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STUDIES ON THE HEPATOTOXICITY OF GALACTOSAMINE/ENDOTOXIN OR GALACTOSAMINE/TNF IN THE PERFUSED MOUSE LIVER

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Abstract—Livers of male albino NMRI mice were perfused *in situ* at a flow rate of 3 ml/min/g liver in a non-recirculation system. The organs remained intact for at least 3 hr as assessed by release of lactate dehydrogenase (LDH) into the perfusate and by constant O_2 consumption. The infusion of the following agents did not cause significant enzyme release or microscopically visible organ injuries: galactosamine (1.8 mg/ml), endotoxin (1 μg/ml), murine recombinant tumor necrosis factor α (0.3 μg/ml/g) or combinations of them. In contrast, *in vivo* pretreatment of the animals with 700 mg/kg galactosamine + 5 μg/kg endotoxin, or 700 mg/kg galactosamine + 15 μg/kg tumor necrosis factor α (TNF) led to a massive LDH release into the perfusate. Infusion of uridine (0.67 mg/ml/g) into perfused livers from animals *in vivo* pretreated with either galactosamine/endotoxin or galactosamine/TNF, prevented LDH release and histologically visible liver injury. We conclude from these findings that *in vivo* pretreatment of the animals resulted in latent and reversible damage of the liver induced by extrahepatic factors which are prevented by intrahepatic events sensitive to uridine.

The understanding of septic shock represents a major problem in human health care which can be approached by the search for the kind and sequence of the mediators involved, starting from a primary endotoxemia to a final multi-organ failure. We studied these events in a special model, i.e. an endotoxin-induced fulminant hepatitis in mice. Based on conclusions by others [1], we recently identified leukotriene D_4 (LTD_4)‡ as a causally involved mediator of galactosamine/endotoxin hepatitis in our model [2]. We presented circumstantial evidence that this potent vasoconstrictor may lead to a transient ischemia of the hepatic microcirculation which might be followed after its release by a reflow and reoxygenation phase [3] leading to a reperfusion injury [4]. Since tumor necrosis factor (TNF) was shown to induce lethal shock in mice indistinguishable from endotoxin shock [5] we investigated whether this cytokine participates also in GalN/E hepatitis. We demonstrated directly the causal involvement of TNF_α and extended previous findings [5] by showing that blockers of the endotoxin or LTD_4 induced hepatitis such as eicosanoid synthesis inhibitors, antagonists, vasodilators or reactive oxygen scavengers were ineffective against GalN/TNF-induced hepatitis [6].

An important exception, however, exists: in all three models, administration of uridine results in a protective effect when given until 2.5 hr after either endotoxin, LTD_4, or TNF [2, 3, 6].

We now addressed the question, if and how the mediators so far identified interact directly with the liver. To this end, we chose the system of the hemoglobin- (and leukocyte)-free perfused mouse liver and focussed our attention on a possible organ injury caused by infusion of *in vivo* active mediators of hepatitis.

MATERIALS AND METHODS

Male albino mice (strain NMRI, weight 25–35 g) were raised on a stock diet (C 1018. Altromin, Lage, F.R.G.) and had free access to food and water. The mice received intraperitoneally a dose of 700 mg/kg GalN together with 5 μg/kg (*Salmonella abortus equi*) endotoxin or 15 μg/kg murine recombinant TNF-α (activity 4×10^7 units/mg) intravenously instead of endotoxin, or NaCl (0.9%) 1 hr before the perfusion [6]. The mouse was fixed on a temperature-controlled operation table (38°) under mild anaesthesia with ketamine hydrochloride/(2%) xylazine (1.6 mg/30 μl/30 g mouse). The gallbladder was incised after opening the abdomen. The mesenteric vein was cannulated by inserting a 0.8 mm i.d. Teflon catheter (Abbocath T-22 G). Before this, the lower part of the inferior vena cava was opened in order to prevent a pressure increase when starting the perfusion. The liver was left *in situ* and perfused at a flow rate of 3 ml/g liver wet weight/min by Krebs-Henseleit solution at 37° (122 mmol/l NaCl, 5.9

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‡ Abbreviations used: LT, leukotriene; TNF, tumor necrosis factor; LDH, lactate dehydrogenase (E.C.1.1.1.27); GalN, D-galactosamine; E, endotoxin.

