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Acute Hepatotoxicity of *Pseudomonas aeruginosa* Exotoxin A in Mice Depends on T Cells and TNF

Jens Schümann,* Sabine Angermüller,‡ Renate Bang,* Michael Lohoff,† and Gisa Tiegs²*

The most potent virulence factor of *Pseudomonas aeruginosa*, its exotoxin A (PEA), inhibits protein synthesis, especially in the liver, and is a weak T cell mitogen. This study was performed to correlate hepatotoxic and possible immunostimulatory features of PEA in vivo. Injection of PEA to mice caused hepatocyte apoptosis, an increase in plasma transaminase activities, and the release of TNF, IFN-γ, IL-2, and IL-6 into the circulation. Most strikingly, liver damage depended on T cells. Athymic nude mice or mice depleted of T cells by anti-Thy1.2 mAb pretreatment failed to develop acute hepatic failure, and survival was significantly prolonged following T cell depletion. Neutralization of TNF or lack of TNF receptors prevented liver injury. In the liver, TNF was demonstrated to induce liver damage not only by protein synthesis inhibition but also by TNF- and perforin-dependent, Fas-independent, apoptotic signals. *The Journal of Immunology*, 1998, 161: 5745–5754.

*Pseudomonas aeruginosa* is a Gram-negative, opportunistic pathogen that evokes respiratory, urinary tract, or skin infections as well as gastrointestinal disorders associated with enterocolitis and bowel perforation. Moreover, *P. aeruginosa* frequently causes septicemia in immunocompromised patients, and a high incidence of *P. aeruginosa* bacteremia was observed in patients with impaired barrier function of the liver (1, 2). *P. aeruginosa* is the fourth most common cause of primary, hospital-acquired, Gram-negative bacteremia and is usually associated with high resistance to antibiotic treatment and high mortality rates. *P. aeruginosa* bacteremia clinically resembles other forms of Gram-negative sepsis, i.e., common symptoms are fever, hypotension, refractory shock, adult respiratory distress syndrome, and renal failure (1). However, jaundice appears to occur more often than in other forms of Gram-negative sepsis (1).

The two bacterial products most likely implicated in the systemic toxicity of *P. aeruginosa* are its LPS and exotoxin A (PEA) (3). Purified exotoxin A is highly lethal for animals, including subhuman primates, and produces shock in dogs and rhesus monkeys. The biologic significance of exotoxin A for the pathogenicity of *P. aeruginosa* became evident by studies showing that patients with high levels of serum Abs to exotoxin A at the onset of *P. aeruginosa* septicemia have a better chance of survival than those with low Ab titers (1, 3). Moreover, in a mouse model it was shown that PEA-producing *P. aeruginosa* strains were more toxic than nonproducers, and that their toxicity could be weakened by Abs to PEA (4).

Hepatic injury due to systemic inflammatory processes has been reported to occur in the pathophysiology of septic shock (5). LPS from Gram-negative bacteria have been intensively studied with respect to their capacity to induce shock by stimulation of monocyte cells. These cells release TNF and other proinflammatory cytokines that mediate multiorgan failure and lethality in experimental animal models of endotoxic shock. More recently, it became evident that activation not only of macrophages but also of T cells may result in a systemic inflammatory response syndrome and organ injury (6). We recently described two models of T lymphocyte-dependent apoptotic and secondary necrotic liver injury induced by either the anti-mouse CD3 mAb 145 2C11 or the bacterial superantigen *Staphylococcus aureus* enterotoxin B (SEB) in δ-galactosamine (GalN)-sensitized mice (7, 8). In these models the T cell stimuli evoke a cytokine release syndrome. TNF was found to be the central mediator of hepatocellular apoptosis and the ensuing severe liver failure (7). The amino sugar GalN depletes uracil nucleotides selectively in the liver, thereby inhibiting hepatic transcription and translation (9) and sensitizing the liver toward T cell stimuli (7, 8), LPS (10–12), or TNF (13, 14).

Like GalN, PEA inhibits protein synthesis in mammalian cells, e.g., in hepatocytes (15, 16), thus being a bacterial toxin that possibly sensitizes the liver toward proinflammatory cytokines such as TNF. In human ovarian tumor cell lines, PEA induced apoptosis that was accelerated by TNF (17). This property of PEA led to the use of PEA-derived fusion proteins as immunotoxins for cancer treatment (18). In clinical trials in humans, the major side effects of immunotoxins are a vascular leak syndrome, fever, and hepatotoxicity (18, 19).

In the past, several reports have tried to draw a connection between PEA and T cells. In vitro, PEA led to mitogenic activation of T lymphocytes in murine whole splenocyte cultures and generated cytolytic T lymphocytes active against EL4 target cells (20). This apparent lack of an apoptotic signal on T cells may be explained by a minor sensitivity of lymphoid cells to inhibition of protein synthesis by PEA compared with the sensitivity of other mammalian cells (20). Moreover, PEA inhibited properties that

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*3 Abbreviations used in this paper: PEA, *Pseudomonas aeruginosa* exotoxin A; SEB, *Staphylococcus aureus* enterotoxin B; GalN, δ-galactosamine; HSA, human serum albumin; LAL, *Limulus* amebocyte lysate; ALT, alanine aminotransferase.

