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Tumor necrosis factor-induced lethal hepatitis: pharmacological intervention with verapamil, tannic acid, picotamide and K76COOH

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Abstract **Tumor necrosis factor (TNF) induces hepatitis when injected in human beings or in rodents. The molecular mechanism by which TNF induces hepatic distress remains largely unknown, although induction of apoptosis of hepatocytes appears to be an essential step. In order to increase the therapeutic value of TNF, we have studied the protective activity of several molecules and found that four chemically totally different substances confer significant protection in the model of TNF-induced lethal hepatitis in mice sensitized with D-(+)-galactosamine (GalN), but not in mice sensitized with actinomycin-D (ActD) or against anti-Fas-induced lethal hepatitis. Verapamil, a calcium-channel blocker, tannic acid, picotamide, a thromboxane A_2 receptor antagonist, and K76COOH, an inhibitor, amongst others, of complement, protected significantly against induction of lethality, release of the liver-specific enzyme alanine aminotransferase (ALT) and induction of apoptosis in the liver after TNF/GalN, except for K76COOH, which paradoxically increased ALT values after challenge, and which also protected against TNF/GalN in complement-deficient mice. The data suggest that activation of platelets and neutrophils, as well as induction of inflammation occur in the TNF/GalN model, but not in the TNF/ActD or anti-Fas models, in which direct induction of apoptosis of hepatocytes may be more relevant. The protective activity of the drugs may lead to an increase in therapeutic value of TNF.**

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Key words: Complement; Calcium channel; Thromboxane; Platelet; Apoptosis

1. Introduction

The powerful anti-tumor activity of tumor necrosis factor (TNF), combined with interferon-γ and melphalan, is currently used to cure metastatic melanomas and sarcomas with a very high efficiency, albeit only in a system of loco-regional administration [1–3]. Leakage of TNF (and interferon) into the general circulation leads to a life-threatening systemic inflammatory response syndrome (SIRS), characterized by a drop in blood pressure and hepatotoxicity, both of which were considered, after clinical trials with human volunteers, as dose-limiting toxicities [4]. Understanding the mechanism of induction of liver toxicity by TNF may lead to an increase in the therapeutic value of TNF, but also to a more efficient treatment of hepatic disorders in which TNF has proven to play a pivotal role, such as alcoholic hepatitis [5,6].

Several models have been used to study the mechanisms of experimental lethal hepatitis in mice: (a) TNF in combination with D-(+)-galactosamine (GalN), (b) TNF with actinomycin-D (ActD), or (c) anti-Fas. GalN is a hepatotoxin that specifically inhibits transcription and translation in hepatocytes [7]. In combination with GalN, TNF becomes lethal at low doses and causes massive apoptosis of hepatocytes [8,9]. A similar liver damage is obtained when TNF is combined with the general transcription inhibitor ActD [9,10] or when anti-Fas is administered [9,11]. In all three models, the animals succumb relatively short after the challenge, exhibiting extreme apoptosis and secondary necrosis in the liver. However, several differences were found between the three models, suggesting that the TNF/GalN model is more complex than the other two models, and involves activation, amongst others, of serine proteases and platelet-activating factor (PAF) [9,12,13]. In the current paper, we describe our efforts to further investigate the mechanism of induction of lethal hepatitis by TNF in GalN-sensitized mice. We start from the hypothesis that in the TNF/GalN model inflammation occurs prior to apoptosis induction in hepatocytes, while in the TNF/ActD and anti-Fas model direct apoptosis of hepatocytes is evoked. In order to study cells and mediators involved in the TNF/GalN model, we applied inhibitors of platelets, thromboxanes and complement. Application of several chemically different drugs confers protection and illustrates that in the GalN model many more factors are playing a role than in the ActD or anti-Fas models.

2. Materials and methods

2.1. Mice

C57BL/6 and DBA/2 female mice were purchased from Iffa-Credo (Saint Germain-sur-l'Arbresle, France). C3-deficient mice were generated by gene targeting by Dr. H.R. Colten (Washington University School of Medicine, St. Louis, MA, USA) [14]. All mice were kept in a conventional, air-conditioned animal house, in 12-h light/dark cycles, and received food and water ad libitum. Mice were used at the age of 8 weeks (20 g).

