Complement Activation in Acetaminophen-Induced Liver Injury in Mice

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ABSTRACT

Overdose with acetaminophen (APAP) results in acute liver failure in humans and experimental animals. Complement comprises more than 30 proteins that can participate in tissue injury and/or repair, but the role of complement activation in APAP-induced hepatotoxicity has not been evaluated. Treatment of male, C57BL/6J mice with APAP (200–400 mg/kg) resulted in liver injury as evidenced by increased activity of alanine aminotransferase (ALT) in plasma and hepatocellular necrosis. Plasma concentration of the complement component C3 was significantly reduced 6 h after treatment with APAP, indicating complement activation, and C3b (detected by immunostaining) accumulated in the centrilobular areas of liver lobules. Pretreatment with cobra venom factor (CVF; 15 U/mouse) to deplete

complement components abolished APAP-mediated C3b accumulation, and this was accompanied by reductions in plasma ALT activity, hepatocellular necrosis, hepatic neutrophil accumulation, and expression of inflammatory genes (interleukin-6, interleukin-10, and plasminogen activation inhibitor-1) at 24 h after APAP treatment. Loss of hepatocellular GSH was similar in APAP-treated mice pretreated with either saline or CVF, suggesting that CVF pretreatment did not affect APAP bioactivation. Mice with a genetic deficiency in C3 had reduced ALT activity 6 and 12 h after APAP administration compared with wild-type animals. These results reveal a key role for complement activation in hepatic inflammation and progression of injury during the pathogenesis of APAP-induced hepatotoxicity.

Introduction

Acetaminophen (APAP; *N*-acetyl-*p*-aminophenol; paracetamol) is one of the most commonly consumed over-the-counter drugs available on the market, but, unfortunately, it is also the most frequent cause of drug-induced liver failure worldwide (Larson et al., 2005). Approximately 500 fatal cases of acute liver failure are reported annually in the United States alone (Lee, 2004). The mechanisms associated with the initiation of APAP-induced liver injury have been extensively studied over the years in humans and experimental animal models. APAP is metabolized to *N*-acetyl-*p*-benzoquinoneimine (NAPQI) by cytochrome P450. NAPQI is detoxified by conjugation with GSH, and overdose with APAP depletes cellular GSH, resulting in the binding of NAPQI to mitochondrial proteins. This is followed by mitochondrial

dysfunction, ATP depletion, oxidative stress, DNA damage, and oncotic necrosis of parenchymal cells (Kaplowitz, 2004; Jaeschke et al., 2011). The hepatocellular necrosis is accompanied by the release of death-associated molecular pattern molecules, such as high-mobility group box 1 protein, heat shock protein 70, and damaged DNA, which can activate nonparenchymal cells, including Kupffer cells and neutrophils (Scaffidi et al., 2002; Bianchi, 2007; Martin-Murphy et al., 2010). Activation of these cells can exacerbate necrosis and/or participate in recovery from injury. Although mechanisms involved in APAP hepatotoxicity have been studied extensively, nothing has been reported about whether and how complement activation affects the progression of liver injury and hepatocellular repair.

The complement system comprises approximately 35 proteins that are present either as soluble factors in the blood or membrane-associated proteins. The main evolutionary function of complement is to sense danger signals from pathogens and dying cells and activate defenses against tissue damage. Complement activation is a sequential cascade of enzymic reactions that is initiated by one or more of three pathways: classic, alternative, and lectin-associated. Each of these path-

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ABBREVIATIONS: APAP, acetaminophen; ALT, alanine aminotransferase; BrdU, 5'-bromo-2'-deoxyuridine; CVF, cobra venom factor; C3, complement component 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; PAI-1, plasminogen activation inhibitor-1; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PMN, polymorphonuclear neutrophil; Sal, saline; TNF- α , tumor necrosis factor- α .

ways results in C3 activation and leads to the generation of anaphylatoxins C3a and C5a, which activate innate immune cells such as neutrophils, monocytes, mast cells, and basophils. Activation of terminal complement components forms a C5b-9 complex, also known as the membrane attack complex, which can kill microbial pathogens but also damage host tissue (Walport, 2001; Ward, 2004; Markiewski et al., 2007). Although necessary for foreign body clearance and immune cell activation, overactivation of complement components can lead to coagulation activation, tissue necrosis, and multiorgan dysfunction. Complement activation has been implicated in several hepatic disease models, including sepsis (Koleva et al., 2002; Ward, 2004), ischemia reperfusion (He et al., 2009), alcoholic (Cohen et al., 2010) and nonalcoholic steatohepatitis (Rensen et al., 2009), and hemorrhagic shock (Cai et al., 2010); however, its role in APAP-induced liver injury has not been reported. In studies with only a limited number of patients with APAP overdose, complement activation was associated with hepatic dysfunction (Ellison et al., 1990; Clapperton et al., 1997). Given the observation that complement is activated in patients with APAP overdose, we sought to determine its role in APAP-induced liver injury in a mouse model. We tested the hypothesis that complement depletion attenuates liver injury mediated by APAP treatment in mice.

Materials and Methods

Animals. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 9 weeks of age and kept at the animal care facility at Michigan State University. Male, C57BL6J mice were used for most studies. The male C3(−/−) mice (B6.129S4-C3^{tm1Crr}/J) had been backcrossed with C57BL6J mice for five generations, and C57BL6J mice were used as controls for these mice. All animals had access to standard chow diet and spring water ad libitum and were used between 10 and 13 weeks of age. All procedures on animals were performed according to the guidelines of the American Association for Laboratory Animal Science and approved by the Institutional Animal Care and Use Committee at Michigan State University.

