Inhibitory Effect of Rifampicin on the Depressive Action of Interleukin-1 on Cytochrome P-450–Linked Monooxygenase System

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It has been shown that interleukin 1 (IL-1) depresses cytochrome P-450–linked monooxygenases. In the present study, the effects of rifampicin on the depressive action of IL-1 on the activities and gene expression of xenobiotic metabolizing enzymes in liver microsomes were investigated in vivo using Wistar rats. Among the monooxygenases studied, we especially focused on the induction mechanism for CYP2D, known to be depressed by IL-1 and responsible for the oxidation of xenobiotics, debrisoquine, bufuralol, and sparteine. The CYP2D protein and its messenger RNA (mRNA) were quantitated by Western blot and slot blot hybridization analyses in the groups treated with and without rifampicin and IL-1. The results showed that the depressive action of IL-1 on CYP2D was offset by additional administration of rifampicin, and the P-450 (CYP2D)–linked monooxygenase system is up-regulated at the mRNA level by rifampicin. These results show that rifampicin has a blocking effect on the depressive action of IL-1 on the CYP2D subfamily.

RIFAMPICIN IS an antibiotic derived from *Streptomyces* spp that is used for the treatment of tuberculosis worldwide. This antibiotic has a variety of effects on liver functions. Rifampicin is well known as a potent inducer of xenobiotic-metabolizing monooxygenases in the liver. In some cases, this induction of xenobiotic-metabolizing monooxygenases causes physiological side effects. For example, when this drug is used with isoniazid, drug-induced hepatitis often occurs.[1] This phenomenon is thought to be evoked by the toxic metabolic products from isoniazid, which are increased by xenobiotic metabolizing monooxygenases induced by rifampicin.

IL-1 is one of the cytokines released in inflammatory reactions. Although this cytokine induces the production of acute-phase protein in the liver such as amyloid A and haptoglobulin, it depresses P-450s responsible for metabolizing several xenobiotic compounds. We have previously shown that interleukin 1 (IL-1) down-regulated the P-450 (CYP2D)–linked monooxygenase system at the messenger RNA (mRNA) level.[2] Although rifampicin is known to be potent inducer of cytochrome P-450–linked monooxygenases, it is not clear whether rifampicin affects the depressive action of IL-1 on the CYP2D subfamily. Based on these observations, we used IL-1 in combination with rifampicin for this study and investigated whether rifampicin had an effect on the depressive action of IL-1 on CYP2D to elucidate more precisely the mechanism underlying the effects of rifampicin.

The P-450–linked monooxygenase system in the liver comprises multiple P-450 isozymes that are responsible for the oxidation and biotransformation of xenobiotics. These isozymes are classified as the P-450 superfamily based on the sequence of the encoded gene.[3] Although much information has been accumulating concerning the induction effect of rifampicin on P-450 isozymes in the liver through in vivo and in vitro experiments,[4-11] no report has appeared so far regarding the effects of rifampicin on the depressive action of IL-1 on the activities and gene expression of the CYP2D subfamily. Therefore, we especially focused on CYP2D and assessed the effects of rifampicin on the depressive action of IL-1 toward the activities of the CYP2D-linked monooxygenase system as well as the contents of the CYP2D enzyme and its mRNA after administration of rifampicin plus IL-1 to rats. To investigate the effects of rifampicin on the activities of P-450, we used debrisoquine and bufuralol, the substrates catalyzed by CYP2D, as well as 7-ethoxycoumarin and benzphetamine catalyzed by other P-450 isozymes such as CYP2C11 or CYP1A1, to compare the effects of rifampicin on the depressive action of IL-1 on the P-450–linked monooxygenase system.

MATERIALS AND METHODS

Chemicals

Rifampicin (original powder) was generously provided by Daiichi Pharmaceutical Co (Tokyo, Japan), and reduced nicoteinamide adenine dinucleotide phosphate (NADPH) proteinase K, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, benzphetamine, and 7-ethoxycoumarin were purchased from the Sigma Chemical Co (St Louis, MO). Debrisoquine, 4-hydroxydebrisoquine, bufuralol, and 1′-hydroxybufuralol were generous gifts from Dr H. Fukui of the Department of Pharmacology, Osaka University Medical School. The Histofine SAB-PO kit (peroxidase-labeled streptavidin) for Western blot analysis was purchased from Nichirei Chemical Co (Tokyo, Japan). Rabbit anti-human immunoglobulins (IgG+IgA+IgM) were purchased from Funakoshi Chemical Co (Tokyo, Japan). All other chemicals of the highest commercially available grade were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Chemical Co (Kyoto, Japan).