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are similar, but not identical with those of microbial superantigens; PEA selectively stimulated the proliferation of murine thymocytes expressing the Vβ8.2 chain in their TCR (21), and thymocyte proliferation depended on the presence of APCs (22, 23). However, in contrast to conventional superantigens, lymphoproliferative activity of PEA was found by others (25). Hence, the objective of this study was to further investigate the role of TNF and T cells in the toxicity of PEA in vivo.

Materials and Methods

Mice

Six- to 8-wk-old BALB/c mice were obtained from the institute’s internal animal breeding house. TNFR-deficient and the corresponding wild-type (C57BL/6 × 129Sv) mice were provided by Dr. H. Bluethmann, Hoffmann-La Roche (Basel, Switzerland). Perforin knockout mice were provided by Drs. J. Tschopp and M. Schröter, University of Lausanne, Institute of Biochemistry (Épalinges, Switzerland). Athymic BALB/C-nu/nu mice, Fas-deficient Ipr/Ipr mice, and mice of the corresponding wild-type strain MRL/Mp, as well as C3H/HeN and C3H/HeJ mice, were purchased from Charles River (Borchen, Germany). Animals received humane care according to National Institutes of Health guidelines as well as the legal requirements in Germany and were maintained under controlled conditions (22°C, 55% humidity, 12-h day/night rhythm) and were fed a standard laboratory chow (Altromin 1313, Altromin, Lage, Germany) ad libitum.

Animal treatments

All reagents were injected in a total volume of 250 μl/25 g mouse. PEA (Sigma, St. Louis, MO) was injected i.v. in pyrogen-free saline containing 0.1% human serum albumin (HSA). The LPS content of PEA was determined with the help of a commercially available Limulus amebocyte lysate (LAL) kit (Coatst Endotox, Chromogenex, Moheln, Sweden). In some experiments mice were treated with one of the following combinations of reagents:

1) 30 mg/kg i.v. of benzylxycarbonyl-Val-Ala-Asp fluoromethylyketone (Bachem Biochemica, Heidelberg, Germany) in saline/6% DMSO followed by PEA 15 min later; 2) 20 μl/mouse i.v. of the IgG fraction of a sheep anti-mouse TNF polyclonal antiserum (provided by Dr. A. Wendel, University of Konstanz, Konstanz, Germany) in saline/0.1% HSA followed by PEA 15 min later; 3) 200 μl/mouse i.v. of rabbit anti-mouse IFN-γ polyclonal antiserum in saline/0.1% HSA followed by PEA 15 min later; 4) 100 μl/mouse i.v. of anti-mouse TCR 12.1 mAb (IgG2a; Bio-Yeda, Rehovot, Israel); this treatment leads to depletion of 82% of T cells 24 h later (26) in saline followed by PEA 24 h later; 5) 100 μl/mouse i.v. of control Ab, i.e., rat IgG2a (PharMingen, Hamburg, Germany), in saline followed by PEA 24 h later; 6) 50 mg/kg i.v. of cyclosporin A in saline/10% placebo (Sandoz, Nurnberg, Germany); 15 and 1 h before PEA; 7) 2.5 mg/kg i.p. of SEB (Sigma) in saline/0.1% HSA followed by PEA 48 h later; 8) 2.5 mg/kg i.p. of SEB in saline/0.1% HSA followed by GalN/LPS (GalN, 700 mg/kg i.p.; SEB, 2.5 mg/kg i.p.) 48 h later; 9) 2.5 mg/kg i.p. of SEB in saline/0.1% HSA followed by GalN/LPS (GalN, 700 mg/kg i.p.; LPS: from Salmonella abortus equi, S form (Metalon, Ragow, Germany), 10 μg/kg i.p.) 48 h later; 10) 10 μg/kg recombinant murine TNF (provided by Dr. G. R. Adolfs, Bender & Co., Vienna, Austria) i.v. 15 min after injection of PEA in saline/0.1% HSA; 11) 10 μg/kg LPS from S. abortus equi, S form, given i.p. 15 min after injection of 3 μg/kg PEA in saline; and 12) 2.5 mg/kg SEB given i.p. 15 min after injection of 3 μg/kg PEA in saline/0.1% HSA.

Sampling of material

Mice were lethally anesthetized with 150 mg/kg pentobarbital i.v. containing a dose of 15 mg/kg heparin. Blood was withdrawn by cardiac puncture. Livers were perfused via the portal vein for 10 s with cold perfusion buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, and 10 mM EGTA, pH 8.0) before excision. In some experiments, perfusion was performed for 20 s via the right venticle of the heart before excision of liver, lung, ecuim, kidneys, and heart. Cem was luminaally perfused with saline to remove contents. All organs were blotted dry and disintegrated in cold perfusion buffer with an Elvehjem-type homogenizer. The 20% organ homogenate solution was centrifuged at 13,000 × g for 15 min. One part of the supernatant was to detect oligonucleosome-bound DNA fragments (see below). The other part was used to precipitate DNA by addition of 1 ml of ice-cold ethanol plus 50 μl of 3 M sodium acetate.