Experimental Protocols. Complement was depleted by two intraperitoneal injections (7.5 U each in 200 μ l of saline/mouse) of cobra venom factor (CVF; Quidel Corporation, San Diego, CA). The first injection was given 24 h before APAP or saline administration, and the second injection was given 5 h after the first CVF injection. This was done to prevent any adverse effects of rapid loss of complement. Groups of mice ($n = 3-6$) were treated with CVF or its vehicle (saline) and with either APAP or its vehicle (saline).

APAP solution (Sigma, St. Louis, MO; 10 mg/ml) was made fresh before use in saline and incubated at 42°C in water bath for 15 min with intermittent vortexing. Mice were fasted overnight and treated with 300 mg/kg APAP or saline intraperitoneally the next morning. In a dose-response study, mice were given 200 to 400 mg/kg APAP as indicated. Food was returned after APAP administration. All animals were euthanized 24 or 48 h after APAP administration. In a time-course study, mice were anesthetized 12 h after APAP administration, and blood was collected by retro-orbital puncture; the mice were euthanized at 24 h. Blood was collected from vena cava into 3.8% sodium citrate (final concentration, 0.38%), and livers were harvested. The left lateral lobe of liver was fixed in 10% formalin for histopathological analysis, the left medial lobe was snap-frozen in liquid nitrogen for protein and RNA analysis, and the right medial lobe was covered in Tissue-Tek OCT embedding medium on a cork, chilled in isopentane, and frozen in liquid nitrogen for C3b immunohistochemistry.

In separate studies, 1) animals were euthanized 1 h after APAP (300 mg/kg) or saline administration, livers were collected, and GSH

was measured; and 2) C3(−/−) mice and their wild-type controls were treated with APAP (400 mg/kg) or saline; blood was collected by retro-orbital puncture after 6 h, as described above, and the mice were euthanized after 12 h. Blood and livers were collected.

Plasma Measurements. Plasma was collected by centrifuging blood at 4000g for 10 min. Alanine aminotransferase (ALT) activity was determined by using Infinity ALT reagent (Thermo Fisher Scientific, Waltham, MA). Concentrations of C3, active PAI-1, and TNF- α were measured by using an enzyme-linked immunosorbent assay from Alpha Diagnostic International (San Antonio, TX), Molecular Innovations (Southfield, MI), and BD Biosciences (San Jose, CA), respectively.

Histopathological Analysis. Five-micrometer sections of paraffin-embedded liver were stained with hematoxylin and eosin for evaluation of necrosis and hemorrhage. Hepatocellular necrosis in sections from the left liver lobe was quantified by using morphometric methods similar to those described by Aibo et al. (2010). Percentage of hepatic lesion area was estimated as [necrotic area/total area] $\times$ 100.

For analysis of polymorphonuclear neutrophil (PMN) accumulation, sections were stained with a rabbit-anti-PMN Ig as described previously (Yee et al., 2003). The slides were coded, randomized, and then examined without knowledge of treatment with a light microscope. PMN accumulation was quantified by counting PMNs in 10 100 $\times$ fields; the average number of PMNs from each animal was considered a replicate.

5'-Bromo-2'-Deoxyuridine Treatment and Staining. A separate study was performed for the assessment of hepatic proliferation. Animals were treated with APAP with or without complement depletion by CVF, as described above, and given 5'-bromo-2'-deoxyuridine (BrdU; 50 mg/kg i.p.; Sigma) 2 h before harvesting of liver at 24 or 48 h. BrdU becomes incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle. BrdU incorporation in liver sections was determined by immunohistochemical staining performed on paraffin-embedded sections by using a mouse anti-BrdU antibody (BD Biosciences) as described previously (Aibo et al., 2010).

GSH Estimation. Approximately 100 mg of frozen liver tissue were homogenized in 1 ml of cold buffer containing 0.2 M 2-(N-morpholino)ethanesulphonic acid, 50 mM phosphate, and 1 mM EDTA, pH 6.0. Homogenates were centrifuged at 10,000g for 15 min at 4°C, and supernatant fluid was collected. Total hepatic GSH concentration was determined using a kit from Cayman Chemical (Ann Arbor, MI). The data are expressed as μ mol GSH/g of liver tissue.

RNA Isolation and Real-Time PCR. Procedures for RNA isolation and real-time PCR were as described previously (Singhal et al., 2009). In brief, total RNA was isolated from approximately 100 mg of hepatic tissue by using TRI reagent (Molecular Research Center, Cincinnati, OH). One microgram of RNA was transcribed into cDNA by using a one-step cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). SYBR green (2 $\times$ power; Applied Biosystems, Foster City, CA) was applied as a detector. Primers were designed by using the PrimerQuest program (www.idtdna.com; Integrated DNA Technologies, Inc., Coralville, IA) and purchased from the same company. Primer sequences were as follows: GAPDH, forward, TCAACAGCAACTCCCCTCTTCCA, reverse, ACCCTGTTGCTGTAGCCGTTCA; PCNA, forward, AGCCACATTGGAGATGCTGTTGTG, reverse, AATGTTCCCATTCGCAAGCTCTCC; CyclinD1, forward, GCTGCAATGGAAGTCTCTGTT, reverse, TACCATTGGAGGGTG GGTGGAAAT; PAI-1, forward, GCTTGGCAACCCACGTTAAAGGAA, reverse, TCTCTGCTTCTGGATGCCTTGGA; IL-6, forward, ATCCAGTTGCCTTCTTGGGACTGA, reverse, TAAGCCTCCGACTTGTGAAGTGGT; and IL-10, forward, TGCACTACCAAAGCCACAAAGCAG, reverse, AGTAAGAGCAGGCAGCATAGCAGT. A 48-well Step-one Real-Time PCR system (Applied Biosystems) was used to determine the mRNA expression level of each gene. mRNA levels were normalized to that of GAPDH mRNA to control for input RNA.