Animals and Treatments

Healthy male Wistar rats (5 weeks old) weighing 150 g were used throughout this study. They were maintained in a 12/12-hour light/dark cycle in plastic cages with free access to food and water. Sixteen animals were divided into four groups.

Group 1 received 1.5 mL of 50 mmol/L HCl on days 1 through 4 and 1.5 mL saline on day 5 intraperitoneally. Group 2 received rifampicin (20 mg/rat) in 1.5 mL of 50 mmol/L HCl on days 1 through 4 and 1.5 mL saline on day 5 intraperitoneally. Group 3 received rifampicin (20 mg/rat) in 1.5 mL of 50 mmol/L HCl on days 1 through 4 and IL-1 (4 mg) in 1.5 mL saline on day 5 intraperitoneally. Group 4 received 1.5

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mL of 50 mmol/L HCl on days 1 through 4 and IL-1 (4 mg) in 1.5 mL saline on day 5 intraperitoneally.

Preparation of Liver Microsomes

Twenty-four hours after the final administration (day 6), the rats were killed by decapitation. Their livers were immediately perfused with ice-cold 50 mmol/L potassium phosphate buffer (pH 7.4) containing 1.15% (wt/vol) potassium chloride to remove as much blood as possible. The microsomal fractions were prepared at below 4°C according to the method of Mitoma et al.[12]

Determination of Protein Contents

Protein concentrations were determined by the method of Lowry et al[13] using bovine serum albumin as a standard. The molar extinction coefficient of bovine serum albumin of 6.68×10^3 $(mol/L)^{-1}$ cm^{-1} at 280 nm was used.[14]

The content of P-450 was determined spectrophotometrically from the carbon monooxide difference spectrum of a dithionite-reduced microsomes with a molar extinction coefficient of 91×10^3 $(mol/L)^{-1}$ cm^{-1} at 450 nm minus 490 nm.[15] The content of cytochrome b5 was determined from the reduced versus oxidized difference spectrum with a molar extinction coefficient of 185×10^3 $(mol/L)^{-1}$ cm^{-1} at 424 nm minus 409 nm.[15]

Enzymatic Activities

The activity of benzphetamine *N*-demethylase was determined by measuring the formaldehyde formed according to the method of Nash.[16] The activity of 7-ethoxycoumarin was determined by measuring the formation of 7-hydroxycoumarin with a fluorescence detection system (excitation maximum, 368 nm; emission maximum, 456 nm), following the method of Greenlee and Poland.[17] These enzymatic reactions were performed at 37°C for 5 minutes. The activities of debrisoquine and bufuralol monooxygenases were determined by the methods of Kronbach et al[18] and Matsuo et al.[19] These enzymatic reactions were performed at 37°C for 40 minutes. All enzymatic activity measurements were carried out in a final volume of 1 mL of 50 mmol/L potassium phosphate buffer (pH 7.4) containing a substrate, 1 mmol/L benzphetamine, 1 mmol/L 7-ethoxycoumarin, 0.25 mmol/L debrisoquine or 0.25 mmol/L bufuralol, an NADPH-regenerating system (5 mmol/L glucose 6-phosphate, 0.5 IU glucose 6-phosphate dehydrogenase, 4 mmol/L $MgCl_2$, and 0.2 mmol/L NADPH), and 1 mg microsomal protein.