Analysis of liver enzymes and released proteins

Hepatocyte damage was assessed by measuring plasma enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase, and sorbitol dehydrogenase according to Bergmeyer (27) using an automated procedure. The cytokines TNF, IFN-γ, IL-2, and IL-6 were determined by ELISA (PharMingen). For determination of TNF, a polyclonal sheep anti-mouse TNF capture Ab (28), purified on protein G columns (Pharmacia, Freiburg, Germany) was used to replace the PharMingen capture mAb. IL-10, IL-1α, and IL-1β were measured using commercial ELISA kits (IL-10: Genzyme, Cambridge, MA; IL-1α: Endogen, Cambridge, MA; IL-1β: Paesel & Lorenz Co., Hanau, Germany).

DNA fragmentation

DNA fragmentation was quantified (29) by measuring cytosolic oligonucleosome-bound DNA using an ELISA kit (Boehringer Mannheim, Mannheim, Germany). Briefly, the cytosolic fraction (13,000 × g supernatant) from approximately 200 μg of liver was employed as Ag source in a sandwich ELISA with a primary anti-histone Ab coated to the microtiter plate and a secondary anti-DNA Ab coupled to peroxidase. DNA fragmentation was also analyzed semiquantitatively after extraction of the 13,000 × g supernatent (corresponding to ~80 mg of liver) by the phenol/chloroform method, precipitation by ethanol, and electrophoresis on 1.0% agarose gels (30).

Electron microscopic studies

For electron microscopic studies, the livers were fixed by perfusing the portal vein with a fixative containing 0.25% glutaraldehyde and 2% sucrose in 100 mM PIPES buffer at pH 7.4 for 5 min. Sections were postfixed with 2% aqueous osmium tetroxide, dehydrated in graded ethanol, and embedded in Epon 812. Ultrathin sections were counterstained with lead citrate and examined in a Philips EM 301 electron microscope (Eindhoven, The Netherlands).

Immunofluorescent staining and confocal laser imaging

Twelve-micrometer-thick cryostat sections of livers were thawed onto glass slides, air-dried, and fixed in acetone/methanol (1/1) for 10 min at 4°C before they were incubated in PBS containing 3% BSA for 30 min at room temperature. After the slides had been rinsed in PBS, incubation was continued with polyclonal rabbit anti-mouse-TNF Ab (Genzyme Virotech, Rüsselsheim, Germany; 1/750) together with rat mAb against murine pan-macrophage marker (clone BMR, DiaNova, Hamburg, Germany; 1/100), or mouse CD4 (clone RM4-5, PharMingen; 1/50), or mouse CD8 (clone Ly-2, PharMingen; 1/50) in PBS containing 3% BSA overnight at 4°C. After rinsing with PBS, binding sites were detected using swine anti-rabbit-IgG tagged with FITC (Dako, Hamburg, Germany; 1/30) and goat anti-rat IgG tagged with Texas Red (Dianova; 1/200) in PBS containing 3% BSA for 1 h at room temperature. After rinsing with PBS, sections were coveredslipped with TBS/glycerol (1/1), pH 8.6. Sections processed for immunofluorescence were examined by confocal laser scanning microscopy (MRC 1000, Bio-Rad, Richmond, CA).

Statistical analysis

The results were analyzed using Student’s t test if two groups were compared and the Dunnett’s t test if more groups were tested against a control group. If variances were inhomogeneous, the results were analyzed using the Welch test. The significance of prolonged survival was tested by comparison of survival curves with the log-rank test. All data in this study are expressed as the mean ± SEM. p < 0.05 was considered significant.

Results

PEA-induced liver injury

The i.v. injection of PEA into BALB/c mice dose-dependently induced liver cell damage within 12 h as assessed by an increase in the activities of plasma transaminases as well as enhanced activities of the liver-specific enzyme sorbitol dehydrogenase in plasma (Fig. 1). Administration of the highest dose, i.e., 300 μg/kg PEA, resulted in lethality within 16 h. The release of liver enzymes was preceded by the appearance of cytosolic oligonucleosome-bound DNA within the liver that was significantly enhanced 6.5 h following PEA administration (Fig. 2). Intermesosomal DNA fragmentation as a measure of programmed cell death was also
demonstrated on an agarose gel. The appearance of the DNA ladder again preceded the increase in plasma ALT (Fig. 3, lanes 1–3). The unspecific tripeptidic caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk), given in a dose that protected mice from GalN/TNF-induced liver injury (31) (data not shown), also significantly inhibited transaminase release following PEA challenge (buffer plus PEA: ALT, 8970 ± 235 U/l; Z-VAD.fmk plus PEA: ALT, 990 ± 215 U/l (p ≤ 0.05); n = 6). The inhibitor had no effect on PEA-induced TNF production, i.e., Z-VAD.fmk did not reduce the amount of circulating TNF (data not shown). Together these data clearly suggest a role for apoptotic proteases in PEA-dependent liver failure.

To explore whether other organs besides the liver were injured by PEA, we quantified DNA fragmentation in lung, cecum, kidney, and heart (32) compared with that in liver. An increase in cytosolic oligonucleosome-bound DNA was most prominent within the liver and the lung (data not shown).

