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DNA Fragmentation in Mouse Organs during Endotoxic Shock

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The systemic inflammatory response syndrome has still an unpredictable outcome, and patients often die of multiple organ failure despite circulatory stabilization therapy. The still incompletely understood pathophysiological mechanisms include organ damage due to direct toxic actions of cytokines elicited by overactivation of the host response. To study this process of organ failure in experimental septic shock, we injected mice with a lethal dose of endotoxin and examined apoptotic and necrotic tissue damage biochemically, histologically, and ultrastructurally. Endotoxin administration caused oligonucleosomal as well as random DNA fragmentation in liver, lung, kidney, and intestine. In the liver, DNA fragmentation was not restricted to hepatocytes but also occurred in nonparenchymal cells. The DNA fragmentation was mediated by tumor necrosis factor and attenuated by endogenous nitric oxide release. Unlike the situation in D-galactosamine-sensitized mice, in which injection or release of tumor necrosis factor causes massive hepatocyte apoptosis, liver failure due to high doses of endotoxin was characterized by single-cell necrosis, a low incidence of apoptosis, and simultaneous damage to nonparenchymal cells. We conclude that, even though endotoxin causes cytokine-mediated DNA fragmentation in several organs including the liver, hepatocyte apoptosis itself seems to be a minor phenomenon in high-dose endotoxic shock in mice.

The systemic inflammatory response syndrome (SIRS) continues to be a major complication and a frequent cause of death in intensive care units.[1] One of the prime initiators of SIRS is microbial infection[2] that leads to an over-reaction of the nonspecific immune system toward microbes, primarily provoked by their cell wall components. In other words, shock-like hyperinflammatory conditions may be elicited in the absence of live microbes by the interaction of their membrane macromolecules, such as endotoxins from Gram-negative bacteria or certain exotoxins, with immunocompetent cells. Thus, the toxicity of these substances is indirect and due to the release of a wide variety of inflammatory mediators in the host.[3] To study the basic mechanisms underlying systemic inflammation, administration of bacterial constituents, such as endotoxins (lipopolysaccharides; LPS), to experimental animals has been extensively used as a model of SIRS.[4,5]

SIRS is characterized by circulatory failure and multiple organ failure (reviewed in Ref. 6). The different aspects of organ failure were mostly studied by injecting a low dose of LPS to animals previously sensitized by LPS (Shwartzman mechanism), bacterial infection (eg, *Corynebacterium parvum* or *Propionibacterium acnes*) or transcriptional inibitors (eg D-galactosamine (GalN) or actinomycin D). Mice treated in such a way develop a relatively selective liver failure.[7-10] In the models using transcriptional inhibitors, it was found that tumor necrosis factor (TNF)-induced hepatocyte apoptosis is a basic mechanism of pathogenesis.[11] In contrast to this prominent role of organ damage for the overall pathology in the sensitization models, death of animals caused by a dose of LPS that is lethal without sensitization has been related to circulatory failure.[5,12] A major contribution to the detrimental fall in blood

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pressure associated with septic shock has been ascribed to the release of nitric oxide (NO) in response to LPS.[9,13-15] On the other hand, pharmacological treatment with a NO donor has been shown to protect sensitized mice from LPS-induced liver injury,[16] suggesting a beneficial role of NO release in septic complications. In contrast to NO, the central role of TNF as a distal mediator of LPS-dependent lethality has been firmly established.[12,17-20] In particular, TNF is known to cause liver cell death after sensitization with a transcriptional inhibitor.[11,21,22]

The role of apoptosis is very well established for the hematopoietic and the immune system. In addition, cytokine-induced apoptosis was found in tumor cells and parenchymal cells of various organs, such as the liver. In a common low-dose LPS model using sensitization by GalN or actinomycin D, the presence of a transcriptional block has been shown to be a metabolic prerequisite for LPS- and TNF-induced murine hepatocyte apoptosis and ensuing necrotic cell death.[21]

We initiated this study with the aim to clarify the following questions. First, we asked whether, in the high-dose LPS shock model, apoptosis and possibly secondary necrosis associated with tissue destruction would also play a role. As oligonucleosomal DNA fragmentation is an indicator of apoptotic cell death found to be suitable as an early and sensitive marker of organ damage in the liver,[11,21] we followed the time course of DNA fragmentation in four major organs after the treatment of mice with a lethal dose of LPS. To assess the extent of DNA fragmentation, data from such LPS-treated animals were compared with those measured in GalN-sensitized mice challenged with TNF as a reference model for apoptotic parenchymal cell death.[11] Morphological studies were carried out on liver tissue obtained from mice after a lethal dose of LPS and also compared with liver samples from GalN/TNF-treated mice. We then investigated the influence of a lethal dose of LPS on hepatic transcription and translation and examined the contribution of the endogenous mediators NO and TNF to the development of organ damage in LPS shock without prior sensitization.

Materials and Methods

Reagents

LPS (*Salmonella abortus equi* endotoxin) was purchased from Sebak (Aidenbach, Germany). Anti-murine TNF IgG fraction was prepared in our laboratory as described in Ref. 11. Recombinant murine TNF was a gift from Dr. Adolf (Bender & Co., Vienna, Austria). N^G-monomethyl-L-arginine (NMMA) was obtained from Alexis (Grünberg, Germany). GalN was purchased from Roth (Karlsruhe, Germany). Tetramethylbenzidine was from Boehringer (Mannheim, Germany). DNA ladder marker (n × 123 bp) was from Gibco (Berlin, Germany), SYBR green was from Molecular Probes (Eugene, OR). Proteinase K, terminal deoxynucleotide transferase, digoxigenin-11-dUTP, and alkaline-phosphatase-coupled anti-digoxigenin antibody were from Boehringer (Mannheim, Germany). [^{14}C]Leucine, [^{3}H]uridine, and all substances that are not otherwise specified were purchased from Sigma Chemical Co. (Deisenhofen, Germany). All substances applied parenterally to animals were given in pyrogen-free saline.

Animals

BALB/c mice (8- to 10-week-old females) were obtained from the animal house of the University of Konstanz. Animals were kept at 22°C and 55% relative humidity in a 12-hour day/night rhythm with free access to food (Altromin 1313, Altromin Co., Lage, Germany) and water. All animals received humane care in compliance with the National Institutes of Health guidelines as well as with legal requirements in Germany.

Animal Experiments

For LPS shock experiments, mice were treated intraperitoneally (i.p.) with 5 mg/kg LPS. Anti-TNF IgG was diluted in saline containing 0.1% human serum albumin and administered in two doses i.p. and intravenously (i.v.) 1 hour before LPS. NMMA (240 mg/kg each time) was injected i.v. 15 minutes before and 4 hours after administration of LPS, and 6 to 24 hours after LPS challenge, the animals were anesthetized with 100 μl of pentobarbital (45 mg/ml) containing 5 mg/ml heparin, and blood was withdrawn by cardiac puncture. The animals were perfused for 20 seconds with cold perfusion buffer (50 mmol/L phosphate, 120 mmol/L NaCl, 10 mmol/L EDTA, pH 7.4) from the right ventricle of the heart to the vena cava, and lung, liver, kidney, and cecum were excised.