Fig. 1. C3 concentration in plasma from mice treated with APAP. Mice were treated with saline vehicle (0 APAP) or APAP (200, 300, or 400 mg/kg) after treatment with saline or CVF as described under *Materials and Methods*. C3 concentration in plasma was determined 6 h (left) or 24 h (right) later. Data are expressed as mean $\pm$ S.E.M. *, significantly different from respective value in the absence of APAP. #, significantly different from respective value in the absence of CVF. $n = 3-5$ per group, $p < 0.05$.

C3b Immunostaining. Immunohistochemical staining for C3b/iC3b/C3c (C3b) was performed on sections from frozen liver using a protocol described by Roychowdhury et al. (2009). In brief, frozen liver sections were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) and washed three times, 5 min each, in washing buffer (0.1% Triton X-100 in PBS). Sections were then blocked with 2% bovine serum albumin (diluted in PBS) containing 0.1% sodium azide and 0.1% Triton X-100 (blocking buffer) for 1 h followed by overnight incubation at 4°C with rabbit anti-mouse C3b (Cell Sciences, Canton, MA) diluted 50-fold in blocking buffer. All sections were washed with washing buffer, incubated with Alexa Fluor-488-labeled goat-anti-rabbit IgG (Invitrogen, Carlsbad, CA; diluted 250-fold in blocking buffer) for 2 h in the dark at room temperature, washed again in PBS, and mounted with Vectashield containing antifade reagent (Vector Laboratories, Burlingame, CA). Images were acquired by using an Olympus (Tokyo, Japan) IX-70 fluorescent microscope.

Statistical Analysis. Data are expressed as mean $\pm$ S.E.M. All data were analyzed by using SigmaStat version 3.5 (Systat Software, Inc., San Jose, CA). Student's *t* test was used to compare data between two groups. For multiple group comparisons, one-way or two-way analysis of variance was performed followed by Student Newman-Keuls post hoc analysis. The results were considered significant when $P < 0.05$.

Results

APAP Treatment Activates Complement. Complement activation is a complex process and can be triggered by either classic, mannose lectin-binding, or alternative pathways. All complement pathways converge on activating and thereby depleting the central component C3. Therefore, we first determined the effect of APAP treatment on plasma C3 concentration. Saline controls had 1.1 ± 0.13 mg/ml of circulating C3 in plasma (Fig. 1). Treatment with APAP (200–400 mg/kg) significantly reduced C3 in plasma as early as 6 h to ~50% of control; no significant difference was observed among the three APAP doses. C3 concentration remained reduced compared with control at 24 h, which is the peak of liver injury observed in this study.

CVF was used to deplete complement. It has been reported that the CVF treatment regimen used in this study does not result in toxic effects in mice and maintains the depletion of hemolytic activity of complement until 72 h (Alper and Balavitch, 1976; Markiewski et al., 2004). In our hands, CVF treatment alone reduced circulating concentration of C3 in plasma to 0.025 ± 0.005 mg/ml at 0 h, the time at which APAP or saline was administered. This is consistent with previous reports showing that CVF pretreatment reduces C3 levels to less than 5% of control values (Pepys, 1975; Cai et

al., 2010). C3 levels remain depleted by CVF pretreatment until 24 h in both saline- and APAP-treated mice.

Activation of C3 is followed by its cleavage into fragments, which form an integral part of C3 and C5 convertases, resulting in the generation of anaphylatoxins and formation of the membrane attack complex (Rawal and Pangburn, 2001; Walport, 2001). Accordingly, the deposition of C3 fragments C3b/iC3b/C3c, abbreviated here as C3b, was examined in liver by using an antibody that recognizes neoepitopes on the C3b fragment after C3 cleavage (Mastellos et al., 2004; Roychowdhury et al., 2009). Immunofluorescence in frozen liver sections taken 24 h after APAP administration revealed C3b deposition in the necrotic, centrilobular regions only in livers from APAP-treated mice (Fig. 2). Prior treatment with CVF prevented APAP-induced C3b deposition (Fig. 2). CVF/Sal and Sal/Sal treatment groups did not have any specific staining. No positive staining was detected with the use of isotype control antibody in livers from APAP-treated mice, suggesting that the reactivity obtained with anti-C3b antibody is specific to C3b and not a result of nonspecific staining in the centrilobular region (data not shown).

CVF Pretreatment Does Not Alter APAP Metabolism.

Depletion of hepatic GSH after treatment with toxic doses of APAP is a well established marker of APAP bioactivation. APAP is metabolized to *N*-acetyl-*p*-benzoquinoneimine, which then binds to GSH; excessive NAPQI formation in APAP overdose depletes hepatic GSH stores to <20% of normal within 1 h of treatment (Chiu et al., 2003). Mice treated with saline (control) had 3.92 ± 0.38 μ mol GSH/g

Fig. 2. Hepatic C3b deposition. Mice were treated with saline vehicle (0 APAP) or APAP (300 mg/kg) after treatment with saline or CVF as described under *Materials and Methods*. Twenty four hours later livers were harvested and frozen. Frozen liver sections were immunostained with C3b antibody as described under *Materials and Methods*. Arrows represent C3b in the centrilobular area. Images were acquired at 100 $\times$ magnification using an Olympus IX-70 fluorescent microscope.

Fig. 3. GSH concentration in livers from APAP-treated mice. GSH was determined 1 h after APAP (300 mg/kg) or saline administration in mice pretreated with CVF or saline vehicle as described under *Materials and Methods*. Data represent mean $\pm$ S.E.M. ($n = 4$ animals per group). *, significantly different from respective value in the absence of APAP ($p < 0.05$).

liver (Fig. 3). CVF treatment alone did not affect GSH concentration (4.41 ± 0.25 μ mol GSH/g liver). APAP treatment resulted in GSH depletion to 0.83 ± 0.25 and 0.53 ± 0.05 μ mol GSH/g liver within 1 h in saline- and CVF-pretreated mice, respectively (Fig. 3); these values were not significantly different from each other, suggesting that CVF pretreatment had no effect on APAP bioactivation.