To determine which cells in the liver died by apoptosis, we set up transmission electron micrographs of mouse livers excised 12 h following PEA injection. Micrographs clearly show that the dying cells were hepatocytes (Fig. 4, a and b). They died by apoptosis (Fig. 4, a and b) and necrosis (Fig. 4b), whereas neither apoptosis nor necrosis was seen in livers of saline-treated mice (33). Necrotic liver areas contained infiltrated polymorphonuclear neutrophils (Fig. 4b). As a cause of damage, perfusion of the livers with the fixative was incomplete. Hence, blood cells were still located in the hepatic sinusoids (Fig. 4c). Fig. 4c also shows a lymphocyte sticking to sinusoidal endothelium. Some lymphocytes infiltrating into the hepatic tissue were observed. One example is shown in Fig. 4d. Fig. 4, a–d, also demonstrates that hepatocytes of PEA-treated mice contained numerous lipid droplets. The development of a fatty liver most likely results from an inhibition of apolipoprotein synthesis by PEA.

Inhibition of transcription by GalN sensitizes the liver toward LPS (10, 12, 33). Because PEA, like GalN, has protein synthesis inhibitory activities, it had to be excluded that PEA was merely sensitizing the mice to contaminating LPS present either in the PEA preparation or in the intestines of mice. However, the following experiments argue against this idea. 1) As tested by the LAL assay, the contaminating LPS dose (1.4 ng/kg) injected together with PEA (300 μg/kg) was 7000-fold lower than the LPS dose (10 μg/kg) used in our laboratory to induce liver injury in GalN-sensitized BALB/c mice. 2) Translocation of LPS from the intestine was excluded by showing that LPS was below 15 pg/ml in plasma 3.5, 8, and 12 h following injection of 300 μg/kg PEA to mice as determined by the LAL assay. 3) LPS-resistant C3H/HeJ mice were as sensitive as LPS-responsive C3H/HeN mice toward PEA (ALT, 1247 ± 881 vs 833 ± 525 U/l; n = 3). Therefore, the involvement of LPS in the induction of PEA-induced liver damage is very unlikely.

**PEA-induced cytokine release**

As PEA has been described to stimulate the proliferation of T cells in vitro (20, 21), it seemed feasible that the toxin also stimulates cytokine production in vivo. By determination of plasma cytokines following PEA administration to mice we found a time-dependent release of the proinflammatory cytokines TNF and IL-6 as well as of the T cell cytokines IL-2 and IFN-γ (Fig. 5). IL-1α, IL-1β, and IL-10 were not detectable in plasma of PEA-treated animals (IL-1α, ≤6 pg/ml; IL-1β, ≤1 pg/ml; IL-10, ≤15 pg/ml). Thus, cytokine analysis suggests that PEA also activates T lymphocytes in vivo.
TNF as a mediator of PEA hepatotoxicity

Because TNF has been identified as a common mediator of hepatocellular apoptosis and liver injury in experimental mouse models (7, 29, 33, 34), we wondered whether TNF is also a mediator of PEA-induced liver damage.

Although TNF was not detectable in plasma until 12 h after administration of PEA (Fig. 5), i.e., at a point when liver damage had already developed, TNF was locally produced within the liver as early as 3 h after administration of PEA to BALB/c mice. This was shown by means of immunofluorescent staining followed by confocal laser imaging (Fig. 6). TNF was colocalized with resident liver macrophages, which were stained with the BM8 murine pan-macrophage marker (Fig. 6A, PEA 3 h), indicating that TNF was produced by Kupffer cells at this early time point. CD4- and CD8-positive T cells were present within the liver 3 h after PEA injection in amounts comparable to those observed at time zero. These cells did not produce TNF (Fig. 6, B and C, PEA 3 h). TNF was also detectable on the surface of hepatocytes, but not inside these cells, suggesting that macrophage-produced TNF bound to liver parenchymal cells (Fig. 6, PEA 3 h). At later time points, i.e., 6 and 9 h after administration of PEA, hepatic TNF was not detectable by immunostaining (data not shown). Twelve hours after challenge with PEA the number of CD8-positive T cells within the liver was slightly increased (Fig. 6C, PEA 12 h), whereas the number of CD4-positive T cells remained unchanged (Fig. 6B, 12 h). Macrophages had disappeared (Fig. 6A, PEA 12 h), and TNF was still undetectable in liver sections (Fig. 6, PEA 12 h). This indicates that the systemic TNF concentrations observed 12 h following
PEA injection were produced extrahepatically. T cells were essential for TNF production of liver macrophages. In mice depleted from T cells by an anti-mouse Thy1.2 mAb (26) (see also below), there was no detectable TNF production within the liver 3 h after administration of PEA (Fig. 6, anti-Thy1.2/PEA 3 h), whereas macrophage staining remained unaltered (Fig. 6A, anti-Thy1.2/PEA 3 h). CD4- and CD8-positive T cells were completely depleted from hepatic tissue in anti-Thy1.2-pretreated animals (Fig. 6, B and C, anti-Thy1.2/PEA 3 h). Because there was no staining by the anti-TNF Ab within the livers of untreated mice and anti-Thy1.2-pretreated mice 3 h after PEA administration, unspecific binding of this Ab to macrophages can be excluded. Taken together, these results show that T cells are required for Kupffer cell-dependent early TNF production following PEA intoxication in mice.

