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Tumor Necrosis Factor-Induced Hepatocyte Apoptosis Precedes Liver Failure in Experimental Murine Shock Models

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We investigated the role of hepatocyte apoptosis in four different murine models of acute inflammatory liver failure. Liver damage induced in D-galactosamine-sensitized mice by endotoxin injection was initiated by processes typical of apoptosis, ie, chromatin condensation, DNA fragmentation, and formation of intracellular apoptotic bodies. DNA was cleaved into oligonucleosomal fragments in the liver before a significant rise of alanine aminotransferase in plasma occurred. Passive immunization against tumor necrosis factor (TNF) completely inhibited the injury caused by endotoxin. Direct injection of recombinant TNF-α also caused DNA fragmentation followed by alanine aminotransferase release into the plasma. Pretreatment of mice with interleukin-1β, which is known to suppress TNF-induced lethality, completely prevented apoptosis and liver failure in this model. These results demonstrate the causal role of TNF in endotoxin-induced hepatic apoptosis. TNF-inducible hepatocyte apoptosis in vivo was not only observed in D-galactosamine-sensitized mice, but also when the alternative transcriptional inhibitor actinomycin D was used. In mice injected with the TNF-inducing T cell mitogen concanavalin A, hepatic apoptosis was even noticed without requirement of additional sensitizers. We conclude that TNF-induced hepatocyte apoptosis is an early, general, and possibly causal event during experimental liver failure triggered by inflammatory stimuli. (Am J Pathol 1995, 146: 1220–1234)

During the management of trauma or infectious diseases, the systemic inflammatory response syndrome and ensuing multi-organ failure are major clinical complications.[1] In humans, dysregulation and overexpression of cytokines such as tumor necrosis factor (TNF) have been found to be correlated with these pathological events.[2] In animals, the causal relationship between cytokine release and lethality in different experimental models of sepsis has been firmly established.[3–7]

For the study of the pathological mechanisms underlying these models, microbial cell surface antigens, such as the bacterial cell wall constituent lipopolysaccharide (LPS), are most commonly used to initiate the inflammatory response. As rodents are known to be more than a 1000-fold less sensitive toward LPS than humans, they are often sensitized by preceding bacterial infection or, alternatively, pretreatment with the amino sugar D-galactosamine (GalN).[8,9] In a variety of different animal models resulting in the release of the cytokine TNF (eg, endotoxin shock,[10] shock induced by plasmodial antigens,[11] or shock induced by T cell stimulatory superantigens[12–15]), a sensitization of up to 10,000-fold toward the initial stimulus by GalN has been observed.

As GalN is metabolized in rats only in the liver, [16] it causes a selective depletion of uridine nucleotides in this organ and thus leads to a hepatic transcriptional block.[17–20] High doses of the amino sugar cause a fulminant hepatitis in rats that has been claimed to resemble human viral hepatitis.[21,22] As protein synthesis inhibition alone did not sufficiently explain GalN-induced hepatic failure,[23] the hypothesis was put forward that GalN sensitized the liver toward other stimuli. Among these, intestinal endo-

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toxins[24] seemed to be likely candidates as potent pathogenic agents. The basis for this concept are data showing that GalN induced portal endotoxemia[25] and that GalN hepatitis was prevented by either colectomy,[25,26] neutralization of endotoxin with polymyxin B[27] or anti-LPS antibodies,[28] or induction of tolerance in rats to endotoxin.[25] Although GalN sensitizes mice toward the lethal effects of endotoxin more than 1000-fold, it is hardly toxic when given alone.[10] By cell transfer experiments between mouse strains of different LPS sensitivity, it was demonstrated that macrophages mediate the lethal and hepatotoxic effect of LPS in GalN-sensitized mice.[29–31] Finally, it has been demonstrated by inhibition experiments or direct injection that the macrophage-derived cytokine TNF was alone sufficient to mediate lethality and hepatic failure.[32–35]

A large body of histological and biochemical data has accumulated during the early 1970s on GalN hepatotoxicity in rats. Retrospectively, it seems that hepatic apoptosis was a key feature of this model, as chromatin condensation and apoptotic bodies (which were then called acidophilic bodies, shrinkage necrosis, or Councilman bodies[36–38]) were repeatedly described.[21,28,39–41] GalN has also been used in mice particularly as a tool for sensitization toward other inflammatory stimuli. However, the molecular mechanisms of hepatic damage and the cause of lethality have not been resolved in this model at the level of the ultimate target cell. We recently described hepatic apoptosis as a pathological mechanism elicited by TNF in actinomycin D (ActD)-sensitized murine liver cell cultures or mice.[42] Here we characterize *in vivo* the sequelae of biochemical and histological changes after co-injection of mice with GalN and LPS or its terminal mediator TNF. Our results identify apoptosis as an early pathological process during septic organ failure with a possibly general significance for commonly used models of the systemic inflammatory response syndrome.

Materials and Methods

Reagents

Recombinant TNF-α was generously provided by Dr. G. R. Adolf, Bender & Co. (Vienna, Austria), and recombinant human interleukin (IL)-1β was from Ciba Geigy (Basel, Switzerland). GalN was purchased from Roth Chemicals (Karlsruhe, Germany). LPS (*Salmonella abortus equi* endotoxin) was purchased from Sebak (Aidenbach, Germany). An immunoglobulin (Ig)G fraction of ovine anti-murine TNF-α antiserum was prepared by S. Jilg from this laboratory after immunization of a ram with recombinant murine TNF-α.

Unless otherwise specified, all other reagents were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

Animal Experiments and Sampling of Material

Specific pathogen-free male BALB/c or C57BL mice (25 g) were from the internal animal breeding house, University of Konstanz. The 55-kd TNF receptor (CD121a)-deficient mice were generously provided by Dr. W. Lesslauer, Hoffmann LaRoche (Basel, Switzerland). All animals received humane care in adherence to the National Institutes of Health guidelines as well as to legal requirements in Germany. Animal experiments were started at 8 am. ActD (800 µg/kg) or GalN (700 mg/kg) were injected intraperitoneally in saline. Concanavalin A (Con A, 20 mg/kg) was injected intravenously without sensitizer, and TNF was given intravenously 2 minutes after GalN or ActD. LPS (1 µg/kg) was given intraperitoneally together with GalN. Anti-TNF IgG was given intravenously 15 minutes before LPS, and rhuIL-1β was given intravenously 4 hours before the TNF challenge. Uracil (480 mg/kg) or uridine (1000 mg/kg) were given intraperitoneally in corn oil 30 minutes before GalN or Con A. Blood for TNF determinations was sampled from the tail vein 90 minutes after LPS administration. All other samples were obtained after lethal anesthesia of mice (150 mg/kg pentobarbital). After midline laparatomy and opening of the chest, blood was withdrawn by cardiac puncture into heparinized syringes. Livers were perfused for 10 seconds with cold perfusion buffer (50 mmol/L phosphate, 120 mmol/L NaCl, 10 mmol/L EDTA, pH 7.4) before they were excised. One lobe was frozen in liquid nitrogen for subsequent glutathione determination[43] after homogenization in 3% metaphosphoric acid. Small pieces of the large anterior lobe were immediately immersed in Carnoy's solution as a fixation for histological studies. The remaining parts of the liver were disintegrated with three strokes of an Elvehjem-type homogenizer. The 20% homogenate (in perfusion buffer) was centrifuged at 13,000 $\times$ *g* for 20 minutes. The supernatant was either further diluted 270-fold and used directly in an enzyme-linked immunoadsorption assay (ELISA) designed to detect DNA fragmentation, or DNA was precipitated from 400 µl of supernatant by addition of 1 ml of ethanol (−20 C) plus 50 µl of NaCl (5 mol/L) and stored at −20 C for further analysis on an agarose gel.

Analysis of Liver Enzymes and Released Proteins

Hepatocyte damage was assessed by measuring plasma enzymes according to Bergmeyer.[44] Tissue transglutaminase was measured essentially according to Piacentini et al[45] with the following modifications: The incubation mixture contained 15 kBq [^{14}C]-putrescine instead of 37 kBq [^{3}H]putrescine. As enzyme source, we used approximately 0.4 mg of protein from the liver 100,000 × *g* supernatant per incubation. Serum amyloid A (SAA) was determined by an ELISA (BioSource International, Camarillo, TX). TNF bioactivity was determined with WEHI164 clone 13 cells according to Espevik et al.[46] Kupffer cells for the measurement of LPS-induced TNF production were isolated by collagenase digest of livers, differential centrifugation, and plastic adherence. The Kupffer cells were stimulated as described[47] with 10 μg/ml LPS.

