Tumor Necrosis Factor–Induced Apoptosis During the Poisoning of Mice With Hepatotoxins

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Background & Aims: **Treatment with tumor necrosis factor (TNF) induces murine hepatocyte apoptosis in vitro and in vivo when sensitizing concentrations of toxins are present. The aim of this study was to investigate whether endogenously formed TNF contributes to liver failure caused by hepatotoxins.** ***Methods:*** **The extent of liver damage, induced by α-amanitin or actinomycin D (ActD), was examined under various experimental conditions, preventing the action of TNF on hepatocytes.** ***Results:*** **TNF induced apoptosis of murine hepatocytes or human hepatoma cells in the presence of α-amanitin or ActD. TNF and α-amanitin induced such hepatotoxicity also in vivo in a synergistic way. After in vivo administration of high doses of ActD or α-amanitin alone, hepatic TNF-messenger RNA was increased and hepatocytes underwent apoptosis. A neutralizing antiserum against TNF-α prevented the liver injury. Hepatotoxicity of ActD or α-amanitin also was prevented by pretreatment of mice with low doses of the tolerizing cytokine interleukin 1. Mice deficient for the 55-kilodalton TNF receptor were protected from ActD- or α-amanitin–induced toxicity. Endotoxin-unresponsive C3H/HeJ mice also had liver failure after ActD treatment, and this damage was prevented by treatment with anti-TNF antiserum.** ***Conclusions:*** **Hepatotoxins such as α-amanitin may induce liver failure by an indirect mechanism involving sensitization of parenchymal cells toward endogenously produced TNF.**

Apoptosis and necrosis are two fundamentally different modes of cell death.[1,2] During adaptive processes to altered metabolic or physiological situations that are not compatible with the survival of all cells within a tissue, apoptosis is the default demise.[3] It is believed that necrosis follows when the insult to a tissue is so severe that the coordinated process of apoptotic death cannot be activated or the removal capacity for apoptotic cells is exhausted.[4,5] This notion is supported by the fact that apoptosis and necrosis may be induced by one and the same agent depending on the intensity of insult and that they may coexist or follow one another during pathological situations.[4,6–10] Often, the contribution of apoptosis to tissue injury may be underestimated or even overlooked because of efficient phagocytosis of apoptotic cells.[2,11]

Traditionally, acute liver toxicity caused by xenobiotics has been described as predominantly necrotic as a consequence of a direct interaction between the toxin or its metabolites and the hepatocyte.[12] Several lines of evidence suggest that hepatotoxicity may be the result of primary apoptosis. For example, hepatotoxins such as paracetamol, cocaine, or nitrosamine have been shown to induce oligonucleosomal DNA fragmentation in vivo,[13–15] and the formation of apoptotic bodies has been observed after treatment of animals with cocaine,[14] ethanol,[16] dimethylnitrosamine[17] D-galactosamine (GalN),[18] lead,[19] or thioacetamide.[9] Also, overstimulation of immunocompetent cells may play a relevant role in hepatotoxicity. Thus, liver nonparenchymal cells or the mediators they release have been shown to modulate the hepatotoxicity of GalN, diethyldithiocarbamate, phalloidin, acetaminophen, CCl_4, and ethanol. In these cases toxicity was reduced or even prevented by the inactivation of liver macrophages or their secretory products.[20–28] These findings suggest that highly regulated, receptor-operated processes are part of the hepatotoxic mechanism of classical xenobiotics.

The liver itself harbors the largest pool of macrophages in the body, and it may indeed release large amounts of tumor necrosis factor (TNF) after stimulation with lipopolysaccharide (LPS).[29,30] This anatomical situation allows xenobiotics and hepatic cytokines (or LPS) to in-

Abbreviations used in this paper: ActD, actinomycin D; AU, arbitrary unit; ELISA, enzyme-linked immunosorbent assay; GalN, dimethylnitrosamine D-galactosamine; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PCR, polymerase chain reaction; SDH, sorbitol dehydrogenase; TNF, tumor necrosis factor.

teract synergistically in eliciting hepatic toxicity. In fact, lead, ethanol, CCl_4, α-amanitin, actinomycin D (ActD), or GalN cause hepatotoxicity in synergy with endogenous or exogenous LPS.[20,31–35] Direct administration of exogenous TNF together with subtoxic doses of GalN or ActD causes hepatotoxicity via activation of the 55-kilodalton TNF receptor.[36] This toxicity is characterized by a sequential manifestation of apoptosis and necrosis and specifically involves TNF-dependent signal transduction, because a similar synergism was not observed after CD95 stimulation.[4]

For these reasons, we examined whether endogenous TNF may be involved in liver damage induced by putatively "direct" hepatotoxins. We chose two model compounds that cause a transcriptional block within the target cell as well-described mechanism of action. ActD is a toxin known to interact with exogenous TNF, whereas α-amanitin was chosen because of its history in human poisoning as a mushroom component that induces severe hepatic failure after ingestion.[37–41] In addition, we considered hepatocyte apoptosis after in vivo administration of the pure toxins.

Materials and Methods

Materials

Tissue culture material was obtained from Nunc (Wiesbaden, Germany), media and supplements were obtained from GIBCO (Eggenstein, Germany), and serum for cell culture was purchased from Biochrom (Berlin, Germany). Recombinant TNF-α was provided generously by Dr. G. R. Adolf, Bender & Co. (Vienna, Austria), and rhuIL-1β was obtained from Ciba-Geigy (Basel, Switzerland). *Salmonella abortus equi* endotoxin (LPS) was purchased from Sebak (Aidenbach, Germany). n × 123–base pair molecular weight marker was obtained from GIBCO. An immunoglobulin (Ig) G fraction of ovine antimurine TNF-α antiserum was prepared as described.[36] Unless further specified, all other reagents, e.g., α-amanitin (endotoxin content ≤2 ng/mg), were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

Animals

Specific pathogen–free male BALB/c or C57BL mice (25 g) were obtained from the internal animal breeding house, University of Konstanz, Germany. A breeding stock of 55-kilodalton TNF receptor (CD121a)–deficient mice[42] and the corresponding wild-type mice (C57B1 × 129) was provided by F. Hoffmann LaRoche (Basel, Switzerland), and 75-kilodalton TNF receptor (CD121b)–deficient mice were obtained by permission of Dr. M. Moore (Genentech, South San Francisco, CA). Male C3H/HeJ (LPS-resistant) and C3H/HeN (LPS-sensitive) mice were purchased from Harlan (Austerlitz, The Netherlands). Animals were housed at a constant day–night cycle of 12 hours at 22°C and 55% humidity. They were tasted overnight before the in vivo experiments and were allowed free access to food and water after the toxin injection. Generally, experiments were started at 8 AM. All animals received humane care in adherence to the National Institutes of Health guidelines as well as to the legal requirements in Germany.

