Hepatocyte Injury by Activated Neutrophils *In Vitro* Is Mediated by Proteases

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Objective

This study determined the mechanism used by neutrophils (PMNs) to induce hepatocellular injury.

Summary Background Data

Neutrophils have been shown to be potent mediators of cell and tissue injury and have been hypothesized to contribute to the hepatic injury that occurs after trauma and infection. Oxygen radical scavengers protect the liver *in vivo* from inflammatory injury and it has been suggested that PMNs are the source of these toxic oxygen radicals. The specific mechanism used by PMNs to produce hepatocellular damage, however, has not been determined.

Methods

Neutrophils were cultured *in vitro* with hepatocytes (HCs) and stimulated with phorbol 12-myristate 13-acetate (PMA) to induce HC injury in the presence of oxygen radical scavengers and protease inhibitors.

Results

PMA induced a PMN-mediated HC injury that was dependent on the number of PMNs present and the concentration of PMA. Protease inhibitors reduced the extent of HC injury, while oxygen radical scavengers had no effect. Hydrogen peroxide, directly applied, was able to injure HCs, but only at concentrations greater than those that could be produced by PMA-stimulated PMNs.

Conclusions

PMNs are cytotoxic to cultured HCs, predominantly due to the release of proteolytic enzymes, while HCs appear relatively resistant to oxidative injury. Involvement of neutrophil toxic oxygen radicals in hepatic damage *in vivo* may require impairment of HC antioxidant defenses or may involve injury to nonparenchymal liver cells with secondary effects on HCs.

The hepatic failure associated with sepsis and trauma, as an isolated organ failure or as a component of multiple system organ failure, is associated with a mortality rate of approximately 40%.[1,2] The mechanism of this sepsis-induced dysfunction is poorly understood. Stud-

ies of the *in vitro* interactions between macrophages, Kupffer cells (KCs), and hepatocytes (HCs) have led investigators to suggest that the complex interactions between these cell types may contribute to hepatic failure in sepsis.[3,4] KC-dependent changes in HC function occur *in vitro* in response to inflammatory stimuli, resulting in both a reduction in total protein synthesis and an increase in acute phase proteins.[5,6] In addition, lipopolysaccharide (LPS)-stimulated KCs have been shown to induce mild hepatocellular damage when cocultured with HCs at high KC to HC ratios.[7]

The contribution of neutrophils (PMNs) to the alterations in hepatic function in sepsis has received less attention. Neutrophils accumulate in the liver in response to endotoxin or bacterial infusion and are associated with microscopic evidence of hepatic damage and increased plasma levels of hepatocellular enzymes.[8-10] In addition, the use of anti-PMN antibodies in animal models has been shown to reduce the hepatic damage resulting from endotoxin infusion,[11,12] providing direct support for a role for PMNs in mediating hepatic injury in sepsis. Previous *in vitro* studies using a bacterial challenge as an inflammatory stimulus have shown that neutrophil-mediated HC cytotoxicity occurs while no KC-induced damage could be produced.[13] Thus, it appears that PMNs not only contribute to hepatic dysfunction in sepsis, but may mediate more cellular damage than KCs.

Neutrophils possess a number of cellular mechanisms that are crucial to their role in the host defense system. They release potent proteolytic enzymes and generate toxic oxygen radicals that function to kill pathogenic micro-organisms.[14] The same cellular mechanisms that PMNs use to defend against bacterial invasion have been shown to produce cell and tissue injury, potentially leading to the organ dysfunction that occurs after an inflammatory insult.[15,16] In this study, we used a PMN:HC coculture model to stimulate PMN-mediated hepatic injury in sepsis[8-12] and we examined the relative contribution of neutrophil toxic oxygen radicals and proteases to this cellular injury.

MATERIALS AND METHODS

HC Cultures

HCs were isolated from male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, IN) weighing 200 to 300 g using a modification of the Seglen technique[17] as previously described.[18] After perfusion of the liver with collagenase, HCs were separated from nonparenchymal cells by repeated differential sedimentation at 50 g for 2 minutes. Cell viability ranged from 80% to 95% by trypan blue exclusion, with cell yield routinely being 1.5 to 2.5 × 10^{8} cells/liver. Cells were plated in gelatin-coated, 96-well plates at 2 × 10^{5} cells/mL in a volume of 0.1 mL to establish a concentration of 20,000 HCs/well.

Culture media consisted of Williams medium E (Gibco Laboratories, Grand Island, NY) supplemented with 10^{-6} M of insulin, 2 mM of L-glutamine, 15 mM of Hepes, penicillin, and streptomycin, and 10% calf serum. Cells were cultured alone for 24 hours before the addition of PMNs.

