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METABOLISM OF CYCLOSPORINE AFTER ORTHOTOPIC LIVER TRANSPLANTATION

JEAN MICHEL FABRE, ROZANGELA PEDROSA,* ANDRÉE BONARDET,† LYDIANE PICHARD,* BERNARD DESCOMPS,† JACQUES DOMERGUE, HUGUES BAUMEL and PATRICK MAUREL*‡

Service de Chirurgie Digestive C, Hospital Saint Eloi, 34059 Montpellier; *INSERM U128, CNRS, Route de Mende, BP5051, 34033 Montpellier; and †Laboratoire de Biologie D, Hopital Lapeyronie, 34059 Montpellier, France

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Abstract—The aim of this work was to determine whether the extensive metabolism of cyclosporine, acquired in a donor by treatment with an inducer of cytochrome P450 3A (P450 3A) (cyclosporine oxidase), was transmissible to the recipient by orthotopic liver transplantation. For this purpose, male Wistar rats were divided into five groups including: control animals (group C), animals treated with dexamethasone (an inducer of P450 3A, 50 or 300 mg/kg/day, for 4 days, group D), animals transplanted with the livers of control rats (group G) or with the livers of dexamethasone-induced rats (group GD), and animals treated with β-naphthoflavone (an inducer of P450 1A, group B). All animals received a single i.v. dose of 10 mg/kg cyclosporine 24 hr after either the last dose of inducer or the transplantation. For each group of animals, the area under the curve (AUC) of cyclosporine was calculated from the curves of blood cyclosporine levels (by radioimmunoassay) against time; liver microsomes were assayed for cyclosporine oxidase activity by HPLC, erythromycin demethylase and P450 3A level by western blot with specific anti-P450 3A antibodies. The decrease in the AUC in groups D and GD with respect to C and G was correlated with increased level of P450 3A (4–5-fold with respect to control) as well as of microsomal cyclosporine oxidase. In addition, cyclosporine oxidase activity of liver microsomes was specifically inhibited by anti-P450 3A antibodies and troleandomycin. The animals in group B did not exhibit increased metabolism of cyclosporine either *in vivo* or *in vitro*. We conclude that: (1) cyclosporine is predominantly oxidized in the rat liver by a form of P450 from the 3A subfamily; (2) the extensive metabolism of cyclosporine acquired by donor rats after treatment with dexamethasone is transmissible to the recipients through orthotopic liver transplantation.

Cyclosporine is widely used in the post-operative treatment of patients after organ transplantation [1, 2]. The use of this drug is complicated by the fact that it has an extremely variable bioavailability and a low therapeutic index [3]. The level of cyclosporine in the blood has therefore to be carefully monitored to maximize its efficacy and minimize its kidney, liver or brain toxicity [4]. In man and in the rabbit, we and others have demonstrated that cyclosporine is predominantly metabolized through the microsomal hepatic cytochromes P450 3A (P450 3A§‖) [5–7]. Cytochromes P450 constitute a multigenic superfamily of monoxygenases located in the endoplasmic reticulum of most cells including hepatocytes [8, 9]. In the liver, they are mainly involved in the oxidative metabolism of endogenous compounds including steroid hormones, fatty acids, biliary salts and prostaglandins, as well as of xenobiotics including most drugs and environmental pollutants.

Recently, Lucey *et al.* [10] presented the case of a liver-transplanted patient who exhibited serious neurological dysfunction and renal failure, while his blood cyclosporine level was in the "normal" therapeutic range. By analysing several forms of P450 by western blot in a post-mortem liver biopsy, they reached the conclusion that this patient had been transplanted with the liver of an individual who had a defect, presumably genetic, in the cytochrome P450 3A protein or regulation. On the basis of this observation, they suggested that toxic metabolite(s) of cyclosporine could have been generated through an alternative pathway (another P450, not belonging to the 3A subfamily). They concluded that a low level of P450 3A in the donor liver could be deleterious to the recipient.