For experiments with GalN-sensitized mice, female BALB/c mice were fasted overnight. GalN (700 mg/kg) was injected i.p. in a volume of 200 μl per mouse, followed by i.v. administration of TNF (10 μg/kg in saline/0.1% human serum albumin), and 5 and 8 hours after challenge, animals were anesthetized with 100 μl of pentobarbital (45 mg/ml) containing 5 mg/ml heparin. The liver was perfused blood-

free with cold perfusion buffer via the portal vein before it was excised. For light-microscopic studies, liver slices of the large anterior lobe were immediately immersed in buffered formalin as a fixative and embedded in paraplast.

Determination of Protein and RNA Synthesis

At different time points after i.p. administration of 5 mg/kg LPS, mice were i.v. injected with 300 μl of saline containing 40 μCi (~1 nmol) of [^{3}H]uridine and 4 μCi (~13 nmol) of [^{14}C]leucine. Sixty minutes later, mice were anesthetized i.v. with pentobarbital (45 mg/ml) containing 5 mg/ml heparin. Blood was withdrawn and the liver was removed after perfusion with cold perfusion buffer (50 mmol/L phosphate, 120 mmol/L NaCl, 10 mmol/L EDTA, pH 7.4). The liver was blotted dry and weighed before a 10% homogenate in perfusion buffer was prepared. Aliquots of 100 μl (corresponding to 10-mg liver or 2.1 mg of protein) were precipitated with 50 μl of 30% cold trichloroacetic acid and washed three times with 1 ml of cold 10% trichloroacetic acid. The resulting pellet was dissolved overnight at 37°C in 500 μl of lysis buffer (1 mmol/L EDTA, 0.1% Triton X-100, 0.5 mol/L NaOH). ^{3}H and ^{14}C activities were determined simultaneously in a scintillation counter on two different channels. The rates of total translational or transcriptional activities from triplicate samples were compared with untreated control animals.

Determination of Nitrite and Nitrate in Plasma

Nitrite and nitrate in plasma were measured essentially according to the method of Misko et al,[23] using the Griess assay instead of the fluorimetric assay described. Briefly, plasma obtained by cardiac puncture after lethal anesthesia with pentobarbital/heparin was filtered through an Ultrafree-MC microcentrifuge filter unit (Millipore, Bedford, MA) for 1 hour at 14,000 rpm to remove hemoglobin released by cell lysis, which might interfere with the colorimetric assay. As nitrite in serum is mostly oxidized to nitrate by reaction with the iron-heme center of hemoglobin, the resulting nitrate was first reduced to nitrite by incubation with nitrate reductase from *Aspergillus* species. Nitrate reductase (14 mU in 20 mmol/L Tris/HCl, pH 7.6) was added to 10 μl of filtrate, and the reaction was started by addition of NADPH in Tris/HCl buffer to a final concentration of 80 μmol/L and a final volume of 50 μl. After 5 minutes of incubation at room temperature, the reaction was terminated by dilution with 50 μl of distilled water. The whole reaction volume was transferred to 96-well microtiter plates, 10 μl sulfanilamide (1% in 1.2 mol/L HCl) and 10 μl *N*-(1-naphthyl) ethylenediamine (0.1% in H_2O) were added, and absorbance was read after 3 minutes of incubation time at 560/690 nm on an enzyme-linked immunosorbent assay (ELISA) reader.

Enzyme Assays

Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and sorbitol dehydrogenase (SDH) in plasma were determined using an Eppendorf ACP 5040 enzyme analyzer according to the protocols described by Bergmeyer.[24] Data are expressed in units per liter of plasma (means ± SEM).

TNF ELISA

For detection of TNF, a polyclonal capture antibody (protein G plus purified ovine anti-mouse TNF; protein solution of 20 mg/ml with approximately 1 to 3 μg/ml specific activity) and a biotinylated anti-mouse rabbit TNF secondary antibody were used. This ternary complex was visualized by binding of peroxidase-conjugated streptavidin and reaction with tetramethylbenzidine. All incubations were performed in flat-bottomed, high-binding polystyrene microtiter plates (Greiner, Nürtingen, Germany). The detection limit of the TNF ELISA for recombinant murine TNF was 10 pg/ml.

DNA Fragmentation ELISA

Lung, kidney, and the remaining parts of the liver were blotted dry and disintegrated in cold perfusion buffer with an Elvehjem-type homogenizer. Cecum was luminally perfused to remove contents and subsequently homogenized. Liver and kidney were disintegrated by three strokes of the homogenizer, lung and cecum by eight strokes. The 20% organ homogenates were centrifuged at 13,000 × *g* for 15 minutes. The supernatants (cytosolic fractions) were further diluted 200-fold (in the LPS shock model) or 250-fold (in the GalN/TNF model). DNA fragmentation was quantitated by measuring oligonucleosome-bound DNA using an ELISA kit (Boehringer, Mannheim, Germany).[21] Data are expressed as original optical density (OD) readings in milli OD (mOD) (means ± SEM) and additionally as percent increase *versus* untreated control animals.

Table 1. *Time Course of LPS-Induced Liver Injury as Assessed by Plasma Enzyme Activities*

Time after LPS (hours)	ALT (U/L)	AST (U/L)	SDH (U/L)	n
0	33 ± 5	72 ± 5	14 ± 2	9
6	38 ± 11	25 ± 7	38 ± 22	9
8	54 ± 23	105 ± 50	30 ± 11	9
12	160 ± 46	360 ± 130*	56 ± 18	9
24	1010 ± 230*	1260 ± 250*	610 ± 140*	8

LPS was administered i.p. at a dose of 5 mg/kg. Data are expressed as means ± SEM. n, number of animals per group.
*$P < 0.05$ *versus* saline-treated control.

Agarose Gel Electrophoresis

Equal amounts of cytosolic fractions (13,000 × *g* supernatant) of the 20% organ homogenates were treated with 15% (v/v) 3 mol/L sodium acetate and 3 volumes of ethanol to precipitate low molecular weight DNA. After treatment with RNAse (1 mg/ml for 1 hour at 50°C) and proteinase K (10 mg/ml for 18 hours at 50°C), the DNA fragments were purified by phenol/chloroform extraction and analyzed by electrophoresis on a 1% agarose gel. A DNA ladder consisting of 123-bp multimers served as a standard.

Light- and Electron-Microscopic Studies

For light-microscopic studies, one slice of the liver was immersed immediately after removal in 4% buffered formalin and embedded in paraplast. The 2-μm sections were stained with hematoxylin and eosin.

For electron microscopy, livers of mice treated with LPS or with GalN/TNF were fixed by perfusion via the portal vein for 5 minutes with a fixative containing 0.25% glutaraldehyde and 2% sucrose in 100 mmol/L of Pipes buffer (piperazine-*N*,*N*′-bis (2-ethanesulfonic acid)) at pH 7.4. After fixation, the tissue was rinsed in the same buffer, and 50-μm sections were cut with a microslicer (Dosaka EM-Company, Kyoto, Japan) and collected in 100 mmol/L Pipes buffer at pH 7.4. For the demonstration of cytochrome c oxidase activity, the tissue was incubated in a medium containing 2.5 mmol/L 3′,3′-diaminobenzidine and 0.05% cytochrome c in 100 mmol/L Pipes buffer (pH 7.2) at 37°C for 30 minutes. The tissue was postfixed in aqueous 2% osmium tetroxide at 4°C for 1 hour. Ultrathin sections were counterstained with lead citrate for 1 minute.[25]

For *in situ* detection of DNA double-strand breaks, the terminal transferase method (terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling; TUNEL) was applied essentially as described by Oberhammer et al[26] with the following modifications. Tissue sections (2 μm) were dewaxed and immersed in 20% cold acetic acid for 15 seconds. The sections were treated with 10 μg/ml proteinase K for 30 minutes at 37°C before they were incubated with terminal desoxynucleotide transferase (25 U/100 μl) and digoxigenin-11-dUTP (2 nmol/100 μl) in 100 mmol/L cacodylate, 1 mmol/L $CoCl_2$, 100 μmol/L dithiothreitol, and 50 μg/ml bovine serum albumin for 60 minutes at 37°C. Incorporated digoxigenin-11-dUTP was detected by incubation with alkaline-phosphatase-coupled anti-digoxigenin antibody (375 mU/ml) and reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Statistics

Data are expressed as means ± SEM. Data were analyzed by nonparametric analysis of variance (Kruskal-Wallis or Mann-Whitney test). Original uncorrected data were used for these analyses. If differences were found between the groups ($P < 0.05$), these were subjected to two-sided nonparametric multiple comparisons of the control group against all other groups (Dunn's test). $P < 0.05$ was considered to be significant.

