0023-6837/94/7001-0053$03.00/0
LABORATORY INVESTIGATION

Vol. 70, No. 1, p. 53, 1994
Printed in U.S.A.

Activated Neutrophils from Rat Injured Isolated Hepatocytes

PATRICIA E. GANEY, MARC B. BAILIE, SIMI VANCISE, MARIA E. COLLIGAN, B. V. MADHUKAR, J. PAUL ROBINSON, AND ROBERT A. ROTH

Departments of Pharmacology and Toxicology, Medicine, and Pediatrics and Human Development, and Institute for Environmental Toxicology, Michigan State University, East Lansing, Michigan; the Purdue University Cytometry Laboratories and Department of Veterinary Physiology and Pharmacology, Purdue University, West Lafayette, Indiana

BACKGROUND: Activated neutrophils (PMNs) release cytotoxic agents that can damage surrounding tissue. These studies were performed to determine whether activated PMNs from rat could injure isolated, rat hepatic parenchymal cells (HCs) *in vitro*.

EXPERIMENTAL DESIGN: HCs were cocultured with unstimulated rat PMNs or with PMNs activated with either f-met-leu-phe (FMLP) or phorbol myristate acetate (PMA), that stimulate predominantly degranulation or superoxide production, respectively. Toxicity to HCs was evaluated from release of alanine aminotransferase into the medium.

RESULTS: Alanine aminotransferase release was greater in HCs cocultured with FMLP- or PMA-stimulated PMNs compared with unstimulated PMNs. Toxicity was observed by 16 hours after stimulation of PMNs. To test the possible involvement of a soluble mediator released by activated PMNs, HCs were incubated with conditioned medium from PMNs. Compared with unstimulated PMNs, toxicity to HCs was greater in the presence of conditioned medium from FMLP-stimulated PMNs, but not conditioned medium from PMA-activated PMNs. Reactive oxygen species do not appear to be involved in the mechanism by which activated PMNs damage HCs since superoxide dismutase, catalase, superoxide dismutase + catalase, or desferrioxamine failed to prevent the injury. Furthermore, less superoxide anion was detected in PMA-stimulated PMNs when either HCs or HC-conditioned medium was present. Proteolytic enzymes released by stimulated PMNs may play a role in HC damage since an inhibitor of proteases diminished injury due to PMNs activated by either FMLP or PMA.

CONCLUSIONS: These results indicate that activated, rat PMNs damage HCs in culture. The data suggest that reactive oxygen species are not involved in the mechanism, but that release of proteolytic enzymes may play a role in the toxic response.

Additional key words: Injury, Proteases, Superoxide anion.

Activated neutrophils (PMNs) play an important role in host defense against pathogens in part through generation of superoxide anion (O_2^-) and release of mediators such as proteolytic enzymes and arachidonic acid metabolites. The same functions that impart the ability to assist in host defense can also lead to tissue damage under certain circumstances. PMNs have been implicated in complement-mediated pulmonary vascular injury (1), in immune complex-mediated injury to kidney (2) or lung (3), in corneal injury in the eye (4), in reperfusion injury after ischemia in heart (5), and in endotoxin-induced lung damage (6).

It has been demonstrated more recently that PMNs can contribute to injury in the liver. Hepatotoxicity induced in rats by administration of α-naphthylisothiocyanate (ANIT) (7) or endotoxin (8) is attenuated by prior depletion of circulating PMNs. Thus, PMNs can participate in liver injury *in vivo*. In addition, human PMNs activated with phorbol myristate acetate (PMA) damage isolated, rat hepatocytes grown in culture (9). The purpose of this study was to determine whether activated PMNs from the rat injure isolated, rat hepatocytes and, if so, to begin to explore the mechanism(s) involved in the hepatocellular injury.

EXPERIMENTAL DESIGN

Rat hepatic parenchymal cells (HCs) were isolated as described in the "Methods" section, and were plated in 6-well tissue culture plates at a density of 5×10^5 HCs/well. They were allowed to attach for 3 hours, then PMNs isolated from rat peritoneum as described in the "Methods" section were added at the indicated ratio of PMN/HC. After allowing the PMNs to attach for 30 minutes, f-met-leu-phe (FMLP) or PMA was added to the cocul-

tures, and toxicity was assessed (routinely 16 hours later) from release of alanine aminotransferase (ALT) into the medium. ALT is a sensitive and specific indicator of hepatocellular damage in rat (10, 11), and in this model, release of ALT from HCs correlates ($r = 0.998$) with cell death measured by uptake of trypan blue. Viability of PMNs was determined in separate wells from release of lactate dehydrogenase (LDH) into the medium. In addition, cellular damage was examined in fixed, cultured cells using scanning electron microscopy.

