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In vitro study of cyclosporin absorption: vehicles and intestinal immaturity

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Abstract

The intestinal absorption of the immunosuppressant cyclosporin-A (CsA) has been studied in vitro using the Grass chamber technique applied to the proximal small bowel of rat. CsA concentration in the donor-phase was 100 mg/l for either 2-weeks or 6-weeks-old rat intestine. CsA was dissolved in different vehicles, including the oily solution Sandimmun® (CsA-S) and the new microemulsion Sandimmun Neoral® (CsA-SN) together with bile salts and pancreatic lipase. The limit of tissue viability was set at 90 min by monitoring the transepithelial electric resistance. Mucosal histology was studied by both optical and transmission electron microscopy. CsA was determined in the receptor-phase. CsA was partially metabolised by the 6-weeks-old rat intestine. In these experiments no difference was observed between CsA-S and CsA-SN absorption. On the contrary, the 2-weeks-old rat intestine did not allow CsA absorption from CsA-SN. This was related to intestinal immaturity and brush border erosion, as shown by electron microscopy. Hence, CsA absorption from microemulsion may require a mature and viable intestine. Microvilli of absorptive cells may play a role in CsA capture from the microemulsion-modified-micelles, in which CsA concentration is lower than in oily droplets. CsA absorption was not impaired by oily vehicles. Complete lipolysis of olive oil (CsA-S) induced an important decrease in CsA absorption, related to free oleic acid production.

Keywords: Cyclosporin; Intestinal absorption; Oil; Microemulsion; In vitro; Rat

1. Introduction

Cyclosporin-A (CsA) is a neutral, lipophilic, cyclic endecapeptide with a low aqueous solubility. In spite of a good stability in the gastrointesti-

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nal tract and a high partition coefficient, the absolute bioavailability of CsA after oral dosing is low with a high variability (10–60%) (Ptachcinski et al., 1986; Lindholm et al., 1988). This complicates the use of CsA, as the therapeutic index for this drug is low. Solubilization of CsA is obtained with oils and surfactants. In the gastrointestinal fluid, the oily solution Sandimmun® (CsA-S) is emulsified by bile salts. Absorption of CsA-S is modified by the emulsion droplet size (Tarr and Yalkowsky, 1989) and triglyceride digestion by pancreatic lipase (Reymond and Sucker, 1988; Reymond et al., 1988; Benmoussa et al., 1994). The new microemulsion Sandimmun Neoral® (CsA-SN) has a high concentration of polyoxyethylated surfactant which enhances CsA absorption and reduces inter- and intrasubject variability in the drug pharmacokinetics (Kovarik et al., 1994). As CsA-SN forms a micellar dispersion of CsA in the gut, absorption becomes bile independant (Trull et al., 1993; Färber et al., 1994; Kattner et al., 1994; Levy et al., 1994; Trull et al., 1994).

The variability of CsA absorption is related to chemical and physical properties of vehicles (Takada et al., 1985; Jevnikar et al., 1988; Reymond et al., 1988). The effect of food and digestion is not clear. In humans, fatty meals seem to enhance absorption of CsA-S and decrease absorption of CsA-SN (Belli et al., 1994; Holt et al., 1994; Levy et al., 1994; Mueller et al., 1994a,b). Therefore blood trough levels are not significantly different after administration of an equivalent amount of CsA-S or CsA-SN with fatty meal (Browne et al., 1994; Kahan et al., 1994; Kahan et al., 1995). In the rat, partial hydrolysis of triglyceride vehicles enhances CsA absorption (Reymond et al., 1988; Benmoussa et al., 1994) whereas free fatty acids decrease it (Takada et al., 1985). In human, digestion of CsA-S seems to be necessary for drug liberation and absorption (Vonderscher and Meinzer, 1994). On the other hand, CsA can be directly absorbed from non-digested-olive oil emulsion in the rat (Reymond et al., 1988; Tarr and Yalkowsky, 1989). The aim of this study was to compare different vehicles of CsA using an in vitro model for intestinal absorption. The role of intestinal maturity was also investigated.