Results

LPS-Induced Plasma Enzyme Release in Nonsensitized Mice

To investigate the organ damage initiated by a lethal dose of LPS without sensitization, mice were treated i.p. with 5 mg/kg LPS. This dose resulted in a mortality rate of >80% within 72 hours. At different times after LPS injection, animals were sacrificed and their plasma was assayed for enzymes indicative of cell lysis in various tissues. A rise of plasma AST only was detected 12 hours after LPS administration with activities of the liver-specific enzymes ALT and SDH still at control levels (Table 1). This finding suggests that LPS caused tissue toxicity at this early time point that was not restricted to the liver. At 12 hours later, ie, 24 hours after the challenge, liver parenchymal

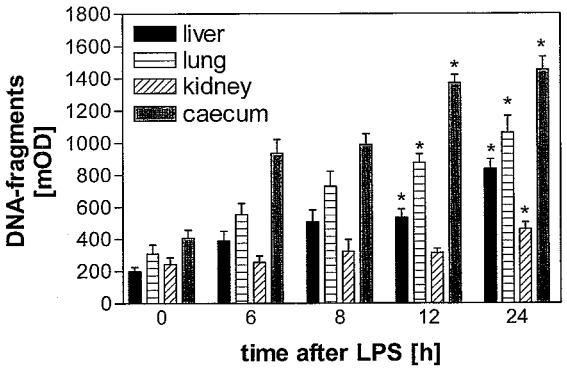

Figure 1. *Time course of LPS-induced DNA fragmentation in different organs of the mouse. Animals were injected i.p. with 5 mg/kg LPS. At various time points after LPS administration, DNA fragmentation was measured by ELISA in the 13,000 × g supernatants of organ homogenates. Data are expressed as mean values ± SEM from nine animals. *P < 0.05.*

cells were severely damaged as concluded from elevated plasma levels of the liver-specific enzymes ALT and SDH (Table 1). Also at this time, the high ratio of AST activity as compared with the activities of the liver-specific enzymes ALT and SDH indicated that LPS-initiated cell death had affected other organs in addition to the liver.

LPS-Initiated DNA Fragmentation in Major Mouse Organs

Murine liver, lung, kidney, and cecum were examined for biochemical indications of cell death at various times after LPS administration. The internal digestion of cellular DNA is a sensitive biochemical marker that allows detection of cell damage in homogenates of whole organs.[11,21] At 12 hours after injection of LPS, a significant increase in the amount of low molecular weight DNA was detected in cytosolic fractions from liver, lung, and cecum as assessed by an ELISA specific for histone-bound DNA (Figure 1). In the kidney, the extent of DNA fragmentation was only significantly increased at 24 hours after LPS challenge. The maximal increase of DNA fragmentation in liver was observed at 24 hours after LPS administration and amounted to 190 ± 60% over untreated control animals. In a parallel experiment, levels of DNA fragmentation due to treatment of GalN-sensitized mice with TNF were quantitated as a reference for apoptotic parenchymal cell death.[11] In this model of inflammatory liver injury, DNA fragmentation was increased by 400 ± 8% (GalN only, 241 ± 25 mOD; GalN/TNF 1198 ± 20 mOD; ALT, 9500 ± 2560 U/L plasma) compared with control animals treated with GalN only, already after 8 hours. Animals treated with GalN or TNF alone have been shown

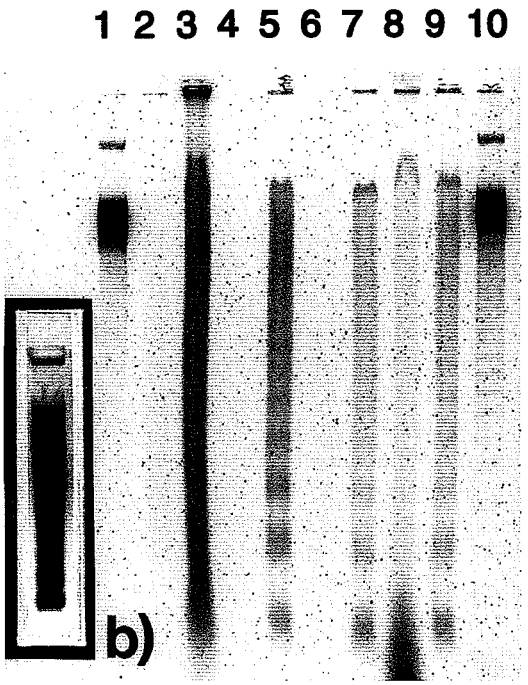

Figure 2. *Detection of LPS-induced DNA fragmentation by agarose gel electrophoresis. Mice were treated i.p. with 5 mg/kg LPS, and 24 hours after LPS challenge, DNA was prepared from cytosolic fractions of organ homogenates and stained with SYBR green after analysis on a 1% agarose gel. Cytosolic fractions of untreated animals served as controls. The lanes of the gel show the following samples:* lane 1, *123-bp multimer DNA ladder;* lane 2, *cecum, untreated;* lane 3, *cecum, 24 hours after LPS;* lane 4, *lung, untreated;* lane 5, *lung, 24 hours after LPS;* lane 6, *kidney, untreated;* lane 7, *kidney, 24 hours after LPS;* lane 8, *liver, untreated;* lane 9, *liver, 24 hours after LPS;* lane 10, *123-bp multimer DNA ladder. In cecum, a very high level of DNA fragmentation was observed when equal amounts of tissue were used for DNA extraction. In* b, *a distinct analysis of cytosolic DNA fragments obtained from the cecum 24 hours after LPS administration is shown, where oligonucleosomal DNA fragmentation is clearly visible due to reduced exposure time of the gel for photographic documentation.*

before to be not significantly different from controls.[11]

To distinguish between necrotic random DNA cleavage and apoptotic oligonucleosomal cleavage in our experiments, cytosolic low molecular weight DNA from LPS-treated mice was analyzed by agarose gel electrophoresis. At 24 hours after LPS challenge, DNA fragmentation ladders of approximately 180-bp multimers, which are considered to be characteristic of apoptotic cell death, were detected in liver, lung, kidney, and cecum of mice (Figure 2).