To prove a functional involvement of TNF in the development of PEA-induced liver injury, we pretreated mice with a specific polyclonal anti-mouse TNF Ab that completely neutralized circulating TNF following PEA challenge (TNF plasma concentration 12 h after PEA alone, 97 ± 18 pg/ml vs undetectable amounts (≤10 pg/ml) in the Ab-pretreated group; n = 5). This Ab significantly inhibited DNA fragmentation as well as ALT release (Fig. 7A). Inhibition of internucleosomal DNA fragmentation in the liver by anti-TNF Ab pretreatment was also demonstrated on an agarose gel (Fig. 3, lanes 3 and 4). Moreover, TNFR1 knockout (tnfr1°) as well as TNFR2 knockout (tnfr2°) mice were significantly protected from PEA-induced liver failure (Fig. 7B). In contrast to PEA-challenged tnfr1° mice, DNA fragmentation in livers of PEA-treated tnfr2° mice was not significantly reduced compared with that in wild-type animals, indicating that hepatic apoptosis is primarily mediated via TNFR1. However, because tnfr2° mice were significantly protected from severe hepatic failure as measured by ALT release, it seems likely that either costimulatory signals provided by TNFR2 (35) or ligand passing (36) are necessary for TNFR1-induced hepatocellular death.

Inhibition of transcription has been described to sensitize mice toward i.v. administered recombinant murine TNF, which, in turn, develop severe liver injury and eventually die (13, 14, 29). To study whether PEA sensitizes the liver against TNF, we treated mice with a subtoxic dose of PEA (3 μg/kg) and recombinant murine TNF (10 μg/kg). Neither PEA nor TNF given alone induced the release of liver enzymes, whereas the combination of both resulted in severe liver injury (PEA alone: ALT, 36 ± 8 U/L; TNF alone: ALT, 46 ± 12 U/L; PEA plus TNF: ALT, 12,795 ± 2,496 U/L (p ≤ 0.05); n = 3). Similar results were obtained when mice were treated with subtoxic doses of PEA (3 μg/kg) plus LPS (10 μg/kg) or PEA (3 μg/kg) plus SEB (2.5 mg/kg; PEA alone: ALT, 36 ± 8 U/L; LPS alone: ALT, 52 ± 6 U/L; PEA plus LPS: ALT, 4744 ± 2227 U/L (p ≤ 0.05); SEB alone: ALT, 10 ± 3 U/L; PEA plus SEB: ALT, 8,663 ± 2,242 (p ≤ 0.05) U/L; n = 3). We conclude that PEA sensitizes the liver toward TNF, e.g., induced by LPS during systemic Pseudomonas infections or coinfections with other bacteria.

**T cell dependence of PEA-induced toxicity**

In vitro, PEA was shown to be a polyclonal activator of cytolytic T lymphocytes, effective against Con A-treated target cells (20). Furthermore, as described above, T cells were required for Kupffer cell-dependent early TNF production following PEA intoxication in mice. To test whether T lymphocytes accounted for part of the in vivo toxicity of PEA, we pretreated mice with the T cell-depleting anti-Thy1.2 mAb (26). This treatment significantly protected mice from PEA-induced liver damage (Fig. 8A) and prevented hepatic internucleosomal DNA fragmentation (Fig. 3, lanes 3 and 5). Pretreatment with control Abs, i.e., rat IgG2a, failed to protect mice from PEA-induced liver failure (data not shown). Similarly, athymic BALB/c nu/nu mice lacking mature T cells were resistant toward PEA-induced liver injury (Fig. 8A). Circulating cytokine levels, including TNF, were significantly reduced or were not detectable in anti-Thy1.2 mAb-pretreated or nu/nu mice (Fig. 8B). In conclusion, the production of TNF and other cytokines as well as liver injury clearly depends on T lymphocytes.

As cyclosporin A is known to suppress T cell activation, the role of T cells in PEA-induced liver injury was supported by our finding that ALT release was inhibited in cyclosporin A-pretreated compared with placebo-pretreated mice following PEA injection (placebo-pretreated: ALT, 1679 ± 247 U/L; cyclosporin A-pretreated: ALT, 544 ± 47 U/L (p ≤ 0.05); n = 3).

Recently, Muraille et al. described a Vβ-unrestricted, T cell-unresponsive state together with an APC defect and a selective decrease in splenic dendritic cell numbers in mice 48 h after SEB injection (37). A similar unresponsive state has been described by Miettke et al., who observed that SEB pretreatment protected mice not only from lethality induced by the very same superantigen, i.e., SEB, but also by a third party superantigen with nonoverlapping Vβ specificity, i.e., toxic shock syndrome toxin-1 (38). Hence, we wondered whether SEB pretreatment would also protect mice from PEA-induced liver injury. Indeed, PEA-induced ALT release was
significantly reduced in SEB-pretreated mice (Fig. 8C). The same SEB pretreatment also protected the animals from GalN/SEB-dependent transaminase release but not from GalN/LPS-induced liver failure (Fig. 8C), again backing up a prominent role of T cells for PEA hepatotoxicity. The release of TNF into the circulation, determined at the time of maximal systemic TNF production (6, 39), was suppressed in SEB-pretreated mice challenged with PEA or GalN/SEB, but not in SEB-pretreated mice challenged with GalN/LPS (Fig. 8C).