Determination of Protein and RNA Synthesis

At different time points after intraperitoneal injection of mice with 700 mg/kg GalN, saline, or Con A, they were intravenously injected with 300 μl of saline containing 1.5 MBq (40 nmol) [^{3}H]uridine and 0.6 MBq (70 nmol) [^{14}C]leucine. After 55 minutes, mice were anesthetized with pentobarbital (150 mg/kg). Another 5 minutes later, liver, spleen, kidney, and heart were removed after perfusion with perfusion buffer. Organs were blotted dry and weighed before a 10% homogenate in perfusion buffer was prepared. Protein contents of the homogenates were determined,[48] and aliquots containing 140 mg of protein were precipitated with 150 μl of 30% trichloroacetic acid and washed three times with 1 ml of 10% trichloroacetic acid. The resulting pellet was dissolved in lysis buffer (1 mmol/L EDTA, 0.1% Triton X-100, 0.5 mol/L NaOH). ^{3}H and ^{14}C activities (absolute values: approximately 30,000 decays per minute in control samples) were determined simultaneously in a scintillation counter on two different channels.

Histology

In each treatment group, three mice were examined after 3.5, 5, or 8 hours. Livers were fixed for histological examination with Carnoy's solution and imbedded in paraplast, and 3- to 5-μ sections were stained with hematoxylin and eosin and photographed at ×1008 magnification. For the evaluation of histological parameters, liver specimens were given randomized numbers and their surface area was quantitated with the interactive image analysis program Cue-2, Olympus (Hamburg, Germany). Apoptotic bodies (only intracellular and chromatin-containing), nuclei displaying chromatin condensation, and immigrated leukocytes were counted in an independent laboratory in a single-blind manner. Necrosis, steatosis, erythrocyte agglutination, and cytoplasmic changes were determined semiquantitatively with five scores for deterioration (none, minimal, mild, moderate, and severe).

DNA Fragmentation

DNA fragmentation was quantitated[42] by measuring cytosolic oligonucleosome-bound DNA using an ELISA kit (Boehringer, Mannheim, Germany). Briefly, the cytosolic fraction (13,000 × *g* supernatant) from approximately 75 μg of liver was employed as antigen source in a sandwich ELISA with a primary anti-histone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase. DNA fragmentation was also analyzed semiquantitatively after extraction of the 13,000 × *g* cell supernatant (corresponding to approximately 50 mg of liver) by the phenol/chloroform method, precipitation by ethanol, and electrophoresis on 1.0% agarose gels.[49]

Statistics

Unless otherwise stated, data are expressed as means ± SD of experiments carried out in triplicate. Statistical significances were determined with the unpaired Student's *t*-test if applicable or with the Welch test if variances were nonhomogeneous (*F*-test < 0.05).

Results

GalN-Induced Selective Transcriptional Block in the Liver

The usual explanation for the fact that GalN causes liver injury in rats is the assumption that this amino sugar gives rise to a selective hepatic depletion of uridine and a consequent transcriptional block in the liver. To check the organ specificity of GalN in mice, we examined its influence on protein or RNA synthesis in liver, kidney, spleen, and heart (Table 1). We observed that the transcriptional rate in murine livers was significantly inhibited 0.5 hour after the injection

Table 1. In Vivo *Transcription and Translation Rates in Different Mouse Organs at Various Times after Intraperitoneal Injection of 700 mg/kg GalN*

Organ	RNA synthesis			Protein synthesis		
	0.5 hour	2 hours	4.5 hours	0.5 hour	2 hours	4.5 hours
Liver	58 ± 4*	55 ± 1*	235 ± 19*	117 ± 16	60 ± 6*	66 ± 3*
Heart	295 ± 42*	197 ± 22*	203 ± 32*	147 ± 7*	112 ± 12	135 ± 9
Kidney	291 ± 24*	432 ± 35*	339 ± 103	147 ± 4*	87 ± 4	98 ± 23
Spleen	88 ± 12	102 ± 2	80 ± 16	79 ± 11	109 ± 3	119 ± 11

RNA synthesis was quantitated by measuring the incorporation of [^{3}H]uridine into acid-precipitable material and is expressed as percentage of untreated controls at the corresponding time points. Protein synthesis was quantitated by measuring the incorporation of [^{14}C]leucine into acid-precipitable material and is expressed as percentage of untreated controls at the corresponding time points. Data are expressed as mean ± SEM ($n = 3$).

*$P < 0.05$ *versus* untreated control.

of 700 mg/kg GalN and that this inhibition persisted for approximately 3 hours. After approximately 5 hours, however, the incorporation rate of uridine into RNA was not only restored to control rate but also showed a 2.4-fold increase compared with controls. The inhibition of transcriptional activity after GalN injection was observed only in the liver. In heart and kidney, we measured a significantly enhanced transcriptional rate, whereas the RNA synthesis in the spleen was not significantly affected by GalN treatment. Also, translational activity in the liver of mice, as measured by quantitation of leucine incorporation into proteins, was significantly and selectively attenuated after GalN treatment. In contrast to the transcriptional block induced immediately after GalN injection, there was a time lag of approximately 2 hours until protein synthesis was significantly depressed. Return of translational activity to control values was not observed until 5 hours after GalN injection, when the experiment was terminated. In the other organs examined, no attenuation of protein synthesis rates was observed. Rather, in heart and kidney a significantly increased translational activity was observed during the first hour after GalN injection. Thus, GalN attenuated both protein and RNA synthesis in murine livers but not in other major organs. As an example of the influence of GalN on proteins secreted from hepatocytes, we measured SAA, the major acute phase protein in mice. Control mice had plasma levels of 2 μg/ml SAA. Injection of the animals with 10 μg/kg LPS or TNF induced within 6 hours an increase of plasma SAA levels to 10 ± 3 or 24 ± 4 μg/ml, respectively. If pretreated with GalN, however, animals challenged with either TNF or LPS had plasma SAA levels < 1 μg/ml. This means that GalN has also systemic effects, such as alteration of the serum composition, that may be consequences of its selective inhibitory action on hepatocyte transcription and translation. In contrast to this, the time course or peak concentrations of LPS-inducible circulating TNF-α (approximately 2 ng of TNF-α per ml of plasma 90 minutes after injection of 1 μg/ml LPS) was not significantly altered by administration of 700 mg/kg GalN. Moreover, LPS (10 μg/ml)-induced *in vitro* TNF production from murine Kupffer cells (peak concentration, 25 ng/10^6 macrophages after 4 hours) was not influenced by GalN concentrations of up to 5 mmol/L. This result provides evidence that, among different hepatic cell populations, GalN affects hepatocytes selectively.

Induction of Apoptosis in LPS-Challenged Mice Pretreated with GalN

Although GalN alone in doses of up to 1 g/kg is not toxic in mice, the combined administration of 700 mg/kg of the amino sugar and low doses (1 μg/kg) of endotoxin causes severe liver failure in this species. We therefore first examined the time course of histopathological changes in the liver after injection of GalN/LPS (Figure 1). A moderate granular condensation of the cytoplasm and significant hepatocellular steatosis with characteristic large lipid droplets was visible at 3.5, 5, and 8 hours after GalN injection. No other significant histological alterations were observed. After injection of LPS alone, steatosis of a similar degree was observed that was also characterized by predominantly large lipid droplets. Besides a mild infiltration of granulocytes, no further changes were observed. After administration of GalN plus LPS, histological changes different from GalN controls were observed after 5 hours. The number of intracellular, DNA-containing apoptotic bodies was significantly raised (130 ± 15/cm^2 *versus* 17 ± 17/cm^2 in untreated controls or 10 ± 10/cm^2 after GalN alone). There was also an increased number of nuclei with chromatin condensation near the nuclear lining. These typical signs of apoptosis were accompanied by minimal to mild multifocal hepatocellular necrosis, by hepatocytes displaying karyorrhexis or nuclear pyknosis, and by a mild to moderate neutrophil infiltration. At 8 hours after GalN/LPS, livers were char-