Protein and RNA Synthesis

For the determination of protein or RNA synthesis the label (8.4×10^4 Bq [^{3}H]uridine or 1.85×10^5 Bq [^{3}H]leucine) was added in a volume of 10 μL to hepatocytes in 24-well plates. After 2 hours, the medium was removed and cells were washed three times with ice-cold 10% trichloroacetic acid (wt/vol) and dried with methanol at −20°C. Then they were lysed for 8–12 hours at 37°C with 300 μL 0.5 mol/L NaOH/1 mmol/L ethylenediaminetetraacetic acid (EDTA)/0.1% Triton. An aliquot of 250 μL was used for detection of acid-precipitable radioactivity by β-scintillation counting. Ten microliters of the remaining lysis buffer was diluted 30-fold and used for protein determination.[43] Typically, with 10 μmol/L α-amanitin hepatocyte RNA synthesis was reduced to <50% within 15 minutes and protein synthesis decreased to <20% (compared with untreated controls) within 18 hours (50% after 6 hours).

In Vivo Experiments

ActD (0.8–3.2 mg/kg) or α-amanitin (0.8–4.5 mg/kg) were injected intraperitoneally in 500 μL of saline. Anti-TNF IgG (sufficient to neutralize TNF serum concentrations of 100 ng/mL) was given in a volume of 200 μL intravenously at the time points indicated, and rhuIL-1β was given intravenously 4 hours before the toxin challenge. After anesthesia, samples were obtained from mice by intravenous injection of 150 mg/kg pentobarbital plus 0.8 mg/kg heparin. Blood was withdrawn by cardiac puncture and immediately centrifuged for 2 minutes at 4°C at 13,000*g* to obtain the plasma. Livers were perfused for 10 seconds with cold perfusion buffer (50 mmol/L phosphate, 120 mmol/L NaCl, and 10 mmol/L EDTA, pH 7.4) and subsequently excised. A slice of the large anterior lobe was immersed immediately in 4% formalin solution as a fixation for histological studies. For RNA preparation, a liver sample (approximately 50 mg of protein) was fixed immediately in buffer (4 mol/L guanidine-isothiocyanate, 25 mmol/L sodium citrate, 0.5% lauroyl sarcosine, and 100 mmol/L β-mercaptoethanol). For determination of DNA fragmentation, the liver was homogenized. A fixed volume of the 20% homogenate (in perfusion buffer) was centrifuged at 13,000*g* for 20 minutes. The supernatant was used to detect DNA fragmentation by enzyme-linked immunosorbent assay (ELISA), or DNA was precipitated with ethanol (−20°C) plus sodium acetate (150 mmol/L final concentration) and stored at −20°C for further analysis of oligonucleosomal DNA fragmentation on agarose gels.

Hepatoma Cell Culture

Human hepatoma cells (HepG2, ATCC HB8065) were cultured routinely in RPMI 1640 supplemented with 5% fetal

calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L alanyl-glutamine at 37°C under a 5% CO_2 atmosphere. Cells were harvested by treatment with 25 mg/mL trypsin plus EDTA (GIBCO) for 4 minutes followed by vigorous tapping of the culture flask. Viability of the detached cells exceeded 95% as determined by trypan blue exclusion. Under normal growth conditions, cells had a doubling time of 20 hours. For toxicity experiments, cells were seeded in 24-well tissue culture plates at a density of 2.5×10^5/mL and grown for 36 hours. The growth medium was then removed and cells were exposed in 500 μL of RPMI 1640.

Hepatocyte Cultures

Hepatocytes were isolated according to Seglen[44] with an initial viability exceeding 80% as determined by the trypan blue exclusion method. Hepatocytes (8×10^4 cells/well) were plated in 200 μL of RPMI 1640 medium containing 10% newborn calf serum in 24-well plates. They were allowed to adhere to culture plates for 5 hours before the medium was exchanged for serum-free RPMI 1640. TNF was added 30 minutes after the medium change. ActD or α-amanitin were added directly after the medium change. Experiments were performed at 37°C in an atmosphere composed of 5% CO_2/40% O_2/55% N_2 for the times indicated.

Cytokine Determination

Samples for determinations of TNF bioactivity or protein content were immediately frozen at −20°C until analysis in the WEHI 164 bioassay[45] or by ELISA.[46] Quantitative polymerase chain reaction (PCR) for determination of hepatic TNF, interferon (IFN) γ, and interleukin (IL) 6 messenger RNA (mRNA) levels was performed as follows[46]: total tissue RNA (1 μg) was reverse transcribed in a volume of 20 μL using random primers and murine Moloney leukemia virus reverse transcriptase according to the supplier's recommendation (GIBCO/BRL, Eggenheim, Germany). Cytokine-specific mRNA expression was quantified by PCR, using a multispecific control fragment as internal standard.[47] Known amounts of control fragment were added in different dilutions to unknown fixed amounts of complementary DNA (cDNA) for competitive coamplification with specific primers. The proportion of PCR products amplified from the control fragment and target cDNA was estimated after separation on 1.5% agarose gels measuring the intensity of ethidium bromide fluorescence by a CCD image sensor and analyzed using the EASY program (Herolab, Wiesloch, Germany). First, the various cDNA samples to be compared were equilibrated according to their β-actin cDNA content. Then, the relative concentration of cytokine cDNA was estimated in each sample from the concentration of the control fragment DNA, which achieved equilibrium between its own amplification and the amplification of the cytokine cDNA. The concentrations were expressed in arbitrary units (AUs). One AU was defined as the lowest concentration of control fragment that yielded a detectable amplification product given the primer pairs and PCR conditions used (1 AU for TNF equals dilution of control fragment $\times 10^{-10}$ L; 1 AU for IFN-γ equals dilution of control fragment $\times 10^{-11}$ L; and 1 AU for IL-6 equals dilution of control fragment $\times 10^{-11}$ L).