2. Materials and methods

2.1. Materials

CsA powder, the oily solution Sandimmun® (CsA-S), and the new oral microemulsion Sandimmun Neoral® (CsA-SN) were kindly provided by Sandoz Laboratories (Rueil Malmaison, France). Both formulations contain 100 mg/ml CsA. The vehicle of CsA-S is a mixture of olive oil, Labrafil® M 1944 CS and absolute ethyl alcohol (40:42:18). CsA-SN is a mixture of a lipophilic solvent (corn oil) and a hydrophilic solvent (propylene glycol), together with absolute ethyl alcohol and a polyoxyethylated surfactant (Levy and Grant, 1994). Oily solutions of 100 mg/ml CsA were also prepared with the medium-chain triglyceride Miglyol 812® (Dynamit Nobel, France), a mixture of medium-chain mono- and diglycerides Imwitor 742® (Hüls, France), glyceryl monooleate (Stepan Europe, France), and oleic acid (Stéarinnerie Dubois, France). Sodium deoxycholate and porcine pancreatic lipase were purchased from Sigma (France). Corn oil and bile extract were supplied by Coopération Pharmaceutique Française (France). All other chemicals used were of analytical grade.

2.2. Donor-phase preparation

Except for CsA-S and CsA-SN, test solutions of drug were prepared by dissolving CsA in the following vehicles: Miglyol 812®, Imwitor 742®, glyceryl monooleate, oleic acid. CsA concentration was 100 mg/ml in vehicles. A drug emulsion was prepared by mixing 100 μl of test solution with 100 ml solution of sodium deoxycholate (3 mM) or bile extract (12 mM) in the hydrogenocarbonate Ringer buffer (pH 7.4) using a magnetic stirrer. It was sonicated for 10 min. CsA concentration was 100 mg/l in emulsion. Sonication was not applied to CsA-SN as a macroscopically monophasic solution was obtained, with an oil droplet size below 100 nm (Vonderscher and Meinzer, 1994). When relevant, 1 μl/ml of corn oil or 1.8 U/ml of pancreatic lipase were added to the buffer.

Table 1
Donor-phase composition

Groups	A	B	C	D	E	F	G	H	I	J	K
Age of rats (weeks)	6	2	6	2	6	6	2	2	2	2	2
Ringer buffer pH 7.4	+	+	+	+	+	+	+	+	+	+	+
Sodium deoxycholate 3 mM	+	+	+	+		+	+	+	+	+	+
Bile extract 12 mM					+						
Corn oil 1 μl/ml						+					
Pancreatic lipase 1.8 U/ml										+	
CsA-S 100 mg/l	+	+									
CsA-SN 100 mg/l			+	+	+	+					
CsA 100 mg/l + MCT							+				
CsA 100 mg/l + MCMD								+			
CsA 100 mg/l + GMO									+		
Digested CsA-S 100 mg/l										+	
CsA 100 mg/l + oleic acid											+

MCT, medium-chain triglyceride;
MCMD, medium-chain mono- and diglycerides;
GMO, glyceryl monooleate.

2.3. Thin-layer chromatography of lipid phase

After lipid digestion of the CsA-S emulsion, the absence of triglycerides was checked by thin-layer chromatography. Lipids were extracted from 20 ml of emulsion during the incubation period in 6 ml of chloroform-methanol (2:1 v/v). Samples obtained after organic phase evaporation were spotted on silica gel plates (60 F-254 Merck, France) next to lipid standards (olive oil, oleic acid, glyceryl monooleate). Lipid separation was performed at room temperature using the following mobile phase: *n*.hexane — *n*.heptane — diethyl ether — acetic acid (63:18.5:18.5:1, v/v) (Schmitz et al., 1984). Lipid spots were developed using the following reagent: manganese chloride (1.6 g) — concentrated sulfuric acid (16 ml) — methanol (240 ml) — deionized water (240 ml) (Schmitz et al., 1984; Magne et al., 1994).