In studies in which the effect of conditioned medium from PMNs was examined, PMNs were plated (5×10^6/well) in tissue culture plates and 30 minutes later were stimulated with FMLP or PMA. After 30 minutes of exposure of PMNs to the stimulus, the medium was removed and spun in a centrifuge, and the cell-free supernatant fluid was added to HCs that had been plated 3 hours earlier. Toxicity was determined 16 hours after addition of conditioned medium.

Generation of O_2^- by PMNs in the absence and presence of HCs was determined as described in the "Methods" section. PMNs were plated as described above, and medium exposed to HCs or containing HCs (5×10^5) was added to the PMNs. O_2^- production was determined after 30 minutes.

STATISTICAL ANALYSIS

Results are presented as the mean ± SEM. For all results presented, N represents the number of repetitions of an experiment, each experiment using cells from different rats. Each value (N) is the average of duplicate determinations. Data were analyzed by Student's t-test (H_2O_2 production) or by analysis of variance. When analysis of variance was employed, individual means were compared using either the least significant difference test (for experiments in which the effect of antiproteases was tested and for time-course data) or Tukey's omega procedure (for all other comparisons). Data expressed as the percentage of total enzyme released were transformed using the arcsin of the percentage before further statistical manipulation. For all studies, the criterion for significance was $p \leq 0.05$.

RESULTS AND DISCUSSION

HC INJURY BY STIMULATED RAT PMNS

In isolated HCs incubated in the absence of PMNs, about 20% of the total cellular ALT appeared in the culture medium after 16 hours (Fig. 1). Addition of unstimulated PMNs in ratios up to 10 PMN/HC did not significantly affect the basal release of ALT; however, at a ratio of 20 PMNs/HC ALT activity released by the HCs was increased slightly to about 30%. In the absence of PMNs, FMLP did not injure HCs (Fig. 1). Stimulation of PMNs with FMLP enhanced hepatocellular injury at all ratios of PMNs/HCs studied.

The surface of HCs cultured with unstimulated PMNs (Fig. 2*A*) or with cytochalasin B and FMLP in the absence of PMNs (not shown) was round and regular in appearance with numerous microvillous projections. When cultured at a ratio of 10 PMNs/HC, most HCs had several PMNs associated with the cell surface (Fig. 2*A*). HCs cultured with PMNs stimulated with FMLP were often irregular in appearance, and outpouches of the cytoplasmic membrane (*i.e.*, blebs) were common (Fig. 2*B*). In addition, some PMNs were associated with what appeared to be remnants of cells or cellular debris.

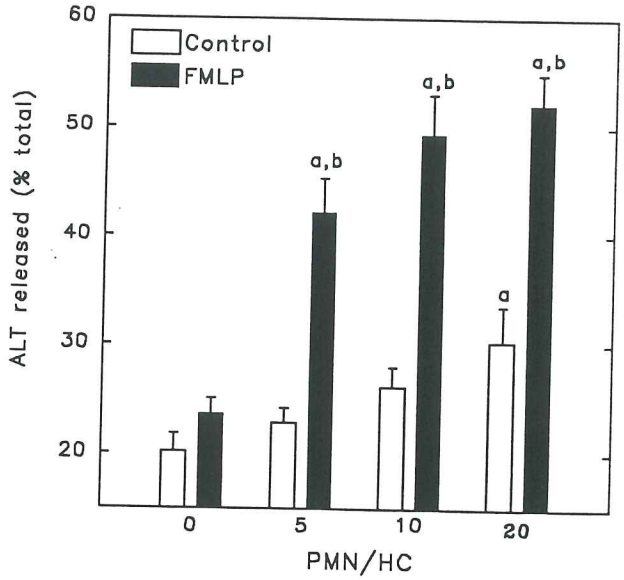

FIG. 1. Release of ALT from HCs in the presence of FMLP-stimulated PMNs. Isolated HCs were plated (5×10^5/well), and after 3 hours the medium was changed and PMNs were added at the ratios indicated (0 PMN/HC refers to wells with HCs only). About 30 minutes later, cytocholasin B (5 μg/ml) was added to each well, followed 5 minutes later by addition of either 100 nM FMLP (■) or its vehicle (□). The activity of ALT was determined in the cell-free medium and the cell lysates 16 hours later, and the percentage of total ALT released was calculated as described in the "Methods" section. $N = 6$. *a*, Significantly different from the respective group without PMNs. *b*, Significantly different from the respective group without FMLP.

In the absence of PMNs, PMA alone did not increase release of ALT from HCs (Fig. 3). When PMA was used as a stimulus for PMN activation, PMNs increased HC injury significantly at a ratio of 10 PMNs/HC. In control cells in the absence of PMA, increasing the ratio of PMNs to HCs caused a modest degree of HC injury.

PMN viability remained relatively constant over 16 hours in culture. In unstimulated PMNs or PMNs activated with FMLP or PMA, LDH release was about 8% after 2 hours in culture, and about 15% after 16 hours in culture. No statistical differences due to stimulation with PMA or FMLP were observed (data not shown).

