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Acute Hepatotoxicity of Pseudomonas aeruginosa Exotoxin A in Mice Depends on T Cells and TNF1

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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 produced by Kupffer cells before hepatocellular death occurred. After T cell depletion, Kupffer cells failed to produce TNF.

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 non-producers, 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 monocytic 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 O-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 exhibited properties that
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 prolif-eration depended on the presence of APCs (22, 23). However, in contrast to conventional superantigens, lymphoproliferative activity required intracellular processing (24), and no superantigenic activity of PEA was found by others (25). Hence, the objective of this study was to further investigate the role of TNE 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 × 129/Sv) 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, Insti-tute of Biochemistry (Eapolias, Switzerland). Atymic BALB/c-nu/nu mice, Fas-deficient lpr/lpr mice, and mice of the corresponding wild-type strain BALB/Mp, as well as C3H/HeN and C3H/HeJ mice, were purchased from Taconic (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 HSA content of PEA was deter-mined with the help of a commercially available Limulus amoeocyte lysate (LAL) kit (Coatst Edostant Toximogenex, Mohrad, Sweden). In some experiments mice were treated with one of the following combinations of reagents: 1) 30 mg/kg i.v. of benzoylcarbonyl-Val-Ala-Asp fluorometh-yketone (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 antisem (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 antisem in saline/0.1% HSA followed by PEA 15 min later; 4) 100 μl/mouse i.v. of anti-mouse Thyl.1.2 mAb (IgG2a; BioYeda, Re-hovot, Israel; this treatment leads to depletion of 82% of T cells 24 h later (26)); 5) 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 min before the first PEA injection; 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.v. of SEB in saline/0.1% HSA followed by PEA 48 h later; 9) 2.5 mg/kg i.p. of GalN/SEB (GalN, 700 mg/kg i.p.; SEB, 2.5 mg/kg i.p.) 48 h later; 10) 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 i.p.); 48 h later; 10) 10 μg/kg recombinant human TNF (provided by Dr. G. R. Adolf, 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. contain-ing 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 with an Elvehjem-type homogenizer. The 20% organ homogenates were centrifuged at 13,000 × g for 20 s via the right ventricle of the heart before excision of liver, lung, cecum, kidneys, and heart. CEC was luminafully 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 homogenates were 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.

Hepatocyte damage was assessed by measuring plasma enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase, and sorbil dehydrogenase according to Bergmeyer (27) using an automated pro-cedure. 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 (Ab (PharMingen), 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 fragmenta-tion was also analyzed semiquantiitatively after extraction of the 13,000 × g supernatant (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 embed-ded 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, Russelheim, Germany; 1/750) together with rat mAb against murine pan-macrophage marker (clone B6, 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 coveredslapped 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 analyses

The results were analyzed using Student’s t test if two groups were com-pared and the Dunnett’s 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 compari-on 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). Internucleosomal 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 ? 2355 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 ?g/kg) was 7000-fold lower than the LPS dose (10 ?g/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 (detection limit) in plasma 3.5, 8, and 12 h following injection of 300 ?g/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-? (Fig. 5). IL-1? (IL-1? and IL-1? were not detectable in plasma of PEA-treated animals (IL-1? ? 6 pg/ml; IL-1? ? 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 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

FIGURE 4. Electron micrographs of mouse livers 12 h after treatment with 300 μg/kg PEA. a. Liver tissue containing an apoptotic cell with typical condensed chromatin at the nuclear membrane, and chromatin emerged outside the nuclear membrane (arrows). Apoptotic bodies (AB) have been phagocytized by neighboring hepatocytes. Hepatocytes contain numerous lipid droplets (L). Magnification, ×3500. b. Necrotic liver area with two polymorphonuclear neutrophils (PMN) and an apoptotic body (arrows). Hepatocytes contain numerous lipid droplets (L). Magnification, ×5200. c. A polymorphonuclear neutrophil (PMN), a lymphocyte (LYM), and two erythrocytes are seen in a liver sinusoid. Hepatocytes contain numerous lipid droplets (L). Magnification, ×3100. d. A lymphocyte (LYM) has moved between two hepatocytes, touching one of them. Hepatocytes contain numerous lipid droplets (L). Magnification, ×9000.
Thy1.2/PEA 3 h]. CD4- and CD8-positive T cells were completely
neutralizing TNF following PEA challenge (TNF plasma concentration
polyclonal anti-mouse TNF Ab that completely neutralized circu-
lation for TNF production of liver macrophages. In mice depleted
PEA injection were produced extrahepatically. T cells were essen-
tial for TNF production of liver macrophages. In mice depleted
PEA-induced cytokine release. BALB/c mice received 300 
μg/kg PEA i.v.; at the time points indicated blood was withdrawn for
cytokine determination. All cytokines (TNF, IL-6, IFN-γ, and IL-2) were
determined by ELISA. Data are expressed as the mean ± SEM (n = 3–6).
*, p < 0.05 vs 0 h.

