Allopurinol and glutamine attenuate bacterial translocation in chronic portal hypertensive and common bile duct ligated growing rats

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Abstract

Background—Spontaneous bacterial infections and septicaemia result in morbidity and mortality in patients with portal hypertension and obstructive jaundice.

Aim—The aim of this study in rats was to investigate the incidence of bacterial translocation in portal hypertension and obstructive jaundice, and to evaluate the effects of allopurinol and glutamine.

Methods—Rats were subjected to sham laparotomy (SL), portal hypertension (PH) by calibrated stenosis of the portal vein, and common bile duct ligation (CBDL). Animals of each group were either treated with allopurinol (50 mg/kg twice a week), glutamine (1 g/kg/d), and allopurinol and glutamine.

Results—After four weeks, significant bacterial translocation in the untreated PH and CBDL rats occurred. Intestinal mucosal malondialdehyde concentrations (MDA), as an indicator for lipid peroxidation, and myeloperoxidase activity (MPO) released from activated neutrophils were also significantly increased ($p < 0.01$). Allopurinol and glutamine in PH and CBDL rats improved bacterial translocation, and decreased MDA and MPO values ($p < 0.01$).

Conclusion—In PH and CBDL rats significant bacterial translocation, ileal mucosal lipid peroxidation, and neutrophil derived MPO activity occurred. Allopurinol and glutamine significantly reduced bacterial translocation, as well as ileal mucosal MDA and MPO activities.

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Keywords: bacterial translocation, portal hypertension, obstructive jaundice, lipid peroxidation, allopurinol, glutamine.

Spontaneous bacterial infections and sepsis resulting in morbidity and mortality have been reported in patients with chronic portal hypertension (PH) and obstructive jaundice.^{1,2} Most of these infectious episodes are spontaneous without apparent sources of infection, and caused by aerobic bacteria normally present in the intestine.³ Chronic PH and obstructive jaundice disrupt the intestinal barrier by producing structural changes in the bowel mucosa,^{4,5} inducing the formation of portal systemic shunts, bacterial overgrowth, by the absence of intestinal bile flow, malnutrition, decreased bacterial clearance by the

reticuloendothelial system, and impaired immunological defence.^{6–10}

PH, either isolated or as a sequence of hepatic diseases leads despite an hyperdynamic splanchnic flow state to intestinal mucosal hypoxaemia by arterial hypoperfusion.¹¹ Decreased intestinal arterial perfusion results in an increased conversion of xanthine dehydrogenase to xanthine oxidase, with subsequent lipid peroxidation.¹² The intestinal mucosa is one of the richest sources of xanthine oxidase.¹³ This initiates an oxidative damage of intestinal mucosal cells and leads to intestinal mucosal atrophy.^{11,13} In this compromised bowel mucosa polymorphonuclear (PMN) neutrophil accumulation occurs triggered by oxygen metabolites and destructive toxins from these cells are released and further damage the intestinal mucosa.^{14,15}

The aim of this study was to estimate intestinal mucosal malondialdehyde (MDA) and myeloperoxidase concentrations (MPO) in chronic PH and common bile duct ligation (CBDL) and to investigate the effects of allopurinol and glutamine on intestinal mucosal lipid peroxidation and PMN neutrophil derived MPO activity on bacterial translocation in chronic PH and CBDL growing rats.

Allopurinol, a competitive xanthine oxidase inhibitor, acts as a free radical scavenger and prevents lipid peroxidation.¹⁶ The amino acid glutamine has received considerable attention in the past, because of new knowledge showing that glutamine is required for mucosal growth and bolsters intestinal barrier function.¹⁷

Methods

Male, 4 weeks old, Sprague-Dawley rats (150 g to 170 g) were used for all experiments. All animals were housed in a controlled environment and permitted free access to food and water. All experiments were approved by the animal research commission of the ministry of science and conformed to guidelines for the care and use of laboratory animals.

Animals were randomly assigned to one of the three groups: sham laparotomy (SL, $n=40$), PH ($n=40$), and CBDL ($n=40$). The rats were anaesthetised with ketamine (100 mg/kg intraperitoneally) and under aseptic conditions a midline laparotomy was performed. In the SL group the common bile duct and the portal vein were dissected free and the abdomen was closed. PH was produced by calibrated stenosis of the portal vein. A 21 gauge blunt tipped needle was placed alongside the vein and both vein and needle were

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ligated with 4-0 silk ligature. The needle was removed, leaving a 21 gauge stenosis in the portal vein.