Cytotoxicity and DNA Fragmentation

Lactate dehydrogenase[48] was determined in culture supernatants, and in the remaining cell monolayer after lysis with 0.1% Triton X-100. The percentage of lactate dehydrogenase release was calculated from the ratio of $S/(S + C)$, where S is culture supernatant and C is the remaining cell monolayer. Alternatively, the capacity to produce formazan from 3-(4,5-dimethyl-thiazol-2-yl)-3,5-diphenyltetrazolium bromide was measured as described recently.[36] Hepatocytolysis in vivo was determined by measuring the plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and sorbitol dehydrogenase (SDH).[48] DNA fragmentation in HepG2 cultures or in murine livers was quantitated by measuring cytosolic oligonucleosome-bound DNA using an ELISA kit (Boehringer, Mannheim, Germany) as described.[49] As antigen source we used either the cytosolic fraction (13,000g supernatant) of about 150 cultured hepatocytes or the cytosolic fraction from about 75 μg liver tissue. Also, DNA fragmentation was analyzed semiquantitatively after extraction of low-molecular-weight DNA by the phenol/chloroform method from the 13,000g supernatant obtained from either 30 mg liver tissue or 2×10^5 cultured hepatocytes. For analysis on 1.0% agarose gels DNA was precipitated by ethanol. Bands in the gel were stained with SYBR-green (Molecular Probes, Eugene, OR), visualized by UV illumination (312 nm), and photographed with Polaroid film (Eastman Kodak Co., New Haven, CT).

Morphology

Liver cell cultures were either stained with 2 μg/mL H-33342 for 6 minutes directly, or they were first fixed with 80% methanol (−20°C) and then stained with 5 μg/mL propidium iodide. The number of apoptotic nuclei was scored under the fluorescence microscope.

Livers were fixed for histological examination 12, 16, 20, and 24 hours after the challenge and imbedded in paraplast. Sections (3–5 μm) were stained with H&E, and photomicrographs were taken on a Leica DM-IRB microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with a 100×/NA 1.2 lens.

Statistics

All experiments with cell culture were repeated in at least three different cell preparations. Statistical differences were determined by an unpaired t test if applicable or with the unpaired Welch test (in the case of inhomogeneous variances).

Results

Facilitation of TNF-Induced Apoptosis in Human Hepatoma Cells by Toxins

Various transcriptional inhibitors, including ActD and the fungal toxin α-amanitin, have been shown

to sensitize murine hepatocytes toward TNF-induced apoptosis.[49] We examined whether similar results would be obtained with human liver cells and therefore used the HepG2 hepatoma line, usually not sensitive toward TNF alone.[50,51] As in murine hepatocytes, α-amanitin alone (≤50 μmol/L) or ActD alone (400 nmol/L) showed no toxicity. However, the toxins dramatically sensitized the cells toward TNF toxicity (Figure 1). The mode of cell death was apoptotic, judged from observations of the nuclear morphology (not shown) and from the fact that oligonucleosomal DNA fragmentation clearly preceded membrane lysis (Figure 1). Thus, various toxins seem to facilitate the well-known induction of apoptosis in human liver cells as well as in murine hepatocytes. This synergism of TNF and toxins may explain the highly hepatotoxic potential of α-amanitin in vivo despite the seemingly low sensitivity of hepatocytes in vitro.

Synergistic Toxicity of α-Amanitin and TNF In Vivo

To investigate whether the synergistic toxicity of TNF and α-amanitin would in fact occur in vivo, we injected mice with a combination of these agents and determined the time course of DNA fragmentation and hepatocytolysis in these animals (Figure 2). The concentrations of TNF[52] and α-amanitin were within a nontoxic range, if administered alone. In agreement with our in vitro results, the combined treatment caused fulminant liver failure, as characterized by massive release of liver enzymes between 5 and 8 hours after the challenge. As expected for a pathological sequence associated with early apoptosis, hepatic DNA fragmentation preceded hepatocytolysis. On the basis of these results we then tested whether α-amanitin or related toxins may induce liver failure by sensitization of hepatocytes toward endogenously released TNF.

Prevention of ActD or α-Amanitin Toxicity by Neutralization of Endogenous TNF

Injection of 0.8 mg/kg ActD, a dose suitable to sensitize animals toward TNF[49] during fulminant short-term (8 hours) experiments, was well tolerated by animals over a period of 24 hours. However, increasing the dose to 1.6 mg/kg was sufficient to elicit fulminant liver failure within 24 hours without concomitant injection of exogenous TNF. This toxicity was prevented by neutralization of endogenous TNF. Also, the toxicity elicited by a higher dose of ActD (3.2 mg/kg) was reduced drastically by passive immunization of mice against TNF (Figure 3*A*), whereas control IgG had no significant effect (Table 1). We then examined whether a single administration

