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Effective peritoneal therapy of acute pancreatitis in the rat with glutaryl-trialanin-ethylamide: a novel inhibitor of pancreatic elastase

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Abstract

The six hour peritoneal lavage with glutaryl-trialanin-ethylamide, a low molecular competitive inhibitor of pancreatic elastase (IC_{50}–8 μmol/l), effectively suppresses the evolution of taurocholate induced acute pancreatitis in the rat. The lavage alone is followed by a marked decrease of fat necrosis and amylase and lipase activity in serum. The area of pancreatic haemorrhage was significantly reduced only after the lavage solution was supplemented with Glt-Ala_3-NHEt. The effect was not enhanced by a bolus injection of the inhibitor before starting the lavage. The combination of Glt-Ala_3-NHEt with aprotinin or nafamstate mesilate produced only marginal greater benefit. The effect of Glt-Ala_3-NHEt on pancreatic haemorrhage is time and dose related even with delayed onset of the lavage. Animals treated with peritoneal lavage without Get-Ala_3-NHEt lived longer than controls ($p<0.05$), but by 60 hours the survival rate of both groups was almost the same (76 *v* 74%). All animals lavaged with Glt-Ala_3-NHEt survived 120 hours and the difference in the survival rate between this and both remaining groups was significant (100% *v* 76% *v* 74% – $p<0.05$). The results were considered favourable and preliminary clinical trials of Glt-Ala_3-NHEt in subjects with acute pancreatitis justified.

Intravital autodigestion of the pancreas as well as of remote tissues in acute pancreatitis is the result of cascade of activated proteolytic and lipolytic enzymes. Pancreatic elastase is responsible for the digestion of intravascular elastin, and through this action it plays an important role in the transition of acute pancreatitis into its severe haemorrhagic and necrotic form.

On the basis of our earlier studies on the optimal structure of the synthetic substrate for pancreatic elastase[1-3] we synthesised and investigated more than 50 elastase inhibitors. These substances are alkylamides of N-acyl or N-alkanoyl-aminoacyl tripeptides.[4,5] They show marked inhibition of pancreatic elastase of various origin in vitro.[6] Their biological effects were studied mainly with the use of glutaryl-trialanin-ethylamide (Glt-Ala_3-NHEt). This compound competitively inhibits pancreatic elastase and displays a suitable value of the inhibition constant (IC_{50}–8 μmol/l).

In a previous communication[7] we found that Glt-Ala_3-NHEt used as a single or repeated bolus injection either alone or in combination with other inhibitors in acute pancreatitis of the rat was mainly effective on intraperitoneal administration. A significant reduction of the area of pancreatic haemorrhage was considered the conclusive criterion of the effectiveness of the elastase inhibitor. At the same time a decrease of fat necroses and ascites was found.

This paper presents the results of peritoneal lavage with Glt-Ala_3-NHEt alone or in combination with other inhibitors in unrestrained rats with taurocholate induced acute pancreatitis.

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Methods

INHIBITORS

Glt-Ala_3-NHEt (Inpankin, Research Institute for Pharmacy and Biochemistry, Prague). Na-Inpankin is the sodium salt of Glt-Ala_3-NHEt. The concentration of Glt-Ala_3-NHEt in the lavage solution amounted to 2 or 4 mg/ml. Na-Inpankin concentration was 4 or 8 mg/ml. A bolus injection of Glt-Ala_3-NHEt included 20 mg of the substance dissolved in 1 ml 0·2 mol/l $NaHCO_3$.

Aprotinin (Antilysin Spofa) – activity 10 000 trypsin inhibiting units per millilitre. The final concentration in the lavage solution was 1000 trypsin inhibiting units per millilitre.

Nafamstate mesilate (6-amidino-2-naphthyl-p-guanidinobenzoate dimethanesulphonate, FUT) was supplied by the courtesy of Dr T Aoyama of Tori Co (Tokyo). The concentration of nafamstate mesilate in the lavage solution amounted to 0·1 mg/ml.

LAVAGE SOLUTION

Peritoneal dialysis solution (Peridial® – Medical Suppliers Co, Prague) supplemented with 4 mmol/l KCl and 1·5% glucose (final osmolarity 370,9 mOsmol) was used alone or with the inhibitors.