Morphological Characterization of LPS-Induced Liver Damage

As the DNA fragmentation data were suggestive of apoptosis, we looked for morphological characteristics of apoptotic cell death and addressed the question of whether or not DNA fragmentation is accompanied or followed by necrotic cell damage as

observed in other models of inflammatory organ injury.[11] For these studies, we concentrated on the liver as one of the major target organs of septic organ failure.[9,27-30]

For gross histological examination, H&E-stained liver sections obtained 3 to 24 hours after LPS administration were studied by light microscopy. At early times after LPS injection (ie, 3 to 6 hours), a high number of leukocytes adhering to blood vessel walls was observed. Liver tissue removed 24 hours after LPS administration showed erythrocyte agglutination, infiltration of granulocytes, and single-cell necrosis, as characterized by karyolysis. The incidence of these histopathological changes did not follow a strict zonation as seen with some low molecular weight model hepatotoxins. Nevertheless, damage was significantly more pronounced in periportal zones as compared with midzonal or perivenous regions of the liver lobule. In addition, several mitotic hepatocytes were found, indicating ongoing liver regeneration processes. An increased occurrence of hepatocytes with chromatin condensed at the nuclear membrane, a characteristic of apoptotic cell death, was observed only at 24 hours after LPS administration but at a very low frequency. Apoptotic cell death was observed only at time points when single-cell necrosis was also visible. This suggests that, in the high-dose LPS shock model, apoptosis and necrosis cannot be dissociated by their time course.

Electron-microscopic studies were performed to obtain unambiguous evidence on the mode of hepatocyte death under conditions of LPS shock. Electron micrographs of mouse liver 20 hours after administration of LPS yielded clear evidence of microcirculatory failure and necrotic hepatocyte death. Sinusoids were filled with activated Kupffer cells, thrombocytes, and erythrocytes (Figure 3A). Figure 3B shows a necrotic hepatocyte as identified by karyolysis and mitochondrial swelling. Infiltrating polymorphonuclear leukocytes were seen in close association with the damaged hepatocyte. It is important to note that neighboring cells frequently showed unaltered, intact morphology.

Apoptotic hepatocytes were not seen in the tissue sections examined by electron microscopy, as might have been expected from the low frequency already observed by light microscopy. To gain some information on the relative importance of apoptosis and necrosis in the high-dose LPS model, we carried out comparative electron-microscopic experiments in livers of GalN-sensitized mice intoxicated with TNF. Tissue sections were taken at two key time points.[11] Samples taken at 5 hours after TNF administration showed that

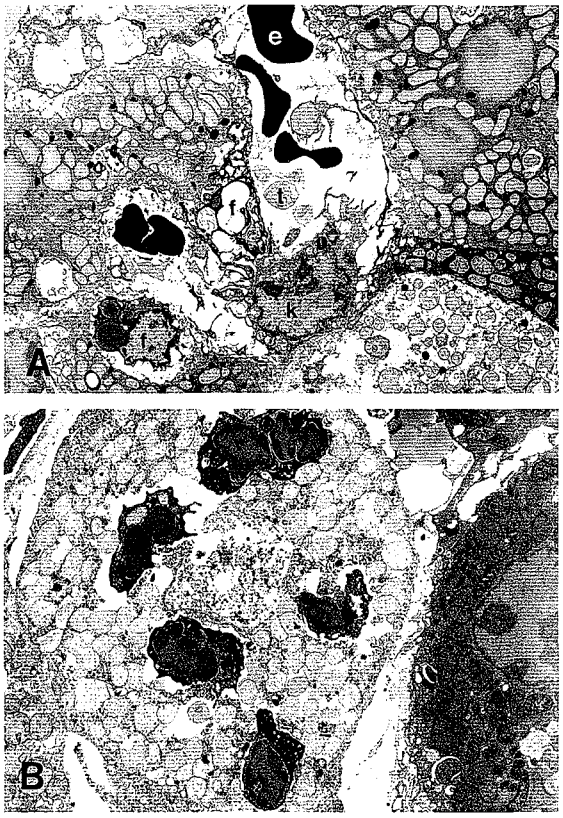

Figure 3. *Electron micrographs of mouse liver 20 hours after LPS. Mice were treated i.p. with 5 mg/kg LPS, and 20 hours later, liver tissue was prepared for electron microscopy.* A: *A sinusoid containing thrombocytes (t), erythrocytes (e), and a Kupffer cell (k) with long cytoplasmic projections and lamellopodia. Two fat-storing cells (f) are seen in the space of Disse. Magnification, ×4800.* B: *Higher magnification (×6100) of a necrotic mouse hepatocyte showing karyolysis, mitochondrial swelling, and invasion of polymorphonuclear leukocytes.*

hepatocyte apoptosis was readily detectable by our methods, even at a time point when there was no accompanying necrosis or cell lysis (Figure 4A). The apoptotic bodies seen on the micrograph still contain functional mitochondria as characterized by cytochrome c oxidase staining. In samples taken after 8 hours, a time point at which concomitant hepatocyte apoptosis and necrosis are known to occur, apoptotic hepatocytes were still easily distinguishable by electron microscopy (Figure 4B). We therefore conclude that our methods were adequate also to identify apoptosis within necrotic tissue. These comparisons between the high-dose LPS and the GalN/TNF model suggest that the contribution of hepatocyte apoptosis to the cell injury induced by a lethal dose of LPS is a relatively minor one.

We then checked whether LPS-induced hepatic DNA fragmentation as detected by ELISA and agarose gel electrophoresis was in fact derived from hepatocytes. For this purpose, DNA double-strand breaks were visualized *in situ* by applying the TUNEL

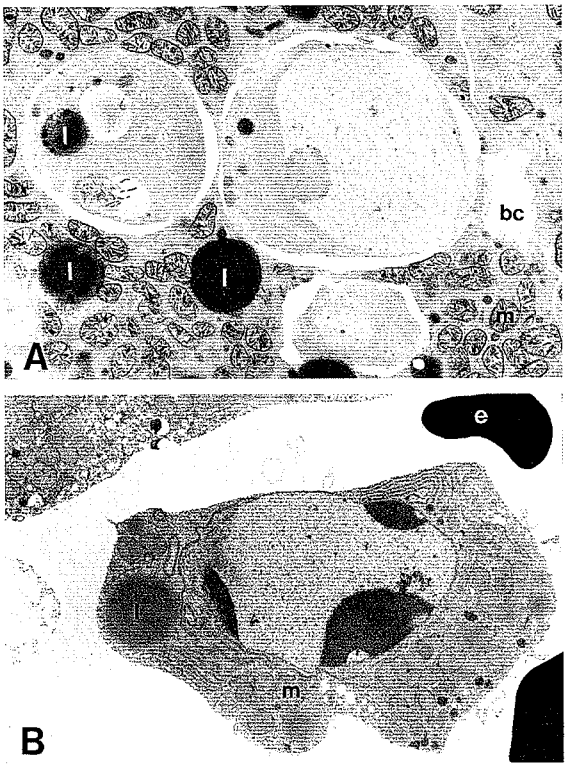

Figure 4. *Electron micrographs of mouse liver after treatment with GalN and TNF. Mice were treated i.p. with GalN (700 mg/kg), followed by i.v. injection of TNF (10 μg/kg), and 5 and 8 hours after challenge, liver tissue was removed for electron microscopy.* A: *Electron micrograph of liver tissue 5 hours after challenge. Three apoptotic bodies phagocytosed by an intact hepatocyte are seen. The integrity of the phagocytosing hepatocyte is shown by staining of mitochondria (m) for cytochrome c oxidase activity. l, lipid droplets; bc, bile canaliculus. Magnification, ×13,000.* B: *Electron micrograph of liver tissue 8 hours after challenge. An apoptotic hepatocyte with chromatin condensed at the nuclear membrane is seen surrounded by a destroyed tissue area. e, erythrocyte; m, mitochondria; l, lipid droplet. Magnification, ×10,000.*

assay.[26] TUNEL staining of formalin-fixed, paraffin-embedded liver tissue 24 hours after LPS challenge showed DNA double-strand breaks not only in parenchymal but also in some nonparenchymal liver cells (Figure 5A). In correspondence to the findings in H&E-stained liver tissue, the incidence of TUNEL-positive hepatocyte nuclei was especially high in periportal regions, ie, 9 ± 4% (data are means ± SD from 20 periportal regions) compared with an average of 5 ± 5% for the entire liver lobule. The majority of TUNEL-positive hepatocyte nuclei contained uniformly compacted chromatin, but there was no margination of chromatin along the nuclear membrane. The subsets of nonparenchymal cells marked by the TUNEL reaction could not be clearly identified by light microscopy. Liver samples from untreated mice showed no positive TUNEL reaction.

