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Effects of aging and cyclosporin treatment on the hepatobiliary efflux of glutathione

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Abstract

The aim of this study was to investigate the effects of cyclosporin (CyA) treatment on biliary glutathione efflux in rats of different ages (1, 2, 4, and 24 months). CyA treatment reduced the liver content of total glutathione in 1-, 2- and 24 month old rats (−30%, −43% and −30%, respectively). By contrast, oxidized glutathione (GSSG) concentration in liver tended to increase, although non significantly, in the rats aged 4 and 24 month (+36% and +28%, respectively). The oxidized-to-reduced glutathione ratio was significantly increased in 2-, 4- and 24 month old animals (+23%, +36% and >100%, respectively). Regarding biliary glutathione, our data indicate that efflux rates of total glutathione in control (untreated) rats increased to a maximum at 4 months, and decreased (−56%) in 24 month old rats, although values were still higher than those from young animals. CyA treatment significantly reduced biliary glutathione secretion except in 24 month old rats (−98%, −66% and −32%, at 1, 2 and 4 month, respectively). In addition, following inhibition of the intrabiliary catabolism of the tripeptide by acivicin, glutathione efflux rates into bile were significantly reduced by the drug only in 1- and 2 month old rats (−29% and −55%, respectively) and even tended to increase, although non significantly, in oldest animals. Our data indicate that inhibition of biliary glutathione efflux by CyA was greater in younger rats and support the view that increased intrabiliary catabolism of the tripeptide and inhibition of its canalicular transport could contribute to the decline in biliary glutathione secretion induced by the drug.

Keywords: Aging; Biliary glutathione; Cyclosporin A; Free radicals; Liver glutathione

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Introduction

Treatment with cyclosporin A (CyA) has contributed to enhancing allograft and patient survival after organ transplantation and to reducing the manifestations of selected autoimmune diseases, but its therapeutic use is, however, often limited by its side effects, which include renal and hepatic dysfunction (Duruibe et al., 1989; Borel et al., 1996; Deters et al., 1997; Galán et al., 1999). It has been reported that CyA interferes the vectorial transport of biliary components from blood to bile, thus causing the inhibition of bile formation and the development of a cholestatic syndrome (Schade et al., 1983; Galán et al., 1995; Chan et al., 1998; Morán et al., 1998) that is characterized by decreases in both the bile acid-dependent and bile acid-independent fractions of secretion (Román et al., 1990; Morán et al., 1998). The mechanisms involved in the formation of the latter are still incompletely know, but various studies have confirmed that glutathione constitutes the primary osmotic driving force in the formation of bile acid-independent flow in different species (Ballatori and Truong, 1989, 1992).

Glutathione is the most important non-protein thiol in living system, the liver being its main site of synthesis and storage and the principal source of plasma and bile in either reduced (GSH) or disulfide form (GSSG) (Ookhtens and Kaplowitz, 1998). Hepatic glutathione turnover is achieved largely by efflux of the tripeptide across canalicular and basolateral membranes of hepatocytes (Lauteburg, 1991) and transport across the canalicular membrane not only contributes to bile formation but also serves to deliver glutathione and its constituent amino acids to the biliary tree and intestinal epithelium and plays an important role in the maintenance of the balance between the formation and removal of reactive oxygen species (Ookhtens and Kaplowitz, 1998). Accordingly, alterations in glutathione hepatic content and export into bile through the canalicular membrane could potentially compromise glutathione-based detoxification processes and protection against oxidative stress. Over the last years there has been accumulating evidence for the role of free radicals and alterations of glutathione homeostasis in the pathogenesis of CyA-induced side effects. It is known that CyA depletes the hepatic pool of glutathione (Duruibe et al., 1989; Deters et al., 1997), and significantly decreases the glutathione contents in the rat kidney (Rikans et al., 1997), while glutathione depletion increases the susceptibility to drug-induced toxicity (Rikans et al., 1997; Rikans and Hornbrook, 1997). CyA also reduces biliary glutathione secretion (Morán et al., 1998) and expression of its canalicular transporter Mrp2 (Bramon et al., 2001).