Isolation of RNA and Slot Blot Hybridization

Liver tissue (approximately 500 mg) was incubated in a solution comprising 4 mol/L guanidine thiocyanate, 25 mmol/L sodium citrate, 0.1 mol/L 2-mercaptoethanol, and 0.5% (wt/vol) sarcosyl for 10 hours at 37°C. Total RNA was then extracted from the livers by the method of Chomczynski and Sacchi.[20] Purified RNA was dissolved in H_2O. Absorbance at 260 nm (OD260) and 280 nm (OD280) was determined on 50 μL of RNA solution using Beckman DU-64 Spectrophotometer. The purity of RNA was estimated from the ratio of OD260 and OD280s. The RNAs of which the value of OD260/OD280 is greater than 1.5 were chosen for the further analysis. Five micrograms of RNA was subjected to slot blot hybridization analysis. Bovine CYP2D complementary DNA (cDNA), cloned and sequenced in this laboratory, was used as a probe.[21] This cDNA clone was found to possess an approximately 70% homologous sequence compared with that of rat CYP2D cDNA. The labeling of CYP2D cDNA with ^{32}P-deoxyadenosine triphosphate (dATP) was carried out using the nick translation method.[22] Hybridization was conducted in 4× SETS buffer (0.6 mol/L NaCl, 120 mmol/L Tris-HCl [pH 7.5], 8 mmol/L EDTA, 10× Denhardt, and 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) containing 50 mg/mL heat-denatured salmon sperm DNA at 60°C for 24 hours. A single washing was conducted in 4× SETS buffer (0.15 mol/L NaCl, 30 mmol/L Tris-HCl [pH 7.5], 2 mmol/L sodium EDTA, and 0.1% [wt/vol] SDS), followed by double washing in 1× SETS buffer at 60°C for 15 minutes.

Western Blot Analyses

Liver microsomes (5 μg of protein per lane) were subjected to SDS–polyacrylamide gel electrophoresis by the established method.[23] The proteins were then transferred electrophoretically to nitrocellulose sheets and immunoblotted with the serum of a patient with autoimmune hepatitis (type II) containing a high titer of LKM-1 autoantibody (anti-CYP2D6 antibody). The serum of the patient with autoimmune hepatitis was a generous gift from Dr Michael P. Manns (Department of Gastroenterology and Hepatology, Hannover Medical School, Germany). The anti-CYP2D6 antibody in this serum has already been confirmed to react with the expressed fusion protein of cDNA in *Escherichia coli.*[24] Low-range molecular weight marker proteins were obtained from Bio Rad Laboratory (Japan).

Statistical Analyses

Mean values ± SD were calculated. Statistical analyses were performed with Macintosh Stat-View 512 software. The statistical significance was determined using one-way ANOVA. A P value of $<.05$ was considered significant.

RESULTS

Effects of Rifampicin and IL-1 on the Spectrophotometrical Contents of Cytochromes P-450 and b5

To investigate the effects of rifampicin on the depressive action of IL-1 on xenobiotic metabolizing monooxygenases in liver microsomes, we prepared four groups of rats treated with rifampicin, IL-1, or both in different manners as described in Materials and Methods. The solutions of rifampicin (20 mg/rat for 4 days) and IL-1 were administered to Wistar rats. First, we attempted to determine the effects of rifampicin on the spectrophotometrical contents of cytochromes P-450 and b5 (Table 1). The contents of cytochromes P-450 and b5 were compared between the group treated with rifampicin plus IL-1 (group 3) and that treated with IL-1 alone (group 4). The results showed that contents of both cytochromes P-450 and b5 in the group treated with rifampicin plus IL-1 were higher than those in the group treated with IL-1 alone. However, the difference between these two groups was remarkable only in the case of P-450, not b5, because the content of b5 was not reduced by the administration of IL-1 compared with that in the untreated group. In

Table 1. Effects of Rifampicin and IL-1 on the Spectrophotometrical Contents of Cytochromes P-450 and b5

	Group 1	Group 2	Group 3	Group 4
P-450	0.81 ± 0.065	1.04 ± 0.034*	0.85 ± 0.074†	0.49 ± 0.12*
b5	0.40 ± 0.015	0.49 ± 0.031*	0.45 ± 0.012	0.40 ± 0.013

NOTE. The contents of P-450 and b5 were spectrophotometrically determined on the liver microsomes from untreated rats (group 1) and rats treated with rifampicin alone (group 2), rifampicin plus IL-1 (group 3), and IL-1 alone (group 4). Statistical significance was calculated by one-way ANOVA.

*$P < .05$ *v* group 1.