Liver sections obtained 5 hours after treatment of GalN-sensitized mice with TNF showed a high incidence of TUNEL-positive hepatocytes with apoptotic morphology, ie, margination of condensed chromatin along the nuclear membranes (Figure 5B). At 8 hours after TNF treatment of GalN-sensitized mice, ie, at a time when pronounced hepatocyte membrane lysis occurs, TUNEL staining was also observed in areas of severe tissue destruction. Not all positive hepatocyte nuclei showed apoptotic morphology at this time point (Figure 5C). In this model, damage was multifocal. A clear zonation of histopathological changes was not detected by a blinded observer. This is mainly diffuse but seemed to start in the perivenous area, sparing the periportal regions initially.[31]

Aggravation of LPS-Induced Liver Failure by Inhibition of Endogenous NO Synthesis

After the characterization of LPS-mediated cell death, our next aim was to clarify the contribution of different mediators known to be released in response to LPS to the development of organ failure. One of the mediators induced by LPS that has been shown to be involved in LPS-dependent shock is NO.[9,14,15,32] The role of NO in LPS-induced organ damage was assessed by the administration of NMMA, a competitive inhibitor of endogenous NO production. Intravenous injection of NMMA (240 mg/kg each time, 15 minutes before and 4 hours after LPS administration) prevented the rise in serum nitrite/nitrate levels caused by administration of LPS alone (Table 2). In the liver, the inhibition of endogenous NO release resulted in a significant increase in both LPS-induced DNA fragmentation as assessed by ELISA (LPS alone, 220 ± 25% increase *versus* untreated control; LPS plus NMMA, 460 ± 90% increase *versus* control animals) as well as hepatocyte membrane rupture as determined by elevated plasma levels of liver-specific enzymes (Table 2). NMMA had no influence on the extent of LPS-induced DNA fragmentation in lung and cecum. Administration of NMMA alone did not result in hepatocyte membrane lysis. As mice treated with a combination of LPS and NMMA showed increased symptoms of severe illness, all animals were sacrificed at 12 hours after LPS challenge, in contrast to the 24-hour time point chosen for all other experiments.

LPS-Induced Cell Death in Liver and Lung but Not in Cecum Is Mediated by TNF

Neutralization of TNF by antibody pretreatment has been shown to prevent lethality caused by a high

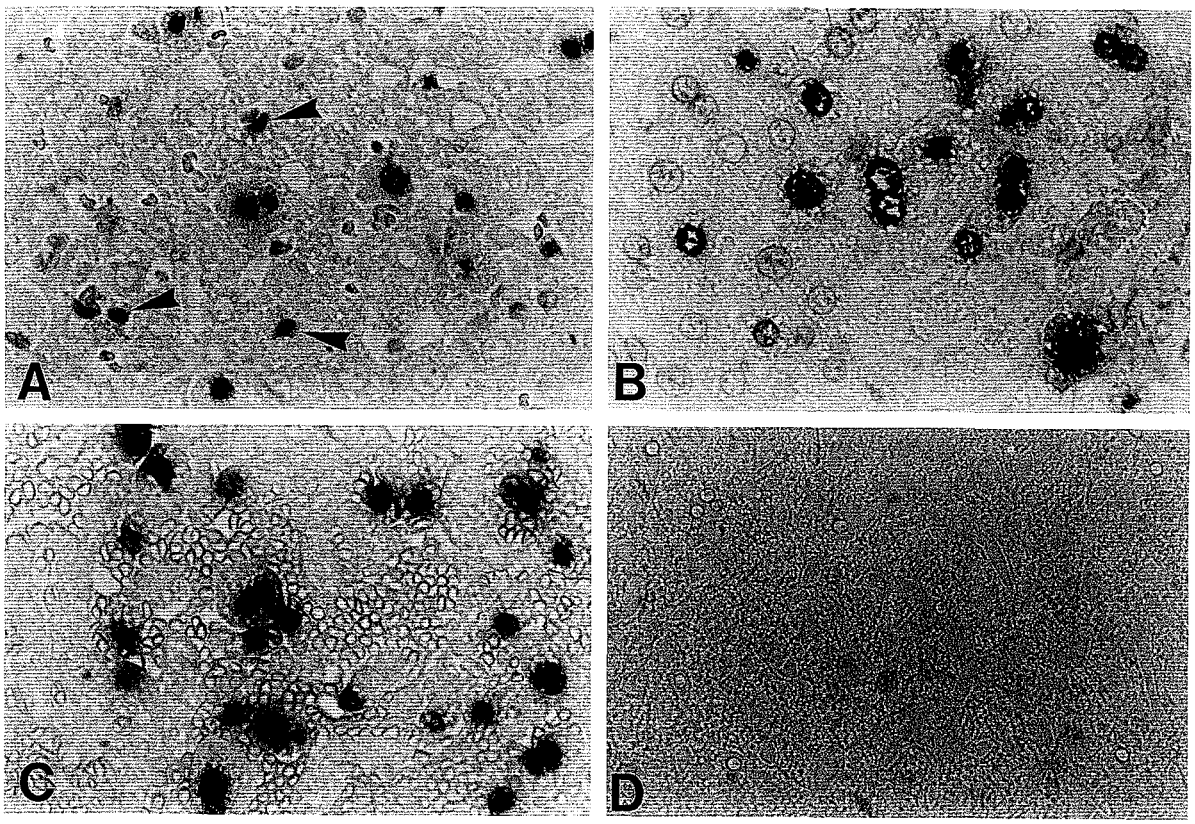

Figure 5. In situ *detection of DNA fragmentation by the TUNEL assay.* A: *Liver tissue removed 24 hours after LPS administration. TUNEL staining is seen in hepatocytes and nonparenchymal cells* (arrowheads). *TUNEL-positive hepatocyte nuclei show compacted chromatin.* B: *Liver tissue obtained 5 hours after GalN/TNF treatment. TUNEL-positive hepatocyte nuclei show margination of chromatin along the nuclear membrane.* C: *Liver tissue removed 8 hours after GalN/TNF treatment. Severe tissue destruction accompanied by erythrocyte agglutination is seen. Not all of the TUNEL-positive hepatocyte nuclei show apoptotic morphology.* D: *Liver tissue of mice treated with anti-TNF antibody before the administration of LPS (24 hours after LPS challenge). No TUNEL-positive cells are visible.*

dose of LPS as well as to protect against LPS-induced liver failure in sensitized mice.[11,12,17,33] We therefore investigated the involvement of TNF in DNA damage and cell lysis observed in endotoxic shock. Pretreatment of mice with neutralizing anti-TNF antibody 1 hour before the administration of LPS protected liver and lung cells from LPS-induced DNA fragmentation (liver, 80% reduction; lung, 75% reduction) but had no influence on the degree of DNA fragmentation in the cecum as assessed by ELISA (Table 3). Pretreatment of mice with anti-TNF antibody also prevented membrane rupture of liver cells, as we conclude from the fact that no elevated plasma levels of liver-specific enzymes were detectable 24 hours after LPS injection (Table 3). Anti-TNF antibody pretreatment also inhibited the LPS-induced rise in plasma AST activity. This indicates that TNF, once elicited by LPS, also gives rise to a general tissue toxicity that is not limited to a certain organ (Table 3).