Since cytochromes P450 3A are inducible in mammalian species, including man [6, 11–13], it is tempting to suggest that pre-induction of the donor, with an appropriate P450 3A inducer, could be of benefit to the recipient when the donor exhibits low hepatic P450 3A activities. The present investigation was undertaken to evaluate the feasibility of this approach, using the Wistar rats as an experimental model. Two main objectives were pursued. The first was to verify whether the kinetics of cyclosporine

‡ Corresponding author. Tel. (33) 67613363; FAX (33) 67523681.

§ Abbreviations: AUC, area under the curve; P450 3A, cytochrome P450 3A.

‖ In the rat, the P450 3A family comprises at least two forms 3A1 and 3A2 which exhibit 89% of similarity in terms of primary sequence [8, 9]. It is not known whether both forms (or only one of them) are (is) involved in cyclosporine oxidase or erythromycin demethylase since the antibodies used here are expected to cross-react with both of them. We accordingly use the term P450 3A, without referring specifically to any of these forms, throughout the paper.

elimination, notably the AUC (area under the curve), were correlated with the hepatic level of P450 3A in control and dexamethasone-induced animals. The second was to determine whether the extensive metabolism of cyclosporine, acquired in the donor by treatment with an appropriate inducer of P450 3A, was transmissible by orthotopic liver transplantation to the recipient.

MATERIALS AND METHODS

Animals. Sixty-four male Wistar rats weighing 250–300 grams were divided into five groups. In group C (control), 10 rats received a single intravenous injection of 10 mg/kg of cyclosporine (CsA, Sandimmun IV, Sandoz, Basel, Switzerland) in the penis vein. Dexamethasone was administered orally by gavage in 2% Tween 20 suspension as recommended [14]. Several protocols of induction, differing in terms of dosing or duration, were tried on animals of groups D and GD. In group D (dexamethasone-induced), 15 rats were treated with dexamethasone for 4 days at 300 mg/kg (rats D1 to D10), or 50 mg/kg (rats D13 to D17). On day 5 they received a single dose of cyclosporin as indicated above. In group G (graft), 11 rats underwent orthoptic allogenic liver transplantation from control donors. Twenty-four hours later, they received a single dose of cyclosporine as indicated above. In group GD (graft from dexamethasone-induced liver donor), 24 rats underwent orthoptic allogenic liver transplantation from donors that had been previously treated with dexamethasone at 300 mg/kg/day for 4 days (group GD1, N = 8) or 2 days (group GD2, N = 5) or for 4 days at 150 (group GD3, N = 5) or 50 (group GD4, N = 6) mg/kg/day. Twenty-four hours later, they received a single dose of cyclosporine as indicated above. In group B (β-naphthoflavone-induced), 5 rats were treated intraperitoneously with a single dose of β-naphthoflavone suspended in ground nut oil at 50 mg/kg. Two days later, they received a single dose of cyclosporine as indicated above.

Liver transplantation. The technique of liver transplantation has been described in detail elsewhere [15, 16]. Briefly, animals were anesthetized under ether. The donor liver and its venous pedicles were skeletonized and the bile duct was cannulated with 0.58-mm diameter polyethylene tube (Guerbet Biochemical, Paris, France). The liver was perfused at low pressure with 10 mL of cold saline solution via the portal vein. It was then removed, stored at 4° in saline and prepared with cuff for the portal vein and the inferior vena cava before implant. Following removal of the recipient liver, the donor liver was placed orthotopically and the suprahepatic anastomosis was performed using a 7/0 uninterrupted suture. Infrahepatic vena cava and portal vein anastomosis were performed using cuff technique and secured with a 6/0 circumferential silk suture. A bile duct stent was introduced into the recipient bile duct and secured with a 6/0 circumferential silk suture. Total time for cross-clamping and portal vein stenting averaged 20 and 15 min, respectively.