PMN:HC Coculture

Human PMNs were obtained from healthy volunteers by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) separation and dextran sedimentation.[19] Contaminating erythrocytes were lysed with ammonium chloride. The cells obtained were consistently 93% to 97% PMNs with viability of more than 98% by trypan blue exclusion. The old media was discarded and PMNs were added to the HC cultures in PMN:HC ratios ranging from 2:1 to 10:1 in fresh Williams medium E, supplemented as above except that 2.5% calf serum was used. The PMNs were allowed to settle onto the HCs for 15 minutes at 37 C and then, where indicated, were stimulated in the presence of protease inhibitors or oxygen radical scavengers. Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co.) was used to stimulate PMN oxygen radical production and degranulation. PMA was stored as a stock solution in DMSO at a concentration of 1 mg/mL and serially diluted in media before use. Protease inhibitors and oxygen radical scavengers (Sigma Chemical Co.) included catalase (46,500 U/mg), superoxide dismutase (SOD) (3600 U/mg), alpha$_1$-antitrypsin (α_1-AT), soybean trypsin-chymotrypsin inhibitor (TCI), phenyl-methylsulfonly-fluoride (PMSF), and deferoxamine myselate. The final volume in each well was 0.1 mL. At the indicated time points, culture supernatants were collected and frozen at −70 C before assay. All cultures were done in triplicate.

Enzyme Determinations

HC cytotoxicity was determined by measuring the release of the hepatocellular enzymes aspartate aminotransferase (AST) and ornithine carbamoyltransferase (OCT) into the culture media. AST is present in the cytosol and in mitochondria, and its release from HC cultures has been shown to correlate well with HC injury.[20] Measurements were performed using an automated procedure on a Technitron RA500 analyzer (Technitron Inc, Tarrytown, NY). OCT is a mitochondrial urea cycle enzyme that is virtually liver-specific and has been shown to be a useful diagnostic tool in hepatocellular disease.[21] Supernatant determinations of OCT were performed according to the method of Oshita et al.[22] Results are presented as the mean ± standard error of the mean of triplicate cultures. The Student's t test was used to determine significance.

Measurement of Hydrogen Peroxide and Superoxide

Hydrogen peroxide production by PMNs was measured by the horseradish peroxidase (HRPO)-dependent oxidation of phenol red as described by Pick and Keisari.[23] Briefly, PMNs were plated at 2×10^5 PMNs/well in 96-well plates with 20 U/mL of HRPO and 0.2 g/L of phenol red in a volume of 0.1 mL. After PMN stimulation by PMA, 1 M of NaOH was added and the change in absorbance at 610 nm was determined using a microplate reader (Vmax, Molecular Devices, Inc., Menlo Park, CA). The concentration of H_2O_2 was determined by extrapolation from a standard curve created using solutions of known H_2O_2 concentrations treated identically. The H_2O_2 concentration of the standard was determined by its absorbance at 230 nm using an absorption coefficient of 81 $M^{-1}cm^{-1}$. Superoxide production was measured by the reduction of cytochrome C by 2×10^5 PMNs stimulated in the presence or absence of superoxide dismutase.[24] The above assays were performed in quadruplicate.

Effect of Nitric Oxide Synthesis

To study the effect of nitric oxide (N = O) production on PMN-induced HC injury, HCs were cultured in isolation as described above with the addition of 10^{-6} M of dexamethasone to the culture media for the initial 24 hours. The HCs were then washed thoroughly with arginine-free media and cultured with either arginine-free media alone or conditioned KC supernatant plus 100 U/mL of interferon-gamma (IFNτ) (Amgen Biologicals, Thousand Oaks, CA) to stimulate HC N = O production.[25] After 14 hours, the HCs were washed and the media replaced with media containing 2 mM of L-arginine (Gibco Laboratories), the substrate for N = O synthesis. N^G-monomethyl-L-arginine (NMA) was prepared as described[26] and added where indicated to inhibit N = O synthesis. PMA (100 ng/mL) was added where indicated, followed by PMNs at a PMN:HC ratio of 10:1 to induce HC damage. Supernatants were collected 8 and 24 hours after the addition of PMNs and analyzed as described.