The incidence and severity of drug-induced toxicity in humans and other species vary with age (Kitani, 1988; Rikans et al., 1997; Rikans and Hornbrook, 1997), and knowledge of the factors that contribute to age-associated differences in sensitivity to toxic agents is important for predicting the effects of drugs in the elderly. We have recently reported that higher CyA-induced oxidative stress and decreases in the antioxidant defense system in aged rats render them more susceptible to the toxic effects of cyclosporin (Palomero et al., 2001). To date, no information exists, however, about the age dependent variations that occur in the biliary secretion of glutathione after CyA treatment, and effects of aging on the susceptibility of factors determining the export of glutathione into bile to the toxic effects of CyA are unknown. The present study was designed to investigate the influence of aging on the inhibition of biliary glutathione efflux induced by CyA. Hepatobiliary transport and hepatic levels of glutathione were studied in young, young-adult, mature and senescent male Wistar rats that were treated for one week with CyA or the CyA-vehicle.

Methods

Reagents

CyA, in powder form, was a gift from Sandoz A.G. (Basel, Switzerland). GSH, acivicin, metaphosphoric acid, NAD, NADPH, 2-vinylpyridine, 5,5-dithio-bis (2-nitrobenzoic acid) and olive oil (3% w/w, highly refined, low acidity, d = 0.91 g/ml) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other standard reagents and chemicals were of the highest quality available commercially.

Animals and treatments

Male Wistar rats were obtained from Charles River, Barcelona, Spain. They were kept on standard rat chow (Panlab, Barcelona, Spain) with free access to tap water, in a temperature- and humidity-controlled animal quarter under a 12-h light–dark cycle. All experiments were performed in compliance with the indications of the Guide to the Care and Use of Experimental Animals, routinely used at our laboratory.

The animals were treated intraperitoneally once daily for one week with CyA, at a dose of 10 mg/kg b.wt per day (CyA groups) or the CyA vehicle -olive oil- (controls) for the same period. The rats were weighed daily and the volumes of solutions administered were adjusted between 0.20 and 0.30 ml, depending on their body weights. Age at the beginning of treatment with CyA o its vehicle was 1 month (young), 2 months (young-adult), 4 months (mature) and 24 months (senescent), respectively.

Experiments were carried out twelve hours after the last injection. They were planned so that they could be initiated for all animals at the same time daily (between 9 a.m. and 10 a.m.) to avoid variation due to the circadian rhythm. Rats were anaesthetized with sodium pentobarbital (50 mg/kg b.wt., i.p.) and kept at a constant temperature of 37.0 ± 0.5 °C. Losses in body temperature were prevented using a rectal probe connected to a thermostatically controlled heating lamp. Routine laparatomy was performed and the common bile duct cannulated with PE-10 polyethylene tubing for collecting bile samples. Animals were fitted with a catheter in the left carotid artery for blood sampling. Bile collection was started 30 min after finishing the surgical procedure, a time estimated as sufficient to allow bile flow stabilization. In some experiments, with the aim of inhibiting γ-glutamyl transpeptidase (γ-GT) activity, an irreversible inhibitor of this enzyme (acivicin) was administered after collecting a 15-min baseline bile sample, acivicin, at a dose of 40 μmol/kg b.wt (in 0.4 ml/kg b.wt of Ringer solution), was administered by retrograde intrabiliary infusion in accordance with previous reports (Ballatori and Truong, 1989; Morán et al., 1998). After this, three additional bile samples were collected at 15 min intervals. Bile was collected in pre-weighed tubes on melting ice containing 15 μl of 5% metaphosphoric acid to prevent oxidation of GSH. At the end of the assays rats were killed by exsanguination, livers were quickly washed in situ with ice-cold 0.154 M NaCl, removed and weighed. Small pieces weighing 0.5 g were harvested from the liver for biochemical determinations and immediately stored in liquid nitrogen until analysis.

Tissue preparation and biochemical analysis

Bile flow was determined gravimetrically, assuming a bile density of 1.0 g/ml. Total glutathione (GSH + GSSG) and GSSG concentrations in bile and liver homogenates were evaluated by the enzymatic

recycling procedure using glutathione reductase and 5,5′-dithio-bis (2-nitrobenzoic acid), according to Griffith (Griffith, 1980).

