Table 4 shows high partition coefficients of 87–88 and 65–68 for CsA in chloroform and hexanes, respectively. Progesterone gave similarly high values.

Table 4. Partition coefficients of ligands. Values expressed as ligand concentration in organic phase/concentration in phosphate-saline buffer, 1 : 1.

	[^{3}H]CsA	[^{3}H]Progesterone
$CHCl_3$/buffer		
50 µg[a]	88[b]	85
500 µg[a]	87	84
Hexanes/buffer		
50 µg	65	78
500 µg	68	79

[a] Amount of ligand added in 1 ml buffer.
[b] Each value is the mean of 3 determinations.

DISCUSSION

The results reported in this study show that CsA or [^{3}H]CsA, when preincubated in-vitro with human serum, is exclusively associated with serum lipoproteins, with HDL binding more than LDL which binds more than VLDL. Chylomicrons, when present, bind CsA and affect total binding. The results show a varying binding of CsA to lipoproteins from individuals with disorders inducing changes in serum lipoprotein profiles. Since those are altered in renal impairment as well as in liver disease, the functional status of the transplanted kidney or liver will influence the profiles and consequently the CsA distribution among serum lipoproteins in transplant patients.

In patients receiving CsA, the results show large individual differences in the distribution among plasma lipoproteins, confirming and extending earlier results (Mraz et al 1983). These variations may reflect individual differences in lipoprotein patterns resulting from the constitution of the lipoproteins, age, sex, diet or disease of the patient. Furthermore, variance in results from different laboratories is expected and is due to differences in method (see Mraz et al 1983). Nevertheless, transplant patients receiving CsA show a distribution among plasma lipoproteins which is similar to the distribution observed in serum previously equilibrated with it.

To understand better the relation between CsA and human lipoproteins, binding studies with isolated lipoproteins were conducted. The results clearly indicate a preferential binding of CsA to HDL over LDL and VLDL with little or no preference between LDL and VLDL. The several-fold higher apparent affinity for HDL may explain the reason for the preferential association of CsA with HDL which is observed in all the present experiments (Tables 1, 2 and 3). On the other hand, the relative abundance of LDL in human serum may explain the reason for the major portion of CsA being carried by LDL.

However, lipoproteins like other proteins can be altered by different separation procedures and by the aqueous phase in the system used for binding studies. In the present study any conclusion based on binding data should be drawn with caution because the interaction between [^{3}H]CsA and lipoproteins is not inhibited by excess of CsA and shows none of the characteristics of the binding of a small molecule to a fixed number of sites on a protein. This, in conjunction with the result that protein-free liposomes bind [^{3}H]CsA in a manner similar to lipoproteins, seems to suggest that the CsA molecules may simply partition into the lipid moiety of the lipoprotein molecules. This hypothesis appears to be supported by the high partition coefficient of CsA in organic solvents and chloroform in particular. Rudman et al (1972) have already shown that lipophilic ligands with a partition coefficient over 11 in chloroform vs buffer actually dissolve in the lipid component(s) of macromolecules. Along these lines there are some reports describing the uptake of hydrophobic drugs by lipoproteins (Nilsen & Jacobsen 1975; Powis 1974) suggesting partition of the hydrophobic substance into the lipid phase without the participation of a protein or a lipid 'receptor'.

The experiments with the liposomes showed that 40–60% of the [^{3}H]CsA could be displaced by incubation in a 500-fold excess of unlabelled CsA. In contrast, a similar displacement was not observed in the association of [^{3}H]CsA with lipoproteins, which seems to suggest that proteins present in lipoproteins are involved in the uptake of CsA by further contributing to an unspecific binding of CsA to lipoprotein molecules. Another possibility is that the protein moiety promotes the uptake of CsA into the lipoprotein molecule and CsA becomes unavailable for exchange.

However, not all binding of CsA to biological macromolecules is unspecific. Ryffel et al (1980, 1982), using [^{3}H]CsA, reported the presence of receptors on human peripheral and mouse lymphocytes. Leoni et al (1978), on the other hand, using [^{125}I]CsA as radiolabelled ligand, failed to demonstrate saturable binding for human peripheral blood lymphocytes. These data suggest that structural changes of the peptide CsA (as those resulting from iodination) play an important role in the binding.

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