Table 2. *Effect of the NO Synthase Inhibitor NMMA on LPS-Induced DNA Fragmentation and Liver Cell Lysis*

Treatment	DNA fragments (mOD)			ALT (U/L)	Nitrite/nitrate* (μmol/L)	n
	Liver	Lung	Cecum			
Saline	270 ± 60	750 ± 15	1020 ± 40	25 ± 4	35 ± 10	3
LPS	860 ± 70	900 ± 100	1670 ± 60	480 ± 75	370 ± 20	5
LPS plus NMMA	1500 ± 240†	860 ± 100	1470 ± 310	1210 ± 420†	60 ± 10†	6‡
NMMA	ND	ND	ND	14 ± 5	ND	5

LPS was given i.p. at a dose of 5 mg/kg. NMMA (240 mg/kg each time) was injected i.v. 15 minutes before and 4 hours after LPS. Data are given as means ± SEM at 12 hours after LPS. n, number of animals per group; ND, not determined.

*Nitrite/nitrate plasma concentrations.

†$P < 0.05$ *versus* LPS control.

‡n = 3 for DNA fragmentation data.

Table 3. *Influence of Pretreatment with Anti-TNF Antibody on LPS-Induced DNA Fragmentation and Liver Cell Lysis in Mice*

Pretreatment	Treatment	TNF (ng/ml)*	DNA fragments (mOD) Liver†	Lung	Cecum	ALT (U/L)	AST (U/L)	n
Saline	Saline	<0.01	160 ± 10	730 ± 70	370 ± 60	38 ± 11	63 ± 6	3
Saline	LPS	28 ± 3	630 ± 130	1400 ± 270	1400 ± 210	1510 ± 300	3680 ± 720	9‡
Anti-TNF IgG	LPS	<0.01	270 ± 30§	910 ± 60§	1560 ± 200	30 ± 10§	110 ± 20§	6

Animals were pretreated with saline or anti-TNF IgG in two doses i.p. and i.v. 1 hour before administration of LPS. LPS was given i.p. in a dose of 5 mg/kg. n, number of animals.

*Data are means ± SEM, 90 minutes after LPS administration.

†Data are means ± SEM, 24 hours after LPS or saline administration.

‡n = 5 for DNA fragmentation data.

§$P < 0.05$ *versus* LPS control.

Application of the TUNEL assay on liver samples removed 24 hours after LPS challenge showed no positive liver cells in mice treated with anti-TNF antibody before LPS administration. Thus, this histological examination confirmed the findings obtained by biochemical methods (Figure 5D).

LPS Does Not Interfere with Hepatic Transcription and Translation

TNF-mediated murine hepatocyte apoptosis *in vivo* as well as *in vitro* has been shown to be dependent on transcriptional arrest.[21] As there was no pronounced parenchymal cell apoptosis detectable in liver sections obtained from LPS-treated animals, we hypothesized that the administration of a lethal dose of LPS should not impair hepatic transcription or translation. To check this hypothesis, the influence of LPS on overall protein and RNA synthesis in mouse liver was determined by measuring the rate of uridine and leucine incorporation at different time points after LPS administration. At the times investigated (3 to 24 hours after challenge), LPS caused no significant alteration of total liver RNA and protein synthesis (data not shown). Thus, the mainly necrotic liver cell damage caused by a lethal dose of LPS was unrelated to a general transcriptional or translational block in mouse liver.

Discussion

In intensive care, immediate death of patients by circulatory failure can be prevented by a variety of interventions, but later on, these patients are frequently threatened by the failure of one or several vital organs such as liver, lung, kidney, and intestine.[34] As early cytotoxicity in parenchymal or endothelial cells was discussed as a potential cause of multiple organ failure,[35] we assessed the involvement of primary organ damage in experimental lethal LPS shock and measured DNA fragmentation as an early indicator of cell death in major shock organs of the mouse.

Indeed, DNA fragmentation seems to be a sensitive marker of LPS-shock-induced tissue injury, as it was significantly increased in liver, lung, and cecum as early as 12 hours after LPS challenge. The pattern of low molecular weight DNA obtained by agarose gel electrophoresis pointed to the involvement of apoptotic cell death in LPS-induced tissue injury. However, no information on the subsets of cells affected and the relative contribution of apoptosis *versus* necrosis is provided by this method.

In sensitized mice challenged with TNF, DNA fragmentation is detectable several hours before hepatocyte membrane lysis.[11,21] In addition, the extent of DNA fragmentation determined by the DNA ELISA was higher and more rapid in onset compared with that observed in nonsensitized mice treated with LPS. The temporal coincidence of DNA fragmentation with hepatocyte cell membrane lysis in high-dose LPS shock suggested that this type of liver damage may include mechanisms different from those in mice sensitized with transcriptional inhibitors. Morphological studies on light- and electron-microscopic levels confirmed this hypothesis. Apoptosis is the predominant mode of cell death at early times after GalN/TNF and clearly precedes necrotic parenchymal cell death seen at later time points.[11,21] In contrast, a lethal dose of LPS in nonsensitized mice caused primarily necrotic cell death, accompanied but not preceded by a low incidence of hepatocyte apoptosis. The difference between the two models was further stressed by the distinct distribution of apoptotic and necrotic parenchymal cell death within the liver lobule. In various *in vitro* models, the induction of either apoptosis or necrosis has been shown to be dependent on the extent and duration of the exposure to cytotoxicity-inducing agents.[36-38] In addition, the succession of necrosis

and apoptosis induced by glutamate in neuronal cells is known to be dependent on mitochondrial function.[37] It is conceivable that, during experimental LPS shock *in vivo*, regional differences in the concentration of cytotoxic mediators as well as the cellular energy status, determined by the oxygen supply, exist within the affected organs. This situation might contribute to the simultaneous occurrence of apoptosis and necrosis observed in LPS shock. *In situ* staining of DNA double-strand breaks by the TUNEL assay 24 hours after LPS administration showed that the number of positive hepatocyte nuclei exceeded the incidence of apoptosis observed in H&E-stained liver tissue. This observation may be explained by findings of a recent study[39] demonstrating that the TUNEL assay under certain conditions fails to discriminate between apoptotic and necrotic cell death. Although the occurrence of DNA fragmentation is considered to be a hallmark of apoptotic cell death when it precedes membrane lysis, DNA fragmentation has also been shown to play a significant role in cell necrosis caused by hepatotoxins[40,41] and in necrotic lymphoma cell death induced by Ca^{2+} ionophore or hypoxia.[42] It appears therefore that DNA fragmentation may sometimes represent a common final pathway for acute cell death by apoptosis as well as by necrosis.[40,41]