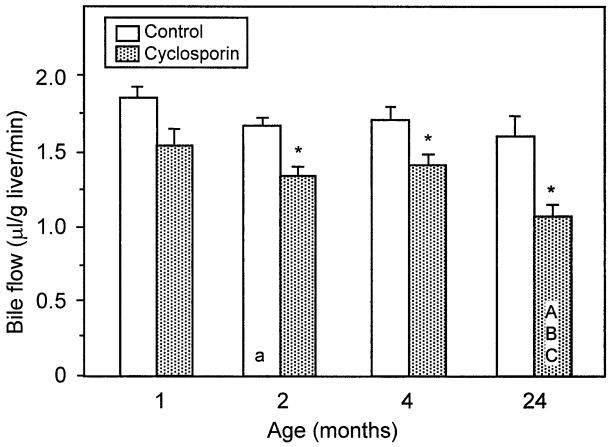
Statistical analysis

Results are expressed as means ± standard error of means (S.E.M.) for all data. The effects of aging and CyA treatment on all parameters studied were tested for significance by a two-way analysis of variance (ANOVA) followed by the Fisher PLSD test. P values less than 0.05 were considered to be statistically significant.

Results

Age-dependent changes and effects of cyclosporin A treatment on bile flow

Fig. 1 shows bile flow in control and CyA-treated rats of different ages. Values were significantly reduced in all groups of treated rats, being the CyA-induced decreases higher in the senescent animals (−34% with respect to their controls) than in young and mature rats. Although the effects of age and treatment were significant, there was no significant interaction between both factors.

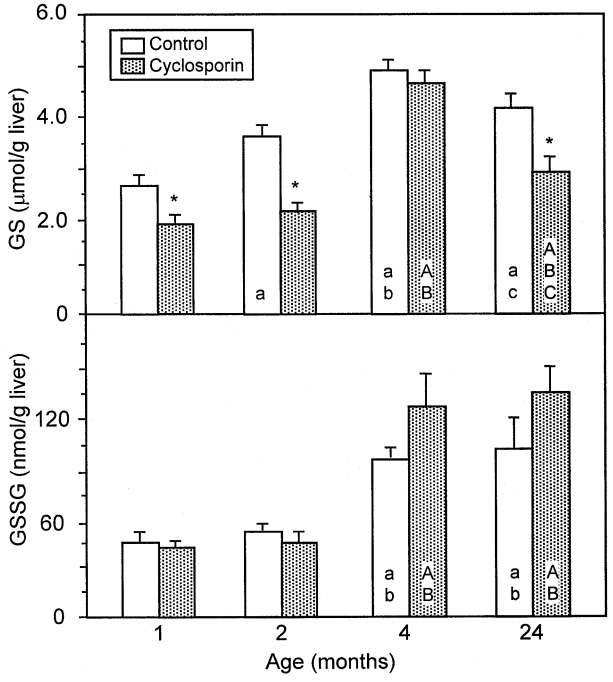


ANOVA	
Ag	$p<.05$
T	$p<.05$
Ag-T	n.s.

Fig. 1. Effects of aging and treatment with cyclosporin A on bile flow. Values are means ± S.E.M. from 6 to 8 animals. *: significantly different from control rats of the same age ($p < 0.05$), *a*: significantly different from 1 month old in control rats, and *A*, *B* or *C*: significantly different from 1-, 2- or 4 month-old in rats treated with CyA, respectively ($p < 0.05$). ANOVA: Ag: effect of age, T: effect of CyA treatment, Ag-T: interaction of age and treatment.

Age-dependent changes and effects of cyclosporin A treatment on liver glutathione

Fig. 2 illustrates the liver concentration of total and oxidized glutathione in the different experimental groups. Total glutathione increased with age in untreated rats, reaching a peak (+83%) in 4 month old animals. When CyA was given, the liver content of total glutathione decreased significantly in 1-, 2- and 24 month old rats (−30%, −43% and −30%, respectively). GSSG levels also increased with age in the controls, being 140% higher in oldest than in youngest rats. After CyA treatment, GSSG concentration tended to increase, although non significantly, in mature and senescent rats (+36% and +28%, respectively) (Fig. 2). Variance analysis proved significance for the effects of age and interaction of age and treatment in both total and oxidized glutathione. The effect of treatment was also significant for glutathione. CyA treatment increased the oxidized/reduced glutathione ratio by 23% and 36% at 2 and 4 months, respectively, and by more than 100% in senescent rats.