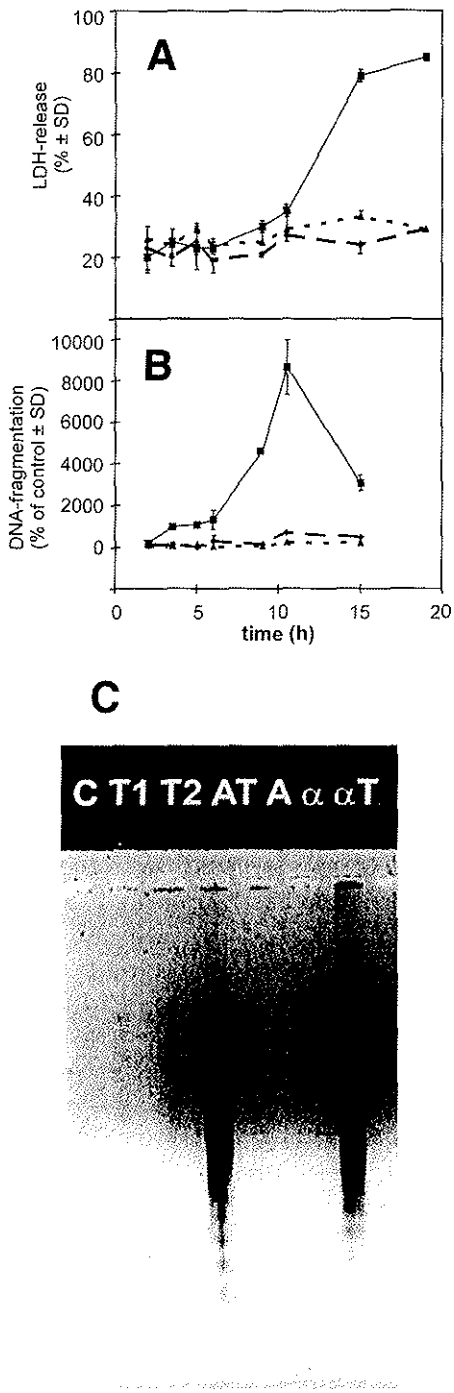

Figure 1. Induction of apoptosis in human liver cells by synergistic action of α-amanitin and TNF. HepG2 cells were preincubated with α-amanitin (10 μmol/L) or saline and subsequently challenged with 1 ng/mL TNF. In sibling cultures the time courses of (*A*) lactate dehydrogenase (LDH) release and (*B*) DNA fragmentation were determined over a period of 24 hours. ♦, Amanitin; ■, amanitin/TNF; ▲, control. Data are means ± SD of triplicate determinations. (*C*) Low-molecular-weight DNA from HepG2 cells was separated on a 1% agarose gel. Cells had been incubated for 8 hours with (C) saline control (c), 10 ng/mL TNF (T1), 1000 ng/mL TNF (T2), ActD plus 10 ng/mL TNF (AT), 400 nmol/L ActD (A), 10 μmol/L α-amanitin (α), and α-amanitin plus 10 ng/mL TNF (αT).

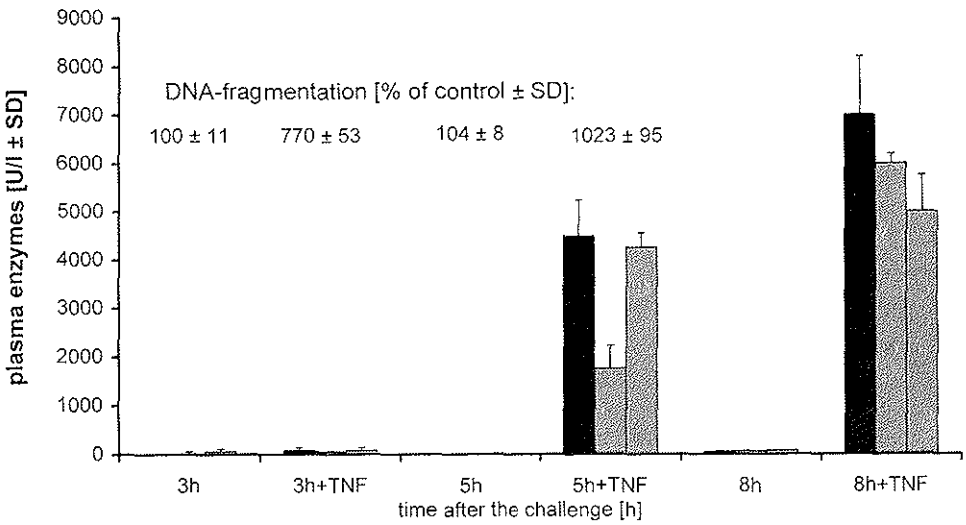

Figure 2. Synergistic hepatotoxicity of α-amanitin and TNF in mice. BALB/c mice were challenged with 0.8 mg/kg α-amanitin or 10 μg/kg TNF plus α-amanitin for 3, 5, or 8 hours before blood was withdrawn and DNA was isolated from the livers. Activities of ALT, ■, AST, ▒, and SDH, ▨ were determined in the plasma. DNA fragmentation (as determined by ELISA after 3 and 5 hours) is indicated in percentage compared with untreated control mice at the corresponding time point. Values are means ± SD from three animals per group.

of α-amanitin doses greater than those used for the TNF sensitization in vivo would cause a TNF-dependent hepatotoxicity. Doses of α-amanitin greater than 2 mg/kg induced fulminant hepatic failure, which was blocked by neutralization of endogenous TNF (Figure 3*B*).

Both ActD and α-amanitin strongly sensitize mice toward LPS.[31] Notably, the toxins used in this study were essentially endotoxin-free as determined by the limulus assay. In complementary experiments we examined the toxicity of ActD in C3H/HeJ mice that are resistant to endotoxin.[53] These mice also developed liver damage 24 hours after ActD challenge, which was comparable in its extent to the one observed in the LPS-sensitive control strain (C3H/HeN) and also was reduced significantly by passive immunization against TNF (Table 1). In addition, we injected anti-TNF IgG 60 minutes after the toxin challenge. In LPS models this time point is coincident with the peak of the TNF production[54] and represents a stage where complete protection by TNF neutralization is no longer possible. However, mice challenged with α-amanitin or ActD were protected by this delayed protocol as efficiently as if the antibody was given before the challenge. Therefore we assume that TNF was produced slowly in this model unlike after treatment of mice with endotoxins.

To get direct evidence on putative cytokine production, we checked whether cytokine mRNAs were upregulated in the target organ itself, i.e., the liver. ActD increased TNF, IFN-γ (Figure 4), and IL-6 (not shown) mRNA levels significantly within 7 hours. Treatment with α-amanitin caused an increase of mRNAs, which was statistically significant in the case of IFN-γ. Thus immunologically mediated toxicity of the hepatotoxins used for this study may in fact be explained by the upregulation of inflammatory cytokines. Finally, we sampled serum at various times to measure TNF that may have been released from the site of production into the circulation. At different times up to 10 hours after the challenge we neither detected TNF bioactivity nor TNF protein in the plasma. This might have been caused by a restriction of TNF production and action to local sites.