To study a positive effect of T cell depletion on survival, mice were pretreated with the anti-Thy1.2 mAb and challenged with 30 $\mu$g/kg PEA i.v. Survival was significantly prolonged by anti-Thy1.2 mAb pretreatment (Fig. 8D). Mice treated with 30 $\mu$g/kg PEA developed liver injury within 24 h (ALT, 1227 ± 271 U/L; n = 5) that was again prevented by anti-Thy1.2 mAb-dependent T cell depletion (ALT, 139 ± 18 U/L; n = 5; p ≤ 0.05). The enhanced survival of the anti-Thy1.2 mAb-pretreated animals points to a prominent role of T cells for PEA-induced toxicity. This finding was corroborated by a significantly prolonged survival of SEB-pretreated compared with nonpretreated mice following intoxication with 30 $\mu$g/kg PEA (Fig. 8D).

Due to the fact that PEA was described to be a polyclonal activator of cytolytic T cells in vitro (20) and our observation of a slight increase in the amount of CD8-positive T cells in the liver 12 h after challenge with PEA (cf, Fig. 6C, PEA 12 h), we wondered whether effector molecules of cytolytic T cells might be involved in PEA-induced hepatotoxicity. Perforin knockout (perforin°) mice (40, 41) were significantly, but not completely, protected from PEA-induced liver injury (Fig. 9). In contrast, mice lacking functional Fas/CD95/APO-1 (lpr/lpr) were more susceptible to PEA than corresponding wild-type mice (Fig. 9). Two of five lpr/lpr mice did not survive the 12 h, while all wild-type MRL/Mp mice were alive. These results suggest that CTL are at least partially involved in PEA-induced hepatotoxicity using the
perforin-dependent pathway. Fas, in turn, expressed by the T cells could possibly slow down the liver damage by mediating activation-induced T cell death (42, 43).

Discussion

Previous experimental work provided evidence for sensitization of mice by inhibitors of hepatic transcription and translation, e.g., the amino sugar GalN, toward either bacterial toxins such as LPS, SEB, and toxic shock syndrome toxin-1 or their common mediator TNF. All these models, which are often referred to as endotoxic or T cell-mediated lethal shock in mice (6, 10, 13, 44, 45), have in common the development of a fulminant hepatitis (7, 11, 12, 14, 29, 33, 45). The mechanism of sensitization has been discussed as inhibition of synthesis of TNF-induced cytoprotective proteins (29). The sensitizing agents used in these studies were either GalN, which specifically inhibits hepatic transcription (9, 33), or actinomycin D (29) or a-amanitin (11), which usually do not affect patients suffering from septicemia.

Our results presented here demonstrate for the first time that a bacterial toxin affecting the host during bacteremia, i.e., the protein synthesis inhibitor PEA, sensitizes the liver and probably also other organs toward either LPS or SEB or their common mediator TNF. This was shown by injection of subtoxic doses of PEA together with recombinant murine TNF, LPS, or SEB to mice and by demonstrating the development of liver damage. Moreover, toxic doses of PEA induced liver damage by themselves and stimulated the release of TNF and other cytokines by the immune system. Experiments using neutralizing Abs clearly identified TNF as a mediator of PEA hepatotoxicity (39, 6), and fulminant hepatitis in GalN-sensitized mice within 8 h (7, 12), PEA-induced plasma TNF was only measurable 12 h after injection of the toxin, i.e., at a time when hepatic necrosis was already detectable. However, circulating cytokine levels only insufficiently reflect local tissue concentrations, a concept that has been described as the tip of the iceberg (46). Indeed, after administration of very high doses of PEA to mice, plasma TNF was detectable by 90 min (data not shown). Furthermore, following injection of 300 µg/kg PEA, local hepatic TNF production was observed by 3 h after intoxication, as shown by means of immunofluorescent staining. This early appearing TNF was produced by Kupffer cells, but not by T cells. However, T cells were required for Kupffer cell-dependent early TNF production, providing evidence of cross-talk between T lymphocytes and liver macrophages. Such cross-talk has been previously suggested to occur upon Con A stimulation of lymphocyte/macrophage cocultures in vitro (47). Interestingly, we observed two phases of TNF production in PEA-treated mice. The first peak occurred after 3 h, and this TNF was found to be locally produced by liver macrophages. The second increase, measurable as systemic TNF, appeared after 12 h. A similar time course of TNF release was recently demonstrated by Toyabe et al. in a T cell-dependent hepatitis model in mice (48). It seems likely that Kupffer cells become activated by resident liver T cells upon PEA stimulation, because there was no additional infiltration of CD4- or CD8-positive T cells into liver tissue following PEA injection. Twelve hours after PEA challenge, Kupffer cells were completely depleted. This is probably a result of PEA-induced ongoing ADP ribosylation of translation elongation factor 2 in these phagocytic cells, which is the mechanism by which PEA inhibits protein synthesis. In contrast, T cells seem to be resistant to direct killing by PEA, because their cell number did not decrease in liver sections even 12 h after PEA injection. This is in line with earlier in vitro findings showing only marginal and short-lived inhibitory effects of PEA on protein synthesis in lymphoid cells (20). The number of CD8-positive T cells in the liver even increased 12 h after PEA challenge, providing a possible explanation for the dependence of liver injury on perforin. These findings support an earlier report describing PEA as a polyclonal activator of cytolytic T lymphocytes in vitro (20). TNF was not detectable in liver sections 12 h after administration of PEA, demonstrating that the systemic TNF measurable at this time point was produced outside the liver. This late TNF might be extensively synthesized by T cells in lymphoid organs such as spleen or lymph nodes.