Measurement of blood cyclosporine level. Six aliquots of whole blood were drawn from the tips of

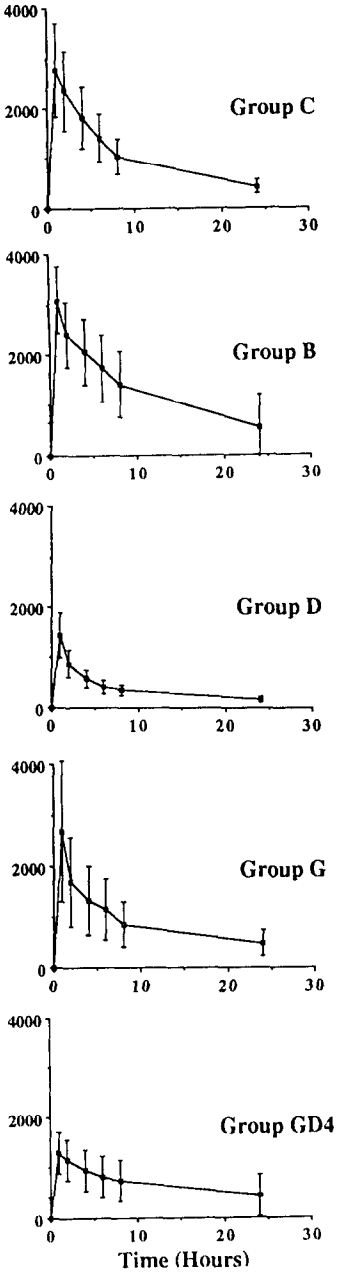

Fig. 1. Blood cyclosporine level against time in rats after a single i.v. injection. Rats from groups C (control), B (β-naphthoflavone-induced), D (dexamethasone-induced), G (grafted with the liver of a control donor) and GD4 (grafted with the liver of a donor induced with dexamethasone at a dose of 50 mg/kg/day for 4 days) received a single intravenous injection of 10 mg/kg cyclosporine in the penis vein, 24 hr after the last dose of inducer (groups B and D) or after transplantation (groups G and GD). The blood cyclosporine level (in ng/mL) was determined by a radioimmunoassay at 0, 1, 2, 4, 6, 8 and 24 hr after the injection. Average values calculated from individual values for all the animals of the respective groups are plotted against time. AUC (area under the curve) values presented in Table 1 were calculated from these curves using appropriate methods. Only the curve characterizing group GD4 is presented here, since all animals from GD groups (GD1–GD4) appeared to exhibit similar behavior with no significant difference in AUC.

Table 1. Cyclosporine AUC and hepatic P450 3A accumulation in control, dexamethasone-treated and transplanted rats

Groups	AUC*	P450 3A†
C (N = 10)	25,305 ± 7430	32 ± 14
D (N = 15)	9328 ± 3796	149 ± 103
G (N = 11)	22,134 ± 8126	51 ± 30
GD‡ (N = 24)	15,383 ± 5904	221 ± 219
B (N = 5)	31,382 ± 12,438	61 ± 10

* These data were determined from the curve of the cyclosporine level against time such as those presented in Fig. 1.

† These values are in arbitrary units (100 U representing the area of the band obtained by western blot with 1 pmol of authentic P450 3A6) and were obtained from densitometric analysis of western blots such as the one presented in Fig. 2.

‡ AUC values for GD1–GD4 were: 16,151 ± 4960; 15,714 ± 8145; 12,319 ± 5302 and 17,196 ± 5677, respectively. See text for statistical significance of differences.

the tails of animals into heparinated plastic tubes, 1, 2, 4, 6, 8 and 24 hr after intravenous administration of cyclosporine. The concentration of cyclosporine in the blood was determined with a radioimmunoassay kit (Cyclo-trac, Incstar) purchased from the Baxter Corporation (Stillwater, MN, U.S.A.). The monoclonal antibody used was specific for unmetabolized cyclosporine and exhibited a weak cross-reaction with the main metabolites, i.e. 1.7% and 0.7% with M1 and M17, respectively. The relative uncertainty was 3.1% for an average concentration of 625 ng/mL. The area under the curve (AUC) was calculated for every animal.