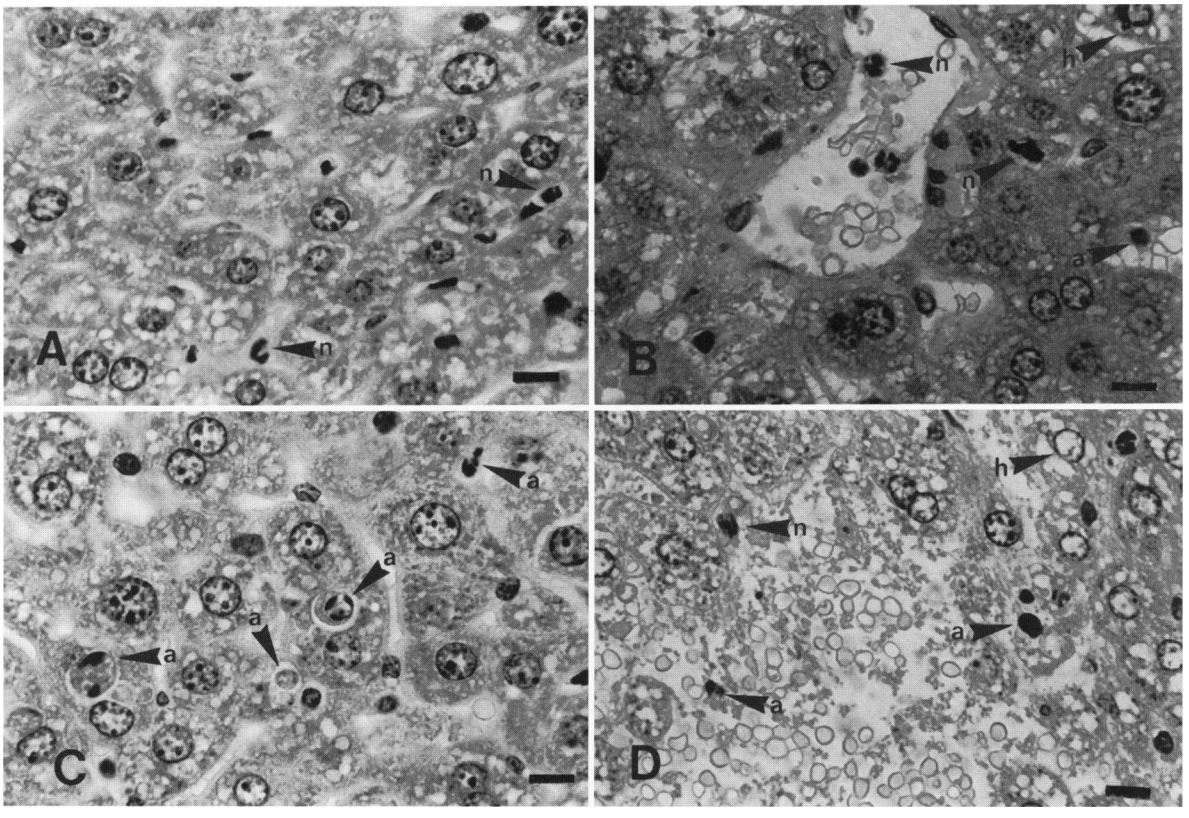

Figure 1. *Histopathology of LPS-induced liver damage in GalN-sensitized mice. Mice were treated with saline (controls) or various combinations of 700 mg/kg GalN and 1 μg/kg LPS for the time periods indicated.* **A:** *Saline, 5 hours. No histological abnormalities.* **B:** *GalN only, 5 hours. A moderate diffuse cloudy swelling indicating cellular edema and accumulation of lipid droplets are evident in the hepatocellular cytoplasm.* **C:** *GalN plus LPS, 3.5 hours. A moderate diffuse cloudy swelling accompanied by accumulation of lipid droplets is evident in the hepatocellular cytoplasm. Apoptotic bodies are not detectable at this stage.* **D:** *GalN plus LPS, 5 hours. Apoptosis manifests by the appearance of intracellular apoptotic bodies (a) and hyperchromatic nuclear membranes (h). n, infiltrating neutrophils. Bars represent 10 μ.*

acterized by severe necrosis, massive neutrophil infiltration, and erythrocyte agglutination. Large numbers of apoptotic bodies were present, but quantitation was not performed because of the widespread destruction of the liver architecture. These data demonstrate that apoptosis occurred as an early event during the development of hepatic failure. To further substantiate this statement, we quantitatively compared the time course of hepatocyte membrane rupture (as a parameter for hepatocyte death) with that of DNA fragmentation (as a marker for apoptosis; Table 2). The enormous rises of plasma transaminases and sorbitol dehydrogenase 8 hours after GalN/LPS indicate that massive tissue destruction

Table 2. *Time Course of Hepatic DNA Fragmentation and the Release of Liver Enzymes into the Plasma Induced by Different Combinations of GalN and LPS*

GalN (700 mg/kg)	LPS (1 μg/kg)	Time (hours)	ALT (U/L)	SDH (U/L)	AST (U/L)	DNA fragmentation (% of control)
+	–	3.5	262 ± 178	4 ± 4	94 ± 40	100 ± 18
+	+	3.5	232 ± 234	5 ± 2	55 ± 32	68 ± 7
–	+	3.5	45 ± 20	8 ± 3	85 ± 39	ND
+	–	5	277 ± 204	38 ± 30	79 ± 30	94 ± 19
+	+	5	164 ± 77	15 ± 11	91 ± 29	748 ± 12†
–	+	5	24 ± 8	12 ± 8	74 ± 23	162 ± 24
–	–	8	50 ± 11	11 ± 5	80 ± 17	100 ± 17
+	–	8	87 ± 38	14 ± 10	87 ± 8	105 ± 20
+	+	8	5290 ± 2290*	2890 ± 1500*	1990 ± 1030*	744 ± 51†
–	+	8	37 ± 24	14 ± 20	58 ± 9	155 ± 4

SDH, sorbitol dehydrogenase; AST, aspartate aminotransferase; –, saline injection; ND, not determined.
*$P < 0.01$ *versus* untreated control; †$P < 0.0001$ *versus* untreated control. Data are mean values ± SD from three animals.

took place within this time. The ratio of the activities of the liver-specific enzymes alanine aminotransferase (ALT) and sorbitol dehydrogenase *versus* aspartate aminotransferase argue in favor of a relatively selective hepatic damage. DNA fragmentation was already strongly augmented after 5 hours, when there was still no significant increase in plasma transaminase activities. Thus, fragmentation of chromatin clearly preceded damage to cell membranes. When we measured the activity of tissue transglutaminase (TG), an enzyme often associated with hepatic apoptosis, in liver homogenates 3.5, 5, or 8 hours after GalN/LPS, we found no significant changes compared with untreated controls (average activity, 199 ± 14 pmoles/mg × minutes). The hepatic glutathione content of 23 ± 2 nmol/mg protein was also not affected until 8 hours after challenge with GalN/LPS. This finding indicates that plasma membranes of liver cells were still largely intact 5 hours after challenge, when DNA fragmentation was already strongly increased.

Induction of Apoptosis in GalN/TNF-Challenged Mice

TNF is thought to be a terminal mediator of the septic liver failure in GalN-sensitized mice.[32,35] We therefore examined whether injection of this mediator itself into GalN-sensitized mice also caused hepatocyte apoptosis. Histological examinations (Figure 2) revealed that TNF, when given alone, caused sinusoidal infiltration of neutrophils, which was significantly different from controls as soon as 3.5 hours after injection. Apart from this, a mild to moderate cytoplasmic condensation and a moderate to severe accumulation of large lipid droplets in hepatocytes was observed. In mice treated with GalN plus TNF, apoptotic bodies and hyperchromatic nuclear membranes were occasionally observed after 3.5 hours. After 5 hours, these hallmarks of apoptosis were significantly increased (170 ± 48 apoptotic bodies/cm^2). However, we also observed a mild erythrocyte agglutination in liver