2.4. In vitro experiments

Sprague Dawley rats (Iffa Credo, France) which were 6-weeks or 2-weeks-old were fasted for 6 or 2 h, respectively, with free access to water, before being killed. A heating unit containing eight diffusion cells as described by Grass and Sweetana (1988), was preheated at 37 ± 0.5°C. From one 6-weeks-old rat 4 cells were prepared, and 3 cells from one 2-weeks-old rat. Duodenojejunal segments were stripped, placed in an ice-cold oxygenated hydrogenocarbonate Ringer buffer (pH 7.4) then successively opened and mounted between 2 half-cells to expose the epithelial side to the donor phase of cells. The absorption surface area was 0.5 cm^2. Peyers patches easily identified in 6-weeks-old rat intestine were placed carefully next to the absorption area. Donor half-cells were filled with 4 ml of the donor-phase preparation, and receptor half-cells with 4 ml of hydrogenocarbonate Ringer buffer (pH 7.4). Both phases were prepared daily and preheated at 37 ± 0.5°C in a water bath. The donor-phase preparation was mixed with a magnetic stir bar before the experiment. The donor-phase composition is given in Table 1 for each experiment. A continuous flow of 40 ml/min O_2/CO_2 (95:5, AGA, France) in both half-cells ensured intestinal segments oxygenation and homogenization of phases for 90 min. Tissue viability was evaluated by measuring the transepithelial electric resistance (TEER) using a multimeter (Millicell®-ERS, Millipore).

2.5. Data analysis

Receptor-phase samples (0.5 ml) were collected after 90 min of incubation. CsA was determined through TDx® fluorescent polarization immunoassay (Abbott, France) using either monoclonal or polyclonal antibodies. The detection limits were 25 μg/l and 60 μg/l, respectively. Results are expressed as the mean ± SEM (standard error of the mean). A statistical analysis was performed using Kruskal and Wallis' test followed by Mann and Whitney's test. Differences were significant at $P < 0.05$.

2.6. Histology

After 90 min of incubation with sodium deoxycholate (3 mM) in a hydrogenocarbonate buffer (pH 7.4), intestinal segments of 2-weeks-old rats were immediately submerged in Bouin's solution (saturated picric acid aqueous solution — formaldehyde 37% — acetic acid; 75:20:5, v/v) for optical microscopy, or in glutaraldehyde 1.5% in phosphate buffer 0.01 M (pH 7.4; 4°C; 1 h) followed by 2% osmium tetroxide (1 h) for transmission electron microscopy (TEM). The tissue was subsequently dehydrated in ethanol and embedded in paraffin for optical microscopy, or Epon 812® araldite plastic (Janning, France) for TEM. Thin sections were stained by standard techniques: HES (Hematein — Erythrosin — Safran) for optical microscopy, or 2% uranyl acetate followed by 0.1–0.4% lead citrate for TEM. Villi and absorptive cells were photographed at magnifications of (× 630) or (× 10 000), respectively. The transmission electron microscope was JEOL® JEM 100 S (Japan).

3. Results

3.1. Viability of mucosa

Table 2 shows the TEER evolution of the intestine. Values measured in 6-weeks-old rats were greater than values in 2-weeks-old-rats. At the end of the incubation period, a decrease of more than 30% from the initial value was a good indication of a leak of CsA through intestinal segments. Microscopy was performed only on the 2-weeks-old rat intestine because of its great weakness. Optical microscopy did not show any villi damage (Fig. 1). Tight junctions were not disrupted and no cytolysis was observed by TEM but microvilli erosion was observed (Fig. 2).

Table 2
Transepithelial electric resistance (mean ± SEM) of rat small bowel in the Grass chamber (Ohm·cm²)

Time (min)	2-Weeks-old rat (n = 35)	6-Weeks-old rat (n = 17)
0	253 ± 6	345 ± 9
90	208 ± 6	260 ± 11
P (t-test)	<0.01	<0.01

3.2. CsA absorption

CsA concentrations in the receptor-phase of all groups of experiments after 90 min of incubation are shown in Table 3. In the 6-weeks-old rat experiments, concentrations determined using monoclonal antibodies were near the limit of detection, whereas polyclonal antibodies gave quantitative results (groups A, C, E, F). A statistical analysis showed that there was no difference between CsA-S and CsA-SN (groups A, C). Substitution of bile extract (12 mM) for sodium deoxycholate (3 mM) did not improve absorption from CsA-SN (groups C, E). Corn oil added to

Fig. 1. Villi of a 2-weeks-old rat duodeno-jejunal segment, after 90 min incubation in a Grass chamber. (Optical microscopy, HES, magnification × 630).