FIGURE 5. PEA-induced cytokine release. BALB/c mice received 300 
μg/kg PEA i.v.; at the time points indicated blood was withdrawn for
cytokine determination. All cytokines (TNF, IL-6, IFN-γ, and IL-2) were
determined by ELISA. Data are expressed as the mean ± SEM (n = 3–6).
*, p < 0.05 vs 0 h.

To prove a functional involvement of TNF in the development
of PEA-induced liver injury, we pretreated mice with a specific
polyclonal 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,
un-specific 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 circu-
lating 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 signif-
ificantly 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
(mifr1°) as well as TNFR2 knockout (mifr2°) mice were signifi-
cantly protected from PEA-induced liver failure (Fig. 7B). In con-
trast to PEA-challenged mfr1° mice, DNA fragmentation in livers of
PEA-treated mfr2° mice was not significantly reduced compared
with that in wild-type animals, indicating that hepatic apo-
poptosis is primarily mediated via TNFR1. However, because mfr2°
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 nec-
essary 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 in-
duced 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-de-
pleting anti-Thy.1.2 mAb (26). This treatment significantly pro-
tected mice from PEA-induced liver damage (Fig. 8A) and pre-
vented 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-Thy.1.2 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 find-
ing that ALT release was inhibited in cyclosporin A-pretreated
compare with placebo-pretreated mice following PEA injection
(placebo-pretreated: ALT, 1679 ± 247 U/L; cyclosporin A-pre-
treated: 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
decay 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 μg/kg PEA i.v. Survival was significantly prolonged by anti-Thy1.2 mAb pretreatment (Fig. 8D). Mice treated with 30 μg/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 μg/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

**FIGURE 6.** Confocal laser images of liver sections from PEA-treated mice following immunofluorescent staining of TNF together with macrophages, CD4+ T cells, or CD8+ T cells. BALB/c mice were treated as indicated (PEA, 300 μg/kg i.v.; anti-Thy1.2 mAb, 24 h before PEA). Twelve-micrometer cryostat sections of livers from these mice were double stained by use of a polyclonal rabbit anti-mouse TNF Ab (secondary swine anti-rabbit IgG tagged with FITC, green fluorescence) together with one of the following Abs: A, rat anti-mouse macrophage, clone BM8 (secondary goat anti-rat IgG tagged with Texas Red, red fluorescence); B, rat anti-mouse CD4, clone RM4-5 (secondary goat anti-rat IgG tagged with Texas Red, red fluorescence); and C, rat anti-mouse CD8, clone Ly-2 (secondary goat anti-rat IgG tagged with Texas Red, red fluorescence). All sections were examined by confocal laser scanning microscopy. Costaining is represented by yellow fluorescence.
mice of the corresponding strain C57BL/6J received 300 μg/kg PEA i.v. 15 min after pretreatment with neutralizing anti-mouse polyclonal TNF Abs. Control animals received 0.1% HSA containing saline instead of Abs. B, *tnfr1°* and *tnfr2°* mice as well as wild-type mice of the corresponding strain C57BL/6 × 129 received 300 μg/kg PEA i.v. A and B. 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 (A, n = 6; B, n = 3). *, p ≤ 0.05 vs control or wild-type mice.

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 α-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 central mediator of this hepatic failure. Liver injury was characterized by early apoptosis that preceded the release of transaminases. A similar effect of TNF has been described in the case of coadministration of actinomycin D and TNF (29), GalN and TNF (33), or GalN and SEB (7). PEA-induced hepatocyte death was inhibited by the unspecific caspase inhibitor Z-VAD.fmk, as it was recently shown for GalN/TNF-induced apoptotic and secondary necrotic liver cell death (31).

In contrast to LPS or SEB, which induce systemic peak concentrations of TNF within 1 or 3 h, respectively (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 re-
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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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 hepatocellular 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. Peripherally 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 hepatotoxicity (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 TNF receptors. 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 stimula-

![FIGURE 10. Proposed sequential stimulation of the immune system by PEA resulting in hepatotoxicity, liver damage, and death following administration to mice. For further explanation, see text.](image-url)