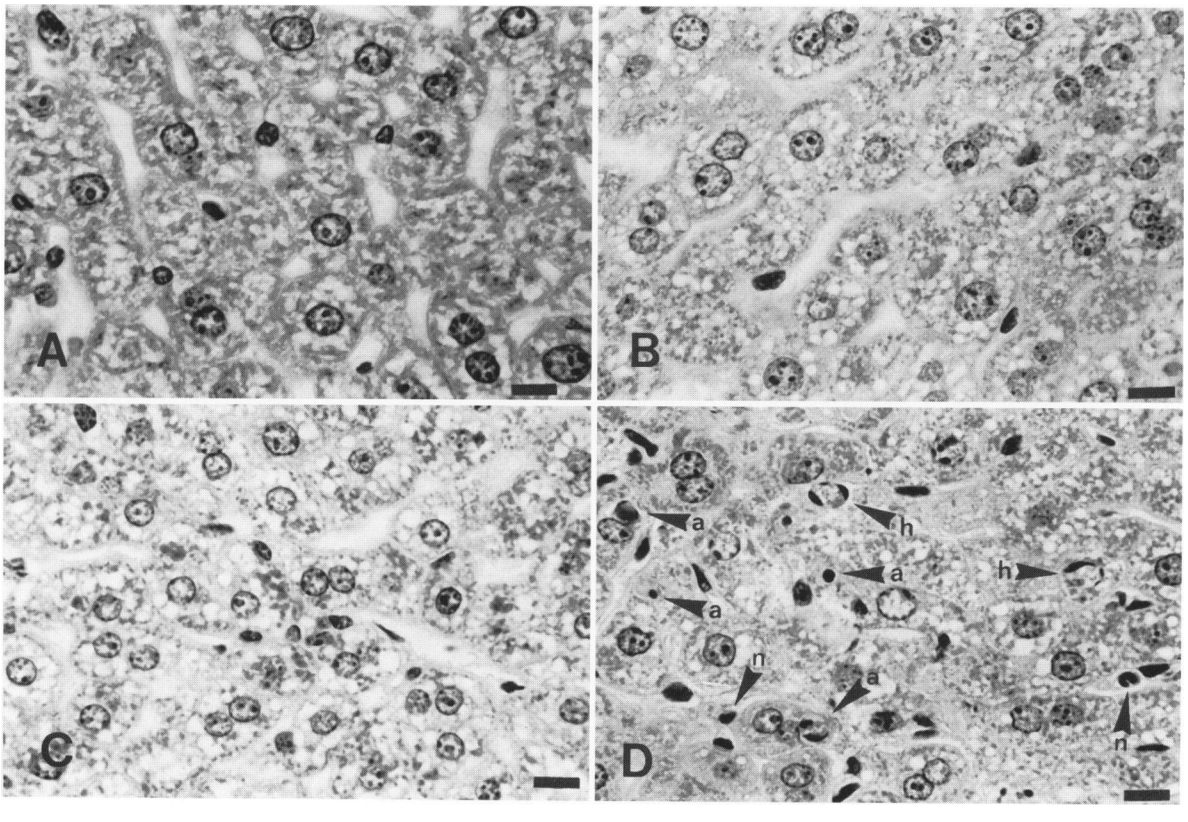

Figure 2. *Histopathology at different times after injection of TNF-α into GalN-sensitized mice. Mice were treated with various combinations of 700 mg/kg GalN and 10 μg/kg TNF-α for the time periods indicated.* A: *TNF only, 5 hours. A moderate diffuse cloudy swelling is evident in the hepatocellular cytoplasm.* B: *GalN plus TNF, 3.5 hours. Hyperchromatic nuclear membranes (h) and apoptotic bodies (a) as signs of hepatocellular apoptosis are visible.* C: *GalN plus TNF, 5 hours. Numerous apoptotic (a) bodies are visible.* D: *GalN plus TNF, 8 hours. Severe agglutination of erythrocytes accompanied by neutrophil infiltration is visible. Hyperchromatic nuclear membranes (h) and apoptotic bodies (a) are seen between necrotic hepatocytes. n, infiltrating neutrophils. Bars represent 10 μ.*

sinusoids and mild to moderate multifocal hepatocellular necrosis. After 8 hours, livers were characterized by severe necrosis, erythrocyte agglutination, and neutrophil infiltration. Apoptotic figures were found throughout the organ. Thus, the histopathology of livers challenged directly with TNF strongly resembled that observed after LPS in GalN-sensitized mice. Compared with the LPS model, the majority of changes occurred earlier in the TNF model, as would be expected from the fact that TNF must be liberated after LPS injection before it can act on hepatocytes. Analogous results were obtained when we determined the time course of biochemical markers of liver damage. As our data demonstrate (Table 3), maximal DNA fragmentation was detectable 5 hours after challenge with GalN plus LPS with little enzyme leakage. At 8 hours, however, when fulminant enzyme release documents that organ disintegration has reached a final state, DNA fragmentation was not further increased. In other words, DNA fragmentation again preceded liver enzyme release. Glutathione content or tissue TG activity remained unaltered until 8 hours after the challenge with GalN/TNF, when they dropped slightly. The ratio of plasma transaminase activities indicated a relatively selective liver failure, as was noted in the LPS model. These biochemical findings demonstrate that TNF-induced cell death is apoptotic. As it has been noticed that hepatic apoptosis is not always associated with DNA fragmentation,[50,51] we also employed the classical technique of agarose gel electrophoresis of low molecular weight DNA extracted from liver homogenates to detect oligonucleosomal DNA fragmentation. The results shown in Figure 3 demonstrate that liver extracts from GalN-sensitized mice prepared 5 hours after challenge with either TNF or LPS exhibit the DNA ladders typical of oligonucleosomal DNA fragmentation upon separation on agarose gels and thus provide additional independent evidence for hepatic DNA fragmentation (Figure 3).

Causal Involvement of TNF in LPS-Induced Apoptosis in GalN-Sensitized Mice

Because we had found that injection of the cytokine TNF induced a similar spectrum of effects as were seen after LPS injection into GalN-sensitized mice and because TNF was induced by LPS, we examined whether blockade of actions of TNF would abolish the apoptosis-inducing effect of LPS in the liver. For this purpose we injected mice with 100 μg of a neutralizing anti-TNF immunoglobulin 15 minutes before injection of GalN/LPS. Transaminase release and pathological alterations were completely prevented in anti-TNF IgG-pretreated mice challenged with GalN plus LPS, ie, values were not significantly different from those obtained from GalN controls (data not shown). DNA fragmentation was also completely suppressed (Figure 4). Thus, TNF is both a sufficient and necessary mediator of LPS for the induction of apoptosis in the liver. Moreover, the close correlation of damage and DNA fragmentation suggests that hepatocyte apoptosis is unlikely to be an epiphenomenon of LPS-induced liver damage that is coincidently also induced by TNF but that it may be causally involved in hepatocyte death.

Induction of Apoptosis in ActD/TNF-Challenged Mice

If it is true that hepatic sensitization by GalN towards endotoxic challenge is caused by the indirect inhibition of transcription via its biochemical effects on the cellular carbohydrate metabolism, then a similar sensitization should be brought about by other inhibitors of RNA synthesis. We checked this by challenging mice with a combination of TNF and an established transcriptional inhibitor that differs both structurally and mechanistically from GalN. Injection of ActD

Table 3. *Time Course of Hepatic DNA Fragmentation and the Release of ALT into the Plasma in Mice Injected with 700 mg/kg GalN and 10 μg/kg TNF*

Treatment	IL-1 pretreatment (*4 hours before TNF*)	Time (hours)	ALT (U/L)	DNA fragmentation (% of untreated control)
GalN/TNF	–	3.5	58 ± 52	83 ± 34
TNF	–	5	25 ± 7	134 ± 26
GalN/TNF	–	5	258 ± 163	840 ± 109*
GalN/TNF	+	5	35 ± 11	127 ± 13†
TNF	–	8	41 ± 8	138 ± 19
GalN/TNF	–	8	10300 ± 3050*	721 ± 51*
GalN/TNF	+	8	30 ± 20†	112 ± 23†

*$P < 0.01$ *versus* untreated controls; †$P < 0.01$ *versus* GalN/TNF-treated animals not pretreated with IL-1. Data are mean values ± SD from three animals.

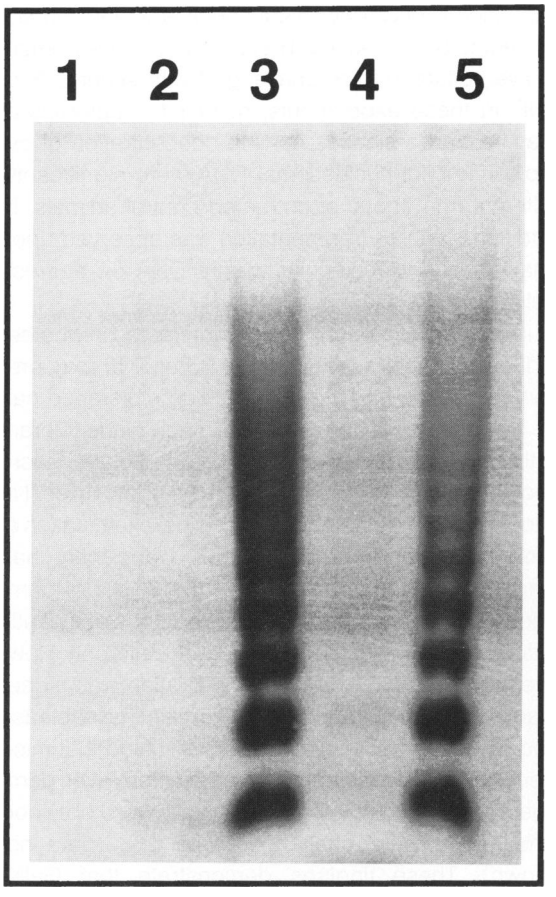

Figure 3. *Hepatic DNA fragmentation after the injection of LPS or TNF-α in GalN-sensitized mice. Mice were treated with different combinations of saline, GalN, LPS or TNF-α for 5 hours. Livers were then perfused blood-free, homogenized, and centrifuged at 13,000 × g. DNA was purified from the supernatants by precipitation with ethanol and extraction by the phenol/chloroform method and subsequently analysed on 1% agarose gels. The lanes of the gel show samples from mice treated as follows:* **1**, *700 mg/kg GalN;* **2**, *10 μg/kg TNF-α;* **3**, *GalN plus TNF;* **4**, *1 μg/kg LPS; and* **5**, *GalN plus LPS.*