Obstructive jaundice was produced by ligation of the common bile duct with a 3-0 silk ligature. The abdomen was closed in two layers with absorbable sutures, and the animals were permitted four weeks recovery with free access to food and water.

Treatment

The three groups SL, PH, and CBDL, were subdivided into four subgroups: (a) 10 animals served as controls; (b) 10 rats received allopurinol (50 mg/kg intraperitoneally) twice a week; (c) in 10 rats glutamine was given (1 g/kg/d orally); (d) 10 rats were treated with allopurinol and glutamine (allopurinol 50 mg/kg twice a week intraperitoneally and glutamine (1 g/kg/d orally).

Experimental design

After four weeks the animals were weighed, and under ketamine anaesthesia and sterile conditions a midline laparotomy was made. The exposed viscera were swabbed with a sterile cotton tipped applicator stick, which was immediately placed on blood agar plates. Portal pressure was measured after cannulating the superior mesenteric vein with a 21 gauge needle connected to a manometer, and the height of the right atrium was taken as zero reference level. Blood samples were withdrawn from the vena cava for the measurement of serum liver enzymes (total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), cholinesterase (CHE), and total serum protein.

Aliquots of portal vein blood and vena cava blood (0.1 ml) were plated onto blood agar plates. The central mesenteric lymph node complex (MLN), the spleen, and the liver were harvested with new sterile instruments and weighed. A 1.5 cm long sample of the terminal ileum was excised, opened on its antimesenteric border, and washed in sterile 0.9% saline solution. The MLN complex, parts of the liver and spleen were cut off, weighed, placed in grinding tubes containing sterile brain-heart infusion 1:9, and homogenised. Serial dilutions of the homogenates were made and 0.1 ml of each dilution was plated on blood, endo, and MRS agar plates and incubated aerobically for 48

hours at 37°C. The plates were evaluated for bacterial growth by standard bacteriological technique. Any growth in the plates was considered positive and expressed as colony forming unit per gram tissue (cfu/g).

For determination of MDA and MPO ileal mucosa was scrubbed off and weighed. All specimens were immediately frozen in liquid nitrogen until assay. MDA was determined as an index of ileal mucosal lipid peroxidation according to the method of Wong *et al.*¹⁸ MPO was assayed as an indicator for activated PMN neutrophils using the method of Krawisz *et al.*¹⁹ MDA values were expressed as nmol/g dry weight of ileal mucosa, and MPO activity was expressed as U/g dry ileal mucosa. Dry weight of ileal mucosa was estimated after incubation of ileal mucosa at 50°C for 48 hours.

Samples of the jejunum, ileum, and liver were taken for histological examination, fixed in buffered formalin, embedded in paraffin wax, cut in 2-3 µm serial sections, and stained with haematoxylin and eosin. Specimens of the liver were also stained with chrom-anilin-blue.

The results were expressed as mean (SEM). Statistical analyses between the means of two groups were performed by the Mann-Whitney U test, and analysis of variance (ANOVA) was used when comparing more than two groups. A *p* value <0.05 or less was considered statistically significant.

Results

After four weeks, all PH animals showed a patent but stenotic portal vein with dilated mesenteric vessels. All CBDL animals became visibly jaundiced and had a cystic common bile duct remnant proximal to the ligature.

Body weight in all SL rats increased from 160 (4.8) g to 355 (3.2) g, and in all PH rats from 155 (3.2) g to 340 (6.4) g. In all CBDL groups malnutrition with decreased weight gain from 165 (3.2) g to 305 (4.8) g was present (*p*<0.05). Portal pressure (mm Hg) increased significantly (*p*<0.01) in all PH and CBDL rats without any differences between the subgroups (Table I). Serum liver enzymes remained normal in all SL and PH rats. CBDL resulted in deterioration of liver function, but in the CBDL groups treated with allopurinol and allopurinol/glutamine significant lower concentrations of bilirubin and AST compared with the other CBDL groups were found (*p*<0.01).

