Complement activation in acetaminophen-induced liver injury in mice.

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**Abbreviations:** APAP, acetaminophen; ALT, alanine aminotransferase; BrdU, 5’-bromod-2’-deoxyuridine; CVF, cobra venom factor; C3, complement component 3; IL-6, interleukin-6; IL-10, interleukin-10; PAI-1, plasminogen activation inhibitor-1; PCNA, proliferating cell nuclear antigen; PMN, polymorphonuclear neutrophils; and TNF-α, tumor necrosis factor- alpha.
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, C57Bl6J 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 6h 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 (IL-6, IL-10 and PAI-1) at 24 h after APAP treatment. Loss of hepatocellular glutathione was similar in APAP-treated mice pretreated with either saline or CVF, suggesting that CVF pretreatment did not affect APAP bioactivation. Mice with genetic deficiency in C3 had reduced ALT activity 6 and 12 h after APAP administration compared to 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 (N-acetyl-p-aminophenol, APAP, 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). About 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 cytochromes (CYPs) P450. NAPQI is detoxified by conjugation with glutathione (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 release of death-associated molecular pattern molecules (DAMPs), such as HMGB1, HSP70, 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 about 35 proteins that are present either as soluble factors in the blood or as 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: classical, alternative and lectin-associated. Each of these pathways 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 (MAC), 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 non-alcoholic 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 III 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 Jackson laboratory (Bar Harbor, ME) at nine 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-C3tm1Crr/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 the Laboratory Animal Science and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

**Experimental protocols.** Complement was depleted by two i.p. injections (7.5 U each in 200 µl 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 Aldrich, Saint Louis, MO; 10 mg/ml) was made fresh before use in saline, incubated at 42°C in water bath for 15 minutes with intermittent vortexing. Mice were fasted overnight and treated with 300 mg/kg APAP or saline intraperitoneally the following morning. In a dose response study, mice were given 200 – 400 mg/kg APAP, as indicated. Food was returned after APAP administration. All animals were euthanized after 6 or 24 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 4000 x g for 10 minutes. Alanine aminotransferase (ALT) activity was determined using Infinity™ ALT reagent (Thermo Fisher Scientific, Middletown, VA). Concentrations of C3, active PAI-1, and TNF-α were measured using ELISA kits from Alpha Diagnostic International (San Antonio, TX), Molecular Innovations (Novi, MI), and BD biosciences (Franklin Lakes, NJ), respectively.

**Histopathological analysis.** Five micrometer sections of paraffin embedded liver were stained with hematoxylin and eosin (H & E) for evaluation of necrosis and hemorrhage. Hepatocellular necrosis in sections from the left liver lobe was quantified using morphometric methods similar to those described by Aibo et al. (2010). Percent hepatic lesion area was estimated as \([\text{Necrotic area}/\text{Total area}] \times 100\).

For analysis of 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 using a light microscope. PMN accumulation was
quantified by counting PMNs in 10 100X fields; the average number of PMN from each animal was considered a replicate.

**BrdU 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 were given 5’-bromodeoxyuridine (BrdU, 50 mg/kg i.p.; Sigma Aldrich) 2 hours 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 using a mouse anti-BrdU antibody (BD Biosciences, San Jose, CA) as described previously (Aibo et al., 2010).

**Glutathione 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 10,000 g for 15 minutes at 4°C, and supernatant fluid was collected. Total hepatic glutathione (GSH) concentration was determined using a kit from n (Ann Arbor, MI). The data are expressed as µmol 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). Briefly, total RNA was isolated from approximately 100 mg of hepatic tissue using TRI reagent (Molecular Research Center, Cincinnati, OH). One microgram RNA was transcribed into cDNA using a one-step cDNA synthesis kit (Bio Rad, Hercules, CA). 2X Power SYBR green (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) was applied as a detector. Primers were designed using PrimerQuest program (www.idtdna.com, Integrated DNA Technologies, Coralville, IA) and purchased from the same
primer sequences are as follows: GAPDH: Forward (F) –
TCAACAGCAACTCCCACTCTTCCA, Reverse (R) – ACCCTGTGCTGTAGCCGTATTCA;
PCNA: F – AGCCACATTGGAGATGCTGTGTG, R –
AATGTTCCTTGGGAGATGCTGTGTG, R –
GCTGCAAATGGAACCTCTTTGCTGT, R – TACCATGGAGGGGTGGTTGGAAT; PAI-1:
F – GCTTGGCAACCCACGTTAAAGGAA, R – TCTCTGCTTCTGGATGCCTTGGAA; IL-6
F – ATCCAGTTGCTTTCTTGGGACTGA, R – TAAGCCTCCGACTTGTGAAGTGTT; and
IL-10: F – TGCACTACCAAAGCCACAAAGCAG, R –
AGTAAGAGCAGGCAGCATAGCAGT. A forty eight-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.