2.2. Reagents

Recombinant murine TNF was produced and purified in our laboratory. It had a specific activity of 1.3×10^8 IU/mg and contained 1.8 ng of endotoxin per mg protein, as assessed by a chromogenic substrate test (Chromogenix, Stockholm, Sweden). An agonistic hamster anti-mouse Fas monoclonal antibody was purchased from PharMingen (San Diego, CA, USA) and had an endotoxin contamination of 0.05 ng/mg protein. GalN, DMSO, ActD, verapamil and tannic acid were from Sigma Chemical Co. (St. Louis, MA, USA). Picotamide and K76COOH were kindly provided by Dr. M. Milani (Sandoz, Milano, Italy) and Dr. W. Miyazaki (Otsuka Pharmaceuticals, Osaka, Japan), respectively.

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2.3. Injections and blood collections

All reagents were diluted in low endotoxin PBS prior to injection except for picotamide, which was dissolved and injected in sterile DMSO, and K76COOH, which was dissolved in sterile 50 mM Tris–HCl pH 8. I.p. injections had a volume of 0.5 ml, except for DMSO, which was injected in a 0.1 ml volume. Blood samples were obtained by retro-orbital bleeding under light ether anesthesia.

2.4. Analysis of serum alanine aminotransferase (ALT)

Serum ALT was measured using a colorimetric assay from Sigma Chemical Co.

2.5. Evaluation of liver apoptosis

Apoptosis was quantified either in a cell death ELISA kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Basel, Switzerland) or by evaluating DNA ladder patterns after agarose gel electrophoresis. Samples were prepared as follows: mice were anesthetized and dissected; the liver was perfused for 10 s with 50 mM phosphate/120 mM NaCl/10 mM EDTA, pH 7.4 buffer. A 20% homogenate was made in perfusion buffer with a homogenizer (Heidolph-Elektro, Kelheim, Germany; model RZR 2020, position II-10) and centrifuged at $13\,000\times g$ for 20 min. Cell death ELISA was based on the principle of sandwich ELISA. Briefly, plates were coated with an anti-DNA antibody; as an Ag source the cytosolic fraction of 25 μg of liver was used. An anti-DNA peroxidase-conjugated antibody was used as a second antibody. The absorbance for apoptotic controls was regarded as 100%. The percentage of apoptosis was calculated as follows: $(A_{\text{sample}}-A_{\text{blank}})/(A_{\text{control}}-A_{\text{blank}})\times 100$. For gel electrophoresis, DNA was prepared from 500 μl of liver homogenate. The proteins were extracted with phenol/chloroform/isoamyl alcohol (50/49/1) and subsequent chloroform/isoamyl alcohol (50/50) extraction. DNA was precipitated overnight with 5 M ammonium acetate and ethanol at −20°C. DNA was dissolved in TE buffer containing RNase. DNA was run on a 1.8% agarose gel and stained with ethidium bromide.

2.6. Statistics

Mean values and S.D. were compared using an unpaired Student's *t*-test. Survival curves (Kaplan-Meier plots) were evaluated with a log rank test; final survival was determined with a chi^2 test.

3. Results

3.1. Protection against TNF/GalN-induced lethality

In C57BL/6 mice, the LD_{100} of murine TNF in combination with 20 mg GalN is reached with as little as 0.1 μg TNF per mouse [15]. To evaluate protection by chemical drugs, we routinely use a dose of 0.5 μg TNF per mouse and 20 mg GalN ($5\times LD_{100}$). In preliminary experiments, we first established the minimal dose and optimal time point needed to protect. Minimal doses were 1 mg, 10 mg, 10 mg and 7.5 mg, whereas optimal time points were −4 h, −2 h, −2 h and −3 h for verapamil, tannic acid, picotamide and K76COOH, respectively. When other doses and time points were used, protection against lethality was less clear or lacking, indicating that a tight schedule of dose and time of administration is required.