Inhibition of ActD or α-Amanitin Toxicity by Prevention of Actions of Endogenous TNF

To get further insights on the role of TNF in the hepatotoxic actions of ActD or α-amanitin, we used two different approaches. First, we examined the toxicity of these substances in mice deficient for either TNF receptor. Hepatotoxicity was only observed in mice having a functional 55-kilodalton TNF receptor, in accordance with the fact that this receptor is obligatory for inducing direct TNF-induced toxicity in sensitized hepatocytes.[36] Mice lacking the 75-kilodalton TNF receptor were not protected from the toxins (Table 1).

A second approach consisted of the desensitization of mice against the hepatotoxic effects of TNF by pretreatment with IL-1.[55] Also, this immunological intervention against TNF toxicity prevented completely the hepatotoxicity of α-amanitin or ActD, respectively (Table 1).

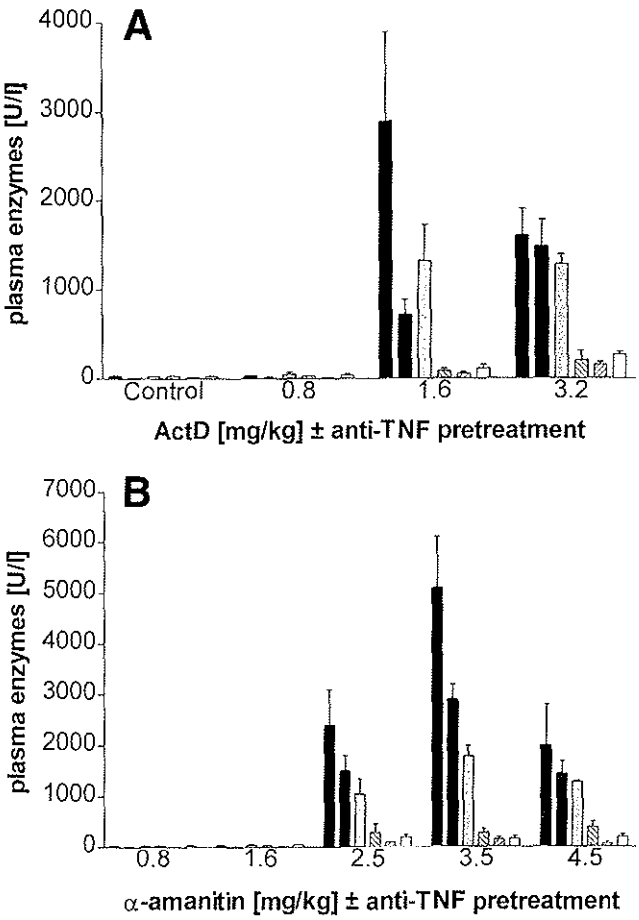

Figure 3. Prevention of ActD or α-amanitin–induced hepatotoxicity by neutralization of endogenous TNF. Mice were pretreated for 10 minutes with saline (*minus*) or ovine anti-murine–TNF IgG (*plus*) and then challenged with different concentrations of (*A*) ActD or (*B*) α-amanitin. Plasma activities of liver enzymes were determined after 24 hours. Control ovine IgG had no protective effect. Values are means ± SD from 4 animals per group. ▩, ALT-minus; ▨, SDH-minus; □, AST-minus; ▧, ALT-plus; ▨, SDH-plus; □, AST-plus.

Hepatocyte Apoptosis in ActD or α-Amanitin Toxicity

Our in vitro data and the evidence from the in vivo experiments suggest that cytokines may contribute to toxic liver failure by the induction of hepatocyte apoptosis. Thus, we followed the time course of DNA fragmentation and cell lysis after treatment of mice with the hepatotoxins (Figure 5*A*). In both experimental models alanine aminotransferase release as a parameter of loss of membrane integrity was preceded by DNA fragmentation, i.e., an indicator for apoptotic cell death. We verified the occurence of apoptosis by two further approaches. First, we investigated whether DNA fragmentation was of the oligonucleosomal type (Figure 5*B* and *C*). Indeed, a distinct laddering of DNA was observed on analysis on agarose gels already 12 hours after the challenge in livers derived from mice that had no significantly elevated plasma transaminase activities.

Second, we examined the histological alterations after injection of α-amanitin or ActD, respectively (Figure 6). The most conspicuous change 12 hours after injection of either toxin was the appearance of individual strongly eosinophilic cells with hyperchromatic nuclei that were condensed, had marginated lumpy chromatin, and adopted an elipsoid shape. Neutrophil invasion or hemorrhage was minimal or undetectable. Eosinophilic hepatocytes, indicating an early stage of apoptosis, were sometimes organized in small patches. About 16 hours after injection of α-amanitin, strongly shrunken nuclei and distinct apoptotic bodies were detectable. At later time points polymorphonuclear leukocyte invasion was marked and necrotic foci appeared, partially associated with hemorrhages. Some hepatocytes displayed macrovesicular vacuolization and karyolysis. The structural organization of the lobes was preserved largely and the numbers of strongly condensed hyperchromatic nuclei and apoptotic bodies were increased. After ActD application changes were similar but occurred more rapidly.