Preparation of liver microsomes. Animals from groups C, D and B were anesthetized under ether and killed by decapitation, after the last aliquot of blood had been collected. Since the survival of animals from groups G and GD was evaluated after transplantation, microsomes were prepared from a liver biopsy (not exceeding 10% of the whole liver mass) collected on the right lobe of the donor, before transplantation. In all cases, liver samples were frozen in liquid nitrogen until further experiments were performed. Microsomes were isolated by ultracentrifugation as described elsewhere [17], resuspended in 0.1 M potassium phosphate buffer pH 7.4, 0.1 mM EDTA and 20% glycerol, and stored at −80°. Protein concentration was determined by the bicinconinic acid method using the kit from the Pierce Biochemical (Rockford, IL, U.S.A.) with bovine serum albumin as standard.

Immunoquantitation of cytochromes P450 3A and 1A by western blot. Cytochromes P450 3A and 1A were quantitated by western blot analysis of 10 or 20 μg samples of microsomal protein as described elsewhere [17]. Polyclonal antibodies raised against rabbit P450 3A6 and 1A2, previously shown to cross-react with the orthologous forms from the rat [11], were used in this work. The relative concentration of both cytochromes was evaluated from a densitometric analysis of the blot with a Dual-Wavelength Scanner (Shimadzu, Kyoto, Japan).

Monoxygenase activities. Erythromycin demethylase and cyclosporine oxidase were determined by the Nash method and HPLC, respectively, as described previously [12]. Microsomes were suspended at 1 mg/mL in 0.1 M potassium phosphate buffer pH 7.4, in the presence of 500 or 25 μM of erythromycin or cyclosporine, respectively. After 3 min at 37°, the reaction was initiated by the addition of 1 mM NADPH, and allowed to proceed for 30 min. Both activities are expressed in nmol/min/mg of microsomal protein. A relative uncertainty of ± 15% was estimated for these activities, from measurements in triplicate of several different preparations. In some experiments, cyclosporine oxidase was determined using microsomes previously exposed to either anti-P450 1A1 or 3A6 antibodies (5 and 10 mg/mL) (6) or to 20 μM troleandomycin, a P450 3A-specific inhibitor [14].

Histological studies. Liver tissue collected from the animals of groups D and GD at death or time of killing was fixed with Bouin reagent and colored with hematein eosine. Glycogen and bacterial clusters were revealed with periodic acid Schiff reagent and Gram.

Statistical analysis. Comparative analysis of the means for the different groups of animals was carried out according to the Student's *t*-test for survival time an the Kruskal–Wallis test for the blood cyclosporine AUC. Correlation between blood cyclosporine AUC and P450 3A, and between microsomal erythromycin demethylase and cyclosporine oxidase, was evaluated according to the method of Spearman with simple regression curves.

RESULTS AND DISCUSSION

All animals received a single i.v. injection of 10 mg/kg of cyclosporine and the blood cyclosporine level was determined at 1, 2, 4, 6, 8 and 24 hr thereafter. Animals from groups D and B were injected 24 hr after the last dose of inducer, while those from groups G and GD were injected 24 hr after liver transplantation. In the first experiments, animals were treated with 300 mg/kg of dexamethasone. Because of the poor survival, we decided to decrease the dosing of dexamethasone to 150 and 50 mg/kg in subsequent series (lower dosing resulted in no induction of cyclosporine oxidase, not shown). The average curves representative of groups C, D, G, GD4 and B are presented in Fig. 1. In all groups the curve of cyclosporine level against time displays a similar pattern. The cyclosporine AUC derived from these curves is reported in Table 1. The AUC values are not significantly different for animals from groups GD1–GD4 ($P = 0.52$), so these subgroups were considered as a single group GD. The difference is highly significant between groups C and D ($P = 0.0002$), and D and GD ($P = 0.006$), and significant between groups G and GD ($P = 0.022$). These results suggest that: (1) treatment of rats with dexamethasone, a known inducer of P450 3A2 in this species [14], accelerates the elimination (metabolism) of cyclosporine, *in vivo* (compare D with C), whatever the dose of the inducer (300, 150 or 50 mg/kg); (2) recipients of a dexamethasone-induced liver exhibit a significantly increased rate of