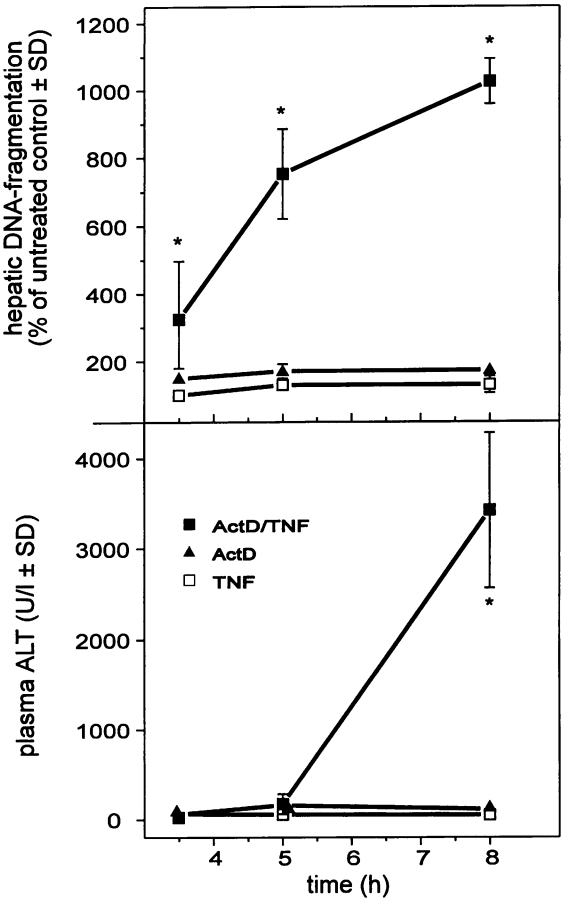

Figure 4. *Time course of hepatic DNA fragmentation and plasma ALT levels in ActD-sensitized mice injected with TNF-α. Mice were treated with saline, 800 μg/kg ActD, 3.3 μg/kg TNF, or a combination of ActD plus TNF. After the time periods indicated, animals were anesthesized. Blood for the enzymatic determination of ALT* (**A**) *was withdrawn by cardiac puncture and livers were perfused blood-free and homogenized. The 13,000 × g supernatant of this homogenate was used for the determination of DNA fragmentation* (**B**).

alone resulted in only minimal to mild steatosis and cytoplasmic condensation. In livers from animals treated with ActD plus TNF, apoptotic body formation was observed already after 3.5 hours and became pronounced after 5 hours (170 ± 76/cm^2 *versus* 27 ± 25 after ActD alone). Necrosis associated with pyknotic nuclei and karyorrhexis was minimal to moderate after 5 hours and very severe after 8 hours. As in the GalN/TNF model, in which neutrophil immigration started earlier than in the GalN/LPS model, an early onset of infiltration was seen also in the ActD/TNF model. Erythrocyte agglutination was less severe than in the GalN/TNF model. Analysis of DNA fragmentation 5 hours after challenge by agarose gel electrophoresis revealed a typical ladder pattern in livers from animals challenged with ActD plus TNF but not in those from mice that received the single compounds. These findings show that, after use of a sensitizer other than GalN, TNF induces apoptosis in the liver, provided that transcription is blocked. The time course of biochemical alterations induced by TNF in ActD-pretreated mice, such as late transaminase release in comparison with early occurrence of cytosolic oligonucleosome-bound DNA (Figure 5) again suggests that DNA fragmentation preceded cell death.

Prevention of Liver Damage and Hepatic Apoptosis by Pretreatment of Mice

We used four different intervention strategies to check the correlation between hepatic apoptosis and liver damage. As demonstrated above, passive immunization of mice with ovine anti-murine TNF antibodies

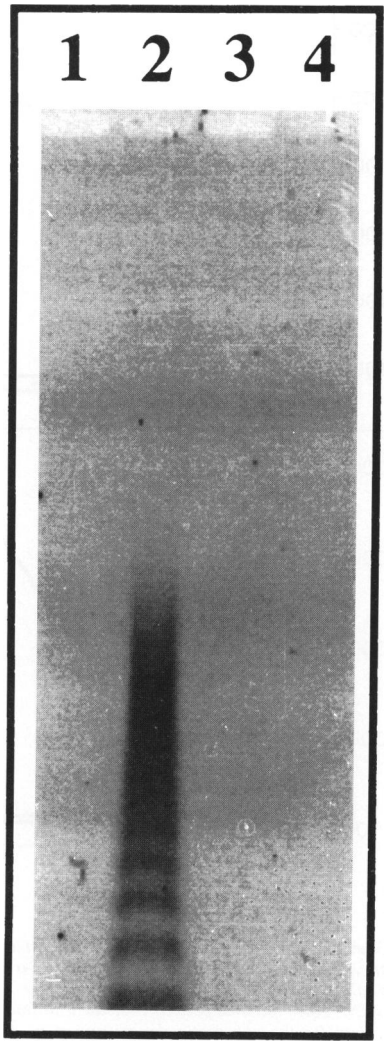

Figure 5. *Prevention by passive immunization against TNF-α of LPS-induced hepatic DNA fragmentation in GalN-sensitized mice. Mice were injected intravenously with control Ig or anti-murine TNF-α Ig. Animals were injected intraperitoneally 15 minutes later with 700 mg/kg GalN and either saline or 1 μg/kg LPS. Five hours later, livers were perfused blood-free, homogenized, and centrifuged at 13,000 × g. DNA was purified from the supernatants by precipitation with ethanol and extraction by the phenol/chloroform method and subsequently analyzed on 1% agarose gels. The lanes of the gel show samples from mice treated as follows:* **1**, *control Ig;* **2**, *control Ig and GalN plus LPS;* **3**, *anti-TNF-α Ig and GalN plus LPS; and* **4**, *anti-TNF-α Ig.*

prevented apoptosis and liver damage caused by LPS shock in GalN-sensitized mice. When the TNF response was blunted by using 55-kd TNF receptor-deficient mice, challenge of the animals with GalN plus TNF failed to induce significant transaminase release after 8 hours or to produce any morphologically detectable signs of liver damage or any mortality (data not shown).

A third approach was to overcome sensitization of GalN-pretreated mice caused by transcriptional arrest by replenishing uridine pools in these animals. Co-injection of GalN with equimolar amounts of uracil or uridine (4.1 mmol/kg) prevented the development of liver failure in mice challenged with either LPS or TNF. In these experiments, neither the activities of plasma transaminases nor the concentration of cytoplasmic oligonucleosomal DNA were significantly different from those of untreated control animals. In addition, no DNA fragmentation was observed upon analysis of low molecular weight DNA on agarose gels (data not shown).

In a fourth approach, we were interested in whether induction of tolerance against LPS or TNF may prevent apoptosis and liver failure. Such tolerance can be induced by IL-1 pretreatment. Mice made tolerant in this way and sensitized with ActD[52] or GalN[53] were shown to be protected against lethal endotoxin or TNF shock. In our experiments, histological examination of livers from mice pretreated with IL-1 and then challenged with TNF revealed that the number of damaged or apoptotic hepatocytes was not significantly different from that of mice treated with GalN only. However, the degree of neutrophil infiltration was higher than in controls. Quantitation of transaminase release and DNA fragmentation (Table 3) revealed that mice were indeed significantly protected from liver damage caused by TNF. Analogous data were obtained when mice were challenged with LPS (data not shown). These findings demonstrate that GalN-sensitized mice made resistant to apoptosis by IL-1 pretreatment were also protected from the development of hepatic failure and lethality in shock models with LPS or TNF as stimuli.

Induction of Hepatic DNA Fragmentation by Con A

Finally, we examined the relevance of DNA fragmentation in a cytokine-dependent systemic inflammatory response syndrome model with naive unsensitized mice. Intravenous injection of Con A into mice was shown to lead to cytokine release and TNF-dependent[5,54] hepatic failure.[55] First we checked whether Con A has an influence on hepatic transcription or translation. Incorporation of uridine or leucine into livers was not significantly altered until 5 hours after injection of Con A, when it declined by 40% as a result of the onset of liver damage. Notably, RNA synthesis in heart or kidney (0.5 to 5 hours after Con A injection) were increased by 50 or 300%, respectively, whereas protein synthesis was only marginally increased by 20%. Secretion of acute phase proteins by the liver was also not inhibited, as serum SAA levels reached 30 ± 2 μg/ml 6 hours after Con A injec-

tion. Thus, the mechanism of hepatic sensitization toward TNF in this model is not likely to involve transcriptional inhibition. Nevertheless, we observed oligonucleosomal DNA fragmentation and apoptotic bodies (73 ± 20/cm^2) 5 hours after Con A injection, which preceded transaminase release (Table 4). Similar results were obtained upon analysis of DNA on agarose gels. Passive immunization of mice against TNF completely inhibited this fragmentation, whereas uridine pretreatment affected neither DNA fragmentation nor ALT release (data not shown). In this model, the temporal dissociation between the first appearance of apoptosis and the final necrotic liver failure was especially striking. DNA fragmentation (Table 4) was increased 5 hours after injection of Con A, plasma transaminase activities were significantly increased after 8 hours, but widespread necrosis was observed only at later time points.[54] Thus, in this model also, apoptosis and liver failure occur in a time sequence that supports the assumption of a cause-and-effect relationship between these pathological changes.