In Fig. 1A–C, survival curves are shown of mice pretreated with verapamil, tannic acid and picotamide, or solvents, and challenged with 0.5 μg TNF/20 mg GalN or 0.1 μg TNF/20 mg GalN in the case of picotamide pretreatment, where no protection was observed against 0.5 μg TNF/20 mg GalN (data not shown). These experiments illustrate that all three products conferred significant ($P=0.001$, 0.002 and 0.008 for the three drugs, respectively) protection in this model. Diltiazem and nicardipine, calcium-channel blockers like verapamil, were tested in different doses and schedules, but had no protective ability whatsoever (results not shown). To exclude an endotoxin-induced tolerance due to possible endotoxin contamination of the solutions used, the experiments were also performed in endotoxin-resistant C3H/HeJ mice, and similar results were obtained (data not shown).

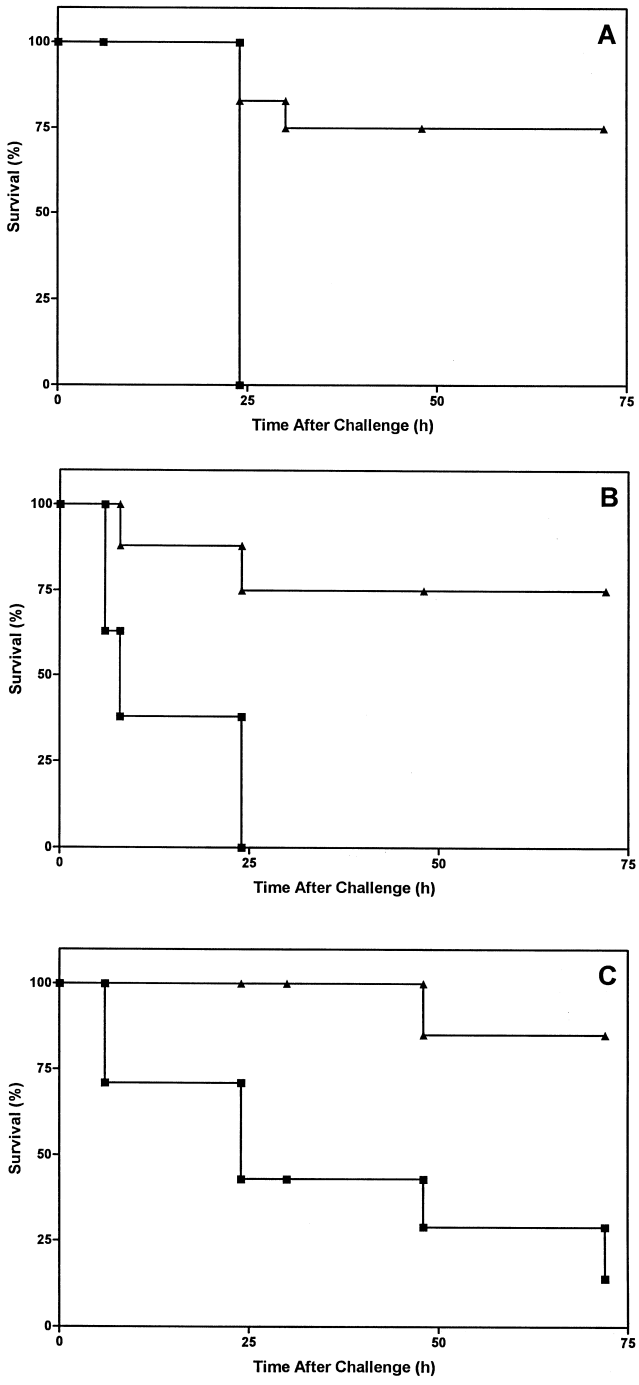

Fig. 1. Protection by verapamil (A), tannic acid (B) and picotamide (C) against a lethal challenge of TNF/GalN. Mice were pretreated with verapamil (1 mg, −4 h), tannic acid (10 mg, −2 h) or picotamide (10 mg, −2 h) i.p. and challenged with 0.5 μg TNF/GalN (verapamil, tannic acid) or 0.1 μg TNF/GalN (picotamide). Lethality was assessed over a period of 72 h (no further deaths occurred). Groups and *P* values were: (A) eight controls, 12 verapamil ($P<0.001$); (B) eight controls, eight tannic acid ($P=0.0019$); (C) seven controls, seven picotamide ($P=0.0075$). All data are pooled results of two experiments.