Conditioned KC supernatant was prepared as described.[25] Briefly, rat KCs were isolated using pronase digestion and elutriation (> 90% purity by peroxidase staining) and cultured in 25-cm² flasks at 2×10^6 KC/mL with 100 U/mL of IFNτ. After 24 hours, the media was changed to arginine-free media with 100 U/mL of IFNτ and 10 μg/mL of LPS (from *Escherichia coli* 0111:B4, Sigma Chemical Co.). The conditioned supernatant was collected 8 hours later, filtered, and stored at −70 C until use.
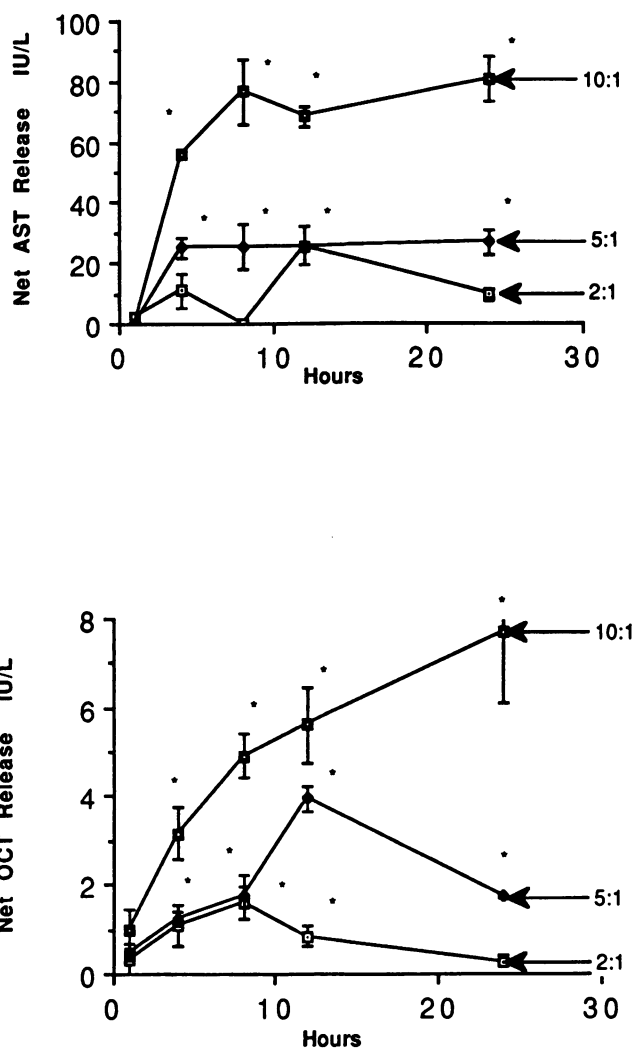

Figure 1. Effect of PMN:HC ratio on HC cytotoxicity. (Top) The net AST release [(cultures with indicated PMN:HC ratio)-(cultures with HCs alone)] in supernatants after stimulation with 100 ng/mL of PMA. (Bottom) The net OCT release. Values represent the mean ± SEM of triplicate cultures from one of three similar experiments. Asterisk indicates p < 0.05 compared to HCs alone. PMNs without stimulation were associated with little enzyme release (10.00 ± 0.82 vs. 34.00 ± 0.58 IU/L of AST and 0.86 ± 0.05 vs. 1.48 ± 0.12 IU/L of OCT, HCs alone vs. HCs with unstimulated PMNs at 10:1 ratio, measured at 12 hours).

Coculture N = O production was determined by measuring the production of nitrite and nitrate (NO_2^-/ NO_3^-), the endproducts of N = O metabolism, as described.[25,26]

RESULTS

Neutrophil-Mediated HC Damage

HC damage secondary to PMA-stimulated PMNs was evident as elevations of both AST and OCT in culture supernatants. The extent of HC injury was dependent on both the number of PMNs and the duration of coculture (Fig. 1). Significant elevations in AST and OCT were
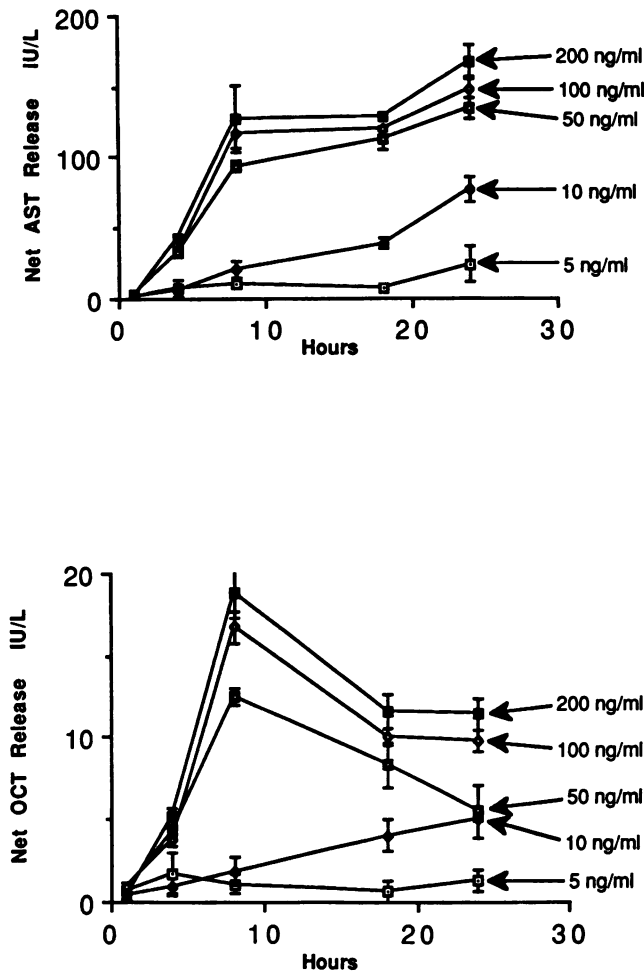
Figure 2. Effect of PMA concentration on HC cytotoxicity. (Top) The net AST release [(PMN:HC cultures with indicated PMA dose)-(PMN:HC cultures without PMA)] into cultures with the indicated PMA concentrations. The PMN:HC ratio was 10:1 for all cultures. (Bottom) The net OCT release. Values represent the mean ± SEM of triplicate cultures from one of three similar experiments.

present after 4 hours of coculture when PMN:HC ratios of 5:1 and 10:1 were used. At the PMN:HC ratio of 10:1, AST release was virtually complete at 8 hours with little additional enzyme release at 24 hours. OCT release, in contrast, continued to increase beyond 8 hours of coculture. When a PMN:HC ratio of 2:1 was used, small elevations in OCT were present after 8 and 12 hours, while increased AST was present at 12 hours only. PMNs alone without stimulation were associated with a small increase in AST and OCT in culture supernatants. This slight increase required at least 12 hours of coculture and a PMN:HC ratio of 10:1. Unstimulated PMNs at ratios of 2:1 and 5:1 were not associated with increased cytotoxicity up to 24 hours of coculture (data not shown).