mmol/l KCl, 1.2 mmol/l Na_2SO_4, 1.2 mmol/l $CaCl_2$, 1.18 mmol/l $MgCl_2$, 1.2 mmol/l KH_2PO_4, 18 mmol/ $NaHCO_3$, 5 mM/l glucose equilibrated with O_2/CO_2, 19:1 v/v; pH 7.4). The pressure in the portal vein was 10.0 ± 2.0 cm of water. The non-recirculating perfusate ran off the lower part of the inferior vena cava *via* an inserted 1.5 mm i.d. Teflon catheter (Abbocath T-18 G). A ligature around the higher part of the inferior vena cava was tightened. To prevent the liver from drying during perfusion it was routinely covered with a gauze pad soaked in warm effluent perfusate. This was removed at intervals for inspection of the liver. The effluent perfusate passed a Clark-type oxygen electrode with a recorder and was collected in sequential samples of 1 ml. LDH activity in the perfusate was assayed according to Ref. 7. Total LDH activity was measured by determining enzyme activity in homogenates of untreated, 10 min-perfused livers. Further components (galactosamine, endotoxin, TNF, or combinations) were infused by a separate pump in a volume less than 2% of the total perfusate. At the end of the experiment, the liver tissue was kept in 4% formalin for histological examination. Data are expressed as means ± SE.

RESULTS

When perfused *in situ* with buffer and no further additions, the system described here allowed perfusion to extend for at least 3 hr without any signs of organ injury. Release of the enzyme LDH into the perfusate was absent or negligible under these conditions (c.f. Table 1). Oxygen consumption was steady and amounted to 146 ± 36 μmol/g liver/hr (N=12). At the end of a 150 min perfusion (as shown in Figs 1 and 2) liver weight had increased by 0.05 ± 0.02 g (N=6) i.e. less than 3% of the liver weight indicating negligible swelling.

Table 1 summarizes experiments where different *in vivo* hepatotoxic agents were directly infused into mouse liver either alone or in combination at doses comparable to the ones given *in vivo*. In none of these perfusion experiments was a significant liver injury observed as assessed by enzyme release or change in oxygen consumption. These results suggest that none of these mediators acts directly as such on the liver. Table 2 shows for comparison two *ex vivo* experiments, i.e. perfusions of livers from animals which had been pretreated *in vivo* with hepatoxic doses of either endotoxin or TNF and galactosamine. When perfusion of these livers started 1 hr later, a LDH release occurred within 150 min of perfusion indicating substantial cell injury.

We therefore continued these experiments by perfusing livers of animals which had received, 1 hr before perfusion, an *in vivo* dose known to induce hepatitis.

Indeed, GalN/E as well as GalN/TNF pretreatment of these mice led to a condition where the liver is rapidly injured when later perfused (Figs 1 and 2, closed symbols). The maximum enzyme

Table 1. Lactate dehydrogenase release of perfused mouse liver within 150 min following infusion of galactosamine, endotoxin or combinations, and galactosamine plus tumor necrosis factor α

	Amount infused per ml‡	Release of LDH (units/150 min/g)	% of total§	N
Control	0	0.6 ± 0.4*†	0.3 ± 0.2*†	3
GalN	1.8 mg	4.0 ± 2.3*†	1.7 ± 0.6*†	3
Endotoxin	1 μg	1.6 ± 1.2*	0.7 ± 0.5*	3
GalN/Endotoxin	1.8 mg/1 μg	5.6 ± 5.7*	2.4 ± 2.3*	3
GalN/TNF_α	1.8 mg/0.3 μg	8.0	3.4	1

* Significance $P < 0.05$ compared to GalN/E (*in vivo*), see Table 2 (Student's *t*-test).
† Significance $P < 0.05$ compared to GalN/TNF (*in vivo*), see Table 2.
‡ Flow rate: 3 ml/min/g
§ Total LDH per gram mouse liver equal to 235 ± 18 units (N=9).
‖ Single experiment due to financial reasons.

Table 2. Lactate dehydrogenase release of perfused mouse liver following *in vivo* pretreatment of the animals with galactosamine, endotoxin, tumor necrosis factor α, or combination of these agents

	Dose†	Release of LDH (units/150 min/g)	% of total liver LDH	N
GalN	700 mg/kg i.p.	9.9 ± 9.8	4.2 ± 4.1	3
TNF	15 μg/kg i.v.	8.5 ± 7.8	3.6 ± 3.6	3
Endotoxin	5 μg/kg i.p.	3.8 ± 2.3	1.6 ± 1.0	3
GalN/Endotoxin	700 mg/kg/5 μg/kg	75.9 ± 37.8*	32.3 ± 16*	3
GalN/TNF_α	700 mg/kg/15 μg/kg	91.6 ± 40.3*	39.0 ± 14*	3

* Significance $P < 0.05$ compared to administration of single compounds.
† Time of administration: 1 hr prior to perfusion.