†$P < .05$ *v* group 4.

addition, contents of both cytochromes P-450 and b5 were significantly increased by the repeated administration of rifampicin alone. These contents were elevated to approximately 1.3 times those in liver microsomes from untreated rats.

Effects of Rifampicin on the Activities of Xenobiotic Metabolizing Monooxygenases

Subsequently, the effects of rifampicin and IL-1 were investigated on the activities of several xenobiotic-metabolizing monooxygenases responsible for the detoxification of debrisoquine, bufuralol, benzphetamine, and 7-ethoxycoumarin. Repeated administration of rifampicin caused increases in the activities of all of the xenobiotic-metabolizing enzymes examined in this study (Fig 1). The induction rates were 30% to 40% higher than those in the untreated group. As shown on analyses of the cytochrome P-450 and b5 contents, the differences in the enzymatic activities between the group treated with rifampicin plus IL-1 and that treated with IL-1 alone were also examined. Especially in the cases of debrisoquine and bufuralol monooxygenases, significant changes in the specific activities were observed between these two groups. By contrast, in the cases of 7-ethoxycoumarin *O*-deethylase and benzphetamine *N*-demethylase, no significant changes in the specific activities were detected between these groups.

Effects of Rifampicin and IL-1 on Levels of CYP2D mRNA and Protein

Based on the results for the enzymatic activities, we performed quantitative analyses of the CYP2D enzyme and its mRNA as the next step to elucidate further the mechanism of induction of xenobiotic-metabolizing monooxygenases by rifampicin (Fig 2) CYP2D mRNA was clearly elevated by the treatment with rifampicin. The induction rates of CYP2D mRNA and protein paralleled each other by densitomerical analyses (data not shown). Furthermore, the level of CYP2D mRNA in the group treated with rifampicin plus IL-1 was equal to that in the group treated with rifampicin alone. In addition, the level of CYP2D mRNA in the group treated with rifampicin plus IL-1 was substantially higher than that in the group treated with IL-1 alone. These results were consistent with results for