Complement Depletion Reduces APAP-Induced Liver Injury. Severity of hepatocellular injury was assessed from

ALT activity in plasma and from histopathology. Treatment with APAP (200, 300, and 400 mg/kg) resulted in an increase in ALT activity in plasma at 6 h (Fig. 4A). CVF pretreatment reduced ALT activity in mice treated with 400 mg/kg APAP; however, no effect of CVF pretreatment was observed on APAP-induced increase in ALT activity at 6 h after doses of 200 or 300 mg/kg. One of six mice in the 400 mg/kg APAP group died between 6 and 24 h, and two others became moribund near 24 h. By contrast, no mortality was observed in APAP-exposed mice that were pretreated with CVF. CVF pretreatment reduced APAP (300 and 400 mg/kg)-induced increase in ALT activity at 24 h; in contrast, no effect of CVF pretreatment was observed on the increase in ALT activity in mice given 200 mg/kg APAP. CVF treatment alone did not change ALT activity compared with saline vehicle either at 6 or 24 h after mice were given the vehicle for APAP (i.e., saline). APAP treatment (200, 300, or 400 mg/kg) resulted in centrilobular necrosis at 6 h (data not shown) and 24 h (Fig. 4B). Moreover, mice treated with either 300 or 400 mg/kg APAP that were pretreated with CVF had smaller necrotic areas and less hemorrhage than those treated with APAP alone 24 h after APAP treatment. No effect of CVF pretreatment was observed on liver histopathology in groups treated with 200 mg/kg APAP (Fig. 4B). Treatment of mice with APAP (300 mg/kg) resulted in a time-dependent increase in plasma ALT activity (Fig. 4C). CVF pretreatment had no

Fig. 4. Liver injury after APAP treatment. Mice were treated with saline vehicle (0 APAP) or APAP (200, 300, or 400 mg/kg) after treatment with saline or CVF as described under *Materials and Methods*. A, plasma ALT activity at 6 h (left) or 24 h (right) after APAP. B, representative images from hematoxylin and eosin-stained liver sections from livers harvested at 24 h. a to d, treatment with APAP [0 (vehicle) (a), 200 (b), 300 (c), or 400 (d) mg/kg] after treatment with saline. e to h, treatment with APAP [0 (vehicle) (e), 200 (f), 300 (g), or 400 (h) mg/kg] after treatment with CVF. C, ALT activity in plasma at 6, 12, or 24 h after treatment with CVF or saline and APAP (300 mg/kg) or saline vehicle as described under *Materials and Methods*. Values for saline/saline are obscured by values for CVF/saline. Data are expressed as mean $\pm$ S.E.M. *, significantly different from respective value in the absence of APAP. #, significantly different from respective value in the absence of CVF. $n = 3$ –5 per group, $p < 0.05$.

Fig. 5. Liver injury in C3(-/-) mice treated with APAP. A, plasma ALT activity at 6 or 12 h after APAP (400 mg/kg) administration. Data are expressed as mean $\pm$ S.E.M. *, significantly different from wild type at the indicated time ($p < 0.05$). $n = 3-5$ per group. B, representative liver sections stained with hematoxylin and eosin. Wild-type (a) or C3(-/-) (b) mice were treated with APAP (400 mg/kg), and livers were harvested 12 h after APAP administration.

significant effect on the increase in ALT activity at 6 or 12 h, whereas a marked reduction was observed at 24 h.

The importance of complement in APAP-induced liver injury was confirmed by using C3(-/-) mice. For this study, an APAP dose of 400 mg/kg was chosen, and the animals were examined at 12 h, because in the previous study some APAP-treated animals died by 24 h at this dose. Plasma ALT activity was attenuated by 70 and 50% at 6 and 12 h, respec-

tively, in C3(-/-) mice treated with 400 mg/kg APAP compared with wild-type controls (Fig. 5A). Liver histopathology also revealed reduced centrilobular necrosis and hemorrhage in C3(-/-) mice at 12 h compared with wild-type controls (Fig. 5B). Morphometric analysis of hepatic lesions at 12 h indicated that the percentage of the area that was necrotic was reduced in APAP-treated C3(-/-) mice ($22.7 \pm 3.5\%$) compared with APAP-treated wild-type mice ($36.9 \pm 2.6\%$;

Fig. 6. Hepatic neutrophil (PMN) accumulation after APAP administration. A, representative liver sections from mice treated with saline (a) or CVF (b) then given APAP (300 mg/kg); livers were harvested 24 h after treatment with APAP. Liver sections were immunohistochemically stained for infiltrating PMNs (red chromagen; arrows) and counterstained with hematoxylin as described under *Materials and Methods*. B, morphometric determination of PMN accumulation in sections of liver 6 or 24 h after APAP treatment. Data represent mean $\pm$ S.E.M. of PMNs counted in 10 microscopic fields at $100\times$ magnification. *, significantly different from respective value in the absence of APAP. #, significantly different from respective value in the absence of CVF.

$P < 0.05$). APAP treatment resulted in reduction of hepatic GSH to 0.21 ± 0.08 and 0.23 ± 0.03 μmol GSH/g liver at 50 min post-treatment in C3(–/–) mice and controls, respectively (no significant difference), suggesting that C3 gene deficiency did not affect the bioactivation of APAP.