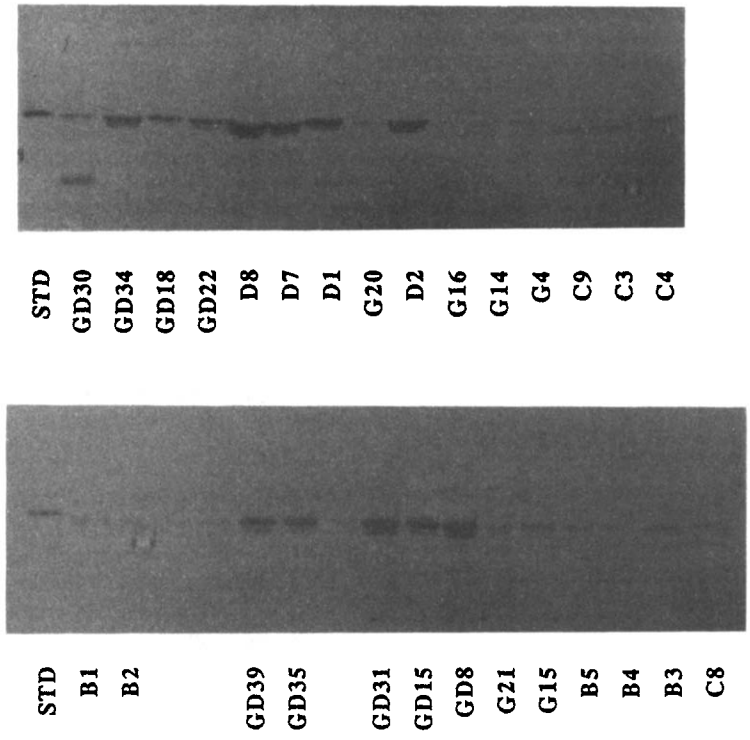

Fig. 2. Cytochrome P450 3A level in rat liver microsomes. Liver microsomes from rats from groups C (control), B (β-naphthoflavone-induced), D (dexamethasone-induced), G (grafted with the liver of a control donor) and GD (grafted with the liver of a donor induced with dexamethasone) were prepared by differential centrifugation. Twenty micrograms of microsomal protein were submitted to electrophoresis on SDS–polyacrylamide 10% gel, transferred to a nitrocellulose filter and revealed with a specific anti-P450 3A6 polyclonal antibody. Animals from groups B and D, were killed 72 and 48 hr after the last dose of inducer, respectively. For animals of group G and GD, a liver biopsy (not exceeding 10% of the whole liver mass) was resected from the right lobe of the donor during transplantation, and used to prepare microsomes. STD refers to a sample of 1 pmol of authentic P450 3A6 used as a relative quantitation standard. The number following letters C, B, D, G or GD allows identification of each animal within the group. Animals GD8, GD15, GD18, GD30 and GD31 were from group GD1; animals GD34, GD35 and GD39 were from group GD2 (see Materials and Methods). Only partial results (N = 28) are presented here, although this analysis was carried out with liver microsome preparations from all the animals studied in this work (N = 65) with similar results.