Discussion

The first descriptions of morphological changes in the liver suggestive of apoptosis date back to the late 1960s,[21,37–41,56–58] but no attempts have been made since then to evaluate the role of this phenomenon as a mechanism of acute liver failure. Our studies show that DNA fragmentation is a salient feature of hepatic injury in various murine models of inflammatory liver failure, as is chromatin condensation that occurs in all of these models. Both phenomena are hallmarks of programmed cell death of apoptotic morphology. In murine livers, chromatin condensation and DNA fragmentation have been shown to be independently regulated.[59] Thus, the histological evaluation of chromatin condensation and the biochemical measurement of DNA fragmentation yield independent and complementary information. The latter parameter was quantitated in this study by a novel ELISA method that is two to three orders of magnitude more sensitive than analysis of DNA on agarose gels. With either analytical method, we obtained a correlation between morphological changes typical of apoptosis and of oligonucleosomal DNA fragmentation in murine liver failure. Our findings that hepatocyte apoptosis is associated with DNA fragmentation is in agreement with previous reports that cleavage of nuclear DNA into oligonucleosomal fragments can be induced in hepatocyte nuclei[60] or in isolated hepatocytes.[61] DNA fragmentation in murine livers was also observed after acetaminophen intoxication.[62] On the other hand, our findings differ from those obtained in some other animal models that use transforming growth factor-β or ocadaic acid as initiators, in which apoptosis proceeded without any significant DNA fragmentation.[50,51]

Table 4. *Time Course of Hepatic DNA Fragmentation and the Release of ALT into the Plasma in Mice Injected with 20 mg/kg Con A*

Treatment	Time (hours)	ALT (U/L)	DNA fragmentation (% of untreated control)
Con A	3.5	92 ± 3	257 ± 15*
Con A	5	93 ± 38	360 ± 29*
Con A	8	2550 ± 525*	645 ± 90*
Con A + uridine	8	2230 ± 470	620 ± 50
Saline	8	63 ± 18	100 ± 6

*$P < 0.01$ *versus* untreated controls. Data are mean values ± SD from three animals.

A different parameter often associated with hepatic apoptosis is an increased activity of tissue TG.[63,64] In our models, we never observed significant changes in the activity of this enzyme. This was not necessarily because of the sensitizing pretreatment with GalN or ActD, as TG activity also remained unchanged in the Con A model with unsensitized naive animals. Therefore, our data suggest that an increased activity of TG is not always involved in hepatic apoptosis and that it is unlikely to be a necessary condition for subsequent liver damage.

The hepatotoxicity of GalN was first characterized in the rat.[21] Subsequently the biochemical effects of this amino sugar, such as a liver-specific depression of transcription, have been examined extensively in the same species. With respect to GalN hepatotoxicity, however, there are significant differences between rats and mice, eg, in rats, GalN as such causes liver damage, whereas mice are resistant to GalN alone at doses exceeding 1 g/kg. One of the most probable explanations for this difference is the natural endotoxemia in rats, which is enhanced by GalN administration[25,65] and which is not observed in mice. Moreover, mice have a more potent capacity to resynthesize uridine.[66] This latter capacity is shared by weanling rats and partially hepatectomized rats, which are both insensitive to the hepatotoxic actions of GalN.[40] The data from our study show that GalN treatment selectively suppresses hepatic transcription and translation also in mice. The increase of these parameters in some other organs may be an indirect effect of GalN. The stronger increase in RNA synthesis compared with protein synthesis in heart and kidney may be explained by a change in the RNA pattern transcribed, which does not necessarily cause an overall increase in net protein synthesis. The depres-

sion of protein or RNA synthesis in the liver by approximately 50% may explain the sensitization toward TNF, as transcriptional arrest of a comparable extent was shown to be a condition for TNF-induced apoptosis in primary hepatocyte cultures.[42] Selectivity of protein synthesis inhibition between different liver cells is suggested by the unaltered secretion of the Kupffer cell product TNF compared with the nearly complete abolition of production of the hepatocyte-derived protein SAA after GalN treatment. These findings are in accordance with other reports on the cell type specificity of GalN.[67,68] GalN affects cells in various other ways besides depleting the uridine pool. It might also sensitize mice by altering the synthesis of glycoproteins.[17,18] From data obtained *in vitro*[42] as well as from the following findings, we conclude that the major reason why GalN sensitizes mice toward inflammatory mediators is its capability to create a transcriptional block. First, because the chemically unrelated transcriptional inhibitor ActD had effects similar to GalN and second, because replenishment of GalN-depleted uridine pools prevented TNF toxicity. It has to be stressed that inhibition of protein synthesis alone causes neither hepatotoxicity[17,23,69] nor qualitative or quantitative changes in the release of inflammatory mediators after challenge with different stimuli. In this respect, GalN is superior as a sensitizer compared with ActD that lacks target cell selectivity and may also suppress cytokine release from macrophages.

Although many different cytokines are involved in inflammatory organ failure, TNF is a dominant and terminal mediator: in GalN-sensitized mice, TNF alone was sufficient to induce hepatic apoptosis. Inhibition studies with anti-TNF IgG showed that this cytokine was also necessary for induction of hepatitis after the injection of different inflammatory stimuli. A peptide that is biologically and structurally related to TNF is the CD95 ligand.[70] Stimulation of its receptor (CD95) with an agonist antibody caused hepatic apoptosis without sensitization by GalN.[71] In our models, however, CD95 obviously does not play a significant role, as *lpr* mice, which do not express functional fas antigen (CD95),[72] did not react significantly different compared with wild-type mice, when challenged with TNF after sensitization with GalN or when challenged with Con A without any additional sensitization (data not shown).

Different kinds of intervention studies were performed to test the close association of DNA fragmentation and subsequent liver failure. Hepatic apoptosis was inhibited when either TNF action was prevented or when GalN sensitization was antagonized by uridine supplementation. These experiments show that neither TNF nor transcriptional block alone are sufficient to induce inflammatory liver failure and hepatic apoptosis and that both components are necessary for their strongly synergistic toxic action on the liver. An important feature of inflammatory reactions is the development of tolerance states under certain conditions. Tolerance involves not only actions on the level of macrophages that produce a reduced spectrum of cytokines but also more distal mechanisms, such as prevention of cytokine action on the target cell. IL-1, which is one of the most powerful tolerance-inducing agents,[52,53] protected GalN-sensitized mice completely from LPS- or TNF-induced liver damage and DNA fragmentation. Thus, in four mechanistically different experimental approaches, a consistent concordance between induction of DNA fragmentation and liver damage was observed.

Previous reports[56] as well as the data from our Con A experiments suggest that TNF-induced apoptotic liver failure is not exclusively observed after sensitization with transcriptional inhibitors and not linked solely to a transient uridine nucleotide deficiency. Starvation may predispose to organ failure as TNF release is increased[73] and the acute phase response is decreased in such a metabolic situation.[74] The most relevant condition associated with hepatic apoptosis induced by endogenous mediators, however, may be viral infection. Viruses sensitize different cell lines toward TNF toxicity.[75] Hepatocyte apoptosis was described in murine and human hepatitis,[38,57,58,76,77] and injection of viral antigens causes fulminant hepatic failure in pregnant rabbits.[78] In addition, viral infection sensitizes the liver toward LPS-dependent hepatocyte destruction,[78,79] and mice were sensitized toward TNF-dependent hepatotoxicity by the expression of viral antigen in their liver cells.[80]

In experimental shock models with the sensitizer GalN, liver damage has been suggested to play the pivotal role in lethality.[13,81] This suggestion is corroborated by the findings that death was prevented by hepatocyte transplantation[82] and also by our experience, based on several thousand mouse experiments, that ALT levels 8 hours after the challenge always correlated with lethality. Therefore, we terminated the experiments in this study after 8 hours and quantitated liver damage as an endpoint by the measurement of plasma ALT. This protocol not only improved the reproducibility of the *in vivo* experiments but also reduced animal suffering as is commonly witnessed during shock experiments extending over a period of several days.