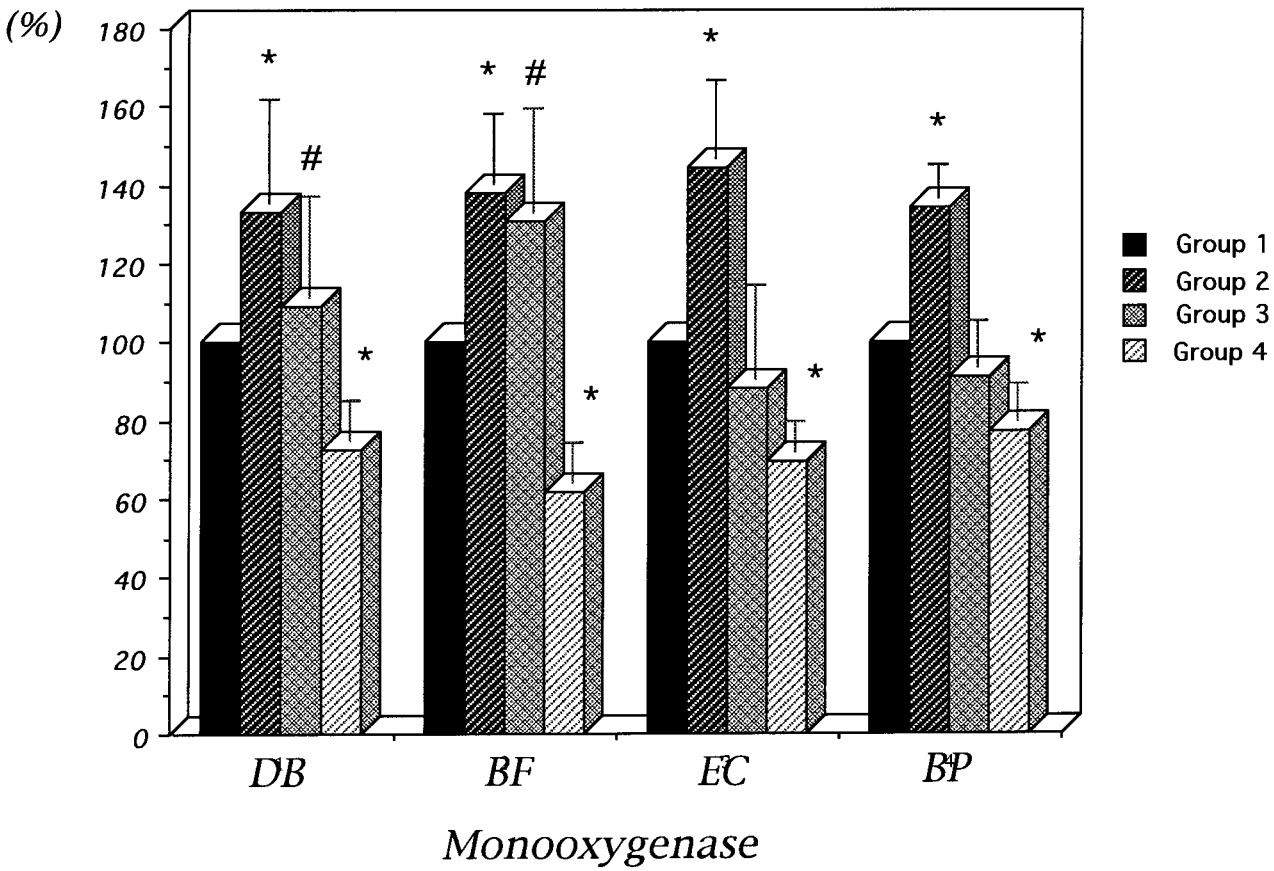

Fig 1. Effects of rifampicin on the activities of debrisoquine monooxygenase (DB), bufuralol monooxygenase (BF), 7-ethoxycoumarin *N*-demethylase (EC), and benzphetamine *N*-demethylase (BP) in rat liver microsomes. These enzymatic activities are expressed as percentages of the mean values in the untreated group (group 1). Each value represents the mean ± SD for four rats. * Significantly different from those in the untreated group (group 1); $P < .05$. # Significantly different from those in the group treated with IL-1 alone (group 4); $P < .05$. The mean values of the activities of debrisoquine monooxygenase, bufuralol monooxygenase, 7-ethoxycoumarin *O*-deethylase, and benzphetamine *N*-demethylase obtained for the untreated group (group 1) were 0.33 ± 0.04, 0.58 ± 0.029, 3.06 ± 0.28, and 3.51 ± 0.38 nmol/min/mg microsomal protein (pH 7.4 and 37°C), respectively.

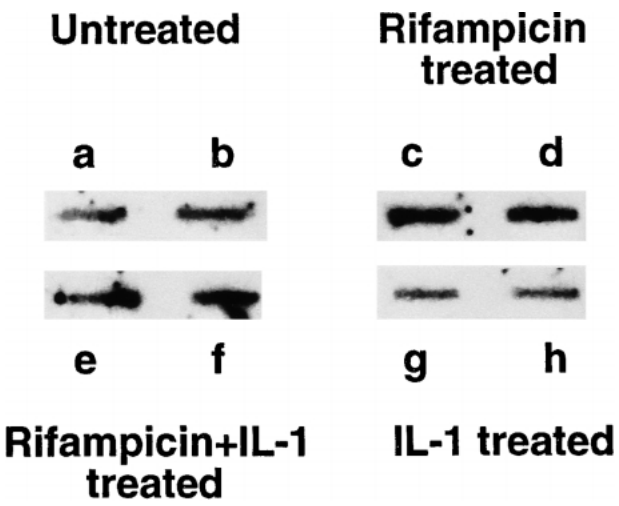

Fig 2. Slot blot analysis of CYP2D mRNA of liver from rifampicin– and/or IL-1–treated rats. Duplicate RNA samples from individual livers are shown. Total liver RNA (5 μg) was bound to a nylon filter and then hybridized with the CYP2D cDNA, as described in Materials and Methods. Lanes a and b, RNA from group 1; lanes c and d, RNA from group 2; lanes e and f, RNA from group 3; lanes g and h, RNA from group 4.

the spectrophotometrical content of P-450 and enzymatic activities.