Fig. 2 shows the protection conferred by K76COOH, when

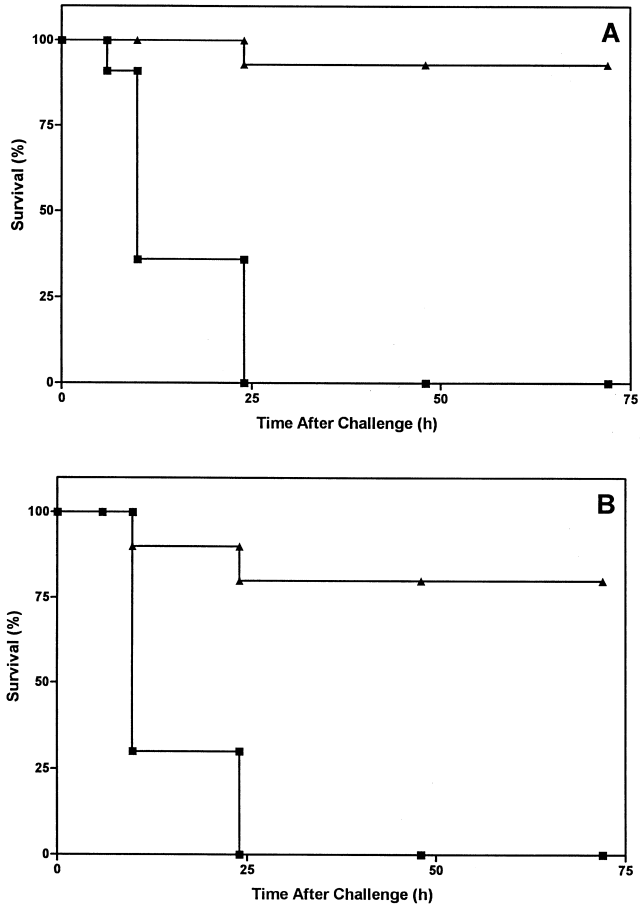

Fig. 2. Protection by K76COOH in wild-type C57BL/6 mice (A) and in $C3^{0/0}$ mice (B). Mice were pretreated with K76COOH (7.5 mg, −3 h) or PBS (0.5 ml, −3 h) and challenged with 0.5 μg TNF/GalN. Lethality was assessed over a period of 72 h (no further deaths occurred). Groups and P values were: (A) 15 controls, 14 K76COOH ($P < 0.0001$); (B) 10 controls, 10 K76COOH ($P < 0.0003$).

given 3 h prior to 0.5 μg TNF/GalN. K76COOH significantly ($P < 0.0001$) protects C57BL/6 mice (Fig. 2A) as well as complement factor C3-deficient mice ($P = 0.0003$) (Fig. 2B), which demonstrates that the target of K76COOH in this model is not the complement convertase. Similar results were obtained using C5-deficient DBA/2 mice (results not shown). None of the four drugs was able to confer any protection against LD_{100} of TNF+ActD or anti-Fas, when applied at the same doses and administration schedules as in the TNF/GalN model (Table 1).

Table 1
Lack of protective effect of verapamil, tannic acid, picotamide or K76COOH in other models of acute lethal hepatitis

Challenge[a]	Pretreatment[b]	Final lethality[c]
TNF+ActD	–	8/9
	verapamil	4/4
	tannic acid	4/4
	picotamide	4/4
	K76COOH	4/4
Anti-Fas	–	8/8
	verapamil	3/3
	tannic acid	3/3
	picotamide	5/5
	K76COOH	5/5

[a]0.1 μg TNF+20 μg ActD were injected together i.p. Anti-Fas (10 μg) was given i.v.
[b]Doses and schedules were as described in the legends to Figs. 1 and 2.
[c]Scored after 20 h in the TNF/ActD model and after 4 h in the anti-Fas model.