Complement Depletion in APAP-Treated Mice Reduces Hepatic Neutrophil Infiltration and Cytokine Expression. Activation of C3 and C5 results in formation of the C3a and C5a peptides. C3a and C5a are chemotactic for neutrophils (PMNs) and macrophages and can activate these cells in addition to endothelial cells, platelets, basophils, and mast cells (Walport, 2001; Ward, 2004). Accordingly, PMN sequestration in liver was assessed in APAP-treated mice that were depleted of complement. Treatment with APAP caused significant PMN accumulation in livers at 6 h, and this increased by 24 h (Fig. 6). Although CVF treatment by itself resulted in very modest PMN accumulation, it significantly reduced APAP-induced PMN accumulation at both 6 and 24 h compared with APAP treatment alone (Fig. 6). A similar result was observed in C3(–/–) mice treated with 400 mg/kg APAP: C3(–/–) mice had significantly reduced (50 ± 3 PMN/field) APAP-induced PMN accumulation at 12 h compared with wild-type controls (126 ± 4 PMN/field; $P < 0.05$).

Increased expression of cytokines occurs during the pathogenesis of APAP-induced liver injury (Blazka et al., 1995). Hepatic mRNA expression for IL-6 and IL-10 was increased by APAP (Fig. 7A). CVF treatment alone did not affect expression of these cytokines but significantly reduced the increase in their expression caused by APAP. Hepatic mRNA expression of IL-6 and IL-10 was also reduced in APAP-treated C3(–/–) mice by 60% ($P = 0.075$) and 84% ($P = 0.05$), respectively, compared with control mice.

Plasma concentrations of TNF- α in saline- and CVF-treated mice were below the limit of detection (16 pg/ml) of the enzyme-linked immunosorbent assay. APAP treatment increased TNF- α concentration in plasma at 6 and 12 h (Fig. 7B), and this increase was significantly attenuated by CVF pretreatment. As reported previously (Ganey et al., 2007; Bajt et al., 2008), APAP treatment increased PAI-1 concentration in plasma at 6 and 24 h; this increase was reduced by CVF pretreatment by 85% at 24 h. Complement depletion did not affect the increase in PAI-1 at 6 h after APAP administration (Fig. 7C).

Complement Depletion Enhances Hepatocellular Viability. In a separate study, we evaluated progression of liver injury and regeneration between 24 and 48 h. Consistent with previous reports (Dambach et al., 2002; Chiu et al., 2003; Aibo et al., 2010) activity of ALT in plasma decreased between 24 and 48 h after treatment with APAP (Fig. 8A). CVF pretreatment resulted in significant reduction in APAP-mediated increase in plasma ALT activity at both of these times compared with APAP treatment alone. The mRNA expression of biomarkers of cell cycle activation, i.e., PCNA and CyclinD1, was significantly elevated by CVF pretreatment in APAP-exposed mice, whereas expression of p21, a protein involved in inhibition of cell cycle, was reduced by CVF pretreatment (Fig. 8B). In a separate study, BrdU was administered 2 h before euthanizing mice at 24 or 48 h after APAP treatment to identify cells undergoing DNA synthesis at those times. At 24 h, BrdU incorporation was not significantly affected by APAP treatment, but a significant increase in BrdU-positive cells was observed in CVF/APAP-treated

Fig. 7. Cytokine mRNA expression or protein concentration after APAP administration. Mice were pretreated with CVF or saline then given APAP (300 mg/kg) or saline vehicle. A, mRNA expression of IL-6 and IL-10 was determined in livers harvested 24 h after APAP administration. Expression levels were normalized to that of the GAPDH house-keeping gene and represented as percentage of saline control. B, TNF- α concentration in plasma was determined 6 or 12 h after administration of APAP. C, PAI-1 concentration in plasma was determined 6 or 24 h after administration of APAP. Data represent mean $\pm$ S.E.M. *, significantly different from respective value in the absence of APAP. #, significantly different from respective value in the absence of CVF. $n = 3$ –5 per group; $p < 0.05$.

mice at 24 h, suggesting that more cells were replicating in these mice compared with those treated with APAP alone. At 48 h, the number of BrdU-positive cells was markedly greater in livers of APAP-treated mice, and the BrdU incorporation was not different in mice cotreated with CVF (Fig. 8,

Fig. 8. Hepatocellular viability after APAP treatment. Mice were pretreated with CVF or saline then given APAP (300 mg/kg) and killed 24 or 48 h after APAP administration. A, plasma ALT activity measurement. B, hepatic mRNA expression 24 h after APAP administration. Expression levels were normalized to that of the GAPDH housekeeping gene and represented as percentage of Sal/Sal (not shown) control. C, mice pretreated with APAP (300 mg/kg) or saline were given BrdU (50 mg/kg) 2 h before euthanasia at 24 or 48 h after APAP administration. Liver sections were immunohistochemically stained for nuclear incorporation of BrdU (brown chromagen) and counterstained with hematoxylin as described under *Materials and Methods*. a and b, mice treated with Sal/APAP with their livers harvested at 24 h (a) and 48 h (b). c and d, mice treated with CVF/APAP with their livers harvested at 24 h (c) and 48 h (d). D, morphometric determination of BrdU-positive cells in section of livers 24 or 48 h after APAP treatment. Data represent mean $\pm$ S.E.M. of BrdU-positive cells counted in 10 microscopic fields at 100 $\times$ magnification. In A, B, and D, data represent mean $\pm$ S.E.M. *, significantly different from respective value in the absence of APAP. #, significantly different from respective value in the absence of CVF. $n = 3-5$ per group; $p < 0.05$.

C and D). Liver sections from mice treated only with saline or CVF had few BrdU-positive cells at either 24 or 48 h.

Discussion

Although studies of patients suffering from APAP overdose have been reported extensively over the past 30 years, surprisingly few reports have examined complement activation. In this study, we present evidence in a murine model of APAP overdose that 1) APAP treatment activates the complement pathway, 2) complement activation contributes to the progression of liver injury from hepatotoxic doses of APAP and 3) hepatic regeneration in response to APAP-induced injury occurs more rapidly in complement-depleted mice.