Because FMLP-stimulated PMNs damaged HCs in ratios as low as 5 PMNs/HC, studies were performed to determine a threshold for this effect. No increase in HC injury was observed with either FMLP- or PMA-stimulated PMNs when equal numbers of PMNs and HCs were cocultured (data not shown).

The development of injury in HCs incubated with unstimulated PMNs or with FMLP- or PMA-activated PMNs was determined. No significant release of ALT was observed after 8 hours in culture (Fig. 4). By 16 hours, injury from FMLP-activated PMNs was significantly greater than control. HC damage caused by PMA-stimulated PMNs was significantly greater than control after 24 hours.

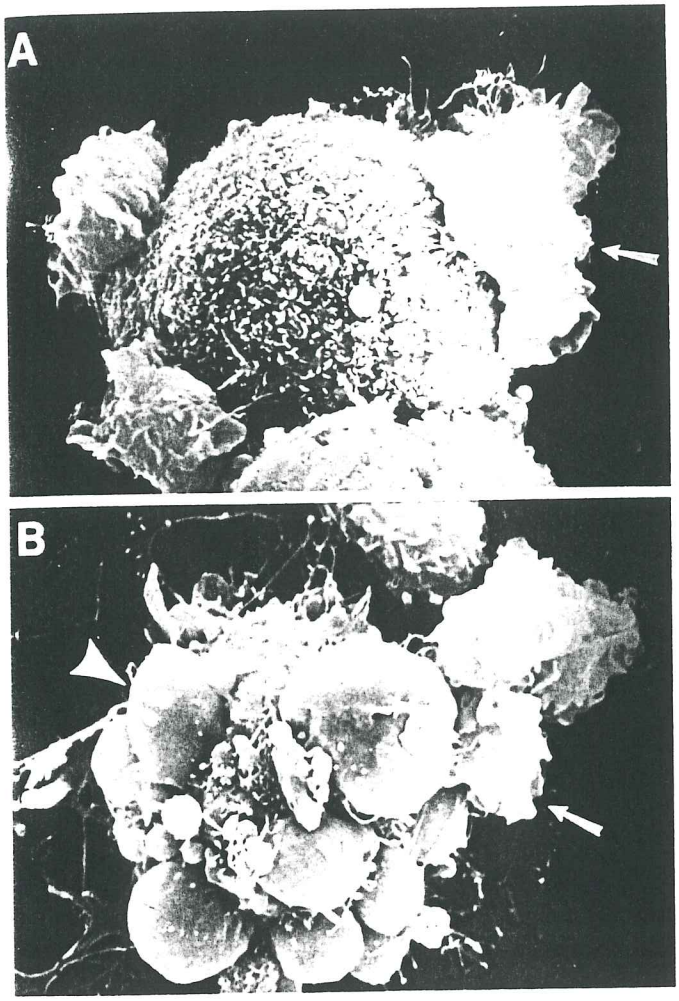

FIG. 2. Scanning electron micrograph of HCs cultured with PMNs in the absence (*A*) and presence (*B*) of FMLP. HCs and PMNs were isolated and plated at a ratio of 1 to 10 on fibronectin-coated glass coverslips. Cells were treated with FMLP (100 nM) or medium as control, and 16 hours later were fixed and prepared for scanning electron microscopy as described in the "Methods" section. *A*, In the absence of FMLP, the surface of the HC is regular. Several PMNs (*small arrow*) can be seen associated with the HC. *B*, In the presence of PMNs (*small arrow*) stimulated with FMLP, numerous cytoplasmic blebs (*larger arrowhead*) are present on the surface of the HC. Figure 2A, ×4,250; *B*, ×4,000.

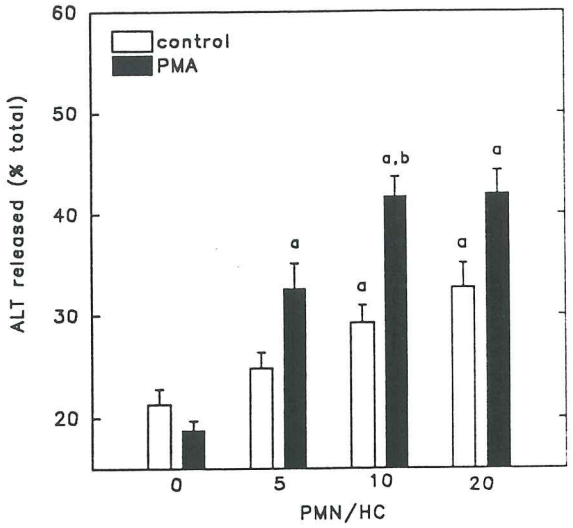

FIG. 3. Release of ALT from HCs in the presence of PMA-stimulated PMNs. HCs were isolated and PMNs were plated as described in the legend to Figure 1 except that no cytochalasin B was present. One hundred nanograms/ml of PMA (■) or its vehicle (□) was added, and after 16 hours, release of ALT was determined as described in the "Methods" section. $N = 5$. *a*, Significantly different from respective group without PMNs. *b*, Significantly different from respective group without PMA.