cyclosporine elimination in comparison to control animals or rats transplanted with liver from control donors (compare GD with C and G); (3) the rate of cyclosporine elimination in dexamethasone-induced rats is significantly higher than in recipients of a dexamethasone-induced liver (compare D with GD); (4) finally, treatment of rats with β-naphthoflavone, a known inducer of P450 1A1 and 1A2 [8], does not affect the metabolism of cyclosporine (compare B with C). Thus, the extensive metabolism of cyclosporine acquired in the donor by induction with dexamethasone is transmissible to the recipient through orthotopic liver transplantation. The heavy surgical stress, caused by such a critical operation, that had been suspected previously to reduce the liver monoxygenase activities [18], has apparently no important influence here. The finding that animals from groups D exhibited a significantly lower AUC for cyclosporine than those from group GD could be ascribed to the fact that in Group D, the whole animals were induced by dexamethasone, while in rats from group GD only the liver was induced. Since the level of hepatic P450 3A was not lower in animals from group GD with respect to those from group D (see Table 1), the difference in AUC between animals from groups D and GD could result either from surgical stress in transplanted rats (GD) with a moderately reduced ability to metabolize cyclosporine, or from the relative contribution of extrahepatic tissues to the metabolism of cyclosporine, in rats from group D. The problem raised by this last alternative is crucial because it could account for the wide inter-individual variability of pharmacokinetic parameters observed in man with this drug [2, 3]. One likely candidate for this contribution is the gut. Watkins and co-workers [19, 20] have clearly shown that one or several form(s) of P450 3A is (are) expressed and inducible in rat and human intestine. In addition, these authors recently showed that cyclosporine administered p.o. to two patients undergoing liver transplantation was oxidized during the anhepatic phase of the operation

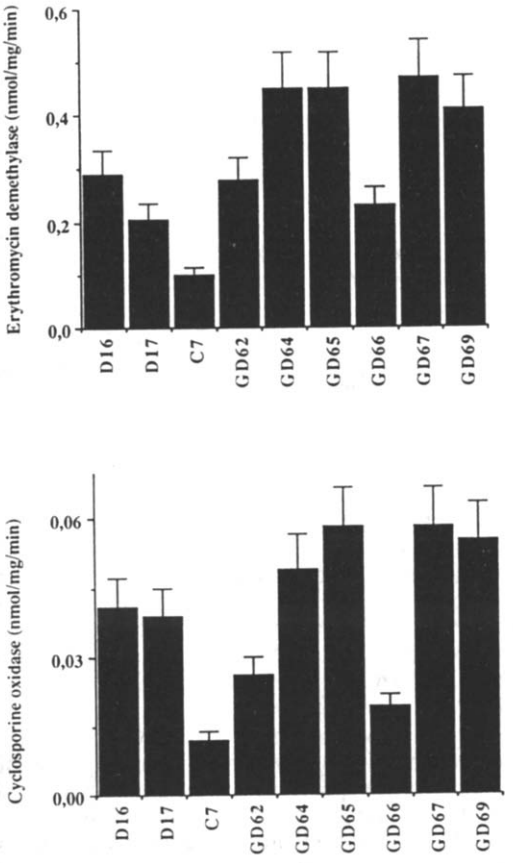

Fig. 3. Erythromycin demethylase and cyclosporine oxidase activities of rat liver microsomes. Liver microsomes were resuspended at 1 mg/mL in 0.1 M potassium phosphate buffer pH 7.4, in the presence of 500 or 25 μM of erythromycin or cyclosporine, respectively. After 3 min at 37°, the reaction was initiated by the addition of 1 mM NADPH, and allowed to proceed for 30 min. Erythromycin demethylase and cyclosporine oxidase activities were monitored by the Nash method and by HPLC, respectively [6]. Both activities were expressed in nmol/min/mg of microsomal protein. A relative uncertainty of ±15% was estimated for these activities, from measurements in triplicate of several different preparations. C, D and GD refer to animals from corresponding groups; rats GD62 to 69 were from subgroup GD4 (see Materials and Methods), but are representative of all animals from group GD.