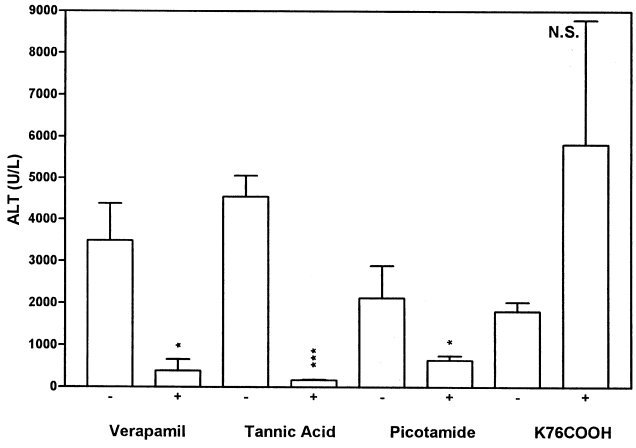

Fig. 3. Effect of verapamil, tannic acid, picotamide and K76COOH on TNF/GalN-induced serum ALT levels. Mice were pretreated with verapamil (1 mg, −4 h), tannic acid (10 mg, −2 h), picotamide (10 mg, −2 h) or K76COOH (7.5 mg, −3 h) and challenged with 0.5 μg TNF/GalN (verapamil, tannic acid, K76COOH) or 0.1 μg TNF/GalN (picotamide). Mice were bled 6 h after the challenge and ALT was measured. Group sizes and P values were: three controls, three verapamil ($P = 0.0146$); five controls, three tannic acid ($P = 0.0003$); six controls, six picotamide ($P = 0.0442$); five controls, five K76COOH ($P = 0.1085$).

3.2. Effect of the inhibitors on serum ALT

6 h after injection of mice with TNF/GalN, very high values of ALT and aspartate aminotransferase are detected. The ALT values usually reach 2000–3000 U/l, illustrating fulminant necrosis in the liver. To evaluate the effect of the drugs on ALT release, mice were pretreated with verapamil, picotamide, tannic acid and K76COOH, or solvents, and challenged with 0.5 μg TNF/20 mg GalN or 0.1 μg TNF/20 mg GalN (for verapamil-pretreated mice and controls). 6 h after the challenge, blood was withdrawn at the retro-orbital plexus for serum preparation and consequent ALT determination. As shown in Fig. 3, pretreatment of mice with a protective dose of verapamil, tannic acid or picotamide significantly reduced the serum ALT levels (Fig. 3, P values). Tannic acid was most potent in inhibiting ALT release. In contrast, K76COOH, although always significantly protecting against a lethal outcome, does not reduce, but even increases serum ALT values (Fig. 3).

3.3. Effects of the drugs on liver apoptosis

To analyze the effect of the drugs on TNF/GalN-induced apoptosis of hepatocytes, livers were excised 6 h after the challenge (from the same mice) and the amount of apoptosis was evaluated using an apoptosis-specific ELISA system. The mean value of apoptotic samples was regarded as 100%; apoptosis of samples was determined as described in Section 2. A protective dose of all four inhibitors proved to inhibit the occurrence of apoptosis in the liver. As illustrated in Fig. 4A, verapamil, tannic acid and picotamide pretreatment almost