DNA fragmentation and chromatin condensation were consistently associated with TNF-induced liver failure in any of our experiments and also in other systemic inflammatory response syndrome models with LPS, staphylococcal enterotoxin B, or anti-CD3 monoclonal antibodies as inflammatory stimuli [56,83,84] Apoptosis always preceded membrane disintegration, and prevention of apoptosis always protected against liver damage. The temporal sequence of apoptosis and subsequent necrosis and the close correlation of these two processes under different experimental conditions suggest a causal relation, but does not prove it. A direct target cell toxicity of TNF is suggested by the *in vitro* findings of TNF-induced apoptosis in liver cell cultures. Alternative explanations for TNF-induced liver failure suggest only a passive role of parenchymal cells and do not consider a direct interaction of TNF and hepatocytes. Rather, infiltrating polymorphonuclear leukocytes (PMNs) are thought to initiate liver damage.[85] This idea is challenged by reports showing that mice lacking a functional gene for intracellular adhesion molecule-1 were still sensitive to GalN/LPS-induced liver damage[83] and that inhibition of PMN immigration into the liver by turpentine pretreatment did not prevent GalN/TNF-induced organ damage.[86] Moreover, mice deficient for the 55-kd TNF receptor (CD121a) were protected against GalN/LPS-, GalN/SEB-, or GalN/TNF-induced liver failure[15,87] (this study), although PMNs mainly display the 75-kd TNF receptor (CD121b).[88] We therefore suggest the following sequence of pathogenic event. Direct TNF-induced hepatocyte apoptosis may act as a primary initiating step of organ injury. Subsequently, neutrophils may be attracted by endogenous mediators and activated by dying hepatocytes not removed swiftly enough under this pathological condition. GalN may act on this stage, in addition to its sensitization of hepatocytes, by preventing the rapid uptake of apoptotic cells by neighboring cells. The activation of PMNs may be the final process leading to necrotic organ destruction. The fact that injection of TNF without GalN into mice caused strong PMN infiltration but no significant liver damage argues in favor of the view that dying hepatocytes are required to maintain or trigger leukocyte activation. This view implies that TNF-dependent hepatic DNA fragmentation is a common pathological process in murine SIRS models and may be a general mechanism relevant to a variety of pathological syndromes. The animal model of hepatic apoptosis allows investigation of possible pharmacological interventions for septic liver failure and might also contribute mechanistic information on a widespread mechanism of liver disease.

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References

1. Bone RC, Balk RA, Cerra FB, Dellinger RB, Fein AM, Knaus WA, Schein RMH, Sibbald WJ: Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest 1992, 101:1644–1655
2. Waage A: Presence and involvement of TNF in septic shock. Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. Edited by B Beutler. New York, Raven Press, 1993, pp 275–283
3. Beutler B, Grau GE: Tumor necrosis factor in the pathogenesis of infectious diseases. Crit Care Med 1993, 21:S423–S435
4. Tracey KJ, Cerami A: Tumor necrosis factor: an updated review of its biology. Crit Care Med 1993, 21: S415–S421
5. Gantner F, Leist M, Lohse AW, Germann PG, Tiegs G: Concanavalin A-induced T cell-mediated hepatic injury in mice: the role of tumor necrosis factor. Hepatology 1995, 21:190–198
6. Vassalli P: The pathophysiology of tumor necrosis factors. Annu Rev Immunol 1992, 10:411–452
7. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ, Zentella A, Albert JD, Shires GT, Cerami A: Shock and tissue injury induced by recombinant cachectin. Science 1986, 234:470–474
8. Redl H, Bahrami S, Schlag G, Traber DL: Clinical detection of LPS and animal models of endotoxemia. Immunobiology 1993, 187:330–345
9. Wendel A: Biochemical pharmacology of inflammatory liver injury in mice. Methods Enzymol 1990, 186:675–680
10. Galanos C, Freudenberg MA, Reutter WR: Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc Natl Acad Sci USA 1979, 76:5939–5943
11. Bate CA, Taverne J, Playfair JH: Soluble malarial antigens are toxic and induce the production of tumor necrosis factor *in vivo*. Immunology 1989, 66:600–605

12. Miethke T, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H: T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. J Exp Med 1992, 175:91–98
13. Nagaki M, Muto Y, Ohnishi H, Yasuda S, Sano K, Naito T, Maeda T, Yamada T, Moriwaki H: Hepatic injury and lethal shock in galactosamine-sensitized mice induced by the superantigen staphylococcal enterotoxin B. Gastroenterology 1994, 106:450–458
14. Miethke T, Duschek K, Wahl C, Heeg K, Wagner H: Pathogenesis of the toxic shock syndrome: T cell mediated lethal shock caused by the superantigen TSST-1. Eur J Immunol 1993, 23:1494–1500
15. Pfeffer K, Matsuyama T, Kündig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Krönke M, Mak TW: Mice deficient for the 55kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. Cell 1993 73:457–467
16. Decker K, Keppler D: Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. Rev Physiol Biochem Pharmacol 1974, 71:77–100
17. Anukarahanonta T, Shinozuka H, Farber E: Inhibition of protein synthesis in rat liver by D-galactosamine. Res Commun Chem Pathol Pharmacol 1973, 5:481–491
18. Shinozuka H, Martin JT, Farber JL: The induction of fibrillar nucleoli in rat liver cells by D-galactosamine and their subsequent re-formation into normal nucleoli. J Ultrastructure Res 1973, 44:279–292
19. Shinozuka H, Farber JL, Konishi Y, Anukarahanota T: D-galactosamine and acute liver cell injury. Fed Proc 1973, 32:1516–1526
20. Konishi Y, Shinozuka H, Farber JL: The inhibition of rat liver nuclear ribonucleic acid synthesis by galactosamine and its reversal by uridine. Lab Invest 1974, 30:751–756
21. Keppler D, Lesch R, Reutter W, Decker K: Experimental hepatitis induced by D-galactosamine. Exp Mol Pathol 1968, 9:279–290
22. El-Mofty S, Scrutton MC, Serroni A, Nicolini C, Farber JL: Early, reversible plasma membrane injury in galactosamine-induced liver cell death. Am J Pathol 1975, 79:579–596
23. Farber E: Response of cells to inhibition of synthesis of DNA, RNA and protein. Biochem Pharmacol 1971, 20:1023–1026
24. Nolan JP: Intestinal endotoxins as mediators of hepatic injury: an idea whose time has come again. Hepatology 1989, 10:887–891
25. Grün M, Liehr H, Rasenack U: Significance of endotoxemia in experimental "galactosamine-hepatitis" in the rat. Acta Hepato-Gastroenterol 1976, 23:64–81
26. Iwaki Y, Noguchi K, Tanikawa K, Tsuda K: Role of endotoxin in the development of galactosamine-induced hepatic injury. Cells of the Hepatic Sinusoid. Edited by E Wisse, DL Knook, K Decker. Rijswijk, The Netherlands, Kupffer Cell Foundation, 1989, pp 351–352
27. Mihas AA, Ceballos R, Mihas TA, Hirshowitz BI: Modification of the hepatotoxicity of D-galactosamine in the rat by an anti-endotoxin. J Med 1990, 21:301–311
28. Czaja MJ, Xu J, Ju Y, Alt E, Schmiedeberg P: Lipopolysaccharide-neutralizing antibody reduces hepatocyte injury from acute hepatotoxin administration. Hepatology 1994, 19:1282–1298
29. Shiratori Y, Tanaka M, Hai K, Kawase T, Shiina S, Sugimoto T: Role of endotoxin-responsive macrophages in hepatic injury. Hepatology 1990, 11:183–192
30. Chojkier M, Fierer J: D-galactosamine hepatotoxicity is associated with endotoxin sensitivity and mediated by lymphoreticular cells in mice. Gastroenterology 1985, 88:115–121
31. Freudenberg MA, Keppler D, Galanos C: Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect Immun 1986, 51:891–895
32. Lehmann V, Freudenberg MA, Galanos C: Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. J Exp Med 1987, 165:657–663
33. Wendel A, Tiegs G: Pharmacological intervention studies against lipopolysaccharides or TNF-α toxicity in mice. Tumor Necrosis Factor: Molecular and Cellular Biology and Clinical Relevance. Edited by W Fiers, WA Buurman. Basel, Karger, 1993, pp 120–125
34. Tiegs G, Niehörster M, Wendel A: Leukocyte alterations do not account for hepatitis induced by endotoxin or TNF-α in galactosamine-sensitized mice. Biochem Pharmacol 1990, 40:1317–1322
35. Tiegs G, Wolter M, Wendel A: Tumor necrosis factor is a terminal mediator in D-galactosamine/endotoxin-induced hepatitis in mice. Biochem Pharmacol 1989, 38:627–631
36. Kerr JF, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–247
37. Kerr JFR: Shrinkage necrosis: a distinct mode of cellular death. J Pathol 1971, 105:13–22
38. Klion FM, Schaffner F: The ultrastructure of acidophilic "councilman-like" bodies in the liver. Am J Pathol 1966, 48:755–765
39. Medline A, Schaffner F, Popper H: Ultrastructural features in galactosamine-induced hepatitis. Exp Mol Pathol 1970, 12:201–212
40. Reutter W, Bauer CH, Lesch R: On the mechanism of action of galactosamine: different response to D-galactosamine of rat liver during development. Naturwissenschaften 1970, 57:674–675
41. Reutter W, Lesch R, Keppler D, Decker K: Galactosamine-hepatitis. Naturwissenschaften 1968, 55:497
42. Leist M, Gantner F, Bohlinger I, Germann PG, Tiegs G, Wendel A: Murine hepatocyte apoptosis induced *in*