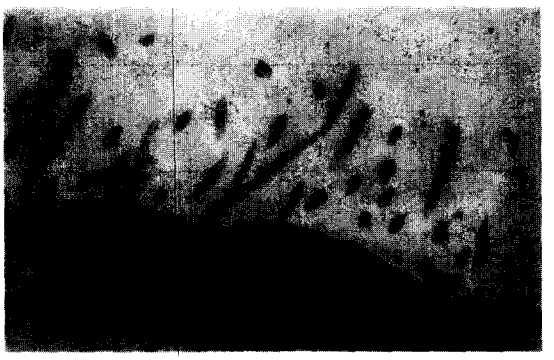

Fig. 2. Microvilli erosion in apex of a representative absorptive cell of a 2-weeks-old rat duodeno-jejunal segment, after 90 min incubation in a Grass chamber. (Transmission electron microscopy, uranyl acetate and lead citrate, magnification × 10 000).

the donor-phase significantly decreased CsA absorption from the microemulsion (groups C, F).

In the 2-weeks-old rat experiments, CsA levels determined using monoclonal antibodies were quantitative and allowed statistical comparison. There was no difference between glyceride vehicles, but a trend towards a greater absorption with triglycerides (groups B, G) compared with mono- and diglycerides (groups H, I). Long-chain triglycerides (group B) gave greater but not significant CsA concentrations than medium-chain triglycerides (group G). Complete digestion of CsA-S was obtained after 4 h of incubation with pancreatic lipase, as shown by the complete disappearance of the olive oil spot in thin-layer chromatography. Digested CsA-S significantly decreased CsA absorption compared with non-digested CsA-S (groups B, J). Oleic acid was the main lipolysis product. This free fatty acid as a vehicle induced the same decrease in CsA absorption as digested CsA-S (groups B, K). CsA-SN induced lower CsA absorption than CsA-S (groups B, D), whereas equivalent values were obtained with 6-weeks-old rat intestine.

4. Discussion

4.1. Limits of the in vitro system

Although such an in vitro system has been used for over 240 min (Grass and Sweetana, 1988), TEER decrease monitoring and microscopy studies limited the incubation time at 90 min in our study. A leak of CsA towards the receptor-phase occured in most of the experiments for longer incubation times. Absorption was represented by CsA concentration in the receptor-phase at 90 min. The CsA concentration in the donor-phase was set at 100 mg/l because the maximum absorption rate in rat bile was previously obtained for this value (Vonderscher and Meinzer, 1994). The main limit of this system was the absence of blood in the receptor-phase. It probably reduced CsA absorption as the drug affinity towards erythrocytes and plasma lipoproteins is high (Ptachcinski et al., 1986). CsA was not detected by monoclonal antibodies in the 6-weeks-old rat experiments. The detection only by polyclonal antibodies suggests an intestinal metabolism of CsA. Such a metabolism has already been described in vivo (Kolars et al., 1991) and in vitro (Tjia et al., 1991). The Grass chamber technique may be interesting for intestinal metabolism studies.

4.2. Comparison of glyceride vehicles

Our findings show that CsA could be directly absorbed from non-digested-oil emulsion in the rat. This is supported by other studies (Reymond et al., 1988; Tarr and Yalkowsky, 1989). A comparison of CsA-S and medium-chain triglyceride (MCT) confirms that the long-chain triglyceride is a better vehicle for CsA absorption, as already shown by Reymond et al. (1988). Similar results on the absorption of 2,2,bis(*p*-chlorophenyl)1,1,1-trichloroethane (DDT) (Palin et al., 1982) and probucol (Palin and Wilson, 1984) were previously obtained by comparing plasma concentrations after oral dosing in the rat. On the contrary, plasma concentration after oral dosing of isotretinoin in the rat was greater with MCT than with long-chain triglycerides (Nankervis et al., 1995). Irrespective of the fatty acid chain length, the degree of esterification of glycerides may influence CsA absorption, as triglycerides are better