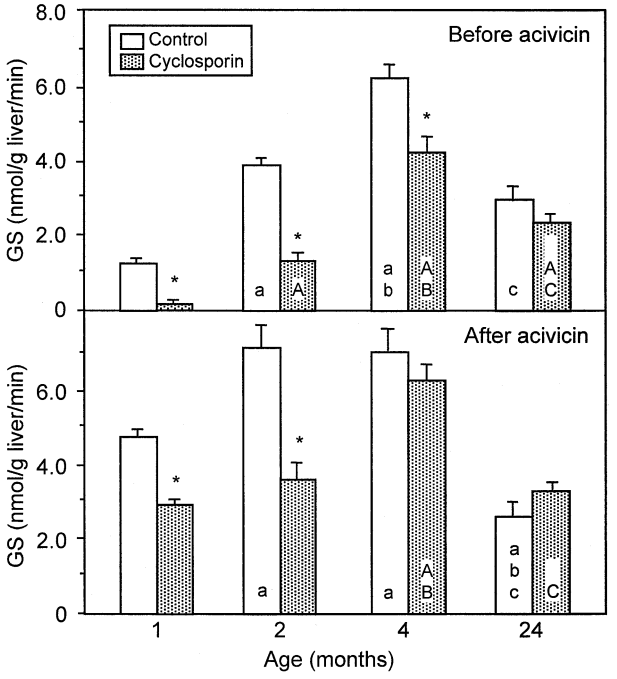


ANOVA	GS	GSSG
Ag	*p*>.05	*p*>.05
T	*p*>.05	n.s.
Ag-T	*p*>.05	*p*>.05

Fig. 2. Effects of aging and treatment with cyclosporin A on liver total glutathione (GS) and oxidised glutathione (GSSG) concentrations. Values are means ± S.E.M. from 6 to 8 animals. *: significantly different from control rats of the same age ($p < 0.05$), *a*, *b* or *c*: significantly different from 1-, 2- or 4-month-old in control rats, and *A*, *B* or *C*: significantly different from 1-, 2- or 4-month-old in rats treated with CyA, respectively ($p < 0.05$). ANOVA: Ag: effect of age, T: effect of CyA treatment, Ag-T: interaction of age and treatment.

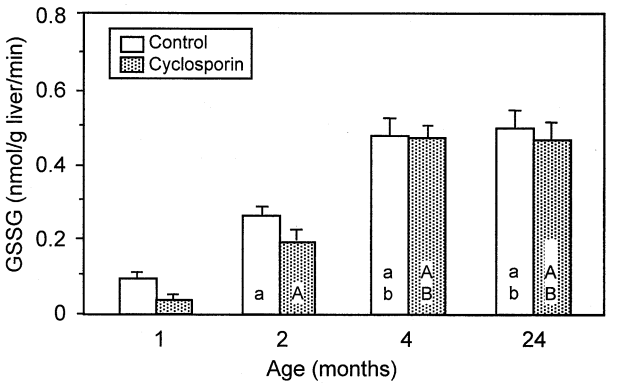
Age-dependent changes and effects of cyclosporin A treatment on the hepatobiliary efflux of glutathione

In untreated rats, total glutathione secretion increased to a maximum at 4 months, and decreased (− 56%) in 24 month old rats, although values were still higher than those from young animals (Fig. 3). CyA treatment significantly reduced the total glutathione concentration in 1-, 2- and 4 month old rats (− 98%, − 66% and − 32% vs controls of the same age, respectively), but not in 24-month old rats. Following inhibition of the intrabiliary catabolism of the tripeptide by acivicin, glutathione secretion was significantly lower in untreated senescent animals when compared to rats of other ages. Values were reduced by CyA only in 1- and 2-month old rats (− 29% and − 55% vs controls of the same age, respectively) and even tended to increase, although non significantly, in oldest animals (Fig. 3). Analysis of variance revealed significance for age, treatment and the interaction between age and treatment. GSSG secretion increased significantly with age in 2 and 4