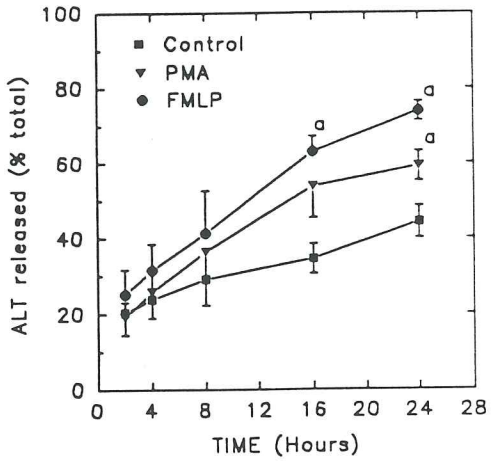

FIG. 4. Time course of injury in HCs cocultured with PMNs. HCs and PMNs were isolated and were plated at a ratio of 10 PMNs/HC as described in the legend to Figure 1. After 2, 4, 8, 16, and 24 hours, the activity of ALT was determined in the cell-free medium and the cell lysates as described in the "Methods" section. $N = 3$ to 4. *a*, Significantly different from control at the same time.

EFFECT OF CONDITIONED MEDIUM FROM STIMULATED PMNS ON HCS

To determine whether PMN-dependent HC damage required direct cell contact, PMNs were stimulated with FMLP or PMA, then the cell-free conditioned medium was incubated with HCs for 16 hours. Over this period, HCs incubated in naive medium released about 25% of total ALT (Fig. 5). The release of ALT was increased to about 40% in the presence of conditioned medium from unstimulated PMNs. Conditioned medium from FMLP-stimulated PMNs significantly enhanced cell damage, increasing ALT release to about 55%, whereas injury seen with conditioned medium from PMA-stimulated PMNs was the same as that caused by conditioned medium from unstimulated PMNs.

To determine whether the injurious factor in conditioned medium from FMLP-stimulated PMNs was stable to heat, conditioned medium was boiled for 5 minutes then allowed to cool before addition to HCs. The increased injury was abolished by boiling; boiled conditioned medium from FMLP-stimulated PMNs caused ALT release comparable to that seen with conditioned medium from unstimulated PMNs (data not shown).

SUPEROXIDE ANION PRODUCTION BY PMA-STIMULATED CELLS

Superoxide generation by PMA-stimulated PMNs was measured in the absence and presence of HCs. In the absence of PMA, PMNs did not produce O_2^- (Fig. 6). PMA stimulation of PMNs resulted in production of

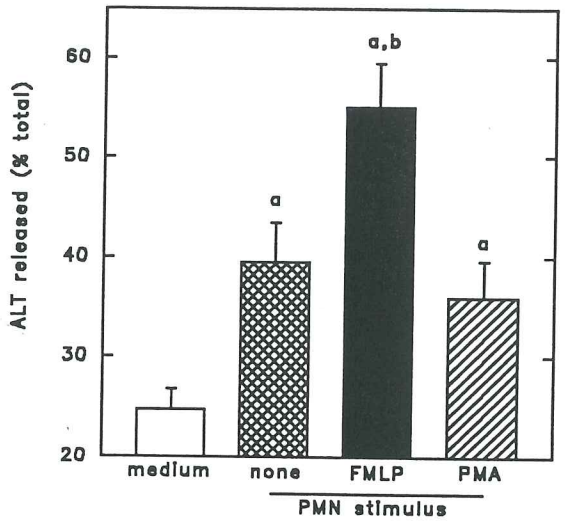

FIG. 5. HC injury due to conditioned medium from stimulated PMNs. HCs were isolated and plated (5×10^6/well), and after 3 hours the medium was removed and replaced with naive medium (□) or with cell-free conditioned medium from PMNs (5×10^6/well) plated separately from the HCs 60 minutes earlier. The PMNs were either not stimulated (■) or were stimulated with 100 nM FMLP (■) or 100 ng/ml PMA (▨) for 30 minutes before collection of conditioned medium. After 16 hours of exposure of HCs to conditioned medium, the release of ALT was determined as described in the "Methods" section. $N = 4$. *a*, Significantly different from medium alone. *b*, Significantly different from no PMN stimulus.

substantial O_2^-. The amount of O_2^- measured was attenuated in the presence of suspended HCs.