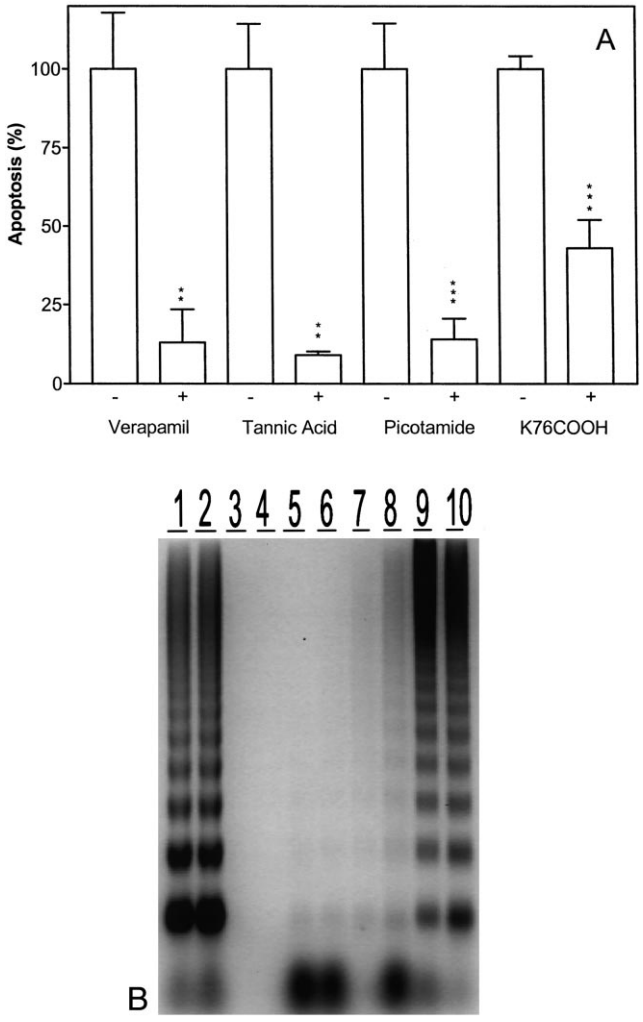

Fig. 4. Protective effect of verapamil, tannic acid, picotamide and K76COOH on TNF/GalN-induced apoptosis in the liver. Mice were pretreated with verapamil (1 mg, −4 h), tannic acid (10 mg, −2 h), picotamide (10 mg, −2 h) or K76COOH (7.5 mg, −3 h) and challenged with 0.5 μg TNF/GalN (verapamil, tannic acid, K76COOH) or 0.1 μg TNF/GalN (picotamide). Livers were excised 6 h after the challenge. A: Apoptosis was quantified using apoptosis-specific ELISA. Group sizes and P values were: three controls, three verapamil ($P = 0.0068$); five controls, three tannic acid ($P = 0.0076$); four controls, four picotamide ($P = 0.0008$); six controls, six K76COOH ($P < 0.0001$). B: Apoptosis was evaluated by agarose gel electrophoresis and staining with ethidium bromide: mice were treated with 0.5 μg TNF/GalN and pretreated with PBS (lanes 1 and 2), verapamil (lanes 3 and 4), tannic acid (lanes 5 and 6), picotamide (lanes 7 and 8) or K76COOH (lanes 9 and 10).

completely prevented apoptosis and K76COOH reduced apoptosis by 60%. These data were confirmed when apoptosis was evaluated by extracting DNA and visualization of ladder patterns (Fig. 4B).

4. Discussion

The study of the induction of liver destruction by TNF and members of the TNF receptor superfamily, such as Fas, has become exceedingly popular as it was found to be preceded by the induction of apoptosis of hepatocytes. Liver cell necrosis has proven to be one of the dose-limiting toxicities in clinical trials with TNF and was also recognized as a life-threatening complication in endotoxic shock, largely mediated by cytokines such as TNF [4,16]. Moreover, induction of liver necrosis by alcohol [5] or even by hepatitis B virus [17] was found to correlate with increased TNF levels. Identification and inhibition of the key players is therefore needed to increase the therapeutic value of TNF and to efficiently cure other diseases with liver necrosis complications.

Several model systems have been developed in mice, in which liver necrosis develops after a bolus injection of (a) TNF in combination with GalN [18], (b) TNF with ActD [8], an anti-Fas agonistic antibody [11] and, recently, (c) concanavalin A [19]. The first model has been studied in detail by several groups [15,20–22]. It was found that a number of drugs were able to inhibit liver apoptosis and secondary necrosis in this model, but not in the second and third models [9]. Amongst these inhibitors are serine protease inhibitors (B. Wielockx, unpublished data) and a PAF receptor antagonist [12], illustrating that one or more serine proteases and PAF play a pivotal role in this model, and that, in contrast to the other models, the TNF/GalN model involves induction of inflammation as well as apoptosis in the liver. Also in the TNF/GalN model, very pronounced lymphopenia and neutrophilia were observed [23], a great number of polymorphonuclear cells being attached to the portal and arterial endothelium in the liver (our unpublished results). Although we previously reported that WEB2170, a PAF receptor antagonist, protects in the TNF/GalN model [12], we were uncertain about the role of PAF and therefore continued to search for more protective drugs.