ACUTE PANCREATITIS

Induction

Male Wistar rats (body mass 180–240 g) were used throughout the experiments. 0·4 ml 2% sodium taurocholate (Calbiochem or Sigma) was injected into the common choledochopancreatic duct of ether anaesthesied rats according to Lankisch.[8]

Evaluation

Six hours after taurocholate injection a second

ether anaesthesia was given. The cannula was withdrawn from the peritoneal cavity of lavaged rats. In all animals a midline laparotomy was then carried out. In controls the ascites was aspirated and its volume measured. After thoracotomy the animals were bled by intracardial puncture. Fat necroses in epididymal, perirenal, and mesenteric fat were counted, while in peripancreatic fat they were determined semiquantitatively because of their confluent character (0-absent, 1-slight, 2-moderate, 3-severe). Haemorrhagic areas in the pancreas were measured (mm^2). Thereafter the pancreas was fixed in 10% formol. Tissue sections stained with haematoxylin and eosin were semiquantitatively evaluated under low magnification for the presence of oedema, cellular infiltration, haemorrhage and necrosis (0-absent, 1-slight, 2-moderate, 3-severe). Enzyme activities were measured both in serum (amylase, lipase) and ascites (amylase, lipase, elastase, trypsin).

PERITONEAL LAVAGE

Before induction of acute pancreatitis a polyethylene cannula was inserted into the pouch of Douglas where the line was passed through a subcutaneous tunnel in the spinal region traversing the skin in the neck according to Abrahamian.[9] All animals were kept in single cages and had free access to water. The lavage was started after induction of acute pancreatitis immediately, 60 or 90 minutes later. The lavage period (360, 300, and 270 minutes) was divided into four equal time intervals. At the beginning of each 10 ml lavage solution were introduced during five minutes into the peritoneal cavity. At the end of each time interval the fluid was slowly removed (in 10 minutes) and its volume measured. With delayed onset of therapy the ascites was aspirated and measured before the instillation of the first dose of the lavage solution. The aspirates were examined for enzymic activities (amylase, lipase, partly trypsin, and elastase) as well as for the residual concentration of Glt-Ala_3-NHEt.

SURVIVAL

The survival time and rate were studied in rats with acute pancreatitis induced by retrograde injection of 0·4 ml 3·5% sodium taurocholate. In these experiments controls with acute pancreatitis also had the cannula inserted into the peritoneal cavity. The lavage solution used was free of glucose and isoosmotic (295·6 mOsmol). The lavage was started immediately after induction of acute pancreatitis with the lavage solution alone or with the addition of 4 mg/ml Na-I. Controls as well as treated animals received an intraperitoneal injection of 1 mg neomycin and 75 U bacitracin at six and 24 hours after induction of acute pancreatitis to prevent early abdominal infection. After 48 hours the rats had free access to food pellets. The survival effect was followed for 120 hours.

LABORATORY METHODS

Blood, ascites, and lavage aspirates were centrifuged at 1000 *g* for 15 minutes (+4°C) and stored at −20°C. The analyses were performed within four days.

ENZYMES

Amylase

Chromogenic starch, end point method, photometer Beckman DU 5, 620 nm-U/l.

Lipase

Olive oil, turbidimetric method, reaction rate analyzer LKB 2086, 400 nm-U/ml.

Elastase

Glt-Ala_4-pNA, kinetic method, Beckman DU 8B, 405 nm-U/l.

Trypsin

pGlu-His-Arg-pNA, kinetic method, Beckman DU 8B, 405 nm-U/l.

Glt-Ala_3-NHEt (I)

Lavage aspirates were deproteinated with 20% trichloracetic acid and centrifuged for 45 minutes at 5000 g. Trichloracetic acid concentration in the final sample amounted to 0·4%. Glt-Ala_3-NHEt was determined by high performance liquid chromatography (Perkin-Elmer) in acetonitrile gradient with 0·1% trifluoroacetic