To test whether the decrease in O_2^- measured in the presence of HCs was due to decreased generation of reactive oxygen species or was due to interference by the HCs in the ability to detect reduction of cytochrome C, experiments were performed using medium conditioned by exposure to HCs. PMNs suspended in HC conditioned medium and stimulated with PMA (100 ng/ml) produced less O_2^- than control PMNs not exposed to HC conditioned medium (Fig. 7). PMNs activated with a lower concentration (20 ng/ml) of PMA during exposure to HC conditioned medium also produced less O_2^-, but this difference was not statistically significant.

In a separate experiment, intracellular generation of H_2O_2 was measured in PMNs using laser cytometry to detect fluorescence of dichlorofluorescein (DCF). When PMNs plated alone were stimulated with PMA, DCF fluorescence (H_2O_2 production) in PMNs increased to 1.8 ± 0.1 times the baseline value. In the presence of HCs, DCF fluorescence after PMN stimulation remained relatively constant (1.0 ± 0.1 times baseline), indicating a lack of H_2O_2 production. The difference in H_2O_2 production in the absence and presence of HCs was statistically significant ($p < 0.05$).

EFFECT OF SUPEROXIDE DISMUTASE (SOD), CATALASE (CAT), OR DESFERRIOXAMINE (DFX) ON PMN-DEPENDENT HC INJURY

To test the possible involvement of reactive oxygen species in the HC injury caused by activated PMNs, the effects of SOD, CAT, and DFX were examined. The concentration of SOD used effectively eliminated all O_2^- produced by 5×10^6 PMNs stimulated with 100 ng PMA/ml. This concentration of SOD did not afford protection from cytotoxicity induced by FMLP- or PMA- activated PMNs (Table 1). Similarly, no protection against PMN-dependent injury was observed by addition of CAT, even though the concentration of CAT used eliminated a concentration of H_2O_2 10 times greater than the amount

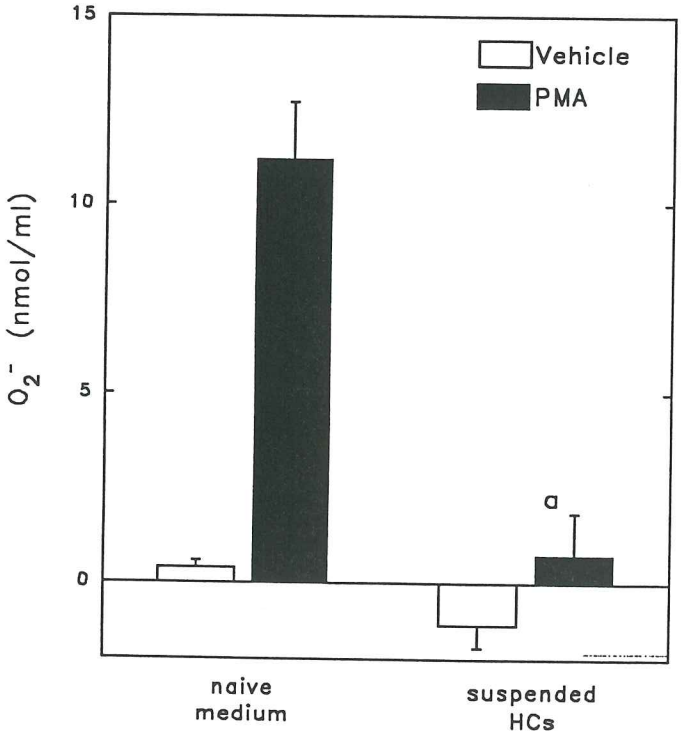

FIG. 6. Superoxide generation by PMNs in the absence and presence of HCs. PMNs (5×10^6/well) were plated, and either naive medium or medium containing suspended HCs (5×10^5) was added to the wells. PMA (100 ng/ml) was added and superoxide (O_2^-) generation over 30 minutes was measured. $N = 4$. *a*, Significantly different from respective value in naive medium.

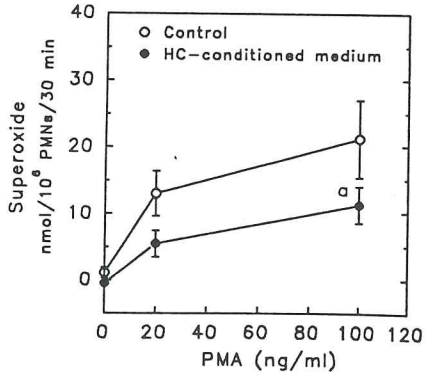

FIG. 7. Generation of superoxide by PMNs in the absence and presence of conditioned medium from HCs. PMNs (5×10^6) were suspended in either naive medium or medium previously exposed for 30 minutes to plated HCs. PMA was added at the concentration indicated, and generation of O_2^- over 30 minutes was measured. $N = 5$. *a*, Significantly different from control at the same concentration of PMA.