The degree of cytotoxicity at a PMN:HC ratio of 10:1 varied with the concentration of PMA (Fig. 2). Increasing concentrations of PMA produced a corresponding increase in AST and OCT release. A PMA concentration

of 50 ng/mL resulted in a significant increase in enzyme release as early as 4 hours of coculture ($p < 0.05$), while PMA at 10 ng/mL required 8 hours to produce increased AST release and 18 hours for increased OCT release. PMA doses below 10 ng/mL produced no measurable cytotoxicity. When cultured with HCs in the absence of PMNs, PMA concentrations up to 200 ng/mL did not result in increased hepatocellular enzyme release (data not shown). HCs in suspension at a concentration of 2×10^5 HCs/cc, subjected to 3 cycles of rapid freeze-thaw using liquid nitrogen, released 885.00 ± 10.79 and 86.07 ± 1.49 IU/L of AST and OCT, respectively. Neutrophils in suspension at a concentration of 2×10^6 PMNs/cc, subjected to the same freeze-thaw procedure, released 5.30 ± 0.67 and 0.27 ± 0.07 IU/L of AST and OCT, respectively. Thus, the enzyme elevations measured were due to PMN effects on HCs and were not due to enzyme release from the PMNs themselves. For the remaining experiments, OCT measurements alone were performed.

Effects of Oxygen Radical Scavengers

Neutrophils in coculture with HCs were stimulated in the presence of oxygen radical scavengers to determine the contribution of these reactive oxygen species to HC damage. Catalase and SOD were used to neutralize hydrogen peroxide and the superoxide ion, respectively. Ascorbate detoxifies superoxide and hydroxyl radicals, while deferoxamine prevents hydroxyl radical formation. When culture supernatants were assayed 8 hours after PMN stimulation, at which time near-maximal enzyme release occurred, no significant protective effect with oxygen radical scavengers could be demonstrated (Table 1). This lack of protection was also evident if supernatants were collected as early as 4 hours or as late as 24 hours after PMN stimulation with PMA. Catalase and SOD in combination failed to protect the HCs from PMN-induced damage. Higher concentrations of catalase (4000 U/mL) or SOD (600 U/mL) alone were not protective (data not shown). When either cultured alone with HCs or in the presence of unstimulated PMNs, these scavengers were not associated with changes in OCT levels (data not shown).

PMN-Generated H_2O_2/O_2^- and the Effect of Reagent H_2O_2

It has been shown that hydrogen peroxide generated from glucose oxidase is cytotoxic to cultured HCs.[27] We found that the addition of H_2O_2 directly to HC cultures resulted in cellular damage (Fig. 3). This damage, however, required high concentrations of H_2O_2 (800 uM or greater), was completely neutralized with 2000 U/mL of

Table 1. EFFECT OF OXYGEN RADICAL SCAVENGERS ON NEUTROPHIL-MEDIATED HEPATOCYTE CYTOTOXICITY

	4 Hours*	8 Hours*	24 Hours*
PMN:HC alone	100.00 ± 3.84	100.00 ± 3.93	100.00 ± 2.43
Catalase (2000 U/mL)	111.13 ± 7.47	97.13 ± 9.65	94.66 ± 10.07
SOD (200 U/mL)	94.96 ± 5.91	107.07 ± 9.21	106.90 ± 15.16
Deferoxamine (10 mM)	87.71 ± 6.62	107.60 ± 20.93	121.56 ± 14.43
Ascorbate (5 mM)	ND	165.80 ± 26.95	108.34 ± 7.23
Catalase + SOD	ND	91.67 ± 9.50	92.40 ± 6.87

ND: not determined.
* Results expressed as a percentage of the OCT release in PMN:HC cultures without oxygen radical scavengers (control). The PMN:HC ratio was 10:1 with a PMA concentration of 100 ng/mL in all cultures. Values represent the mean ± SEM of at least three separate experiments. Control OCT release was 10.67 ± 0.62 IU/L, 14.54 ± 1.50 IU/L, and 19.43 ± 1.20 IU/L at 4, 8, and 24 hours, respectively.