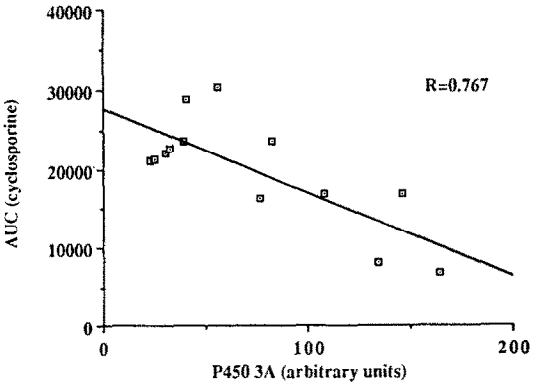

Fig. 4. Correlation between the AUC of cyclosporine and the level of hepatic P450 3A quantitated by western blot in rats. For all animals (N = 65), AUC and P450 3A levels were determined from the results such as those presented in Figs 1 and 2. The level of P450 3A was estimated from densitometric analysis of the western blots, using authentic P450 3A6 as a standard (100 arbitrary U/pmol). In order to avoid staining variability from blot to blot, the correlation was investigated only for those animals analysed in the same western blot. The correlation presented here (r = 0.767) was obtained with animals corresponding to the lower blot of Fig. 2. Similar correlation plots were generated with other animals from the various groups (r varied between 0.667 and 0.869).

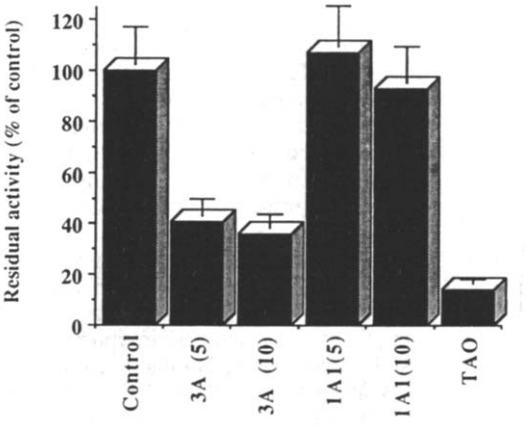

Fig. 5. Immunoinhibition and inhibition by triacetyloleandomycin (TAO) of cyclosporine oxidase activity in rat liver microsomes. Liver microsomes from rat GD66 were resuspended at 1 mg/mL in 0.1 M potassium phosphate buffer pH 7.4, in the absence (control) or presence of 5 or 10 mg/mL of anti-P450 3A (3A) or 1A1 (1A1) antibodies. After 20 min at room temperature, 25 μM of cyclosporine was added to the suspension. After 3 min at 37°, the reaction was initiated by the addition of 1 mM NADPH, and allowed to proceed for 30 min. In the experiments with TAO (20 μM), this compound was added to the microsomal suspension just before cyclosporine and the reaction was allowed to proceed as indicated above. Cyclosporine oxidase activities were monitored by HPLC [6] and expressed in % of control activity. A relative uncertainty of ±15% was estimated from measurements in triplicate of different preparations.

[21]. Intestinal P450 3A is therefore likely to be involved in the metabolism of cyclosporine (and of other drugs). We are currently using the experimental approach presented here (comparative studies between control animals transplanted with an induced liver versus induced animals transplanted with a control liver) to evaluate the actual relative contributions of the liver and gut to the metabolism of cyclosporine administered p.o.

In order to determine whether the effect of the treatment with dexamethasone on the AUC of cyclosporine was related to increased hepatic metabolism of the drug, liver microsomes prepared from the animals of the various groups were analysed