TABLE I Serum liver enzymes and portal pressure after sham laparotomy (SL), portal hypertension (PH), and common bile duct ligation (CBDL)

	SL	PH	PH +Allo	PH +Glu	PH +Allo/Glu	CBDL	CBDL +Allo	CBDL +Glu	CBDL +Allo/Glu
Bilirubin (µmol/l)	4.2 (0.5)	3.9 (0.6)	3.7 (0.8)	3.9 (0.1)	4.3 (0.5)	136.8 (5)*	72.7 (6)*†	141.9 (3)*	63.3 (5)*†
AST (U/l)	43 (5)	47 (4)	51 (3)	42 (6)	55 (4)	567 (37)*	101 (18)*†	241 (9)*†	173 (9)*
ALT (U/l)	27 (4)	37 (6)	31 (3)	29 (5)	34 (4)	101 (10)*	105 (6)*	114 (5)*	94 (4)*
AP (U/l)	231 (11)	267 (10)	273 (12)	254 (9)	243 (11)	638 (19)*	683 (37)*	643 (29)*	656 (31)*
CHE (U/l)	331 (19)	271 (13)	271 (13)	306 (10)	295 (12)	148 (9)*	164 (9)*	141 (5)*	173 (11)*
Portal pressure (mm Hg)	7.6 (2.9)	19.2 (0.4)*	19.6 (0.3)*	18.9 (0.5)*	19.3 (0.7)*	20.5 (0.6)*	18.9 (0.6)*	19.3 (0.9)*	19.1 (0.5)*

**p*<0.01 *v* SL; †*p*<0.01 control group *v* allopurinol (Allo), glutamine (Glu), and allopurinol/glutamine (Allo/Glu) treated groups. Data shown as mean (SEM).

TABLE II Incidence of bacterial translocation after SL, PH, and CBDL

	Peritoneum	Portal vein	Vena cava	Liver	Spleen	MLN
SL	ND	ND	ND	ND	1/10	4/10
SL+Allo	ND	ND	ND	ND	2/10	3/10
SL+Glu	ND	ND	ND	ND	1/10	3/10
SL+Allo/Glu	ND	ND	ND	ND	2/10	4/10
PH	1/10	3/10	1/10	4/10	2/10	5/10*
PH+Allo	ND	ND	ND	4/10	2/10	7/10
PH+Glu	ND	ND	ND	ND	2/10	4/10
PH+Allo/Glu	ND	ND	ND	ND	ND	4/10
CBDL	2/10	5/10*	2/10	9/10*	4/10	9/10*
CBDL+Allo	ND	ND	ND	3/10	1/10	5/10
CBDL+Glu	ND	ND	ND	3/10	3/10	5/10
CBDL+Allo/Glu	ND	ND	ND	3/10	1/10	4/10

* $p < 0.05$ control groups *v* Allo, Glu, Allo/Glu groups. Abbreviations as Table I. ND = not detected.

TABLE III Ileal colonisation after SL, PH, and CBDL

	SL	PH	CBDL
Gram positive	$1.6 (0.3) \times 10^4$	$2.5 (1.1) \times 10^5$ *	$4.9 (0.9) \times 10^5$ *
Gram negative	$3.9 (0.4) \times 10^3$	$6.3 (1.0) \times 10^3$ *	$3.8 (0.7) \times 10^4$ *

* $p < 0.01$ *v* SL. Abbreviations as Table I. Data shown as mean (SEM).

Bacterial translocation and ileal colonisation (Table II)

After SL, bacterial translocation occurred in 8% to 10% of the MLN and spleen without significant differences between the control group and allopurinol, glutamine, and allopurinol/glutamine treated animals. Translocating bacteria cultured in the spleen and MLN were lactobacilli species in 80% and *Escherichia coli* in 20%.

In PH, the incidence of bacterial translocation increased to 23% in the control group, and bacterial translocation to the peritoneum, vena cava, portal vein, liver, spleen and MLN occurred. Allopurinol and glutamine treatment decreased the rate of bacterial translocation to 10% and 7% ($p < 0.05$ *v* control). Lactobacilli, streptococci, enterococci species, and *E. coli* were the most commonly cultured bacteria.