catalase (data not shown), and was mild compared to that inflicted by stimulated PMNs. Using the HRPO method, 2×10^5 PMNs could generate only 218.66 ± 2.73 uM of H_2O_2 when exposed to 100 ng/mL of PMA for 3 hours. Exposure beyond 3 hours did not result in additional H_2O_2 release from stimulated PMNs (data not shown). To determine if superoxide was being completely scavenged by SOD, superoxide production by 2×10^5 PMNs stimulated by 100 ng/mL of PMA was measured in the presence and absence of 200 U/mL of SOD. This concentration of PMA resulted in a large generation of O_2, while the presence of SOD effectively inhibited the reduction of cytochrome C (Table 2) to below that produced by resting (unstimulated) PMNs.
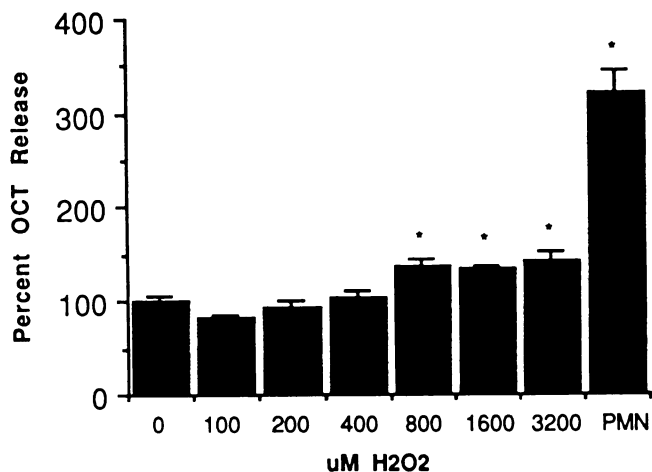

Figure 3. Effect of H_2O_2 on HC cytotoxicity. Concentrations of H_2O_2 were varied as indicated and OCT release was measured after 24 hours. Results are expressed as percentage of control (HCs alone without H_2O_2). Values represent the mean ± SEM of triplicate cultures from one of three similar experiments. Enzyme release induced by a 10:1 PMN:HC ratio with 100 ng/mL of PMA is expressed as a percentage of PMN:HC culture without PMA (ten experiments). Catalase (2000 U) completely prevented the increased OCT release with 800, 1600, and 3200 μM of H_2O_2 (not shown). Asterisk indicates p < 0.05 compared to control.

Effect of Protease Inhibitors

The protease inhibitors α_1-AT, TCI, and PMSF were tested for their ability to protect HCs from PMN-mediated damage. All protease inhibitors tested were able to reduce the amount of OCT released in 4-, 8-, and 24-hour cultures (Table 3). The protective effect was greatest at 4 and 8 hours with TCI, and decreased slightly with time. Bovine serum albumin was not protective, showing that the reduction in OCT release was not due to a nonspecific effect of added protein. The protective action of α_1-AT could be abolished by heating the preparation in a boiling water bath before adding to coculture, demonstrating the requirement for the active enzyme. Higher concentrations of protease inhibitors did not contribute additional protection (data not shown).

Sublethal doses of H_2O_2 cause endothelial cells to be more susceptible to proteolytic attack.[28] To determine if H_2O_2 was acting in a similar fashion in HC cultures, combinations of catalase, SOD, and α_1-AT were studied. Neither catalase nor SOD provided any additional protective effect beyond that provided by α_1-AT alone when cultured together for 8 hours (Fig. 4). Similar results were found at 24 hours (data not shown).

Effect of N = O Synthesis

It has been previously demonstrated that KCs can induce minor HC cytotoxicity in coculture by an N = O–

Table 2. EFFECT OF SUPEROXIDE DISMUTASE ON NEUTROPHIL PRODUCTION OF SUPEROXIDE

	nmol O_2^-/2 × 10^5 PMN/120 min
PMN	4.13 ± 0.32
PMN + PMA (100 ng/mL)	23.24 ± 1.84
PMN + PMA + SOD (200 U/mL)	0.19 ± 0.08

Table 3. EFFECT OF PROTEASE INHIBITORS ON NEUTROPHIL-MEDIATED HEPATOCYTES CYTOTOXICITY

	4 Hours*	8 Hours*	24 Hours*
PMN:HC alone	100.00 ± 3.84	99.82 ± 2.93	99.99 ± 2.07
α_1-AT (1 mg/mL)	69.52 ± 5.24†	52.67 ± 4.55†	84.30 ± 4.07‡
PMSF (1 μM)	ND	67.68 ± 5.48†	73.94 ± 5.15‡
TCl (2 mg/mL)	54.68 ± 3.90†	55.38 ± 7.30†	65.37 ± 11.37‡
Bovine serum albumin (1 mg/mL)	ND	134.44 ± 29.50	ND
α_1-AT, heat inactivated	ND	98.63 ± 17.48	ND