ANOVA	Before	After
Ag	$p<.05$	$p<.05$
T	$p<.05$	$p<.05$
Ag-T	$p<.05$	$p<.05$

Fig. 3. Effects of aging and treatment with cyclosporin A on biliary secretion of glutathione (GS) before and after acivicin administration. Values are means ± S.E.M. from 6 to 8 animals. *: significantly different from control rats of the same age ($p < 0.05$), *a*, *b* or *c*: significantly different from 1-, 2- or 4 month old in control rats, and *A*, *B* or *C*: significantly different from 1-, 2- or 4 month old in rats treated with CyA, respectively ($p < 0.05$). ANOVA: Ag: effect of age, T: effect of CyA treatment, Ag-T: interaction of age and treatment.



ANOVA	
Ag	$p<.05$
T	n.s.
Ag-T	n.s.

Fig. 4. Effects of aging and treatment with cyclosporin A on biliary secretion of oxidized glutathione (GSSG). Values are means ± S.E.M. from 6 to 8 animals. *: significantly different from control rats of the same age ($p < 0.05$), *a* or *b*: significantly different from 1- or 2 month old in control rats, and *A*, *B* or *C*: significantly different from 1-, 2- or 4 month old in rats treated with CyA, respectively ($p < 0.05$). ANOVA: Ag: effect of age, T: effect of CyA treatment, Ag-T: interaction of age and treatment.

month old untreated rats (+88% and +278% vs 1 month old animals). No significant effect of CyA was found (Fig. 4).

Discussion

The vulnerability of organs to the toxic effects of drugs varies with age in humans and other species and it is well known that the extent of the liver damage induced by some hepatotoxins is enhanced in the elderly (Kitani, 1988; Rikans et al., 1997). We have recently shown that glutathione depletion and decreased antioxidant enzyme activities contribute to the hepatotoxic effects of CyA in rats of different ages, although oxidative stress is a relatively late age-associated (Palomero et al., 2001). CyA also induces a cholestasis related to inhibition of both bile acid and glutathione secretion into bile (Román et al., 1990; Morán et al., 1998), but potential effects of aging and factors involved require to be identified.

Our data confirm that treatment with CyA induces impairment of bile formation and of the biliary efflux of glutathione. This inhibition of biliary glutathione secretion could theoretically be attributed to different defects.

Hepatic depletion of glutathione

First, low bile glutathione levels might be related to a hepatic depletion of glutathione, since hepatic glutathione regulates its biliary secretion and liver efflux of glutathione has been reported to decrease when its hepatic content is reduced (Lauteburg, 1991; Deleve and Kaplowitz, 1990; Oude Elferink et al.,

1995). We have previously shown this effect in 2 month old rats treated with CyA for 7 days (Morán et al., 1998). However, a tendency to increased hepatic glutathione has been found in association to the biliary defect in different models of cholestasis (Bouchard et al., 1994, 2000), and biliary glutathione secretion is rapidly reduced following a single i.v. dose of CyA, even when the hepatic content of the tripeptide remains unaltered (Morán et al., 1998). In addition, in our experiments treatment with CyA significantly reduced liver total glutathione content of senescent rats in a similar percentage to young and young-adult rats, but did not significantly modify its biliary secretion. This is a further indication that CyA did not cause inhibition of biliary glutathione efflux only through the depletion of glutathione.

Increased γ-GT activity

Glutathione is known to be unstable in bile, as it is degraded in the biliary tree by a process initiated by γ-GT (Ballatori et al., 1988). Increased γ-GT activity could enhance the intrabiliary breakdown of glutathione and reduce its biliary content (Ballatori et al., 1986; Lauteburg, 1991; Oude Elferink et al., 1991). We have previously reported that CyA treatment increases γ-GT activity in both hepatocyte canalicular plasma membrane vesicles and liver homogenates from 2 month old rats (Galán et al., 1999). As has been previously proposed in different experimental situations (Ballatori et al., 1986; Lauteburg, 1991), this effect, if present in animals of different ages, could increase the intrabiliary hydrolysis of glutathione and reduce biliary glutathione content. When hepatic γ-GT activity was blocked with acivicin we observed that: (i) intrabiliary catabolism of glutathione was reduced, because biliary glutathione secretion increased in all groups, except in treated-senescent rats, (ii) the magnitude of this effect diminished with age, (iii) treatment with CyA increased γ-GT activity, since increases in biliary glutathione secretion were higher than those reached in untreated animals, and (iiii) CyA effect was greater in younger rats, because following acivicin treatment increases were progressively lower with age (from 86% in young rats to only 51% in senescent animals).