CBDL resulted in a bacterial translocation rate of 52% in the control group. Positive cultures of the peritoneum, vena cava, portal vein, liver, spleen, and MLN were found. Lactobacilli, streptococci, enterococci, and *E. coli* were the main translocating bacteria. Allopurinol and glutamine treatment significantly reduced the incidence of bacterial translocation to 18% and 13% in CBDL animals

($p < 0.05$ *v* control). The quantitative analysis of bacteria colonising the ileum showed a significant ($p < 0.01$) Gram positive and Gram negative overgrowth in all PH and CBDL animals (Table III). This overgrowth was shown by higher cfu/g ileum of Gram positive lactobacilli species, streptococci, and Gram negative *E. coli*, enterococci, and pasteurilla strains.

MDA in the ileal mucosa (Fig 1)

After SL ileal mucosal MDA concentrations were between 31.2 (0.3) nmol/g and 36.6 (2.1) nmol/g in the control and glutamine supplemented groups. Treatment with allopurinol and allopurinol/glutamine resulted in significant lower values ($p < 0.05$) of ileal MDA, 19.2 (2.1) nmol/g and 17.1 (1.3) nmol/g respectively. PH led to a significant increase of MDA values compared with the SL group ($p < 0.01$). Treatment with allopurinol, glutamine, and allopurinol/glutamine significantly attenuated MDA concentrations ($p < 0.01$) compared with the PH control group (control 98.7 (1.6) nmol/g, allopurinol 49.2 (1.4) nmol/g, glutamine 51.0 (3.6) nmol/g, allopurinol/glutamine 32.4 (2.6) nmol/g).

After CBDL, the MDA value in the control group increased to 102.2 (1.4) nmol/g ($p < 0.01$ *v* SL). In the allopurinol, glutamine, and allopurinol/glutamine treated CBDL animals significantly decreased MDA values ($p < 0.01$) were present (allopurinol 66.1 (2.4) nmol/g, glutamine 67.2 (1.3) nmol/g, allopurinol/glutamine 52.1 (2.4) nmol/g).

MPO in the ileal mucosa (Fig 2)

Ileal mucosal MPO activities after SL showed a mean value of 286 (4.8) U/g without any significant differences between the subgroups.

PH resulted in a significant increase ($p < 0.01$) of MPO activity in the control group to 866 (5) U/g. Allopurinol and glutamine treatment significantly decreased MPO activities to a mean of 326 (14) μ /g ($p < 0.01$).

In the CBDL animals MPO activity increased to 1016 (7.1) U/g in the control group. Treatment with allopurinol and glutamine significantly reduced MPO activities to a mean of 369 (8) U/g ($p < 0.01$).

Histological examination

Microscopically, the ileum was normal in all SL, PH, and CBDL rats. In the jejunum, the mucosa showed a mild atrophy with decreased villus height in untreated and allopurinol treated PH and CBDL animals, but these differences were not statistically significant compared with the SL groups and glutamine treated animals. The liver of untreated and glutamine treated rats showed a fibrosis grade III with periportal inflammation and bile duct proliferation (Fig 3). The liver of allopurinol and allopurinol/glutamine treated animals had a lesser degree of fibrosis, classified as grade I-II (Fig 4), and this was statistically significant ($p < 0.05$).

Figure 1: Malondialdehyde values in ileal mucosa (SL = sham laparotomy, PH = portal hypertension, CBDL = common bile duct ligation). A = control, B = allopurinol, C = glutamine, D = allopurinol/glutamine. * $p < 0.01$ *v* SL; † $p < 0.01$ A *v* B, C, D.

Figure 2: Myeloperoxidase activities in ileal mucosa. A=control, B=allopurinol, C=glutamine, D=allopurinol/glutamine. * $p < 0.01$ v SL; † $p < 0.01$ A v B, C, D. Abbreviations as in Figure 1.