TABLE 1. EFFECT OF SOD, CAT, AND DFX ON PMN-DEPENDENT CYTOTOXICITY TO HCS

Addition	ALT released (% total)		
	Stimulus		
	None	FMLP	PMA
None	32 ± 3	46 ± 7	33 ± 3
SOD	32 ± 4	48 ± 9	35 ± 4
CAT	34 ± 5	50 ± 6	31 ± 2
SOD + CAT	34 ± 3	50 ± 5	40 ± 3
DFX	38 ± 5	51 ± 7	44 ± 3

Isolated HCs were plated (5×10^6/well), and after 3 hours, the medium was changed and PMNs (10 PMN/HC) were added to the wells. SOD (300 units/ml), CAT (3,000 units/ml), or DFX (20 mM) were also added at this time. After 30 minutes, FMLP (100 nM) or PMA (100 ng/ml) were added. The activity of ALT was determined in the cell-free medium and in the cell lysates, and the percentage of total ALT released was calculated as described in the "Methods" section. N = 3 repetitions, each using cells from different rats. No statistically significant effects of SOD, CAT, SOD + CAT, or DFX were observed.

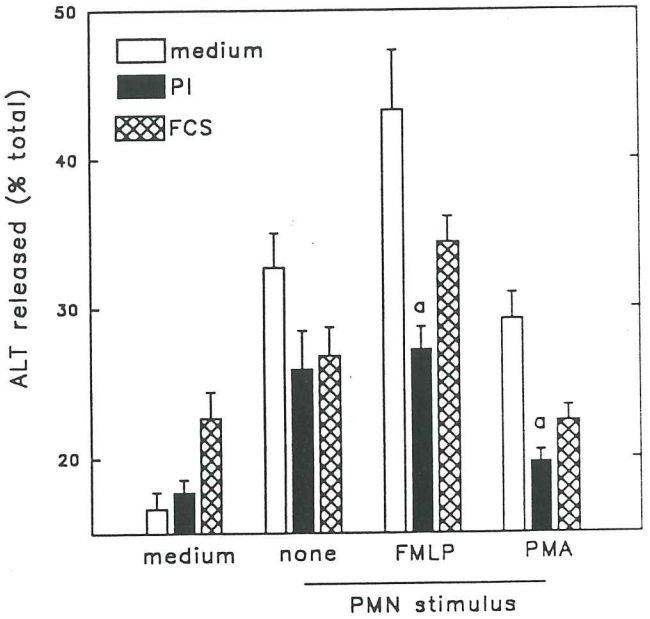

FIG. 8. Effect of antiproteases on PMN-dependent HC damage. The experiment was performed as described in Figure 5 except that protease inhibitor (2 ng/ml; ■) or FCS (10%; ■) were added concurrent with addition of PMN conditioned medium. N = 4. a, Significantly different from respective medium control.

expected if all O_2^- produced was converted to H_2O_2. In addition, the combination of SOD and CAT did not affect PMN-induced cytotoxicity to the HCs. The concentration of DFX used was chosen based on reports of effectiveness in preventing cytotoxicity from reactive oxygen species in another study (12). DFX did not affect HC injury caused by activated PMNs.

EFFECT OF ANTIPROTEASES ON PMN-DEPENDENT HC INJURY

The effect of addition of an antiprotease or of fetal calf serum, that contains antiproteases (13), to the cell-free conditioned medium from stimulated PMNs was examined. Protease inhibitor (Sigma P0787), which inhibits calcium-activated neutral protease (14) but not trypsin or α-chymotrypsin (15), diminished injury to HCs caused by conditioned medium from FMLP- or PMA- stimulated PMNs (Fig. 8). Protease inhibitor tended to decrease ALT release in cells incubated with conditioned medium from unstimulated PMNs, but this effect was not statistically significant. A nonsignificant trend toward decreased ALT release was also observed with fetal calf serum.

DISCUSSION

A central role has now been established for PMNs in the hepatotoxicity caused in rats *in vivo* by the cholestatic agent ANIT (7) and that caused by bacterial endotoxin (8). The mechanism by which PMNs contribute to liver damage in these models is not understood. ANIT stimulated O_2^- production by PMNs *in vitro* (16); however, this mechanism may not be involved in ANIT-induced toxicity *in vivo* because administration of SOD and CAT failed to attenuate the hepatotoxic response (7). With respect to endotoxin, the PMN is likely one component of a complex network of cellular and soluble mediators that contribute to injury. For example, whereas PMNs accumulated in livers of endotoxin-treated rats and depletion of circulating blood PMNs diminished hepatotoxicity (8), elimination of tumor necrosis factor-α also attenuated liver injury but did not decrease accumulation of PMNs in liver (17). Thus, accumulation of PMNs in livers of endotoxin-treated rats is necessary but not sufficient for the production of liver injury. Other factors are clearly critical. Studies with these two models of liver injury indicate that PMNs can contribute to liver injury *in vivo* by a variety of mechanisms that are incompletely understood.