Discussion

The results of this study strongly suggest that endogenous TNF and the induction of hepatocyte apoptosis are key phenomena in the hepatotoxicity of ActD or α-amanitin in mice. Either of the two toxins is commonly assumed to inhibit transcription of mamma-

Table 1. Immunomodulation of α-Amanitin and ActD Hepatotoxicity

Strain/genotype	Treatment	Challenge with[c]: α-Amanitin (3 mg/kg): ALT (*U/l*)	Challenge with[c]: *ActD* (2 mg/kg): ALT (*U/l*)
C57Bl-wt	—	950 ± 170 (3)	2084 ± 680 (7)
C57Bl-tnfr-1°	—	150 ± 20 (3)[d]	238 ± 370 (3)[d]
C57Bl-tnfr-2°	—	ND	3050 ± 790 (7)
C3H/HeJ	—	ND	2530 ± 370 (6)
C3H/HeJ	Neutralizing anti-TNF IgG[a]	ND	110 ± 35 (3)[d]
C3H/HeN	—	ND	2060 ± 200 (6)
C3H/HeN	Neutralizing anti-TNF IgG[a]	ND	190 ± 40 (3)[d]
BALB/c	Control IgG	2880 ± 370 (4)	3032 ± 518 (8)
BALB/c	Neutralizing anti-TNF IgG[b]	130 ± 20 (4)[d]	170 ± 35 (4)[d]
BALB/c	IL-1 (*10 μg/kg*; 4 hours before toxins)	40 ± 15 (4)[d]	30 ± 10 (4)[d]

NOTE. Data are means ± SEM; n is indicated in parentheses; ND, not determined.
[a]Anti-TNF IgG was injected 10 minutes before the toxin challenge.
[b]Anti-TNF IgG was injected 60 minutes after the toxin challenge.
[c]Serum was withdrawn 24 hours after the challenge.
[d]$P \leq 0.05$.

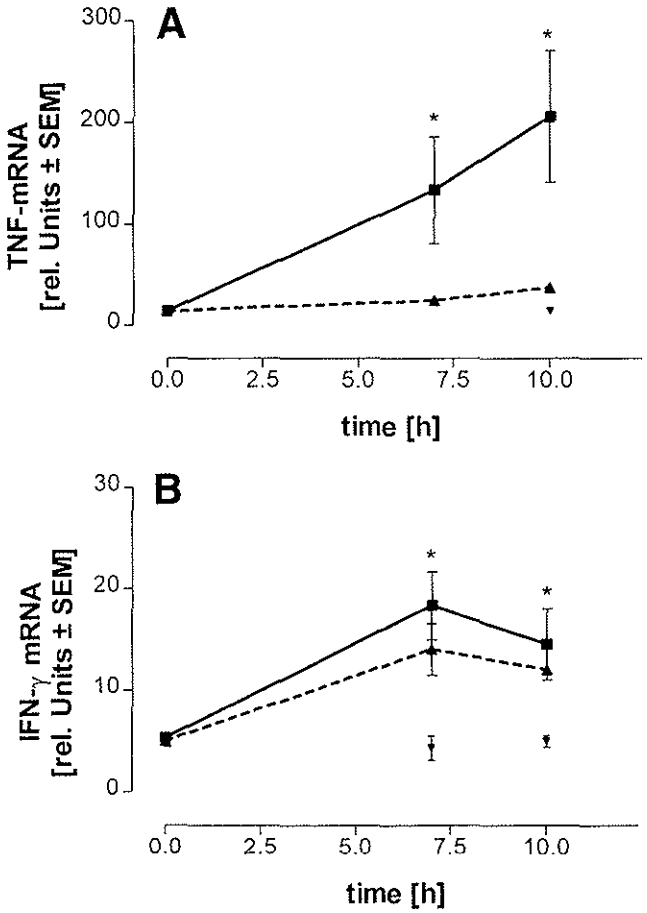

Figure 4. Increased cytokine mRNA in hepatotoxin-treated murine livers. RNA was prepared from livers of mice treated with ActD (2 mg/kg) or α-amanitin (3 mg/kg) after the time periods. The (*A*) TNF mRNA and (*B*) IFN-γ mRNA were quantitated by competitive PCR. Data are means ± SEM from 3 animals/group. ■, ActD; ▲, amanitin; ▼, control.