Two clinical studies reported a reduction in complement proteins in serum from APAP-overdosed patients. Ellison et al. (1990) compared total hemolytic activity, expressed in CH50 units, and the concentrations of complement components in serum from 15 patients with liver disease caused by alcohol toxicity and only 1 patient with APAP hepatotoxicity. In comparison with healthy subjects, patients under study had reduced CH50 values and small concentrations of C1q, C3, C4, and C5 in serum (Ellison et al., 1990). Clapperton et al. (1997) studied 14 patients with acute liver failure caused by APAP overdose and found a significantly smaller C3 concentration in plasma from patients (0.270 ± 0.078 mg/ml) compared with healthy control subjects (1.041 ± 0.059 mg/ml). Similar to these clinical findings, mice treated with APAP (200–400 mg/kg) exhibited reduced plasma C3 concentration (Fig. 1). This was accompanied by accumulation of the C3 activation product, C3b/iC3b/C3c, in the necrotic centrilobular regions of livers (Fig. 2). Together, these results suggest a deficiency in plasma

complement as a result of persistent complement activation during APAP hepatotoxicity.

APAP treatment resulted in a dose- and time-dependent centrilobular necrosis and hemorrhage (Fig. 3). At the smallest dose of APAP used in these studies (200 mg/kg), complement was activated, but prior complement depletion did not affect hepatotoxicity. In contrast, at a markedly toxic APAP dose (400 mg/kg) that was lethal to some animals, complement was activated and contributed to the early progression of injury, as evidenced by the observation that complement depletion by CVF pretreatment reduced injury by 6 h. At an intermediate APAP dose (300 mg/kg) that also resulted in pronounced liver injury, complement seemed to contribute only to the later progression of injury, that is, the increase in severity that occurred between 12 and 24 h (Fig. 4C). Protection afforded by CVF was not a result of reduced bioactivation of APAP because GSH depletion was similar in APAP-treated mice pretreated with saline or CVF (Fig. 3). In support of the CVF results, C3 gene deletion also reduced liver injury at 6 and 12 h after a dose of 400 mg/kg APAP (Fig. 5). These results indicate that the participation of complement in the progression of APAP-induced hepatocellular injury clearly depended on the dose of APAP, with early contribution at a markedly hepatotoxic dose, later contribution at an intermediate dose, and no contribution despite complement activation at a mildly toxic dose.

APAP hepatotoxicity is associated with cytokine release and influx of inflammatory cells, including PMNs (Blazka et al., 1995; Lawson et al., 2000; Ju et al., 2002). Complement depletion reduced, but did not completely abolish, APAP-mediated hepatic PMN accumulation as well as gene expression of IL-6 and IL-10 and plasma concentration of TNF- α . PAI-1, which can be released by Kupffer cells in response to C5a (Kastl et al.,

2006), was also increased in plasma by APAP treatment and reduced by prior complement depletion. Taken together, these results suggest an important role of complement in APAP-induced liver injury, PMN accumulation, and cytokine release. The reduced accumulation of PMNs and release of cytokines is consistent with the reduction in the severity of APAP-induced liver injury by complement depletion, but cause and effect cannot be assigned. Evidence has been presented for roles of Kupffer cells, inflammatory cytokines, and PMNs in the progression of APAP-induced liver injury (Blazka et al., 1995; Laskin et al., 1995; Liu et al., 2006). However, contrasting results suggesting ameliorative influences of these factors have also been reported (Bourdi et al., 2002; Ju et al., 2002; Gardner et al., 2003; James et al., 2003; Masubuchi et al., 2003; Bajt et al., 2008), and their roles continue to be debated (Jaeschke, 2008; Jaeschke et al., 2011). Indeed, it remains a possibility that these and other factors have contrasting roles at different times and/or concentrations during the pathogenesis. Nevertheless, our results suggest that complement activation influences the hepatic accumulation of PMNs and the generation of cytokines during the progression of APAP hepatotoxicity. It is also possible that formation of the membrane attack complex plays a role in APAP hepatotoxicity; this will be investigated in future studies.

It is likely that some alteration in, or injury to, hepatocytes caused early and directly by APAP exposure is required for complement activation. For example, the classic pathway of complement activation can be initiated by compromised mammalian cells undergoing apoptosis or release of heat shock proteins (Prohászka et al., 2002; Navratil et al., 2006). That neither complement depletion with C5 nor genetic deficiency in C3 protected completely against APAP toxicity suggests that additional factors play a role in the progression of injury. It is noteworthy that hepatocytes damaged by APAP release various death-associated molecular pattern molecules, and evidence has been presented for a role for these in the progression of APAP-induced hepatocellular necrosis (Imaeda et al., 2009; Martin-Murphy et al., 2010; Dear et al., 2011). Accordingly, complement activation is likely only one of several events responsible for injury progression in APAP overdose.

Treatment with 300 mg/kg APAP resulted in pronounced liver injury by 24 h followed by cell proliferation (i.e., BrdU incorporation into DNA) that was associated with reduced ALT activity in plasma at 48 h (Fig. 8A). In addition to reducing the progression of necrosis, complement depletion resulted in greater numbers of replicating cells in and around the centrilobular lesions. This was evident by the greater numbers of BrdU-positive cells, increased mRNA expression of markers of cell cycle activation (PCNA and CyclinD1 markers of G₁/S transition), and reduced expression of the cell cycle inhibitor p21 (Fausto, 2000; Bajt et al., 2008; Aibo et al., 2010) at 24 h (Fig. 8). This suggests that attenuated liver injury associated with complement depletion enables early tissue regeneration and complement activation delays this reparative response to APAP-induced injury. It is noteworthy that this observation contrasts with effects of complement activation on liver regeneration in other models. For example, complement (C3 or C5)-deficient mice were found to have reduced pro-survival signaling, leading to impaired liver regeneration after partial hepatectomy (Markiewski et al., 2004) or carbon tetrachloride administration (Mastellos et al., 2001). In a study of partial hepatectomy, deficiency in complement components reduced the induction of

cytokines IL-6 and TNF- α , which have been shown to be required for the priming phase of liver regeneration in that model (Markiewski et al., 2009). In studies presented here, cell cycle activity was greater in APAP-treated mice pretreated with C5F despite reduced expression of IL-6 and TNF- α . A possible explanation for this difference is that the concentrations of these proregenerative cytokines, although reduced after complement depletion, are sufficient enough to stimulate hepatocyte proliferation. In any case, complement depletion was associated with a reduction in hepatocellular necrosis as well as an enhanced proliferative response.