ND: not determined.
* Results expressed as a percentage of the OCT release in PMN:HC cultures without protease inhibitors (control). The PMN:HC ratio was 10:1 with a PMA concentration of 100 ng/mL in all cultures. α_1-AT was heat inactivated by placing in a boiling water bath for 60 minutes. Values represent the mean ± SEM of at least three separate experiments. Control OCT release was 10.67 ± 0.62 IU/L, 16.68 ± 1.48 IU/L, and 20.27 ± 1.91 IU/L at 4, 8, and 24 hours, respectively. OCT release in PMN:HC cultures without PMA (spontaneous release) was 30.89 ± 2.57% and 29.82 ± 2.72% at 8 and 24 hours, respectively. Spontaneous release in PMN:HC cultures at 4 hours was 50.92 ± 4.74% of control (4.85 ± 0.35 IU OCT/L), the same as HC cultured alone without PMNs (47.99 ± 2.64% of control; 4.67 ± 0.35 IU OCT/L).
† p < 0.001.
‡ p < 0.01.

mediated mechanism.[7] PMNs can synthesize N = O,[29] as can HCs.[25] To determine if N = O synthesis by either PMNs or HCs was involved in PMN-induced HC cytotoxicity, NMA was used to inhibit N = O production in PMN:HC coculture. HCs were stimulated to synthesize NO_2^-/NO_3^- with conditioned KC supernatant,[25] while PMA was used to active the PMNs. PMN:HC cocultures, not pretreated with conditioned KC supernatant, produced little NO_2^-/NO_3^- (8 hours: 0.8 ± 0.4 μM of NO_2^-/NO_3^-; 24 hours: 1.6 ± 0.8 μM of NO_2^-/NO_3^-). The addition of PMA to activate PMNs had no effect on supernatant NO_2^-/NO_3^- (8 hours: 1.1 ± 0.4 μM of

Figure 4. Effect of combinations of oxygen radical scavengers and α_1-AT. Results expressed as percentage of control OCT release (PMN:HC cultures without inhibitors) measured after 8 hours. PMA concentration was 100 ng/mL in all cultures. Values represent the mean ± SEM of the means from five experiments. Control OCT release was 14.54 ± 1.50 IU/L. OCT release was significantly reduced for all groups compared to control (p < 0.001), but was not reduced by combinations of oxygen radical scavengers with α_1-AT compared to α_1-AT alone (p = 0.472 and p = 0.212 for α_1-AT/catalase and α_1-AT/SOD, respectively).

NO_2^-/NO_3^-; 24 hours: 0 μM of NO_2^-/NO_3^-). Pretreatment of HCs with conditioned KC supernatant resulted in increased NO_2^-/NO_3^- synthesis (8 hours: 24.3 ± 1.6 μM of NO_2^-/NO_3^-; 24 hours: 30.5 ± 2.2 μM of NO_2^-/NO_3^-) that was inhibited with NMA (8 hours: 3.2 ± 1.2 μM of NO_2^-/NO_3^-; 24 hours: 0.8 ± 0.4 μM of NO_2^-/NO_3^-). While NMA effectively decreased NO_2^-/NO_3^- synthesis, it had no effect on PMN-induced HC injury (Fig. 5).

DISCUSSION

Neutrophils have been implicated in a variety of disease processes involving inflammation including rheumatoid arthritis and ulcerative colitis.[16] They have also been linked to some of the pathophysiologic conditions associated with sepsis and trauma. They are thought to mediate, at least in part, the lung parenchymal damage associated with systemic complement activation and have been associated with the pathophysiology of the adult respiratory distress syndrome.[30,31] They are also thought to participate in damage secondary to hemorrhagic shock and reperfusion.[32,33]