**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). Briefly,
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 hour 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 (Molecular Probes, 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 anti-fade reagent (Vector Laboratories, Inc., Burlingame, CA). Images were acquired
using Olympus IX-70 fluorescent microscope.
Statistical analysis: Data are expressed as mean ± SEM. All data were analyzed using
SigmaStat version 3.5. Student's t test was used to compare data between two groups. For multiple group comparisons, one-way or two-way analysis of variance (ANOVA) 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 classical, 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 to 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 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, centrlobular 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 centrlobular region (data not shown).

**CVF pretreatment does not alter APAP metabolism.** Depletion of hepatic glutathione (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 glutathione; excessive NAPQI formation in APAP overdose depletes hepatic glutathione stores to < 20% of normal with in 1 h of treatment (Chiu et al., 2003). Mice treated with saline (control) had 3.92 ± 0.38 µmol GSH/g liver (Fig. 3). CVF treatment alone did not affect GSH concentration (4.41 ± 0.25 µmol GSH/g liver). APAP treatment resulted in GSH depletion to 0.83 ± 0.25 and 0.53 ± 0.05 µmol GSH/g liver within 1 hr 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 increase in ALT activity in plasma at 6 h (Fig 4A). CVF pretreatment reduced ALT activity from 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 out 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 which 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 to 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 centrlobular necrosis at 6 (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 affect of CVF pretreatment was observed on liver histopathology in groups treated with 200 mg/kg APAP (Figs. 4B). Treatment of mice with APAP (300 mg/kg) resulted in a time-dependent increase in plasma ALT activity (Figs. 4C). CVF pretreatment had no 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 using C3-/- mice. For this study, an APAP dose of 400 mg/kg was chosen, and the animals were examined at 12 h, since in a 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, respectively, in C3-/- mice treated
with 400 mg/kg APAP compared to wild type controls (Fig. 5A). Liver histopathology also revealed reduced centrilobular necrosis and hemorrhage in C3-/- mice at 12 h compared to 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 ± 3.5%) compared to APAP-treated wild type mice (36.9 ± 2.6%, 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 6h, and this increased by 24 h. Although CVF treatment by itself resulted very modest PMN accumulation, it significantly reduced APAP-induced PMN accumulation at both 6 and 24h compared to APAP treatment alone (Fig. 6A and B). 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 to 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 to control mice.

Plasma concentrations of TNF-α in saline- and CVF-treated mice were below the limit of detection (16 pg/ml) of the ELISA assay. APAP treatment increased TNF-α concentration in plasma at 6 and 12h (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 48h. 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 to 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 (Figure 8B). In a separate study, BrdU was administered two hours 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 mice at 24 h, suggesting that more cells were replicating in these mice compared to 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 8C 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. compared total hemolytic activity, expressed in CH50 units, and the concentrations of complement components in serum from 15 patients with liver disease due to alcohol toxicity and only 1 patient with APAP hepatotoxicity. In comparison to healthy subjects, patients under study had reduced CH50 values and small concentrations of C1q, C3, C4, and C5 in serum (Ellison III et al., 1990). Clapperton et al. studied 14 patients with acute liver failure due to APAP overdose and found a significantly smaller C3 concentration in plasma from patients (0.270 ± 0.078 mg/ml) compared to healthy control subjects (1.041± 0.059 mg/ml) (Clapperton et al., 1997). 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 appeared 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 MAC plays a role in the 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 classical pathway of complement activation can be initiated by compromised mammalian cells undergoing apoptosis or release of heat shock proteins (Prohaszka et al., 2002; Navratil et al., 2006). That neither complement depletion with CVF nor genetic deficiency in C3 protected completely against
APAP toxicity suggests that additional factors play a role in the progression of injury. Interestingly, hepatocytes damaged by APAP release various DAMPs, 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 (ie., 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 that complement activation delays this reparative response to APAP-induced injury. Interestingly, 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 prosurvival 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 CVF 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 (NAC), 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 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.
Reference List


Footnote

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Figure Legends

Figure 1. C3 concentration in plasma from mice treated with APAP. Mice were treated with saline vehicle (0 APAP) or with APAP (200, 300 or 400 mg/kg) after treatment with saline or CVF as described in Methods. C3 concentration in plasma was determined 6 (left) or 24 (right) hours later. Data are expressed as mean ± SEM; * 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.

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

Figure 3. Glutathione (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 in Methods. Data represent mean ± SEM, n = 4 animals per group. * significantly different from respective value in the absence of APAP (p<0.05).

Figure 4. Liver injury after APAP treatment. Mice were treated with saline vehicle (0 APAP) or with APAP (200, 300 or 400 mg/kg) after treatment with saline or CVF as described in Methods. (A) Plasma ALT activity at 6 (left) or 24 (right) hours after APAP. (B) Representative images from H&E-stained liver sections from livers harvested at 24 h. Images – a, b, c, and d represent...
treatment with APAP [0 (vehicle), 200, 300, or 400 mg/kg], respectively, after treatment with saline, and images – e, f, g and h represent treatment with APAP [0 (vehicle), 200, 300, or 400 mg/kg], respectively, 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 in Methods. Values for Saline/Saline are obscured by values for CVF/Saline. Data are expressed as mean ± SEM. * 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.