Discussion

In patients with PH and obstructive jaundice an increased incidence of bacterial infections, spontaneous bacterial peritonitis, and sepsis is described.^{1,2} The frequency of these complications is reported to be between 8% and 25% with a mortality of 78%.^{1,20} Some 95% of these infections are caused by an aerobic organism of intestinal origin.³ Clinical and experimental studies have undertaken to define the mechanism that leads to disruption of intestinal mucosal barrier function, and to translocation of indigenous intestinal bacteria to extra-intestinal organs in PH and obstructive jaundice.^{4,5,21,22} Factors that have been proposed to promote bacterial translocation from the gut in chronic PH and CBDL, include physical disruption of the intestinal mucosa, intestinal bacterial overgrowth, and impaired host immune defence.^{23,24}

Isolated portal hypertension is characterised by an abnormal increase of portal pressure, and the formation of a network of porto-systemic shunts, diverting the portal blood stream to the systemic circulation, bypassing a normal liver.²⁵ It is proposed that in PH there is a comparative shunting of the bloodstream from the mucosa to the submucosa, leading to intestinal mucosal hypoxaemia.^{4,13} PH therefore disrupts the intestinal mucosal barrier by vascular congestion of the mucosa, thus

Figure 3: Histological examination of the liver, haematoxylin and eosin staining, X250; fibrosis grade III was present in the control CBDL group.

Figure 4: Histological examination of the liver, haematoxylin and eosin staining, X250; fibrosis grade I-II in the allopurinol treated CBDL animals.

increasing intestinal permeability and favouring the transmural passage of bacteria.^{4,26} Intestinal bacterial overgrowth with destruction of the ecological balance of the normal indigenous microflora is reported in cholestatic diseases.²⁷ It is supposed that the absence of intestinal bile acids and sIgA, which have bacteriostatic properties, promote intestinal bacterial overgrowth.²⁸ Furthermore, PH and obstructive jaundice leads to malnutrition, and an association between malnutrition and infectious complications has been reported.²⁹ Protein undernutrition disrupts the normal ecology of the gut microflora and leads to intestinal mucosal atrophy.^{30,31} The intestinal mucosa is an extremely active metabolic tissue that exhibits high rates of epithelial replication. It is supposed that protein malnutrition increases enteric bacterial overgrowth, and influences the composition of the intestinal flora.⁸ Chronic obstructive jaundice, the absence of intestinal bile flow, impaired hepatic reticuloendothelial system function, intestinal bacterial overgrowth, and physical disruption of gut mucosal barrier explain the high incidence of infectious complications in this disease.^{5,21} PH impairs systemic immunity by shunting the portal blood away from the liver to systemic circulation.³ PH also occurs as a result of hepatic fibrosis with increased vascular resistance by compression of hepatic venules by regenerative nodules.³² Lipid peroxidation and the generation of oxygen derived free radicals has also been associated with hepatic fibrogenesis.³³ In obstructive jaundice high concentrations of lipid peroxides are believed to be an important mediator for liver fibrosis, intestinal bacterial overgrowth, intestinal mucosal atrophy, and neutrophil infiltration into the bowel.^{14,34} Increased plasma concentrations of lipid peroxides have been reported in patients with cholestatic diseases.^{35,36} Several studies were undertaken to investigate mechanisms that initiate and favour bacterial translocation in PH and obstructive jaundice.

PH, either isolated or as a result of liver fibrosis, leads despite an hyperdynamic splanchnic circulatory state, to intestinal mucosal hypoperfusion and hypoxaemia.¹¹ Hypoxaemia can initiate an increased conversion of xanthine dehydrogenase to xanthine oxidase (XO) with subsequent formation of

oxygen derived free radicals.¹⁶ The intestinal mucosa is one of the richest sources of XO. In several studies the role of XO induced lipid peroxidation on intestinal permeability and subsequent generation of oxygen derived free radicals triggering bacterial translocation has been elucidated in intestinal ischaemic conditions.^{12 14 33–35} These oxygen derived free radicals also trigger attractants like activated PMN neutrophils, which have the ability to release a complex of reactive oxygen metabolites that can destroy normal cells and can dissolve connective tissue.³⁷ Therefore increased XO values generated by mucosal hypoperfusion change intestinal mucosal membranes by lipid peroxidation, and promote PMN neutrophil infiltration into the bowel initiated by XO derived free radical metabolites. Both XO and PMN neutrophil accumulation are believed to mediate mucosal damage in themselves, leading to bacterial overgrowth, mucosal atrophy, and increased intestinal permeability.^{15 37 38}