Verapamil is a well-known calcium-channel antagonist of the phenylalkylamine group, used to treat cardiovascular diseases [24]. We found that this drug conferred significant protection against TNF/GalN-induced apoptosis, transaminase release and lethality. Such protective effects were not observed using nicardipine or diltiazem, from the dihidropyridine and benzothiazepine group, respectively. Comparable differences in response between these three calcium-channel blockers were found in the inhibition of platelet aggregation, which was much more outspoken in the case of verapamil [25]. We believe that these data, but also our previous data obtained with the platelet aggregation-inhibiting α_1 acid glycoprotein [26] suggest that platelet aggregation plays an important role in this model. Activation of platelets may cause release of thromboxane A_2 (Tx-A2), which again stimulates platelets in an autocrine fashion. Activated Kupffer cells can also release Tx-A2, but depletion of these cells using gadolinium chloride had no protective effect in our models (unpublished results). Picotamide, a specific Tx-A2 antagonist [27], was found to inhibit significantly the effects of TNF/GalN. However, the protective power of picotamide was clearly inferior compared to the other drugs, since no protection was observed against a higher dose of TNF/GalN (data not shown). We believe that these data indicate that Tx-A2 may play a role in propagating the inflammatory response, but that other phenomena are of relatively greater importance. An involvement of platelet activation in the GalN model has been suggested before and was found to be a protective response using a platelet-depleting antibody [28].

The protective capacity of K76COOH was initially studied because, by comparing the sensitivity to TNF/GalN of different inbred strains of mice, we believed that activation of the complement system played an important role. K76COOH is a

quite strong inhibitor of C5 as well as C3 convertases and has proven to be effective in prolonging survival in models of interspecies xenograph transplantation, experimental immune complex glomerulonephritis and ulcerative colitis [29]. We found that K76COOH protected very well against TNF/GalN-induced apoptosis and lethality, but that ALT values are not reduced. So far, we have no explanation for this apparent contradiction, but the data seem to indicate that mice manage to survive because apoptosis is inhibited, although necrosis still occurs. By affecting the complement, K76COOH may also lead to reduced formation of reactive oxygen species (ROS), being the mechanism of protection. This is rather unlikely, since we also found that C3-deficient mice, which are still fully responsive to TNF/GalN [30], though completely devoid of complement activation [14], can still be protected by K76COOH. These data suggest that the target of K76COOH is not a complement convertase. Involvement of ROS can be further excluded, since other inhibitors of ROS formation, such as allopurinol and SOD, fail to confer any protection in the TNF/GalN model (unpublished data). Other activities of K76COOH are inhibition of histamine release from mast cells and inhibition of neutrophil activation [29]. It has been shown that neutrophils play a central role in TNF-induced hepatitis [31]. As picotamide, verapamil and K76COOH act on neutrophils, it is possible that besides platelets also neutrophils play an important role in the TNF/GalN model. Tannic acid, finally, has been shown to inhibit the induction of apoptosis in cultures of endothelial cells by a variety of agents [32]. We found that it also protects in our model of acute lethal hepatitis, but believe the mechanism is not at the level of apoptosis of hepatocytes, since we found no protection in the ActD or anti-Fas models. Indeed, none of the four inhibitors mentioned here was able to protect in the TNF/ActD or anti-Fas model. This may be ascribed to direct apoptosis of hepatocytes in these models, while in the GalN model also inflammation is induced; the latter phenomenon leads to release of a number of harmful mediators which, together with TNF, eventually cause apoptosis and secondary necrosis. A further step to investigate the involvement of inflammation in the TNF/GalN model could be by studying the activation of the proinflammatory transcription factor NF-κB and secretion of, for example, soluble ICAM-I, which will be undertaken in the near future. We believe that application of the drugs described here, alone or in combination therapy, may lead to an efficient treatment of inflammatory liver disorders and might increase the therapeutic value of TNF in the treatment of cancer.

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