Figure 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 ± SEM; * significantly different from wild type at the indicated time (p<0.05). N = 3-5 per group (B) Shown are representative liver sections stained with H & E. Wild type (a) or C3-/- (b) mice were treated with APAP (400 mg/kg), and livers were harvested 12 h after APAP administration.

Figure 6. Hepatic neutrophil (PMN) accumulation after APAP administration. (A) Shown are 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 in Methods. (B) Morphometric determination of PMN accumulation in sections of liver 6 or 24 h after APAP treatment. Data represent mean ± SEM of PMNs counted in ten microscopic fields at 100X magnification. * significantly different from respective value in the absence of APAP; # significantly different from respective value in the absence of CVF.
Figure 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 GAPDH housekeeping gene and represented as percent 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 ± SEM. * 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.

Figure 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 GAPDH housekeeping gene and represented as percent of sal/sal (not shown) control. (C) and (D) Mice pretreated with CVF or saline and then treated 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 in Methods. Images a and b are from mice treated with Sal/APAP. Livers were harvested at 24 and 48 h, respectively; images c and d are from mice treated with CVF/APAP. Livers were harvested at 24 and 48 h, respectively. (D) Morphometric determination of BrdU positive cells in section of livers 24 or 48 h after APAP
treatment. Data represent mean ± SEM of BrdU positive cells counted in ten microscopic fields at 100X magnification. In panels A, B and D, data represent mean ± SEM. * 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.
Figure 1.

Plasma C3 (mg/ml) at 6 h and 24 h for different APAP doses (mg/kg) for Saline and CVF groups.

- * indicates a statistically significant difference between groups.
- # indicates a statistically significant difference from the 0 dose within the same group at the same time point.

APAP dose (mg/kg):
- 0
- 200
- 300
- 400

Plasma C3 (mg/ml) at:
- 6 h
- 24 h
Figure 1 (left panel).

![Graph showing plasma C3 levels at different APAP doses (mg/kg) at 6 hours.](image)

- **X-axis:** APAP dose (mg/kg)
- **Y-axis:** Plasma C3 (mg/ml)
- **Legend:** Saline (black bars) and CVF (gray bars)
- **Significance:**
  - *: Statistically significant difference
  - #: Not statistically significant
Figure 1 (right panel).

![Bar graph showing plasma C3 levels at different APAP doses (mg/kg) after 24 hours. The graph compares saline and CVF groups.](image-url)
Figure 2.
Figure 3.
Figure 4a.
Figure 4a (left panel).
Figure 4a (right panel).

The graph shows the plasma ALT (U/L) levels at 24 hours after administration of different doses of APAP (mg/kg). The bars represent the mean plasma ALT levels for saline and CVF treatment groups. The asterisks (*) indicate statistically significant differences compared to the 0 mg/kg dose, and the hash (#) indicates a significant difference between the saline and CVF groups.
Figure 4b.
Figure 4c.
Figure 5a.

Plasma ALT (U/L) vs. Hours after APAP administration.

- **Wild type**
- **C3-/-**

Bar graph showing the plasma ALT levels in wild type and C3-/- mice at 6 and 12 hours after APAP administration. Significant differences are indicated by asterisks (*) for each time point.
Figure 6b.
Figure 7a.

![Graph showing mRNA expression (% control) for IL-6 and IL-10 across different treatment groups.]

- Sal/Sal
- CVF/Sal
- Sal/APAP
- CVF/APAP

Significance markers:
- * indicates a significant difference from control
- # indicates a significant difference between Sal/APAP and CVF/APAP
Figure 7b.

![Graph showing Plasma TNFα (pg/ml) levels over time after APAP administration.]

- **X-axis:** Hours after APAP administration (6 and 12 hours).
- **Y-axis:** Plasma TNFα (pg/ml).

- **Legend:**
  - Black: Sal/APAP
  - Gray: CVF/APAP

- At 6 hours:
  - Sal/APAP: Approximately 60 pg/ml
  - CVF/APAP: Approximately 30 pg/ml
  - Significance indicated by #.

- At 12 hours:
  - Sal/APAP: Approximately 120 pg/ml
  - CVF/APAP: Approximately 30 pg/ml
  - Significance indicated by #.
Figure 7c.

[Graph showing Plasma PAL-1 levels in ng/ml for different conditions: Sal/Sal, CVF/Sal, Sal/APAP, and CVF/APAP at 6 and 24 hours after APAP administration. The bars are labeled with asterisks (*) indicating significant differences. The graph includes error bars to indicate variability.]
Figure 8a.

![Graph showing plasma ALT (U/L) levels at 24 and 48 hours after APAP administration for different groups: Sal/Sal, CVF/Sal, Sal/APAP, CVF/APAP.](image-url)
Figure 8b.

The graph shows the mRNA expression of PCNA, Cyclin D1, and p21 in response to Sal/APAP and CVF/APAP treatments. The expression levels are presented as a percentage of control. A significant difference is indicated by the symbol #.
Figure 8d.