The mechanisms involved in the γ-GT increase by CyA and the differential effects with age cannot be easily judged with the results obtained here. It is likely that these changes could be related to the density/amount of the enzyme protein anchored into the biliary epithelium and to the alterations induced by CyA in biliary bile acid and phospholipid secretion (Román et al., 1990; Fernández et al., 1995), which determine the efficacy to remove lipids and proteins from the canalicular membrane (Verkade et al., 1995). A lower bile acid-dependent solubilization and extraction of γ-GT is consistent with the observed increase in its activity in liver homogenates and could also explain the lowered biliary excretion of the enzyme and its reversal by S-adenosylmethione that we have previously reported (Galán et al., 1999).

There are, however, two important facts that allow to suggest the presence of additional factors responsible for the inhibition of biliary glutathione efflux after cyclosporin treatment: (i) except in senescent rats, biliary glutathione secretion following acivicin administration was lower in CyA-treated than in untreated rats, and (ii) in a previous study it was shown that i.v. acute administration of CyA rapidly reduces biliary glutathione content (Morán et al., 1998), and it seems improbable that enzyme induction could be responsible for such effect.

Inhibition of glutathione canalicular transport and oxidative stress

Another potential cause of the low biliary GS levels is related to inhibition of its canalicular transport. Oxidized glutathione is preferentially excreted into bile via a canalicular multispecific organic anion

transporter (cMOAT or Mrp2) (Muller et al., 1996; Keppler and Arias, 1997), which might also be involved in the biliary secretion of GSH (Ballatori and Rebbeor, 1997; Paulusma et al., 1999). It is known that CyA inhibits the activity of Mrp2 in canalicular membrane vesicles Bohme et al., 1994) or reduces the biliary excretion of substrates from Mrp2 (Román et al., 1990; Galán et al., 1991), and very recently, it has been suggested that CyA cholestasis could be related to a pronounced reduction in Mrp2 mRNA (Rost et al., 1999). Oxidative damage of membrane lipids and proteins leads to alteration in membrane-bound enzyme activities (Vendemiale et al., 1999) and this could contribute to inhibition of glutathione transporters. In the present study the liver GSSG/GSH ratio, an indicator of oxidative stress (Palomero et al., 2001), was significantly increased by CyA in 2-, 4- and 24-month-old animals. Although GSSG biliary secretion was not significantly reduced by CyA in mature and senescent animals, this does not rules out the possibility of an impairment of its canalicular transport and could be explained by the larger amounts of GSSG generated in the liver. However, the intensity of the change in the GSSG/GSH ratio was age-dependent, the values being increased by 23% and 36% at 2 and 4 month old, respectively, and by more than 100% in senescent rats, being the latest those in which total glutathione biliary secretion was not significantly affected by CyA. This indicates that oxidative stress could not solely explain the inhibition of glutathione canalicular transport induced by CyA.

Recent studies have given evidence for the importance of vesicular transcytosis in the canalicular supply of proteins, including canalicular transporters (Bouchard et al., 1994). CyA administration also results in inhibition of vesicular transcytosis as assessed by HRP biliary secretion (Román et al., 1990), which could lead to accumulation of the P-glycoproteins and of Mrp2, as has been reported in other experimental models of cholestasis (Vendemiale et al., 1999). Intracellular internalization of canalicular transporters could result in a rapid loss of transport expression protein at the canalicular membrane level and contribute to CyA-induced decline in glutathione secretion.

In summary, our data confirm the inhibition of biliary glutathione efflux by subchronic administration of CyA and indicate that this effect was greater in younger rats. Results obtained support the view that increased degradation of glutathione and inhibition of its canalicular transport could contribute to the decline in biliary glutathione output induced by the drug. The defect in the canalicular secretion of glutathione could alter not only bile formation but also the turnover of hepatic glutathione and the interorgan homeostasis of cysteine and potentially compromise glutathione-based detoxification processes. These effects should be considered during immunosuppressive therapy with CyA.

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