The detection of DNA fragmentation in nonparenchymal liver cells 24 hours after LPS treatment might account for some of the oligonucleosomal DNA cleavage observed by ELISA and agarose gel electrophoresis. LPS- or TNF-induced endothelial cell apoptosis has been described in various experimental settings.[43-47] In addition, apoptosis of activated granulocytes and macrophages appears to play an important role in the resolution of tissue inflammation.[48,49]

Apart from exerting direct cytotoxicity, LPS-induced inflammatory cytokines are also known to contribute to the development of disseminated intravascular coagulation frequently observed in septic patients. Clearly, the formation of microvascular thrombi in various organs is involved in the pathogenesis of multiple organ failure (reviewed in Ref. 50). In our study, microcirculatory failure due to blockade of the sinusoids by activated Kupffer cells, thrombocytes, and erythrocytes was observed in liver tissue obtained 20 hours after LPS administration. A contribution of microcirculatory failure to the liver damage observed in LPS shock would also account for the aggravating effect of NMMA treatment on LPS-induced DNA fragmentation and hepatocyte membrane lysis. This is in line with previous evidence that endogenous and exogenous NO has hepatoprotective functions.[16,29,51] The protective effects of endogenously produced NO may be due to scavenging of polymorphonuclear-leukocyte-derived reactive oxygen as well as to the direct inhibition of neutrophil superoxide anion formation.[52] In addition, NO produced by constitutive NO synthase in endothelial cells inhibits platelet aggregation and improves blood flow in the microcirculation that is important for the supply of cells with oxygen and nutrients.[51,53,54] As NMMA inhibits all known isoenzymes of NO synthase, it might increase LPS-mediated organ injury by impairing the local blood flow, thus contributing to hypoxic cell damage.[55]

In contrast to endogenous NO, TNF was found here to be a detrimental mediator of LPS-induced tissue destruction. LPS-induced lethality in experimental animals as well as apoptotic and necrotic liver parenchymal cell injury in GalN-sensitized mice has been shown to be mediated by TNF.[11,12,17,19,20] In this study, we used passive immunization of mice with a neutralizing anti-TNF antibody before a lethal LPS challenge to show that TNF causes DNA fragmentation and membrane lysis in liver parenchymal cells of nonsensitized mice. As TNF-induced hepatocyte apoptosis *in vitro* as well as *in vivo* has been shown to be dependent on transcriptional arrest[11,21] and as apoptosis was not the dominant mode of cell death in LPS shock, we hypothesized that hepatic transcription is not impaired by the administration of a lethal dose of LPS. In a previous study, hepatic incorporation of uridine and leucine was shown to be significantly reduced by treatment of mice with GalN.[11] Employing an analogous experimental setup, no reduction of hepatic uridine and leucine incorporation was observed under conditions of septic shock. Nevertheless, it is conceivable that the large increase in the synthesis of acute-phase proteins observed in septic shock[56] could well have masked a selective impairment of the synthesis of individual proteins that normally protect hepatocytes against TNF-mediated apoptosis.

LPS-induced lung injury was also significantly reduced by neutralization of TNF. Pulmonary dysfunction observed in SIRS is believed to be at least partly dependent on neutrophil infiltration.[57,58] As the sequestration of neutrophils to the lung is mediated by TNF,[59] this could explain the protective effect of anti-TNF antibody pretreatment on LPS-induced lung injury. The unaltered cecal injury in mice pretreated with anti-TNF antibody points to the involvement of other LPS-inducible endogenous mediators, eg, interleukin-1, interferon-γ, prostaglandin E_2, or platelet-activating factor in the development of organ damage. The contribution of platelet-activating fac-

tor to LPS-induced bowel necrosis has been shown previously.[60-62] The occurrence of TNF-independent organ damage in LPS shock is in accordance with clinical studies in humans, in which passive immunization against TNF alone failed to prevent death of patients with septic shock.

Our study demonstrates that beyond circulatory failure a lethal dose of LPS causes early and pronounced cellular injury in major shock organs of nonsensitized mice. Although LPS-induced liver injury was shown to be dependent on TNF and reduced by NO as in inflammatory liver injury in GalN-sensitized mice, the mode of cell death was different in LPS shock. In this situation, hepatocytes died mainly by necrosis unlike the predominance of apoptosis caused by TNF under transcriptional arrest. The lack of correlation between biochemical detection of oligonucleosomal DNA fragmentation and morphological proof of parenchymal cell apoptosis emphasizes the indispensability of accompanying histological studies for the definition of the relative importance of apoptosis for the disease state or experimental model under examination.