In conclusion, complement activation is associated with APAP hepatotoxicity, and complement depletion reduces the progression of APAP-induced liver injury. Current therapy for APAP overdose includes treatment with *N*-acetylcysteine, which is an effective antidote if given early after APAP ingestion. Our results suggest that complement plays a role later in the pathogenesis and raise the possibility that interference with its activation or effects may reduce the progression of liver injury.

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Authorship Contributions

Participated in research design: Singhal, Ganey, and Roth.

Conducted experiments: Singhal.

Performed data analysis: Singhal.

Wrote or contributed to the writing of the manuscript: Singhal, Ganey, and Roth.

References

- Aibo DI, Birmingham NP, Lewandowski R, Maddox JF, Roth RA, Ganey PE, Wagner JG, and Harkema JR (2010) Acute exposure to ozone exacerbates acetaminophen-induced liver injury in mice. *Toxicol Sci* **115**:267–285.
- Alper CA and Balavitch D (1976) Cobra venom factor: evidence for its being altered cobra C3 (the third component of complement). *Science* **191**:1275–1276.
- Bajt ML, Yan HM, Farhood A, and Jaeschke H (2008) Plasminogen activator inhibitor-1 limits liver injury and facilitates regeneration after acetaminophen overdose. *Toxicol Sci* **104**:419–427.
- Bianchi ME (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* **81**:1–5.
- Blazka ME, Wilmer JL, Holladay SD, Wilson RE, and Luster MI (1995) Role of proinflammatory cytokines in acetaminophen hepatotoxicity. *Toxicol Appl Pharmacol* **133**:43–52.
- Bourdi M, Masubuchi Y, Reilly TP, Amouzadeh HR, Martin JL, George JW, Shah AG, and Pohl LR (2002) Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase. *Hepatology* **35**:289–298.
- Cai C, Gill R, Eum HA, Cao Z, Loughran PA, Darwiche S, Edmonds RD, Menzel CL, and Billiri TR (2010) Complement factor 3 deficiency attenuates hemorrhagic shock-related hepatic injury and systemic inflammatory response syndrome. *Am J Physiol Regul Integr Comp Physiol* **299**:R1175–R1182.
- Chiu H, Gardner CR, Dambach DM, Brittingham JA, Durham SK, Laskin JD, and Laskin DL (2003) Role of p53 tumor necrosis factor receptor 1 in acetaminophen-induced antioxidant defense. *Am J Physiol Gastrointest Liver Physiol* **285**:G959–G966.
- Clapperton M, Rolando N, Sandoval L, Davies E, and Williams R (1997) Neutrophil superoxide and hydrogen peroxide production in patients with acute liver failure. *Eur J Clin Invest* **27**:164–168.
- Cohen JJ, Roychowdhury S, McMullen MR, Stavitsky AB, and Nagy LE (2010) Complement and alcoholic liver disease: role of C1q in the pathogenesis of ethanol-induced liver injury in mice. *Gastroenterology* **139**:664–674, 674.e1.
- Dambach DM, Watson LM, Gray KR, Durham SK, and Laskin DL (2002) Role of CCR2 in macrophage migration into the liver during acetaminophen-induced hepatotoxicity in the mouse. *Hepatology* **35**:1093–1103.
- Dear JW, Simpson KJ, Nicolai MP, Catterson JH, Street J, Huizinga T, Craig DG, Dhaliwal K, Webb S, Bateman DN, et al. (2011) Cyclophilin A is a damage-associated molecular pattern molecule that mediates acetaminophen-induced liver injury. *J Immunol* **187**:3347–3352.
- Ellison RT 3rd, Horsburgh CR Jr, and Curd J (1990) Complement levels in patients with hepatic dysfunction. *Dig Dis Sci* **35**:231–235.
- Fausto N (2000) Liver regeneration. *J Hepatol* **32** (Suppl 1):19–31.
- Ganey PE, Luyendyk JP, Newport SW, Eagle TM, Maddox JF, Mackman N, and