by western blot, to evaluate their P450 3A content, and assayed for two monoxygenase activities, cyclosporine oxidase and erythromycin demethylase, the latter being a well-known P450 3A-related monoxygenase activity [14]. Western blots are presented in Fig. 2. Clearly, the level of P450 3A was increased in the livers of animals from groups D and GD with respect to the level in animals from groups C, G and B. In some but not all samples two bands of similar molecular weight were revealed by the antibodies. The low molecular weight band could represent P450 2B1 (slight cross-reaction with our anti-P450 3A6 antibodies) which is inducible by dexamethasone in the rat [8]. Note that in all animals, the liver was obtained after the last blood aliquot had been collected following cyclosporine administration, that is, 48 hr after the last dose of dexamethasone for animals from group D and 24 hr for animals from group GD (biopsy of liver donor at transplantation). In spite of this, there was no significant difference in the level of P450 3A between groups D and GD. Since all the microsome samples could obviously not be run on the same blot, a standard amount of 1 pmol of rabbit P450 3A6 was run together with microsomal samples on each blot. This allowed a comparative study to be made of the level of P450 3A in all groups. Average values are presented in Table 1. Cyclosporine oxidase and erythromycin demethylase activities of liver microsomes from animals from groups C, D and GD are reported in Fig. 3. Both activities were significantly increased in liver microsomes from animals of groups D and GD ($P < 0.005$) with respect to group C, but were not significantly different between groups D and GD. These activities appear to be linearly correlated in liver microsomes from tested animals ($r = 0.903$). In addition, when the AUC of cyclosporine determined in all animals tested in this work was plotted against the corresponding level of P450 3A determined from Western blots, linear correlations were found, r values being between 0.667 and 0.869, as presented for one series of data in Fig. 4. Finally, cyclosporine oxidase activity of liver microsomes from animals of group GD was specifically inhibited by anti-P450 3A6 antibodies (but not by anti-P450 1A1 antibodies) as well as by triacetyloleandomycin, a specific inhibitor of P450 3A in the rat [14], as reported in Fig. 5. In summary, these results suggest that P450 3A is the major form(s) involved in the oxidation of cyclosporine in rat liver microsomes and *in vivo*. This is in close agreement with previous studies with rabbit and human liver microsomes and hepatocytes in culture [5–12], and with rat enterocytes [20].

The survival time of animals from groups G and GD1–GD4 was evaluated after transplantation. No immunosuppressive treatment was administered to the animals, except for the single injection of cyclosporine 24 hr after surgery. Hepatic transplantation in the inbred Wistar strain is well tolerated, as observed here with animals of group G for which the time of survival was on average 24.4 days (with extremes at 4 and 60). This time was dramatically decreased ($P < 0.0001$) to 3 days (with extremes at 1 and 8) in animals from group GD. No significant difference was observed between animals from subgroups GD1–GD4 which exhibited survival times of 3.3, (1–8), 3.2 (2–4), 2 (1–3) and 3.4 (2–6) days, respectively. That the death of these animals was not due to liver rejection is suggested firstly by the fact that rejection usually takes place after a much longer time and secondly because no histological signs of rejection were found on post-mortem examination of these animals (groups G and GD). However, the livers from animals treated with dexamethasone (groups D and GD1–GD4) exhibited one or several signs of injury including: microvacuolar steatosis, reaching 80% in some areas, bacterial clusters, distension of centrolobular veins and sinusoids, and centrolobular ischemia. No apparent difference was observed between the liver specimens from animals of group D and the different groups GD. It is therefore likely that the liver of dexamethasone-treated animals failed to function properly after transplantation to the recipient, due to direct toxicity of the inducer, although a high cyclosporine oxidase activity was exhibited by the liver. This suggests that the activity of the drug-metabolizing enzyme systems does not necessarily reflect the quality of the liver, in contrast to previous observations [22, 23]. The poor survival of animals after transplantation of dexamethasone-induced liver did not allow us to determine the period during which the induced state is maintained. This is however of critical importance from a clinical point of view. From previous reports, focusing on the effect of rifampicin on the level of cyclosporine in the blood of transplanted patients, it appears that induction of the metabolism of this drug is maintained from 2 to 3 weeks after cessation of inducer treatment [24, 25].

In conclusion, our results show that pre-induction of cyclosporine oxidase in the donor is transmissible to the recipient through orthoptic liver transplantation. This procedure could accordingly be proposed in man with rifampicin as the inducer [11, 12, 24, 25], in those cases where low P450 3A level would not allow a significant metabolism of cyclosporine after transplantation.

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