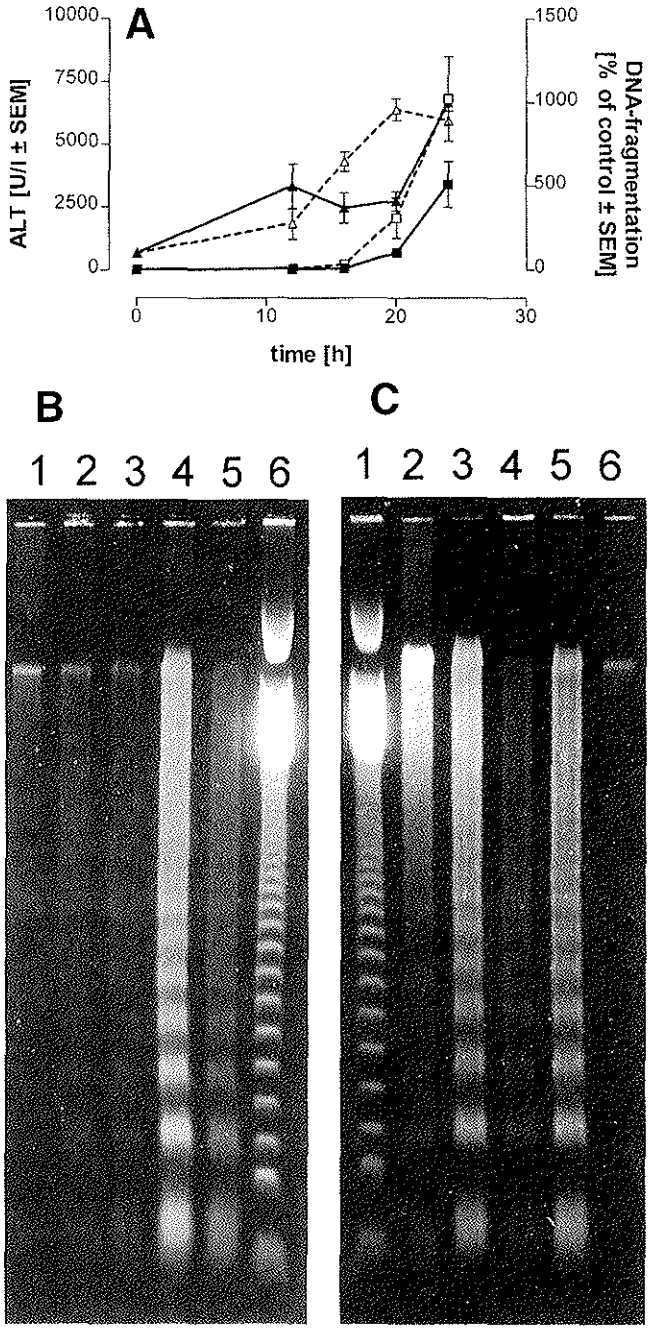

Figure 5. Time course of liver damage in hepatotoxin-treated mice. (*A*) Mice were injected intraperitoneally with 3 mg/kg α-amanitin or 2 mg/kg ActD and the time course of DNA fragmentation (DNA), and liver enzyme release (ALT) was followed over 24 hours. Data points indicate means ± SEM from 3 animals/group. ■, amanitin-ALT; ▲, amanitin-DNA; □, ActD-ALT; △, ActD-DNA. (*B*) Low-molecular-weight DNA was isolated from livers of animals treated with amanitin (2 mg/kg) for 0 hours (1), 12 hours (2), 16 hours (3), 20 hours (4), or 24 hours (5), respectively, and separated on a 1% agarose gel. *Lane 6*, 123 × n–bp molecular weight marker. (*C*) Low-molecular-weight DNA was isolated from livers of animals treated with ActD (3 mg/kg) for 12 hours (2), 16 hours (3), 20 hours (4), 24 hours (5), or 0 hours (6), respectively, and separated on a 1% agarose gel. *Lane 1*, 123 × n–bp molecular weight marker.

lian cells nonselectively and the selective hepatotoxicity of α-amanitin in humans has been explained previously mainly by pharmacokinetic properties. Our data provide a different rationale for the mechanism of toxicity of these compounds: the induction of hepatocyte apoptosis and the subsequent liver necrosis may result from a synergistic action with endogenous TNF. This agrees with and extends previous findings that a liver-specific transcriptional inhibitor, i.e., GalN, induces hepatic apoptosis and necrosis in synergy with endogenous TNF when coadministered with LPS,[52] and that the unspecific transcriptional inhibitor ActD induced hepatic apoptosis synergistically with exogenous TNF.[49]

TNF has different activities, depending on the cell type and the metabolic situation of the cell, i.e., it may induce apoptosis,[56,57] cause necrosis,[56–58] render protection,[51,59] or stimulate proliferation[58] of cells. In HepG2 cells, TNF has been shown to induce toxicity

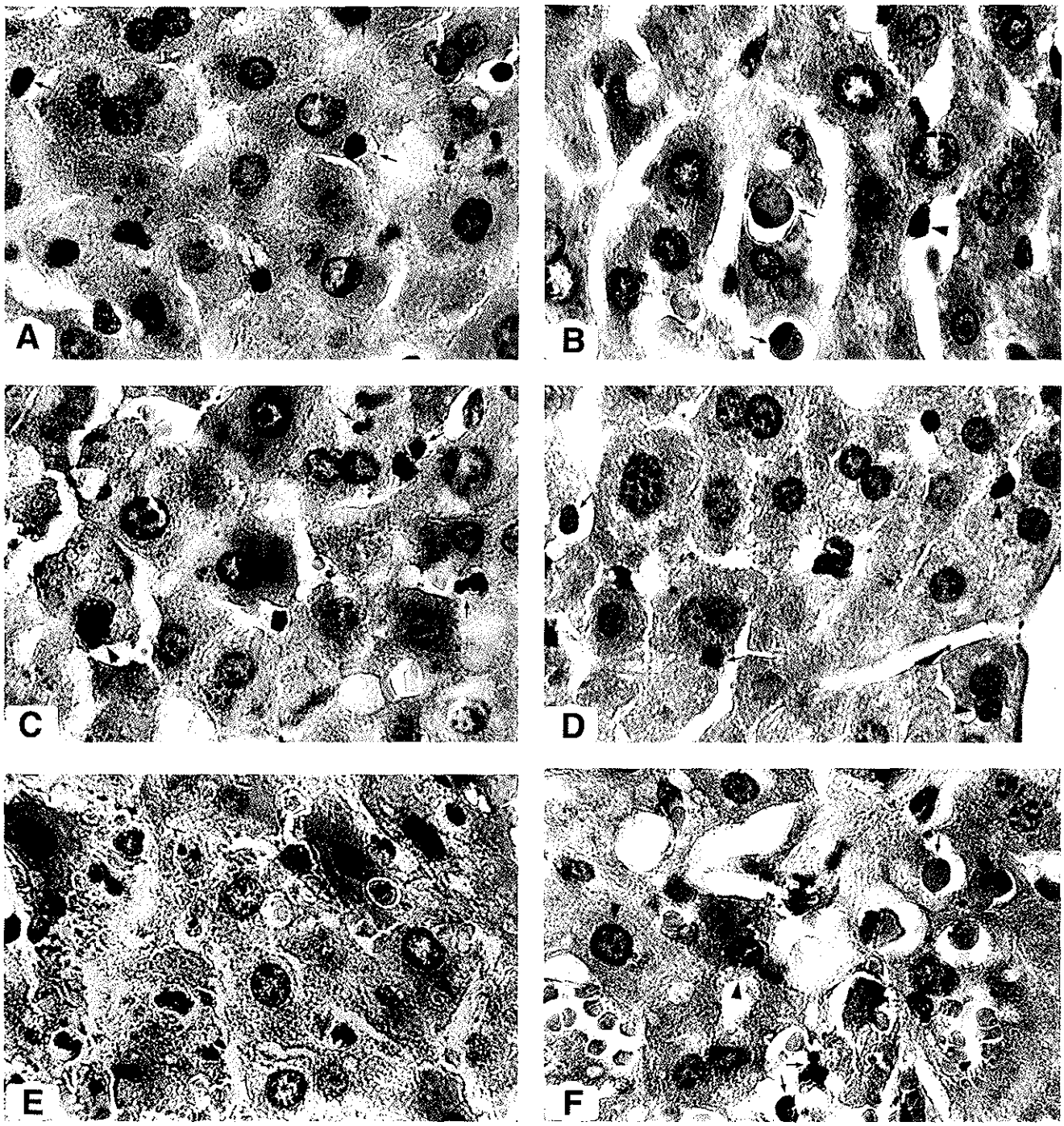

Figure 6. Morphology of α-amanitin– or ActD-induced hepatocyte apoptosis in vivo. Liver sections were prepared (*A* and *D*) 12 hours, (*B* and *E*) 16 hours, or (*C* and *F*) 20 hours after injection of mice with (*A–C*) 3 mg/kg α-amanitin or (*D–F*) 2 mg/kg ActD. Condensing nuclei are indicated by *arrowheads.* Apoptotic nuclei and engulfed apoptotic bodies are indicated by *arrows.* The horizontal width of the white figure label corresponds to 18 μm in the original sections.