- Roth RA (2007) Role of the coagulation system in acetaminophen-induced hepatotoxicity in mice. *Hepatology* **46**:1177–1186.
- Gardner CR, Laskin JD, Dambach DM, Chiu H, Durham SK, Zhou P, Bruno M, Gerecke DR, Gordon MK, and Laskin DL (2003) Exaggerated hepatotoxicity of acetaminophen in mice lacking tumor necrosis factor receptor-1. Potential role of inflammatory mediators. *Toxicol Appl Pharmacol* **192**:119–130.
- He S, Atkinson C, Qiao F, Cianflone K, Chen X, and Tomlinson S (2009) A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice. *J Clin Invest* **119**:2304–2316.
- Imaeda AB, Watanabe A, Sohail MA, Mahmood S, Mohamadnejad M, Sutterwala FS, Flavell RA, and Mehal WZ (2009) Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *J Clin Invest* **119**:305–314.
- Jaeschke H (2008) Innate immunity and acetaminophen-induced liver injury: why so many controversies? *Hepatology* **48**:699–701.
- Jaeschke H, McGill MR, Williams CD, and Ramachandran A (2011) Current issues with acetaminophen hepatotoxicity—a clinically relevant model to test the efficacy of natural products. *Life Sci* **88**:737–745.
- James LP, Lamps LW, McCullough S, and Hinson JA (2003) Interleukin 6 and hepatocyte regeneration in acetaminophen toxicity in the mouse. *Biochem Biophys Res Commun* **309**:857–863.
- Ju C, Reilly TP, Bourdi M, Radonovich MF, Brady JN, George JW, and Pohl LR (2002) Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol* **15**:1504–1513.
- Kaplowitz N (2004) Acetaminophen hepatotoxicity: what do we know, what don't we know, and what do we do next? *Hepatology* **40**:23–26.
- Kastl SP, Speidl WS, Kaun C, Rega G, Assadian A, Weiss TW, Valent P, Hagmueller GW, Maurer G, Huber K, et al. (2006) The complement component C5a induces the expression of plasminogen activator inhibitor-1 in human macrophages via NF- κ B activation. *J Thromb Haemost* **4**:1790–1797.
- Koleva M, Schlaf G, Landmann R, Götze O, Jungermann K, and Schieferdecker HL (2002) Induction of anaphylatoxin C5a receptors in rat hepatocytes by lipopolysaccharide in vivo: mediation by interleukin-6 from Kupffer cells. *Gastroenterology* **122**:697–708.
- Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, Reisch JS, Schiodt FV, Ostapowicz G, Shakil AO, et al. (2005) Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* **42**:1364–1372.
- Laskin DL, Gardner CR, Price VF, and Jollow DJ (1995) Modulation of macrophage functioning abrogates the acute hepatotoxicity of acetaminophen. *Hepatology* **21**:1045–1050.
- Lawson JA, Farhood A, Hopper RD, Bajt ML, and Jaeschke H (2000) The hepatic inflammatory response after acetaminophen overdose: role of neutrophils. *Toxicol Sci* **54**:509–516.
- Lee WM (2004) Acetaminophen and the U.S. Acute Liver Failure Study Group: lowering the risks of hepatic failure. *Hepatology* **40**:6–9.
- Liu ZX, Han D, Gunawan B, and Kaplowitz N (2006) Neutrophil depletion protects against murine acetaminophen hepatotoxicity. *Hepatology* **43**:1220–1230.
- Markiewski MM, DeAngelis RA, Strey CW, Foukas PG, Gerard C, Gerard N, Wetsel RA, and Lambris JD (2009) The regulation of liver cell survival by complement. *J Immunol* **182**:5412–5418.
- Markiewski MM, Mastellos D, Tudoran R, DeAngelis RA, Strey CW, Franchini S, Wetsel RA, Erdei A, and Lambris JD (2004) C3a and C3b activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury. *J Immunol* **173**:747–754.
- Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE, and Lambris JD (2007) Complement and coagulation: strangers or partners in crime? *Trends Immunol* **28**:184–192.
- Martin-Murphy BV, Holt MP, and Ju C (2010) The role of damage associated molecular pattern molecules in acetaminophen-induced liver injury in mice. *Toxicol Lett* **192**:387–394.
- Mastellos D, Papadimitriou JC, Franchini S, Tsonis PA, and Lambris JD (2001) A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration. *J Immunol* **166**:2479–2486.
- Mastellos D, Prechl J, László G, Papp K, Oláh E, Argyropoulos E, Franchini S, Tudoran R, Markiewski M, Lambris JD, et al. (2004) Novel monoclonal antibodies against mouse C3 interfering with complement activation: description of fine specificity and applications to various immunoassays. *Mol Immunol* **40**:1213–1221.
- Masubuchi Y, Bourdi M, Reilly TP, Graf ML, George JW, and Pohl LR (2003) Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease. *Biochem Biophys Res Commun* **304**:207–212.
- Navratil JS, Liu CC, and Ahearn JM (2006) Apoptosis and autoimmunity. *Immunol Res* **36**:3–12.
- Pepys MB (1975) Studies in vivo of cobra factor and murine C3. *Immunology* **28**:369–377.
- Prohászka Z, Singh M, Nagy K, Kiss E, Lakos G, Duba J, and Füst G (2002) Heat shock protein 70 is a potent activator of the human complement system. *Cell Stress Chaperones* **7**:17–22.
- Rawal N and Pangburn MK (2001) Structure/function of C5 convertases of complement. *Int Immunopharmacol* **1**:415–422.
- Rensen SS, Slaats Y, Driessen A, Peutz-Kootstra CJ, Nijhuis J, Steffensen R, Greve JW, and Buurman WA (2009) Activation of the complement system in human nonalcoholic fatty liver disease. *Hepatology* **50**:1809–1817.
- Roychowdhury S, McMullen MR, Pritchard MT, Hise AG, van Rooijen N, Medof ME, Stavitsky AB, and Nagy LE (2009) An early complement-dependent and TLR-4-independent phase in the pathogenesis of ethanol-induced liver injury in mice. *Hepatology* **49**:1326–1334.
- Scaffidi P, Misteli T, and Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418**:191–195.
- Singhal R, Badger TM, and Ronis MJ (2009) Estrogenic status modulates the effect of soy on hepatic responses to 7,12-dimethylbenz(a)anthracene (DMBA). *Toxicol Appl Pharmacol* **234**:89–97.
- Walport MJ (2001) Complement. Second of two parts. *N Engl J Med* **344**:1140–1144.
- Ward PA (2004) The dark side of C5a in sepsis. *Nat Rev Immunol* **4**:133–142.
- Yee SB, Hanumegowda UM, Hotchkiss JA, Ganey PE, and Roth RA (2003) Role of neutrophils in the synergistic liver injury from monocrotaline and bacterial lipopolysaccharide exposure. *Toxicol Sci* **72**:43–56.

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