Besides the protective effect of anti-TNF Ab against PEA, further evidence of the critical role of TNF was shown by the resistance of tnfr1° and tnfr2° mice to PEA-induced hepatotoxicity. As described earlier, the p55 TNFR1 mediates liver injury and lethality in GalN/LPS-treated mice (45, 49) as well as TNF-induced hepatocyte apoptosis (50). In contrast, the blockade or the absence of p75 TNFR2 failed to protect mice from GalN/LPS-induced lethality (49, 51), and tnfr2° mice exhibited enhanced sensitivity to GalN/TNF-induced hepatic damage compared with the corresponding wild-type mice (G. Tieg and G. Künzle, unpublished observations). However, we recently found in a different TNF- and T cell-dependent hepatitis model, which is induced by Con A and is independent of transcriptional inhibition (34), that tnfr1° as well as tnfr2° mice were protected from liver injury. This was explained by providing evidence of a critical role of transmembrane TNF (52) that has previously been described to cooperatively signal via both TNFRs (35). Hence, PEA-induced hepatocellular toxicity is possibly mediated by direct cytolytic action of Kupffer cell-derived transmembrane TNF and its activation of both TNF receptors (35).

An alternative explanation may be given in view of the low TNF concentrations observed following PEA injection and the concept of ligand passing (36) from the high affinity TNFR2 to TNFR1, which, in turn, transduces cytotoxic signaling. We have in vivo evidence for the latter mechanism from experiments proving a significant hepatoprotection of tnfr1°, but enhanced sensitivity of
tnfr2° mice following administration of very high doses of PEA (data not shown).

The PEA-induced hepatotoxicity was clearly dependent on T cells. This was proven by the protective effect of T cell depletion by anti-Thy1.2 mAb, by the inability of nu/nu mice to develop liver injury, by the protective effect of cyclosporin A, and by the prevention of PEA-dependent liver failure following SEB pretreatment. Notably, a substantial inhibition of protein synthesis by PEA in mice was only observed in the liver, whereas the spleen was much less affected (15). Furthermore, T cells were not depleted from the liver by PEA in our experiments. These findings suggest that despite inhibition of hepatic protein synthesis, T cell stimulation by PEA was still possible in vivo. The lack of T cells in nu/nu mice or following anti-Thy1.2 mAb-dependent depletion correlated with significantly reduced levels of plasma cytokines upon PEA challenge, and T cell depletion abolished early TNF production by Kupffer cells, indicating that T lymphocytes are necessary for the production of TNF and other cytokines. Suppression of T cell activation by cyclosporin A provided protection from PEA-induced liver injury, as it did in the case of Con A-induced, T cell-dependent liver failure (26) or lethal toxicity of the superantigen SEB in GalN-sensitized mice (6). Moreover, the induction of an immunosuppressed state characterized by Vβ-unrestricted T cell unresponsiveness and defective APC functions 48 h after SEB pretreatment (37) also provided protection from PEA-dependent liver injury accompanied by suppression of TNF release. Because SEB pretreatment only protects from the deleterious effects of T cell mitogenic agents, not from LPS, this result again emphasizes the key role of T cells in PEA-induced liver failure. Furthermore, the prominent role of T cells in PEA-induced toxicity was shown by the prolonged survival of anti-Thy1.2- as well as SEB-pretreated mice. However, T cell depletion was not life saving. Furthermore, immediately before death, high activities of plasma transaminases were detectable in T cell-depleted mice (data not shown).

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FIGURE 8. T cell dependence of PEA-induced hepatotoxicity, cytokine release, and lethality. A, BALB/c mice pretreated with anti-Thy1.2 mAb 24 h before challenge as well as athymic nude/nude mice (nu/nu) of the strain BALB/c received 300 μg/kg PEA i.v. Twelve hours after challenge blood was withdrawn for ALT determination, and liver homogenates were prepared for quantification of oligonucleosomal hepatic DNA fragmentation with the help of a commercially available cell death detection ELISA. Data are expressed as the mean ± SEM (n = 5). *, p ≤ 0.05 vs wild-type control. B, In the same experiment as that described in A, the blood samples were also employed for cytokine determinations. All cytokines (TNF, IL-6, IFN-γ, and IL-2) were determined by ELISA. Data are expressed as the mean ± SEM (n = 5). *, p ≤ 0.05 vs wild-type control. C, BALB/c mice pretreated with SEB 48 h before challenge received PEA (300 μg/kg i.v.) or GalN/SEB (GalN, 700 mg/kg i.p.; SEB, 2.5 mg/kg i.p.) or GalN/LPS (GalN, 700 mg/kg i.p.; LPS, 10 μg/kg i.p.). Control animals were pretreated with 0.1% HSA instead of SEB. Twelve hours (PEA) or 8 h (GalN/SEB and GalN/LPS) after challenge blood was withdrawn for ALT determination. TNF was determined 12 h (PEA), 3 h (GalN/SEB), or 1.5 h (GalN/LPS) following mouse treatment, respectively.