Table 3
Cyclosporin concentrations in the receptor phase (mean ± SEM)

Vehicle	6-weeks-old rat			2-weeks-old rat	
	Group	MC (μg/l)	PC (μg/l)	Group	MC (μg/l)
CsA-S	A	30 ± 4	105 ± 5	B	278 ± 87
CsA-SN	C	34 ± 5	108 ± 10	D	49 ± 5(b)
CsA-SN	E	43 ± 6	112 ± 11		
CsA-SN	F	26 ± 2	76 ± 6(a)		
CsA-MCT				G	173 ± 48
CsA-MCMD				H	108 ± 25
CsA-GMO				I	155 ± 44
Digested CsA-S				J	64 ± 10(b)
CsA + oleic acid				K	49 ± 13(b)

MC, Monoclonal antibodies analysis;
PC, Polyclonal antibodies analysis;
(a) $P < 0.05$ compared to group C;
(b) $P < 0.05$ compared to group B.

vehicles than mono- and diglycerides. The mechanism involved is still unknown.

4.3. The micellar phase

The mechanism through which CsA is absorbed from the oil emulsion is hypothetic. As other lipophilic drugs, CsA may distribute between oil droplets and a large volume of a water-continuous phase that includes micellar aggregates of bile acids (Jandacek, 1982). The micellar solution may increase the transport of CsA towards absorptive cells. This transport was highly influenced by micellar composition, as CsA absorption was impaired by oil lipolysis, free oleic acid, and CsA-SN surfactant in 2-weeks-old rat.

On the contrary, in vivo studies have shown that oil hydrolysis increases absorption of CsA (Reymond et al., 1988) and digitoxin (Benmoussa et al., 1993). Oil digestion in these experiments was only partial. Hence, the increased absorption may be related to the reduction of oil droplet size (Tarr and Yalkowsky, 1989). This partial lipolysis effect should disappear when free fatty acids mix with micelles. Impairment of drug absorption by free fatty acids has already been observed for CsA (Takada et al., 1985) and isotretinoin (Nankervis et al., 1995).

CsA-SN absorption was impaired only by the 2-weeks-old rat intestine, whereas CsA-S absorption was not. Microemulsions such as CsA-SN contain a high concentration of surfactant (Ritschel et al., 1990, Ritschel, 1991) which mix with bile salts. Micellar composition was modified by the microemulsion CsA-SN. Comparison of CsA-SN absorption in 2-weeks-old and 6-weeks-old rat shows that intestinal maturity is important for CsA absorption from modified-micelles. Microvilli of absorptive cells may also play a role in CsA capture from the modified-micelles, as brush border erosion was observed by TEM in the 2-weeks-old rat intestine. Microemulsion-modified-micelles compete with the oily vehicle, as CsA-SN absorption was decreased by non-digested corn oil. The mechanism could consist in a distribution of CsA in the micellar-to-oil direction based on the partition coefficient (Jandacek, 1982; Benmoussa et al., 1994). The deprivation of the drug from the micellar phase resulted in reduced absorption. There is clinical evidence for this deprivation, as fatty meals decrease absorption of CsA-SN (Belli et al., 1994; Holt et al., 1994; Levy et al., 1994; Mueller et al., 1994a,b). CsA-SN absorption by the 6-weeks-old rat intestine was irrespective of the bile salts composition (groups C, E). This suggests a replacement of bile salts properties by the effect of polyoxyethylated sur-

factant. There is clinical evidence for this bile independence in liver transplant recipients (Trull et al., 1993; Färber et al., 1994; Kattner et al., 1994; Levy et al., 1994; Trull et al., 1994).

Tjia et al. (1991) first studied CsA absorption using the Ussing chamber technique (1% absorption after 3 h incubation). We found an absorption of about 0.1% after 1.5 h with a high variability related to the vehicle used and the intestinal maturity. All the results are consistent with those of Jandacek (1982): oil and micelles are very different in their effect on lipophilic drugs absorption. Furthermore, absorption from micelles should require a specific capture by microvilli of absorptive cells, which may be effective only in the mature and viable small bowel. Whether this mechanism is specific to CsA or common with all lipophilic drugs needs further investigation.

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