Experimentally, direct complement activation induces PMNs to aggregate intravascularly.[34] Aggregates of PMNs may also be found in various organs after bacterial endotoxin infusion or after soft tissue trauma.[34,35] The liver, presumably due to its large vascular bed, is significantly affected by these manipulations and reveals an early deposition of PMNs within the sinusoids. This hepatic PMN influx is associated with elevations of plasma hepatocellular enzyme levels, disturbances of hepatic microcirculatory blood flow, and histologic evidence of organ injury, suggesting a causal relationship between PMN stimulation and alterations in hepatic function.[8,10,13] Evidence for some role for the PMNs in
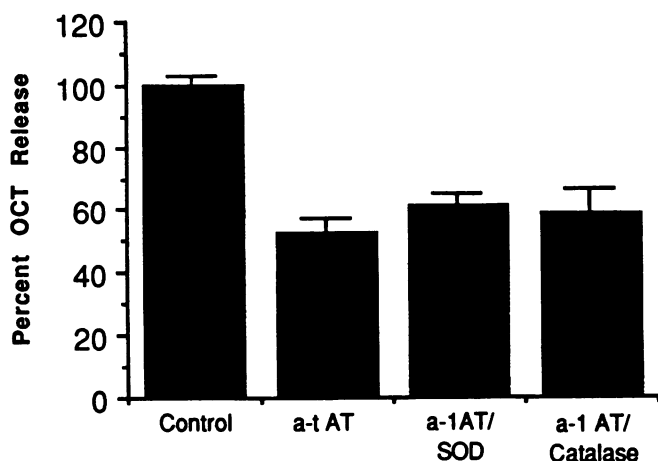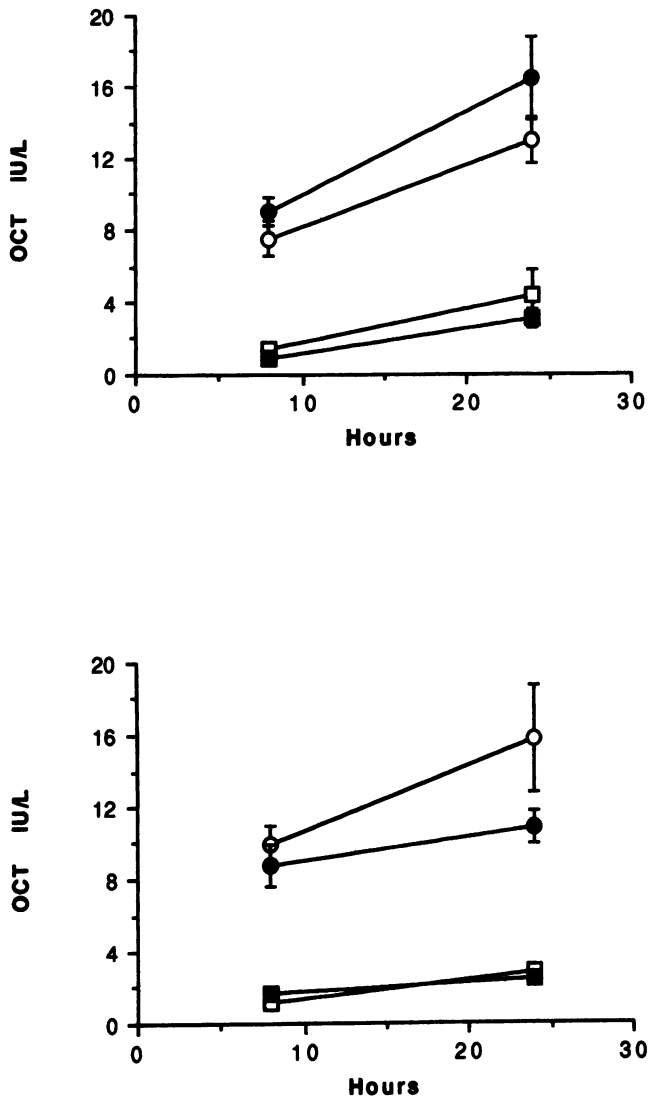
Figure 5. Effects of NMA on PMN-mediated HC cytotoxicity. PMN:HC cocultures were cultured in 2 mM of L-arginine alone (□) or with 1 mM of NMA (■), 100 ng/mL of PMA (O), or NMA/PMA (●), and the degree of HC toxicity was determined. (Top) HCs cultured in arginine-free media alone (no HCs N = O synthesis). (Bottom) HCs cultured in conditioned KC supernatant to stimulate HC N = O synthesis. Differences are not statistically significant.

mediating hepatic injury is found in studies that demonstrate improved survival and hepatic function after PMN depletion in various animal models of sepsis.[11,12,33,36] Our study was conducted to investigate the specific cellular mechanisms involved in PMN-induced hepatic injury.

In vitro, PMNs induce cellular injury by way of their production of toxic oxygen radicals,[37,38] release of proteolytic enzymes,[39] or a combination of the two processes.[28] We have demonstrated that phorbol ester-stimulated PMNs directly damage cultured HCs and that the amount of damage is dependent on both the number of PMNs present and the intensity of the stimulus to the

neutrophil. Low numbers of stimulated PMNs inflict little damage, suggesting that a threshold of toxic PMN products is required for the development of HC injury.

We also demonstrated that the HC damage in coculture is prevented by the addition of protease inhibitors that neutralize PMN proteolytic enzymes. Each protease inhibitor tested was able to reduce the HC injury to a significant degree. The absence of an effect with albumin demonstrated that this protection was not due to a nonspecific effect of added protein. The protective action could be abolished by heating the inhibitor before adding to coculture, confirming that the enzymatic function of the inhibitor was essential. α_1-AT is the predominant protease inhibitor found in human serum and has the broadest spectrum of activity in neutralizing proteolytic activity.[40] It was effective in protecting HCs at all time points studied. The ability of all three protease inhibitors tested to provide a substantial reduction in OCT release suggests that a chymotrypsin-like or neutral protease is responsible for most of the HC injury in coculture. The failure of these protease inhibitors to provide complete HC protection from stimulated PMNs suggests that other proteolytic enzymes may also be involved in mediating HC injury. Other neutrophil lysosomal enzymes that may be involved include acid or alkaline proteases and nucleotidases, ribonucleases, and peroxidases.[41]