In a coculture system *in vitro*, PMNs stimulated with either FMLP or PMA injured HCs (Figs. 1 to 3). Blebs were apparent by scanning electron microscopy on HCs cocultured with FMLP-activated PMNs (Fig. 2*B*). Blebs appear on the surface of HCs in response to a number of cytotoxic agents (18, 19), and blebbing is considered to be common during events leading to irreversible cell injury (18). Freshly isolated HCs were used in these studies and susceptibility of these cells to injury may be different from hepatic parenchymal cells *in vivo* due to the isolation procedure. Despite this, HCs cultured in the absence of stimulated PMNs were regular in appearance and blebbing was uncommon.

FMLP is a more effective stimulus for degranulation of lysosomes than for oxygen radical production. It causes release of proteolytic enzymes from both specific and azurophilic granules. Some experimental evidence suggests that proteolytic enzymes may be involved in the mechanism by which FMLP-stimulated PMNs damage HCs in culture. First, toxicity does not require direct cell contact and the factor responsible for toxicity is relatively stable, since cytotoxicity was observed with conditioned medium from FMLP-stimulated PMNs (Fig. 5). Second, boiling of the conditioned medium abolished the toxic effect. Third, a protease inhibitor attenuated the toxic response (Fig. 8). Although proteolytic enzymes released by the PMNs may be involved in the toxicity to HCs, there may be other, as yet unidentified factors that

contribute to the response. In addition, the mechanism of hepatotoxicity may be different in coculture and in experiments using conditioned medium.

If injury is due to proteolytic enzymes, the observation that 10% serum tended to reduce the cytotoxic effect of conditioned medium from both FMLP- and PMA-stimulated PMNs (Fig. 8) raises the possibility that serum may protect hepatic parenchymal cells *in vivo* from proteolytic damage. However, proteolytic enzymes have been implicated in hepatic injury caused by *Corynebacterium parvum* and endotoxin in rat *in vivo* (20), suggesting that antiproteases present in serum may sometimes be insufficient to afford protection.

PMA is a better stimulus for oxygen radical generation than for degranulation. The observation that cell-free conditioned medium from PMA-stimulated PMNs did not enhance toxicity in HCs (Fig. 5) suggests that direct cell contact is required for HC injury by PMNs stimulated with PMA. Reactive oxygen species may be involved in this response, since these species are short-lived and would likely require cell contact to produce toxicity. However, less O_2^- was measured in PMA-activated PMNs in the presence of HCs and in PMNs exposed to HC-conditioned medium (Figs. 6 and 7), raising doubt as to whether O_2^- would be produced in toxic quantities in cocultures of PMNs and HCs.

In rat PMNs, PMA is a weak stimulus for secretion of azurophilic granules that contain proteases (21). In a limited study, PMA-stimulated PMNs released less than 18% as much β-glucuronidase (a marker of secretion of azurophilic granules) as FMLP-activated PMNs at concentrations of stimulators that produced maximal PMN response. Despite this fact, proteolytic enzymes may be involved in the mechanism by which PMA-stimulated rat PMNs damage HCs, since a protease inhibitor reduced cytotoxicity due to conditioned medium from PMA-activated PMNs (Fig. 8). Similar protection against hepatocellular cytotoxicity was observed with antiproteases when human PMNs activated with PMA were cocultured with rat HCs (9). Once again, the possibility exists that mechanisms of cytotoxicity produced by conditioned medium and in coculture are different.

The observation that less O_2^- was measured in PMNs in the presence of HCs (Fig.6) raises the possibility that HCs affect PMN function. This hypothesis is supported by the observation that less O_2^- was generated by PMA-activated PMNs in the presence of HC-conditioned medium and that inhibition was not overcome by addition of a higher concentration of PMA (Fig. 7). These latter observations indicate that direct cell contact between HCs and PMNs is not required for alterations in O_2^- production and rule out the possibilities that HCs either decrease availability of the stimulant or interfere with detection of reduction of cytochrome *c*. The results suggest that something released by HCs alters PMN function. Furthermore, production of intracellular H_2O_2 by PMA-activated PMNs was depressed in the presence of HCs, suggesting that HCs did not simply scavenge or inactivate PMN-derived O_2^-, but that generation of reactive oxygen species was decreased. The possibility that HCs modulate PMN function is reminiscent of the influence of HCs on functions of Kupffer cells, the resident phagocytic cells of the liver. For example, Kupffer ce production of prostaglandin E_2 is modulated by HC (22), and Kupffer cell-mediated tumor cell cytostasis more efficient in the presence than in the absence HCs (23). Perhaps a similar regulatory influence exist between HCs and PMNs.

Maximal cytotoxicity in cocultures was observed at ratio of PMN/HC of 10:1 (Fig. 1). These results ar consistent with results in a coculture system using hu man PMNs and rat HCs (9). One possibile explanatio for this finding is that cytotoxicity in coculture require intimate cell contact, and due to the available surface o HCs, 20 PMNs/HC provides no more cell-cell contac that 10 PMN/HC.