FIGURE 9. PEA hepatotoxicity in perforin-deficient mice and Fas-mutated lpr/lpr mice. Perforin° mice, lpr/lpr mice, as well as mice of the corresponding wild-type mice (C57BL/6 × 129 and MRL/Mp, respectively) received 300 μg/kg PEA i.v.; 12 h after challenge blood was withdrawn for ALT determination. Data are expressed as the mean ± SEM (n = 3 for perforin°; n = 5 for lpr/lpr). Two of five lpr/lpr mice did not survive 12 h. *, p ≤ 0.05 vs corresponding wild-type mice.
shown). Hence, liver damage might be the cause of death in these mice also. This points to a multifactorial mechanism leading to liver injury and death in PEA-treated mice that includes T cell activation and production of TNF. The release of transaminases in T cell-depleted mice at the time of death could be explained in terms of ongoing ADP ribosylation of elongation factor-2 in hepatocytes (15). Nevertheless, it has to be emphasized that T cell activation significantly contributes to PEA toxicity. In athymic BALB/c nude mice, which were shown to be protected from acute liver injury, survival was not significantly improved compared with that in wild-type BALB/c mice (data not shown). Again, at the time of death, high amounts of plasma transaminases were detectable in nude mice (data not shown). Hence, the results obtained from nude mice corroborate the findings from T cell-depleted mice concerning the role of T cells in acute hepatotoxicity and the significance of liver failure for death. However, the lack of prolonged survival of nude mice points to the existence of additional mechanisms that might have developed in these mice as a consequence of a whole life without T cells. These differences include higher amounts of NK cells and the presence of extrathymically matured T cells, which possibly caused the increased susceptibility of nude mice compared with that of mice only deficient in responsive peripheral T cells.

Previous in vitro findings suggested that PEA is a superantigen, as it selectively stimulated the proliferation of murine thymocytes expressing Vβ8.2 in their TCR (21). PEA induced the proliferation of murine splenocytes (20) (data not shown). However, we failed to observe specific accumulation of either CD4+ or CD8+ T cells expressing the Vβ8.1 or Vβ8.2 chain in their TCR (data not shown). Hence, our preliminary results together with previous findings by others (25) argue against PEA being a superantigen.

Besides TNF, perforin was found to mediate PEA hepatotoxicity, as suggested by a significantly reduced susceptibility of perforin knockout mice to PEA-induced liver injury. TNF and other proinflammatory cytokines may indirectly activate cytotoxic T cells (53–55), thereby being responsible for additional hepatocytotoxic signals by the perforin/granzyme system. In contrast, the second important mediator of cytotoxic lymphocytes, i.e., Fas ligand (40, 41), seems unlikely to induce hepatic cellular damage in our model; lpr/lpr mice lacking functional Fas suffered even more from PEA toxicity than corresponding wild-type mice. This result suggests a functionally different role of Fas in PEA-inducible T cell-dependent liver injury compared with the results obtained by Kondo et al., who demonstrated a protective effect of soluble Fas-Fc in two different murine hepatitis models (56). One possible explanation might be a suppression of Fas-dependent activation-induced T cell death (42, 43) following PEA stimulation, thereby aggravating liver injury via the TNF and perforin pathways. Peripheral clonal deletion of T cells has been shown to be impaired in lpr/lpr mice following SEB (57) or Ag (58) injection in vivo. Our results are well matched with previous findings showing that Fas-deficient lpr/lpr mice were more susceptible than wild-type mice to GalN/SEB-induced lethality (59), which is mediated by T cells as well.

In conclusion, we propose a sequential stimulation of the immune system by PEA resulting in hepatocytotoxicity (Fig. 10): T cell activation by PEA results in the production of the central mediator TNF by Kupffer cells, which, in turn, induces apoptotic signals in hepatocytes via the TNFR1, whereas TNFR2 possibly passes the ligand or costimulatory signals to TNFR1. The inhibitory effect of PEA on protein synthesis sensitizes the liver to the cytotoxic action of TNF. TNF and other cytokines may also activate cytotoxic T cells, providing additional hepatocytotoxic signals by the perforin/granzyme system. The continual immune stimulation and hepatocytotoxic processes finally result in severe liver failure, which causes lethality. Hence, protein synthesis inhibition during Pseudomonas septicemia is likely to be a relevant sensitization mechanism to the cytotoxic action of TNF, which is produced not only upon direct stimulation of monocytes/macrophages by LPS but also via T cell activation with PEA.

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References

Ku¨sters, S., F. Gantner, G. Ku¨nstle, and G. Tiegs. 1996. Interferon-
Methods of Enzymatic Analysis, 14.
Tiegs, G., M. Wolter, and A. Wendel. 1989. Tumor necrosis factor is a terminal
Lehmam, V., M. A. Freudenberg, and C. Galanos. 1987. Lethal toxicity of lipo-
poly saccharide and tumor necrosis factor in normal and ß-galactosamine-
Wendel, A., and G. Tiegs. 1986. A novel biologically active seleno-organic com-
ound. VI. Protection by ebselen (PZ 51) against galactosamine/endotoxin-in-
Iglesiwas, B. H., P. V. Liu, and D. Kabat. 1977. Mechanism of action of Pseudo-
monas aeruginosa exotoxin A: adenosine diphosphate-ribosylation of mamma-
monas aeruginosa exotoxin A. Infect. Immun. 58:978.
Leist, M., F. Gantner, I. Bohlanger, P. G. Germann, G. Ties, and A. Wendel. 1994. Murine hepatic apoptosis induced in vitro and in vivo by TNF-a re-