in conjunction with ActD or cycloheximide.[49,51] To firmly establish that the mechanisms in these human cells were similar to those observed in murine hepatocytes, the mode of cell death was put under scrutiny. Morphological changes as well as the time course of cell lysis vs. DNA fragmentation suggest that the events of cell death were similar to those observed in murine hepatocytes in vitro. It may be speculated that the basis

of our in vivo findings in mice may be extrapolated to the human situation.

Although the direct molecular targets of ActD and α-amanitin are different, their overall action is strikingly similar. As in the case of ActD,[49] a reduction of transcription by about 50% is sufficient to sensitize α-amanitin–treated cells toward TNF-induced apoptosis. If impairment of transcription or translation is indeed a general mechanism to sensitize the liver toward the toxicity of endogenous TNF, then the action of many different toxins may involve this principle of toxicity.[60] Different toxins such as diethylnitrosamine, CCl_4,[12] and *Pseudomonas aeruginosa* exotoxin[61] have been shown to impair partially protein synthesis; therefore, they may eventually cause liver damage synergistically with TNF.

In our experience, it seems that at least two factors confound a direct linear relationship between the extent of a protein synthesis block and TNF-induced apoptosis. First, a complete shutdown of protein synthesis would also inhibit the de novo synthesis of endogenous TNF. The relevance of this phenomenon may be indicated by the fact that the dose-response curves for the two toxins used in this study were consistently bell-shaped (Figure 3; data not shown). Supportive evidence comes also from findings that cycloheximide is a poor sensitizer for TNF in hepatocytes and may even protect under certain situations.[62] Also, complete arrest of protein synthesis by cycloheximide, puromycin, or ricin was shown to prevent the apoptotic action of TNF.[49]

Although our experiments with gene-targeted mice and a specific antiserum against TNF provide strong evidence for the involvement of endogenous TNF in the toxicity of ActD or α-amanitin, we deemed it important to show that endogenous mediators could be produced under such experimental situations. The fact that we found increases of different cytokine mRNAs in the liver indicates that an augmentation of the respective mRNA pool was still possible and may have allowed enhanced production of different peptide mediators. In addition, in the case of TNF it has been shown that macrophages in the presence of ActD released increased amounts of TNF spontaneously and upon stimulation with endotoxin.[63,64] We found that LPS stimulated the subsequent release of TNF from murine Kupffer cells (10 ng/10^6 macrophages after 5 hours) up to 30 minutes after preincubation with ActD. An explanation for these observations may be a posttranscriptional regulation of TNF synthesis.[65] Circulating cytokines can only be detected in serum when there is a massive overflow at the local site of production, which was obviously not the case in our models.

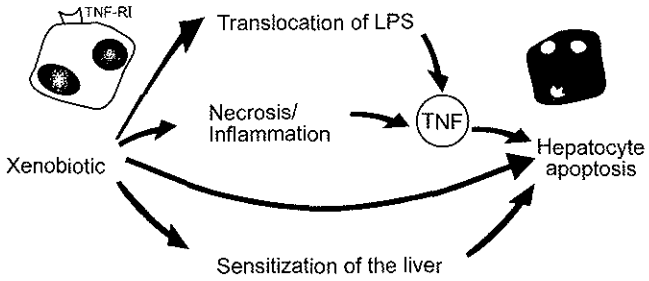

Figure 7. Putative pathways of xenobiotic-induced apoptosis in vivo.

Several agents cause tolerance toward a subsequent LPS challenge, by suppressing cytokine production.[66] IL-1 differs from this insofar as pretreatment with this cytokine selectively prevents TNF-induced apoptosis[55] (but not CD95-induced apoptosis[4]), i.e., it protects the target organ from the terminal mediator of toxicity. Protection of mice from α-amanitin or ActD by IL-1 pretreatment provides an independent line of evidence for the involvement of indirect immune mechanisms in the hepatotoxicity of these agents.

The question of why such detrimental immune mechanisms are activated by toxins cannot be answered by this study. It is established that hepatocytes possess several independent pathways to initiate apoptosis. However, it is not clear whether all deaths are mediated by neighboring cells (Kupffer cells or other hepatocytes), by soluble cytokines, or even by autocrine mechanisms, because hepatocytes themselves may acquire the capacity to synthesize cytokines such as IL-1[67] or TNF.[68] The convergence of different noxious stimuli such as xenobiotics,[14,16,17,69] graft rejection,[70] viral infection,[71,72] and overstimulation of the immune response[5,49,73–77] into a limited number of apoptotic pathways may allow the liver to react in a controlled way without loss of tissue organization, unless the stimulus becomes strong enough to activate secondary processes leading to necrosis and inflammation. The latter case may occur in toxicological model systems like the one used in this study or after ingestion of large amounts of hepatotoxins (e.g., fungal poisoning), but it may represent an exception to the rule that small percentages of cells affected under less severe conditions are removed silently by apoptosis.

To summarize our findings diagrammatically (Figure 7), we suggest two different principles of action for the in vivo toxicity of hepatotoxins. They may directly damage parenchymal cells according to well-established cytotoxic mechanisms deducible from a plethora of in vitro studies. Such damage may both sensitize cells to the attack of cytokines and be the basis for the release of cytokines. Second, they may cause the overproduction of cytokines by allowing LPS mobilization from the intestine,[25,78] by

inducing the formation of local inflammatory foci, or by a direct, yet unknown, interaction with the cytokine-producing cells. The synergistic action of endogenous cytokines such as TNF with xenobiotic-sensitized cells may be the final cause of liver failure as shown in the case of ActD or α-amanitin.

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