We were unable to demonstrate a direct role for superoxide, hydrogen peroxide, or the hydroxyl radical in HC cytotoxicity despite the use of a PMN stimulus, phorbol ester, which is a potent inducer of oxygen radical production. We were able to induce cytotoxicity with reagent hydrogen peroxide when applied directly to HCs, a finding that is consistent with studies that show HC injury by the H_2O_2-producing enzyme glucose oxidase.[27] The amount of hydrogen peroxide required to elicit HC damage, however, was far greater (approximately fourfold) than could be produced by the PMA-stimulated PMNs used in these experiments. In addition, this high concentration of hydrogen peroxide produced relatively little enzyme release compared to that produced by activated PMNs. Oxygen radical scavengers were unable to reduce the PMN-induced injury when added alone or in combination, despite concentrations that were effective in neutralizing these radicals (Table 2 and Fig. 3). They also did not increase the protective effect of protease inhibitors when used in combination with these substances. These data suggest, therefore, that PMN-derived oxygen radicals contribute little to the PMN-induced HC injury. We cannot rule out the possibility, however, that PMN oxygen radicals may have a toxic effect in coculture that may not involve hepatocellular enzyme release from cultured HCs, such as an effect on HC DNA or cellular proteins.[42,43]

Our laboratory has previously shown that KCs may induce HC cytotoxicity and that this injury is mediated

by the conversion of L-arginine to $N = O$.[7] The degree of KC-mediated HC injury was small, however, in agreement with other investigators who could elicit only minimal KC-induced HC damage in response to a phagocytic challenge.[13] This finding is in marked contrast to the profound PMN-mediated HC injury in the current report. $N = O$ can be produced by both KCs[25] and PMNs,[29] although the quantity of $N = O$ produced by PMNs is small and requires indirect techniques for measurement.[29] $N = O$ may potentially have beneficial or harmful effects in PMN:HC coculture because of its ability to react with the superoxide ion.[29] This interaction has been postulated to be protective by detoxifying the O_2^- radical and preventing O_2^--mediated injury.[29,44] It has also been hypothesized to be harmful by leading to the production of the peroxynitrite radical, a very unstable and dangerous species.[45] In the current report, however, we were unable to demonstrate a role for $N = O$ in either promoting or preventing HC injury by activated PMNs.

Oxygen radicals have been implicated as a principal mediator in the hepatic injury that occurs in experimental models of sepsis.[42,46,47] PMNs have been postulated to be one possible source of these injury-producing oxygen radicals,[46,48] and studies that show reduced hepatic damage after PMN depletion in septic animals would appear to support this theory.[11,12] In this report, we studied the ability of PMNs to damage resting HCs *in vitro*. We used a PMN:HC ratio of 10:1 to determine the specific cytotoxic mechanism involved in this coculture system. This ratio is greater than the PMN:HC ratio found in the liver *in vivo* after endotoxin infusion (less than 1:1).[48] Even at this high PMN concentration, however, we were still unable to demonstrate a direct role for PMN-derived hydrogen peroxide, superoxide, or hydroxyl radical in HC cytotoxicity. This finding suggests that under a physiologic *in vivo* condition, PMN-mediated HC injury in unlikely to be caused by the effect of PMN-derived oxygen radicals. HCs contain large amounts of catalase, superoxide dismutase, glutathione, and glutathione peroxidase that function to detoxify oxygen radicals and prevent oxygen radical-mediated cell damage.[42] Our data suggest that even at an extremely high PMN density, resting HC anti-oxidant defenses are sufficient to prevent significant PMN-induced oxygen radical-mediated injury from occurring *in vitro*.

The mechanism or mechanisms involved in PMN-mediated hepatic injury *in vivo*[11,12] have not yet been determined. Our data would suggest that PMN proteolytic enzymes are involved in this injury because they can directly injure coculture HCs. While we could find no role for oxygen radicals in our *in vitro* model of hepatic injury, there may be conditions *in vivo* in which PMN oxygen radicals contribute to hepatic damage. It has been shown that sepsis results in a decrease in intra-

cellular glutathione in HCs,[49] suggesting that a reduction in the ability of HCs to detoxify oxygen radicals may accompany the complex metabolic changes associated with sepsis. It is possible that this reduction in antioxidant capacity in sepsis may make the HCs *in vivo* more susceptible to PMN oxygen radicals than resting HCs are *in vitro*.

A separate hypothesis consistent with findings from other investigators would be that PMN-derived oxygen radicals may produce HC damage *in vivo* but induce this injury indirectly through actions on other cells in the liver. Endothelial cells are known to be highly susceptible to damage from PMN oxygen radicals.[28,37] PMN-mediated endothelial cell damage *in vivo* could potentially lead to endothelial cell disruption with exposure of the subendothelial matrix, promoting fibrin clot deposition and injury to the hepatic microvasculature. This disruption of the normal pattern of hepatocellular perfusion may eventually lead to an ischemia/reperfusion injury that results in hepatocellular damage.[42,46] Further work is needed to clarify the precise role of PMN-derived oxygen radicals in inflammatory liver injury *in vivo*.

These studies demonstrate that cultured HCs are susceptible to injury by PMA-stimulated PMNs and that the cytotoxicity is mediated by the release of PMN proteolytic enzymes. Oxygen radical scavengers did not protect HCs from injury in this coculture model. *In vivo* PMN-induced hepatic damage may therefore be caused by the release of these proteolytic enzymes from stimulated PMNs. Involvement of neutrophil oxygen radicals in HC injury *in vivo* may require a reduction in HC antioxidant defenses or may involve damage to adjacent nonparenchymal liver cells with secondary effects on HCs.

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