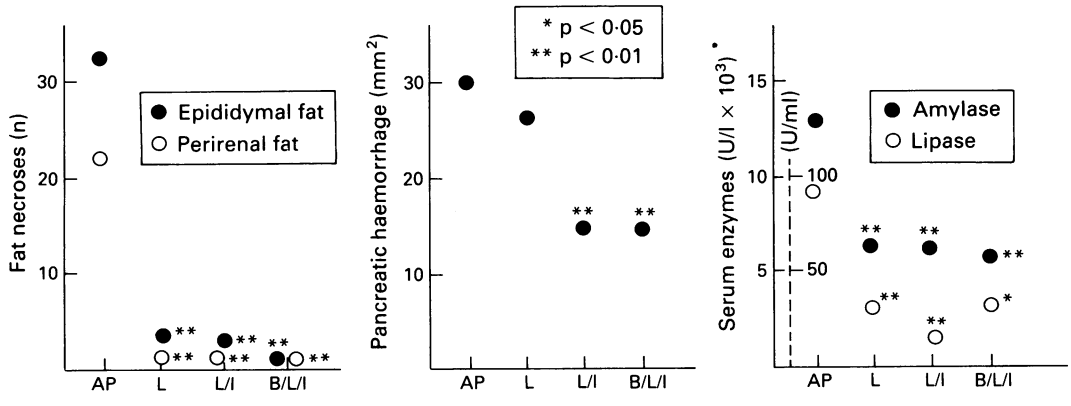

*Figure 1: Acute pancreatitis in rats (AP) (n=eight) treated immediately after induction with peritoneal lavage without (L) (n=eight) or with Glt-Ala_3-NHEt (2 mg/ml, L/I, n=eight), as well as with an additional intraperitoneal bolus injection of Glt-Ala_3-NHEt (20 mg) administered 60 minutes before induction of AP B/L/I (n=eight). Fat necroses: E (●) – in epididymal fat, R (○) – in perirenal fat. Serum enzymes: AM (●) – amylase (U/l), LIP (○) – lipase (U/ml). *p<0·05, **p<0·01.*

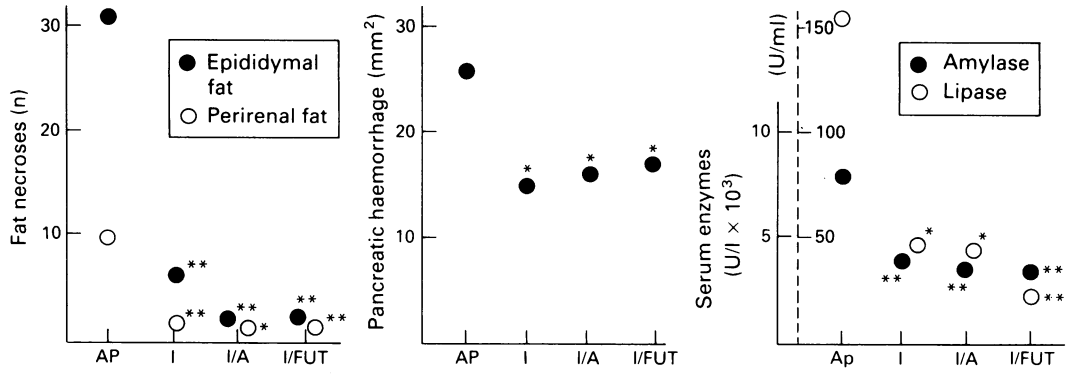

*Figure 2: Acute pancreatitis in rats (AP, n=eight) treated immediately after induction with peritoneal lavage supplemented with Glt-Ala$_3$-NHEt (2 mg/ml, I, n=eight) and its combination with aprotinin (1000 TIU/ml, I/A, n=eight) or nafamstate mesilate (0·1 mg/ml, I/FUT, n=eight). Fat necroses: E (●) – in epididymal fat, R (○) – in perirenal fat. Serum enzymes: AM (●) – amylase (U/l), LIP (○) – lipase (U/ml). *p<0·05, **p<0·01.*

acid on a glass column (Tessek) packed with LiChrosorb RP 18 or Sepharon SGX C 18. Identification and quantification was carried out with the use of software CIT-2 Data System 3600 Perkin-Elmer by integration of the absorption area and comparison with an external standard.

STATISTICAL ANALYSIS

The significance of differences between controls and lavaged groups without or with inhibitor(s) were tested with the use of Wilcoxon's non-parametric test (fat necroses) or the *t* test (other values). The differences in survival time and rate were evaluated with the use of *t* test and Smirnov-Kolmogorov test.