vitro and *in vivo* by TNF-α requires transcriptional arrest. J Immunol 1994, 153:1778–1787
43. Tietze F: Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues. Anal Biochem 1969, 27:502–522
44. Bergmeyer HU: Methods of Enzymatic Analysis, ed. 3. Weinheim, Germany, Verlag Chemie, 1984
45. Piacentini M, Sartori C, Beninati S, Bargagli AM, Argento-Cerù MP: Ornithine decarboxylase, transglutaminase, diamine oxidase and total diamines and polyamines in maternal liver and kidney throughout rat pregnancy. Biochem J 1986, 234:435–440
46. Espevik T, Nissen-Meyer J: A highly sensitive cell line WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods 1986, 95:99–105
47. Hartung T, Wendel A: Endotoxin-inducible cytotoxicity in liver cell cultures. I. Biochem Pharmacol 1991, 42: 1129–1135
48. Bradford, MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72:248–254
49. Wyllie, AH: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980, 284:555–556
50. Oberhammer F, Bursch W, Tiefenbacher R, Fröschl G, Pavelka M, Purchio T, Schulte-Hermann R: Apoptosis is induced by transforming growth factor-β1 within 5 hours in regressing liver without significant fragmentation of the DNA. Hepatology 1993, 18:1238–1246
51. Böe R, Gjertsen T, Vintermyr OK, Houge G, Lanotte M, Döskeland SO: The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp Cell Res 1991, 195:237–246
52. Wallach D, Holtmann H, Engelmann H, Nophar Y: Sensitization and desensitization to lethal effects of tumor necrosis factor and IL-1. J Immunol 1988, 140:2994–2999
53. Libert C, Van Bladel S, Brouckaert P, Shaw A, Fiers W: Involvement of the liver, but not of IL-6, in IL-1-induced desensitization to the lethal effects of tumor necrosis factor. J Immunol 1991, 146:2625–2632
54. Mizuhara H, O'Neill E, Seki N, Ogawa T, Kusunoki C, Otsuka K, Satoh S, Niwa M, Senoh H, Fujiwara H: T cell activation-associated hepatic injury: mediation by tumor necrosis factor and protection by interleukin 6. J Exp Med 1994, 179:1529–1537
55. Tiegs G, Hentschel J, Wendel A: A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J Clin Invest 1992, 90:196–203
56. Levy E, Slusser RJ, Ruebner BH: Hepatic changes produced by a single dose of endotoxin in the mouse. Am J Pathol 1968, 52:477–502
57. Svoboda D, Nielson A, Werder A, Higginson J: An electron microscopic study of viral hepatitis in mice. Am J Pathol 1962, 41:205–224
58. Child P, Ruiz LA: Acidophilic bodies: their chemical and physical nature in patients with Bolivian hemorrhagic fever. Arch Pathol 1968, 85:45–50
59. Sun DY, Jiang S, Zheng L, Ojcius DM, Young JD: Separate metabolic pathways leading to DNA fragmentation and apoptotic chromatin condensation. J Exp Med 1994, 179:559–568
60. Jones DP, McConkey DJ, Nicotera P, Orrenius S: Calcium-activated DNA fragmentation in rat liver nuclei. J Biol Chem 1989, 264:6398–6403
61. Williamson R: Properties of rapidly labelled deoxyribonucleic acid fragments isolated from the cytoplasm of primary cultures of embryonic mouse liver cells. J Mol Biol 1970, 51:157–168
62. Ray SD, Kamendulis LM, Gurule MW, Yorkin RD, Corcoran GB: Ca^{2+} antagonists inhibit DNA fragmentation and toxic cell death induced by acetaminophen. FASEB J 1993, 7:453–463
63. Fesus L, Thomazy V, Falus A: Induction and activation of tissue transglutaminase during programmed cell death. FEBS Lett 1987, 224:104–108
64. Piacentini M, Autuori F, Dini L, Farrace MG, Ghibelli L, Piredda L, Fesus L: "Tissue" transglutaminase is specifically expressed in neonatal rat liver cells undergoing apoptosis upon epidermal growth factor-stimulation. Cell Tissue Res 1991, 263:227–235
65. Liehr H, Grün M, Seelig H, Seelig R, Reutter W, Heine W: On the pathogenesis of galactosamine hepatitis. Virchows Arch B 1978, 26:331–334
66. Pausch J, Keppler D, Decker K: Activity and distribution of the enzymes of uridylate synthesis from orotate in animal tissues. Biochim Biophys Acta 1972, 258: 395–403
67. Mandl J, Mészàros K, Antoni F, Spolaries Z, Garzó T: Reversible inhibition of RNA synthesis and irreversible inhibition of protein synthesis by D-galactosamine in isolated mouse hepatocytes. Mol Cell Biochem 1982, 46:25–30
68. Alcorn JM, Fierer J, Chojkier M: The acute-phase response protects mice from D-galactosamine sensitization to endotoxin and tumor necrosis factor-α. Hepatology 1992, 15:122–129
69. Hwang KM, Yang LC, Carrico CK, Schulz RA, Schenkman JB, Sartorelli AC: Production of membrane whorls in rat liver by some inhibitors of protein synthesis. J Cell Biol 1974, 62:20–31
70. Suda T, Takahashi T, Golstein P, Nagata S: Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993, 75:1169–1178
71. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S: Lethal effect of the anti-fas antibody in mice. Nature 1993, 364:806–809
72. Adachi M, Watanabe-Fukunaga R, Nagata S: Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene

of *lpr* mice. Proc Natl Acad Sci USA 1993, 90:1756–1760
73. Vaisman N, Schattner A, Hahn T: Tumor necrosis factor production during starvation. Am J Med 1989, 87: 115
74. Jennings G, Bourgeois C, Elia M: The magnitude of the acute phase response is attenuated by protein deficiency in rats. J Nutr 1992, 122:1325–1331
75. Wong GHW, Kamb A, Goeddel DV: Antiviral properties of TNF. Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. Edited by B Beutler. New York, Raven Press, 1992, pp 371–381
76. Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, Fusamoto H, Kamada T: Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. Hepatology 1994, 19:1354–1359
77. Kerr JFR, Searle J, Halliday WJ, Roberts I, Cooksley WGE, Halliday JW, Holder L, Burnett W, Powell LW: The nature of piecemeal necrosis in chronic active hepatitis. Lancet 1979, 2:827–828
78. Mori W, Aoki N, Shiga J: Acute hepatic cell necrosis experimentally produced by viral agents in rabbits. Am J Pathol 1981, 103:31–38
79. Gut J, Schmitt S, Bingen A, Anton M, Kirn A: Probable role of endogenous endotoxins in hepatocytolysis during murine hepatitis caused by frog virus 3. J Infect Dis 1984, 149:621–629
80. Gilles PN, Guerrette DL, Ulevitch RJ, Schreiber RD, Chisari FV: HBsAg retention sensitizes the hepatocyte to injury by physiological concentrations of interferon-γ. Hepatology 1992, 16:655–663
81. Chang HR, Vesin C, Grau GE, Pointaire P, Arsenijevic D, Strath M, Pechère JC, Piguet PF: Respective role of polymorphonuclear leukocytes and their integrins (CD-11/18) in the local or systemic toxicity of lipopolysaccharide. J Leukocyte Biol 1993, 53:636–639
82. Hirai S, Kasai S, Mito M: Encapsulated hepatocyte transplantation for the treatment of D-galactosamine-induced acute hepatic failure in rats. Eur Surg Res 1993, 25:193–202
83. Xu H, Gonzalo JA, St Pierre Y, Williams IR, Kupper TS, Cotran RS, Springer TA, Gutierrez-Ramos J: Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. J Exp Med 1994, 180:95–109
84. Gantner F, Leist M, Jilg S, Germann PG, Freudenberg MA, Tiegs G: Tumor necrosis factor-induced hepatic DNA-fragmentation as an early marker of T cell-dependent liver injury in mice. Gastroenterology 1995, in press
85. Jaeschke H, Farhood A, Smith C: Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. Am J Physiol 1991, 261: G1051–G1056
86. Schlayer HJ, Laaf H, Peters T, Schaeffer HE, Decker K: Tumor necrosis factor (TNF) mediates lipopolysaccharide (LPS)-elicited neutrophil sticking to sinusoidal endothelial cells *in vivo*. Cells of the Hepatic Sinusoid, vol 2. Edited by E Wisse, DL Knook, K Decker. Rijswijk, The Netherlands, The Kupffer Cell Foundation, 1989, pp 319–324
87. Rothe J, Lesslauer W, Lötscher H, Lang Y, Koebel P, Köntgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H: Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. Nature 1993, 364:798–800
88. Abe Y, Gatanaga M, Osuka Y, Kimura S, Burger RA, Granger GA, Gatanaga T: Role of 55- and 75-kDa tumor necrosis factor membrane receptors in the regulation of intercellular adhesion molecule-1 expression by HL-60 human promyelocytic leukemia cells *in vitro*. J Immunol 1993, 150:5070–5079