Allopurinol acts as a free radical scavenger, and the proposed mechanism for its protective effects includes XO inhibition and prevention of toxic oxygen radical formation.³⁹ Glutamine has been shown to be the main fuel used by gut mucosal cells as a regulator of cell proliferation and as an essential amino acid for mucosal growth and function.⁴⁰ Glutamine prevents enteric bacterial overgrowth and influences the composition of the intestinal flora, perhaps favouring bacterial species with lack of the characteristics necessary for translocation.⁴¹

In this study we investigated the role of intestinal mucosal lipid peroxidation and PMN neutrophil derived MPO activity on bacterial translocation in growing rats with PH and CBDL. Further, we evaluated possible effects of a treatment with allopurinol and glutamine. In growing rats with PH significant bacterial translocation, increased intestinal mucosal lipid peroxidation, MPO activity, and ileal bacterial overgrowth with Gram negative and Gram positive bacteria occurred. This is in contrast with other studies, where bacterial translocation was only observed in acute PH, or in PH in association with haemorrhagic shock.^{4 38} These reported differences in bacterial translocation might be attributed to the fact, that in our study growing rats were analysed after four weeks of PH, whereas in other studies adult rats were investigated after 14 days of PH. A possible explanation for the differences in bacterial translocation between growing and adult rats might be a decreased resistance of the intestinal mucosal barrier against bacteria during maturation of intestinal integrity.⁴² Both allopurinol and glutamine treatment in PH rats, significantly decreased bacterial translocation, ileal mucosal MDA and MPO values compared with untreated animals.

No differences in ileal and jejunal bacterial colonisation and histomorphology of the jejunum and ileum between untreated and treated PH rats were present. These findings show that in isolated PH mucosal lipid peroxidation and MPO activity contribute to

increased intestinal permeability for bacteria, which could be prevented by allopurinol and glutamine.

CBDL in growing rats resulted in a significant deterioration of liver function, but with significant lower values of bilirubin and AST in the allopurinol/glutamine treated group.

This might be explained by the fact that liver fibrosis is mediated by lipid peroxides, which can be blocked by allopurinol.⁴³ The histological investigation of the liver confirmed this hypothesis, because there was less periportal fibrosis and portal infiltration in the allopurinol/glutamine treated CBDL group compared with the other CBDL animals. In untreated CBDL rats, bacterial translocation occurred in 52% and this could be increased to 13%–18% by allopurinol and glutamine treatment. Significant ileal bacterial overgrowth with Gram positive and Gram negative bacteria was present in all CBDL rats and this was unaffected by all treatment regimens. In contrast, allopurinol and glutamine significantly decreased ileal mucosal MDA and MPO values indicating a protective effect on mucosal lipid peroxidation and intestinal PMN neutrophil infiltration in CBDL rats. Histomorphologically, the ileum and jejunum did not show important differences between untreated and treated animals with CBDL except a mild mucosal atrophy in the jejunum of untreated and allopurinol treated rats.

In summary, chronic PH and CBDL in growing rats are associated with significant bacterial translocation and ileal bacterial overgrowth with Gram positive and Gram negative aerobes. Significant intestinal mucosal lipid peroxidation and PMN neutrophil derived MPO activity occurred, and this might be an additional important mechanism that disrupts the intestinal mucosal barrier function and increases the egress of gut bacteria to extra-intestinal organs.

Allopurinol, a competitive XO inhibitor and free radical scavenger, reduced intestinal mucosal lipid peroxidation by direct inhibition of XO and indirectly decreased intestinal PMN neutrophil derived MPO activity resulting in a considerably lower rate of bacterial translocation in PH and CBDL. Glutamine supplementation in PH and CBDL rats had the same inhibitory effect on bacterial translocation, intestinal mucosal lipid peroxidation, and MPO activity. This might be attributed to the fact that glutamine supports intestinal mucosal growth and cell turnover thus bolstering intestinal barrier function, which makes the intestinal mucosa more resistant to bacterial overgrowth and hypoxaemia caused by PH.

The clinical significance of this study in growing rats remains speculative. Our findings suggest, however, that inhibitors of oxygen derived free radicals like allopurinol, and regulators of intestinal mucosal cell proliferation like glutamine might be useful as prophylactic agents to reduce infectious complications in PH and obstructive jaundice.

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