Results

EFFECT OF PERITONEAL LAVAGE AND GLT-ALA$_3$-NHEt

Peritoneal lavage started immediately after induction of acute pancreatitis with the lavage solution alone caused a marked decrease of fat necroses in all localizations (p<0·01) and of amylase (p<0·01) as well as lipase (p<0·01) activity in serum compared with controls. Nevertheless, the area of pancreatic haemorrhage did not change. It was reduced (p<0·01) only after the lavage solution had been supplemented with Glt-Ala$_3$-NHEt (2 mg/ml). The effect was not further increased by a prophylactic intraperitoneal bolus of Glt-Ala$_3$-NHEt injected 60 minutes before the lavage (Fig 1).

PERITONEAL GLT-ALA$_3$-NHEt LAVAGE COMBINED WITH OTHER INHIBITORS

In parallel experiments peritoneal lavage was started immediately after induction of acute pancreatitis with Glt-Ala$_3$-NHEt alone (2 mg/ml) and combined with either aprotonin (1000 U/ml) or nafamstate mesilate (0·1 mg/ml). In all experiments a similar reduction of the area of pancreatic haemorrhage (p<0·05) was seen compared with controls. The peritoneal lavage with Glt-Ala$_3$-NHEt and nafamstate mesilate was followed by a more extensive reduction of fat necroses in perirenal fat than with the combined use of Glt-Ala$_3$-NHEt and aprotonin (p<0·01 *v* 0·05). The decrease of serum lipase was also more pronounced after the lavage with Glt-Ala$_3$-NHEt and nafamstate mesilate than with the combination of Glt-Ala$_3$-NHEt and aprotonin as well as Glt-Ala$_3$-NHEt alone (p<0·01 *v* 0·05)+ (Fig 2).

TIME-AND-DOSE-RELATED EFFECT OF GLT-ALA$_3$-NHEt LAVAGE

The inhibitory effects were followed with 2, 4, and 8 mg of Glt-Ala$_3$-NHEt per millilitre of the lavage solution. The lavage was started immediately after the induction of acute pancreatitis as well as 60 or 90 minutes later. For preparation of

TABLE I *Effects of peritoneal lavage with Glt-Ala$_3$-NHEt (I) 4 mg/ml or 8 mg/ml/started 90 minutes after induction of acute pancreatitis (AP). Fat necroses, pancreatic haemorrhage, volume of ascites, serum and ascitic enzymes were estimated at the end of the experimental period (six hours after induction of AP). Enzyme activities were also determined in aspirates of the lavage solution obtained at the end of each of the four time intervals of the lavage. The significance of differences was evaluated in relation to the enzyme activities in the first aspirate. Fat necroses were determined in epididymal (E), perirenal (R), mesenteric (M) and peripancreatic (P) fat. AM-amylase, LIP=lipase, EL=elastase, TR=trypsin. NS=not significant, *p<0–05, †p<0·01*

Group	Rats (n)	Fat necroses E	R	M	P	Pancreatic haemorrhage (mm²)	Serum AM U/l	LIP U/ml	Ascites V ml	AM U/l	LIP U/ml	EL U/l	TR U/l
AP	19	23·9	4·4	1·6	1·4	18·2	10798	119·3	2·01	125214	6630	29·1	95·3
									Aspirates of lavage solution				
AP+I (4 mg/ml)	17	4·5†	0·3†	0	0·1†	13·5*	6096†	15·3†	11·4	26988	1180	3·94	7·27
									12·1	20325*	722†	1·69*	5·64
													NS
									11·7	18902*	646†	0·73†	3·94†
									10·9	14884†	609†	0·41†	2·55†
AP+I (8 mg/ml)	17	3·5†	0·1†	0	0	11·5†	6678†	20·0†	10·0	26383	991	1·38	6·33
									10·4	18287†	71NS	0·37†	4·26*
									9·8	13771†	606*	0·38†	3·02†
									12·5	13885†	567†	0·35†	2·19†

TABLE II *Reduction of pancreatic haemorrhage in relation to Glt-Ala$_3$-NHEt concentration in the lavage solution and onset of peritoneal lavage (0, +60, +90 minutes – after induction of acute pancreatitis immediately, 60 or 90 minutes later). NS=not significant, *p<0·05, †p<0·01*