The final question that should be raised is whether the induction effect of rifampicin on CYP2D can be observed at the protein level. We performed quantitative Western blot analysis of the CYP2D enzyme using the anti-CYP2D antibody (Fig 3). The stained protein bands indicated a molecular weight of 52 kd, corresponding to that of the CYP2D enzyme in rat liver microsomes. Enhancement of the content of the CYP2D enzyme was also observed in the samples from the rats treated with rifampicin alone.

DISCUSSION

Many lines of evidence have demonstrated that cytokines related to inflammation such as IL-1, IL-6, and tumor necrosis factor have depressive effects on the cytochrome P-450–linked monooxygenase system.[4,25-30] We also previously showed that IL-1 had a depressive effect on the CYP2D subfamily, and this depressive effect was evoked at the mRNA level.[2] IL-1 is likely to act on the xenobiotic-metabolizing monooxygenases more rapidly than other cytokines do. In a previous study, we observed the depressive effect of IL-1 on the P-450–linked monooxygenase system 24 hours after injection of IL-1. Therefore, we administered IL-1 to rats 24 hours before they were killed.

Rifampicin has induction effects on the components in cytochrome P-450–linked monooxygenase system through in vitro[4,8,10,11,31,32] and in vivo[5,6,9,33,34] studies. In these reports, the induction effect of rifampicin was detected in CYP3A and CYP1A subfamilies. In this study, we examined the effects of rifampicin on the CYP2D subfamily. In addition to the finding that CYP2D was induced by administration of rifampicin alone, addition of injection of rifampicin offset the depressive action of IL-1 on CYP2D. To our knowledge, this report is the first description of the induction effect and inhibition of the depressive action of IL-1 on the CYP2D subfamily by rifampicin.

It is crucial to show whether the inhibitory effects of rifampicin on the action of IL-1 on the CYP2D subfamily are regulated by translational or posttranslational events. In the present study, the quantity of total mRNA corresponding to the CYP2D gene was assessed, and the induction effects of rifampicin on several enzymatic activities and expression of the CYP2D gene were maintained even in the group treated with rifampicin plus IL-1. In addition, some investigators reported that the principal mechanism by which IL-1 reduces the levels of cytochrome P-450 mRNA is suppression of transcriptional activation.[35] Therefore, our finding that the depressive effect of IL-1 on the gene expression of CYP2D was offset by rifampicin may be taken as indirect evidence that rifampicin up-regulates, and transcriptional regulation might be closely related with this induction effect.

To perform quantitative analysis of the CYP2D protein of liver microsomes, we used serum containing the LKM-1 autoantibody obtained from a patient with autoimmune hepatitis (type II). This antibody was shown to react with a 52-kd protein present in rat liver and kidney.[36,37] It has already been shown that the LKM-1 antibody specifically recognizes CYP2D1 and CYP2D2 of rat liver microsomes and CYP2D6 of human liver microsomes.[24,38-42] Therefore, the protein bands stained on Western blot analysis in this experiment should be CYP2D1 and/or CYP2D2 of rat liver microsomes.

In conclusion, we have shown that rifampicin induces CYP2D, and the induction of CYP2D is up-regulated at the mRNA level by rifampicin and overcomes the suppression of CYP2D mRNA expression by IL-1.

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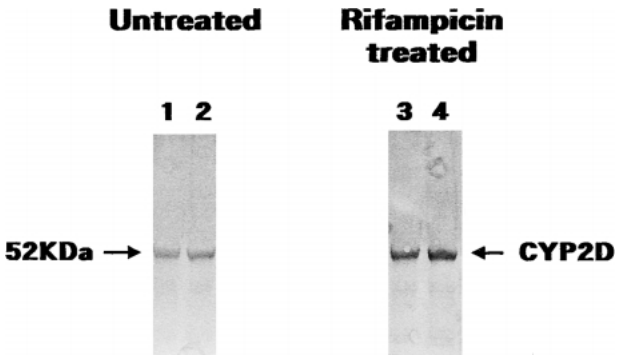

Fig 3. Western immunoblot analysis of CYP2D of rat liver microsomes. The liver microsomal proteins (5 μg) were subjected to SDS-PAGE and transferred to nitrocellulose sheets, then the blots were treated with the anti-CYP2D antibody. The relative molecular mass of the stained protein band is indicated as 52 kd. Lanes 1 and 2, group 1; lanes 3 and 4, group 2.

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