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References

1. Darville T, Giroir B, Jacobs R: The systemic inflammatory response syndrome (SIRS): immunology potential immunotherapy. Infection 1993, 21:279–290
2. Bone RC, Fisher CJ Jr, Clemmer TP, Slotman GJ, Metz CA, Balk RA: Sepsis syndrome: a valid clinical entity. Crit Care Med 1989, 17:389–393
3. Bone RC: The pathogenesis of sepsis. Ann Intern Med 1991, 115:457–469
4. Redl H, Bahrami S, Schlag G, Traber DL: Clinical detection of LPS and animal models of endotoxemia. Immunobiology 1993, 187:330–345
5. Natanson C, Eichenholz PW, Danner RL, Eichacker PQ, Hoffman WD, Kuo GC, Banks SM, Mac Vittie TJ, Parrillo JE: Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. J Exp Med 1989, 169:823–832
6. Bone RC, Balk RA, Cerra FB, Dellinger RP, 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
7. Ohno Y, Shiga J, Mori W: Selective segmental hepatic necrosis produced by the Shwartzman mechanism in rabbits. Virchows Arch A Pathol Anat Histol 1989, 416: 75–80
8. Billiar TR, Curran RD, Harbrecht BG, Stuehr DJ, Demetris AJ, Simmons RL: Modulation of nitrogen oxide synthesis *in vivo*: *NG*-monomethyl-L-arginine inhibits endotoxin-induced nitrite/nitrate biosynthesis while promoting hepatic damage. J Leukocyte Biol 1990, 48: 565–569
9. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie Q.-W, Sokol K, Hutchinson N, Chen H, Mudgett S: Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 1995, 81:641–650
10. Wendel A: Biochemical pharmacology of inflammatory liver injury in mice. Methods Enzymol 1990, 186:675–680
11. Leist M, Gantner F, Bohlinger I, Tiegs G, Germann PG, Wendel A: Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am J Pathol 1995, 146:1220–1234
12. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A: Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 1987, 330:662–664
13. Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW, Levi R, Lodato RF: *NG*-methyl-[scapl-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. Proc Natl Acad Sci USA 1990, 87:3629–3632
14. Nava E, Palmer RMJ, Moncada S: Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? Lancet 1991, 338:1555–1557
15. Petros A, Bennett D, Vallance P: Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. Lancet 1991, 338:1557–1558
16. Bohlinger I, Leist M, Barsig J, Uhlig S, Tiegs G, Wendel A: Interleukin-1 and nitric oxide protect against tumor necrosis factor-α-induced liver injury through distinct pathways. Hepatology 1995, 22:1829–1837
17. Beutler B, Milsark IW, Cerami AC: Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 1985, 229:869–871
18. Mathison JC, Wolfson E, Ulevitch RJ: Participation of tumor necrosis factor in the mediation of Gram negative bacterial lipopolysaccharide-induced injury in rabbits. J Clin Invest 1988, 1937, 81:1925
19. Rothe J, Lesslauer W, Loetscher H, Lang Y, Koebel P, Köntgen F, Althage R, 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
20. 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
21. 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, 153:1778–1788
22. Tiegs G, Wolter M, Wendel A: Tumor necrosis factor is a terminal mediator in D-galactosamine/endotoxin-induced hepatitis in mice. Biochem Pharmacol 1989, 37:627–631
23. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG: A fluorimetric assay for the measurement of nitrite in biological samples. Anal Biochem 1993, 214:11–16
24. Bergmeyer HU: Methods of Enzymatic Analysis, ed 3. Weinheim, Germany, Verlag Chemie, 1984
25. Angermüller S, Schunk M, Kusterer K: Alteration of xanthine oxidase activity in sinusoidal endothelial cells and morphological changes of Kupffer cells in hypoxic and reoxygenated rat liver. Hepatology 1995, 21: 1594–1601
26. 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
27. Boler RK, Bibighaus AJ, Brunson JG: An electron microscopic study of the liver of endotoxin-shocked dogs treated with a combination of propiomazine and levarterenol. Lab Invest 1969, 20:319–325
28. Hewett JA, Jean PA, Kunkel SL, Roth RA: Relationship between tumor necrosis factor-α and neutrophils in endotoxin-induced liver injury. Am J Physiol 1993, 265: G1011–G1015
29. Harbrecht BG, Billiar TR, Stadler AJ, Demetris AJ, Ochoa DJ, Curran RD, Simmons RL: Nitric oxide synthesis serves to reduce hepatic damage during acute murine endotoxemia. Crit Care Med 1992, 20:1568–1574
30. Portoles MT, Ainaga MJ, Pagani R: The induction of lipid peroxidation by *E. coli* lipopolysaccharide on rat hepatocytes as an important factor in the etiology of endotoxic liver damage. Biochim Biophys Acta 1993, 1158:287–292
31. Tiegs G, Wendel A: Leukotriene-mediated liver injury. Biochem Pharmacol 1988, 37:2569–2573
32. Wei X-Q, Charles IG, Smith A, Ure J, Feng G-J, Huang F-P, Xu D, Muller W, Moncada S, Liew FY: Altered immune response in mice lacking inducible nitric oxide synthase. Nature 1995, 375:408–411
33. Jin H, Yang R, Marsters SA, Bunting SA, Wurm FM, Chamow SM, Ashkenazi A: Protection against rat endotoxic shock by p55 tumor necrosis factor (TNF) receptor immunoadhesin: comparison with anti-TNF monoclonal antibody. J Infect Dis 1994, 170:1323–1326
34. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Crit Care Med 1992, 20:864–874
35. Schlag G, Redl H, Hallström S: The cell in shock: the origin of multiple organ failure. Resuscitation 1991, 21: 137–180
36. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA: Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci USA 1995, 92: 7162–7166
37. Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P: Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 1995, 15:961–973
38. Dypbukt JM, Ankarcrona M, Burkitt M, Sjöholm A, Ström K, Orrenius S, Nicotera P: Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. J Biol Chem 1994, 269:30553–30560
39. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R: *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995, 21: 1465–1468
40. 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
41. Fukuda K, Kojiro M, Chiu J: Demonstration of extensive chromatin cleavage in transplanted Morris hepatoma 7777 tissue: apoptosis or necrosis?. Am J Pathol 1993, 142:935–946
42. Collins RJ, Harmon BV, Gobe GC, Kerr JFR: Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. Int J Radiat Biol 1992, 61:451–453
43. Maeda K, Abello PA, Abraham MR, Wetzel RC, Robotham JL, Buchman TG: Endotoxin induces organ-specific endothelial cell injury. Shock 1995, 3:46–50
44. Abello PA, Fidler SA, Bulkley GB, Buchman TG: Antioxidants modulate induction of programmed endothelial cell death (apoptosis) by endotoxin. Arch Surg 1994, 129:134–141
45. Buchman TG, Abello PA, Smith EH, Bulkley GB: Induction of heat shock response leads to apoptosis in endothelial cells previously exposed to endotoxin. Am J Physiol 1993, 265:H165–H170
46. Polunovsky VA, Wendt CH, Ingbar DH, Peterson MS, Bitterman PB: Induction of endothelial cell apoptosis by

TNF-α: modulation by inhibitors of protein synthesis. Exp Cell Res 1994, 214:584–594
47. Robaye B, Mosselmans R, Fiers W, Dumont JE, Galand P: Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells *in vitro*. Am J Pathol 1991, 138:447–453
48. Squier MK, Sehnert AJ, Cohen JJ: Apoptosis in leukocytes. J Leukocyte Biol 1995, 57:2–10
49. Munn DH, Beall AC, Song D, Wrenn RW, Throckmorton DC: Activation-induced apoptosis in human macrophages: developmental regulation of a novel cell death pathway by macrophage colony-stimulating factor and interferon-γ. J Exp Med 1995, 181:127–136
50. Levi M, ten Cate H, van der Poll T, van Deventer SJH: Pathogenesis of disseminated intravascular coagulation in sepsis. JAMA 1993, 270:975–979
51. Harbrecht BG, Billiar TR, Stadler AJ, Ochoa DJ, Curran RD, Simmons RL: Inhibition of nitric oxide synthesis during endotoxemia promotes intrahepatic thrombosis and an oxygen radical-mediated hepatic injury. J Leukocyte Biol 1992, 52:390–394
52. Clancy RM, Leszczynska-Piziak J, Abramson SB: Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. J Clin Invest 1992, 90:1116–1121
53. Radomski MW, Palmer RM, Moncada S: An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA 1987, 87: 5193–5197
54. Natanson C, Hoffman WD, Suffredini AF, Eichacker PQ, Danner RL: Selected treatment strategies for septic shock based on proposed mechanisms of pathogenesis. Ann Intern Med 1994, 120:771–783
55. Cobb JP, Natanson C, Quezado ZM N, Hoffman WD, Koev CA, Banks S, Correa R, Levi R, Elin RJ, Hosseini JM, Danner RL: Differential hemodynamic effects of L-NMMA in endotoxemic and normal dogs. Am J Physiol 1995, 268:H1634–H1642
56. Vary TC, Kimball SR: Regulation of protein synthesis in chronic inflammation and sepsis. Am J Physiol 1992, 262:C445–C452
57. Tate RM, Repine JE: Neutrophils and the adult respiratory distress syndrome. Am Rev Respir Dis 1983, 128:552–559
58. Heflin AC Jr, Brigham KL: Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. J Clin Invest 1981, 68: 1253–1260
59. Colletti LM, Remick DG, Burtch GD, Kunkel SL, Strieter RM, Campbell DA Jr: Role of tumor necrosis factor-α in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. J Clin Invest 1990, 85:1936–1943
60. Hsueh W, Gonzalez-Crussi F, Arroyave JL: Platelet-activating factor: an endogenous mediator for bowel necrosis in endotoxemia. FASEB J 1987, 1:403–405
61. Terashita Z, Imura Y, Nishikawa K, Sumida S: Is platelet activating factor (PAF) a mediator of endotoxic shock? Eur J Pharmacol 1985, 109:257–261
62. Toth PD, Mikulaschek AW: Effects of a platelet-activating factor antagonist, CV-3988, on different shock models in the rat. Circ Shock 1986, 20:193–203