Onset (min)	Glt-Ala$_3$-NHEt (mg/ml) 2	4	8
+90	NS	*	†
+60	NS	*	
0	*		

TABLE III *Recovery of Glt-Ala$_3$-NHEt (I) and its sodium salt (Na-I) from aspirates obtained during the whole lavage period*

Rats (n)	Inhibitor Lavage mg/rat	Aspirates mg/rat	%
8	60 I	10·9	18·2
10	80 I	26·6	33·3
8	160 I	51·0	31·9
4	160 Na-I	49·8	31·1
6	320 Na-I	82·6	25·8

the lavage with 4 mg/ml of Glt-Ala$_3$-NHEt both Inpankin and Na-Inpankin were used with identical results. With respect to its improved solubility, only the sodium salt was used to examine the lavage with 8 mg/ml of Glt-Ala$_3$-NHEt. The inhibitor was found effective with delayed application of up to 25% of the total experimental period. Even with this delay the lavage resulted in significant improvement of individual parameters of pancreatic inflammation, including the enzyme activities in aspirates of the lavage solution (Table I). The reduction of pancreatic haemorrhage was related both to the concentration of Inpankin in the lavage solution and the onset of therapy (Table II).

RECOVERY OF GLT-ALA$_3$-NHET AND NA-I IN ASPIRATES OF THE LAVAGE SOLUTION

The volume of the solution aspirated at the end of each time interval of the lavage was in most cases 9 to 12 ml. From the total quantity of 80–320 mg of Glt-Ala$_3$-NHEt or Na-I applied during the lavage, 26 to 33% were recovered in the aspirates. It is suggested that over this range considerable turnover (absorption and/or degradation) of the inhibitor occurs during the lavage. With 160 mg of the inhibitor the recovery was the same for Glt-Ala$_3$-NHEt as well as Na-I (Table III).

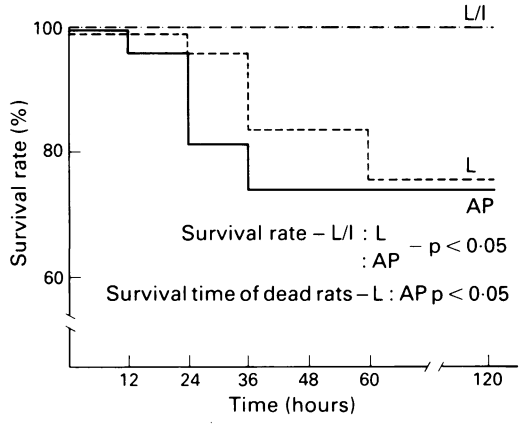

Figure 3: Survival time and rate in control rats (AP, n=27), rats treated with peritoneal lavage without an inhibitor (L, n=25), as well as in rats treated with peritoneal lavage and Na-I (4 mg/ml, L/I, n=25). Acute pancreatitis was induced with 3·5% Na-taurocholate.

MICROSCOPIC ANALYSIS

In experiments with Glt-Ala$_3$-NHEt and Na-I, as well as their combinations with other inhibitors, less advanced histologic changes of the pancreas were seen. Nevertheless, the differences in semiquantitative grading were significant (p<0·05) only for pancreatic haemorrhage in acute pancreatitis treated with Glt-Ala$_3$-NHEt or Na-I lavage delayed for 90 minutes after induction of the disease.

SURVIVAL EFFECT

Animals treated with peritoneal lavage without Na-I lived longer than controls (p<0·05), but by 60 hours the survival rate of both groups was nearly the same (76% *v* 74%). All animals lavaged with Na-I survived at 120 hours and the difference between this and both remaining groups was found significant (100% *v* 76% *v* 74%, p<0·05, Fig 3).

Discussion

Peritoneal lavage has been repeatedly examined in the management of experimental and clinical acute pancreatitis.[10–16] Experimental studies were mostly favourable, whereas clinical work yielded more conflicting results. A similar conclusion may be drawn from the application of various inhibitors of pancreatic proteases during lavage.[11 17–22] These inconsistent data are caused by the differences in aetiology as well as the time course of acute pancreatitis in experimental animals and man.[23] Nevertheless, Imrie[24] in 1985 stated, that 'addition of potentially therapeutic substances such as antiproteases to lavage fluid deserves serious consideration'.