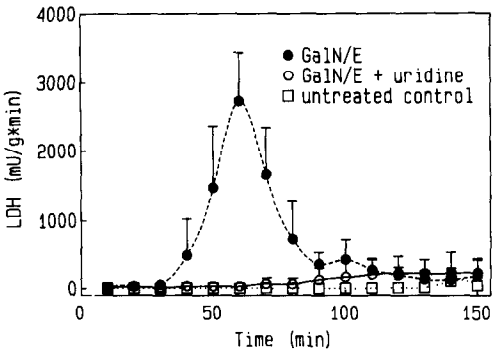

Fig. 1 Release of lactate dehydrogenase into the perfusate from the *in situ* perfused liver of mice which had been pretreated *in vivo* by galactosamine/endotoxin and protection by uridine infusion (*in vivo* doses: *cf.* Table 2; uridine infusion rate 0.67 mg/ml at a total perfusion rate of 3.0 ml/min/g liver net wt).

release upon GalN/E treatment was observed after 60 min of perfusion indicating total deterioration of the organ. The GalN/TNF pretreated livers showed a later maximum LDH activity release into the perfusate after 100 min.

Gross histological examination of the perfused livers from GalN/E or GalN/TNF pretreated animals showed no significant differences between the two groups. In both of them, diffuse large necrosis, an increase in endocytic vacuoles and general destruction of the hepatocytes was observed. In contrast, the livers of untreated animals which had been perfused with the agents compiled in Table 1 showed no detectable histological signs of injury.

Since administration of uridine protected the animals *in vivo* against GalN/E [2] or GalN/TNF-induced hepatitis [6] up to 2.5 hr after intoxication we wondered whether infusion of this agent might be able to protect the perfused organ also. The further experiments shown in Figs 1 and 2 (open symbols) demonstrate that this is the case in both types of injuries. The histological appearance of the uridine-protected, perfused liver was normal and corroborates the conclusion derived from the absence of a significant enzyme release in both cases.

These findings suggest that *in vivo* pretreatment

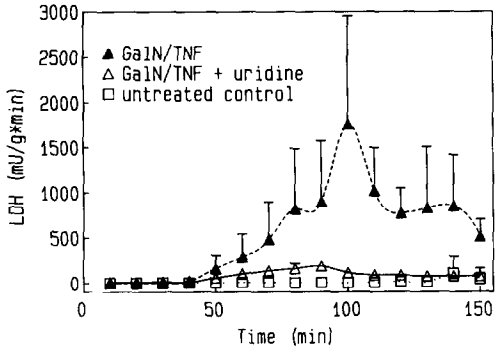

Fig. 2 Release of lactate dehydrogenase into the perfusate from the *in situ* perfused liver of mice which had been pretreated *in vivo* by galactosamine/TNF and protected by uridine infusion (details in legend to Fig. 1 and Table 2).

of mice by GalN/E or GalN/TNF induced a latent damage which further developed when the livers were perfused. This latent injury, however, seems to be fully reversible since it can be prevented later by uridine in the perfusate.

DISCUSSION

The results of this study lead to two major conclusions: the first is that the effect of endotoxin on inducing hepatitis in GalN-sensitized mice is likely to be mediated by participation of extrahepatic factors. The second conclusion implies that the pathogenic role of galactosamine and its counteraction by uridine are intrahepatic processes. The first conclusion seems not very surprising since it is known that the postulated mediators are produced in circulating i.e. non-hepatic cells: peptido-leukotrienes mainly in macrophages and TNF mainly in monocytes. The question was open, however, whether TNF, once released upon an endotoxin stimulus, may directly act upon a target cell, i.e. in our model upon a liver parenchymal cell which had been additionally challenged by galactosamine. This alternative seems clearly not to hold true. Also, addition of endotoxemic serum to the perfusate containing GalN failed to induce a direct detrimental effect on the isolated liver (not shown). On the other hand, the ensuing experiments showed that *in vivo* pretreatment of the animals with either mediator predisposed the organ for a latent endotoxic damage which is expressed when galactosamine is present. Traditionally, the toxic principle of galactosamine on hepatic metabolism is believed to consist mainly in a rapid and consistent depletion of uridine triphosphate [8] which secondarily leads to inhibition of RNA and hence protein synthesis [9]. However, also alternative explanations have been suggested such as disturbance of glycoprotein metabolism [10]. In the previous paper [2] we reasoned that the target of GalN-induced hepatic protein synthesis inhibition might consist in the inability of the organ to synthesize and secrete acute-phase proteins. Indeed, some of these proteins such as α_1-antitrypsin were protective against GalN/E-induced, however not against GalN/TNF-induced hepatitis (Niehörster, Tiegs and Wendel, 1989, unpublished). Since an open perfusion system was used in this study, an assumed protection by secreted proteins cannot account for our results. However, the possibility remains open that uridine supports a protein synthesis-dependent protective event within the hepatocytes.

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