The role of pancreatic elastase in generating vascular injury in experimental and human acute pancreatitis has been studied in detail by Geokas *et al.*[25–28] Pancreatic elastase is inhibited by high molecular natural plasma and tissue inhibitors (α_1-antitrypsin and α_2-macroglobulin), but not by aprotinin and other low molecular synthetic inhibitors recommended for the treatment of acute pancreatitis (gabexate mesilate, camostate, nafamstate mesilate). The molecular mass of natural inhibitors limits their distribution space and their availability in various compartments (peritoneal cavity, pancreatic and vascular tissue) may be impaired in acute pancreatitis.[29] From this point of view a low molecular synthetic inhibitor of pancreatic elastase may be considered a valuable therapeutic agent in acute pancreatitis.

Glt-Ala$_3$-NHEt produced a significant reduction of pancreatic haemorrhage in all experiments presented in this paper. The effect is Glt-Ala$_3$-NHEt-specific and cannot be obtained by instillation of the lavage solution only. Peritoneal lavage is superior to other routes

of Glt-Ala_3-NHEt application (24 hours intravenous infusion in unrestrained rat or intraperitoneal bolus injection),[7 30] It has been shown that the effect of Glt-Ala_3-NHEt on pancreatic haemorrhage is time and dose related and that the oligopeptide is effective even on delayed administration. The recovery study revealed that on the average 70% of Glt-Ala_3-NHEt is either absorbed from or metabolised in the peritoneal cavity during the six hour period of lavage. The favourable effect of peritoneal lavage with Glt-Ala_3-NHEt on other parameters of acute pancreatitis is considered to be mainly because of the wash out phenomenon affecting various toxic substances and other activated enzymes. On the other hand, it cannot be excluded, that if an important reaction is interrupted in the cascade of enzymic proteolysis, other steps of this process may be favourably affected as well.

The combination of Glt-Ala_3-NHEt with aprotinin and nafamstate mesilate displayed only a small beneficial effect of the latter substance. Aprotinin (molecular mass approximately 6000 D) may be absorbed from the peritoneal cavity at a lower rate than low molecular synthetic inhibitor. An additional disadvantage may be an interference between the basic aprotinin and acid Glt-Ala_3-NHEt. It would be worthwhile to combine the intraperitoneal administration of Glt-Ala_3-NHEt with the intravenous application of aprotinin. In fact, in short term experiments we observed an extensive inhibitory effect of the combined administration of both substances through these routes in the form of bolus injections.[7]

The peritoneal instillation of fresh frozen plasma containing natural inhibitors showed significantly longer survival of rats with acute pancreatitis than the instillation of aprotinin.[31] This observation indirectly supports the idea that the inhibition of pancreatic elastase by high molecular natural inhibitors, may also play a significant role in preventing the transition of acute pancreatitis into its severe haemorrhagic and necrotic form. It should be mentioned however, that the complexes of α_2-macroglobulin with elastase (similarly as with other serine proteinases) partly retain their catalytic properties.[32] This does not occur with the synthetic inhibitor Glt-Ala_3-NHEt. Its additional advantages include lack of any toxicity in its molecule, specificity, and easy absorption.

In parallel with similar studies[20 31 33] microscopic analysis displayed less consistent results than macroscopic and biochemical findings. Nevertheless, significant reduction of pancreatic haemorrhage was found with delayed application of Glt-Ala_3-NHEt-lavage.

The survival effect clearly separated animals lavaged with Glt-Ala_3-NHEt from both the group lavaged without Glt-Ala_3-NHEt and controls. Animals lavaged without Glt-Ala_3-NHEt lived longer than controls, but at approximately 60 hours after lavage the mortality was nearly the same as in controls. This finding confirms the clinical findings that the beneficial effect of peritoneal lavage alone may be only transitory at best. On the other hand, the survival rates support the opinion that the six hours peritoneal lavage supplemented with Glt-Ala_3-NHEt effectively suppresses the evolution of taurocholate induced acute pancreatitis in the rat. The results are considered favourable and further evaluation of peritoneal lavage with Glt-Ala_3-NHEt and similar inhibitors in acute pancreatitis is justified.

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