Both degranulation and generation of O_2^- occur rapidly upon stimulation of PMNs with FMLP or PMA, yet ALT release did not increase until 8 to 16 hours after stimulation (Fig. 4). The reason for the delay in expression of toxicity is not understood. It is possible that several hours of exposure to toxic factors are required to cause overt injury to plasma membranes of HCs. Alternatively, increases in release of ALT may not occur until some time after injury occurs. Indeed, when isolated HCs were exposed to an oxygen radical-generating system, changes in cellular morphology and inability to exclude trypan blue preceded increased release of ALT (24).

Coincubation with unstimulated PMNs or with cell-free conditioned medium from unstimulated PMNs caused a modest increase in release of ALT (Figs. 1, 3, and 5). One possibility for this effect is that during elicitation and isolation, PMNs become somewhat activated and release small amounts of proteolytic enzymes. The observation that a protease inhibitor did not diminish release of ALT in HCs exposed to conditioned medium from unstimulated PMNs (Fig. 8) would argue against this hypothesis. In addition, one report indicated that glycogen-elicited PMNs were no more responsive to stimuli than PMNs isolated from blood, suggesting that the effects of the elicitation procedure are minimal (25).

Some chemicals, for example lindane (26, 27), dieldrin (25), and polychlorinated biphenyls (28), stimulate PMNs *in vitro*. The data presented here indicate that stimulated PMNs can damage HCs in culture. PMNs have also been implicated in hepatotoxicity *in vivo* in some models (7, 8). Thus, the observation that activated PMNs can damage HCs raises the possibility that stimulation of PMNs by chemicals might contribute to hepatotoxic injury observed *in vivo*.

METHODS

ISOLATION OF HCS

HCs were isolated from male, Sprague-Dawley rats (CD-Crl:CD(SD)BR VAF/Plus; Charles River Laboratories, Portage, Michigan) weighing 200 to 500 gm according to the method of Seglen (29) as modified by Klaunig (30). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and the portal vein was cannulated. The liver was perfused with approximately 150 ml of Mg^{2+}-free, Ca^{2+}-free Hanks' balanced salt solution followed by perfusion with 250 ml of collagenase type A (0.5 mg/ml; Boehringer-Mannheim Biochemicals, Indianapolis, Indiana). The resulting liver digest was filtered through gauze and spun in a centrifuge

at 50 × *g* for 2 minutes. The HCs were resuspended in Williams medium E (Gibco, Grand Island, New York) containing 10% fetal calf serum (Intergen, Purchase, New York) and 1% gentamicin (Gibco), and cells were plated in 6-well plates at a density of 5×10^5 HCs/well. After an initial 3-hour attachment period, the medium and unattached cells were removed, and fresh medium was added. Experiments were initiated at this time, and the choice of medium was determined by the experimental design (see below). Using this isolation procedure, 98% of the cells in the final preparation were hepatic parenchymal cells: the remaining cells were lymphoid, macrophages, or PMNs. Viability of HCs using this method of isolation was routinely >90%.

Isolation of PMNs

Glycogen-elicited PMNs were isolated from the peritoneum of male, Sprague-Dawley, retired breeder rats (Charles River Laboratories, Portage, Michigan) as described previously (25). Briefly, 30 to 40 ml of 1% glycogen in sterile saline were injected into the peritoneum of rats anesthetized with diethylether. Four hours later, the rats were anesthetized again with diethylether and were killed by decapitation. The peritoneum was rinsed with 30 ml of 0.1 M phosphate-buffered saline containing 1 unit/ml of heparin. The rinse solution was filtered through gauze and was spun for 7 minutes at 500 × *g*. Red blood cells were lysed in 15 ml of 0.15 M NH_4Cl, and the cells were spun for 7 minutes at 300 × *g*. Cells were washed once with phosphate-buffered saline, then they were resuspended in Williams' medium E for coculture studies or in minimum essential medium (Gibco) for studies in which superoxide anion was measured. All media contained 1% gentamicin. The percentage of PMNs in the cell preparation is routinely >95%, and viability is ≥95%. PMNs were stimulated with either FMLP in the presence of cytochalasin B (5 μg/ml) or with PMA. Concentrations of FMLP and PMA were chosen based on previous studies in which these concentrations were shown to stimulate degranulation (25) or O_2^- production (31), respectively.

Determination of HC Injury in HC/PMN Cocultures

PMNs were added to adherent HCs (5×10^5/well) in Williams medium E containing no fetal calf serum in ratios of 0.5, 1, 5, 10, or 20 PMNs/HC. Wells contained HCs plus PMNs, HCs only, or PMNs only. PMNs were allowed to attach for 30 minutes, then PMN stimuli were added. After a 16-hour incubation at 37° C in 92.5% O_2/7.5% CO_2, the medium was collected. The cells remaining on the plate were lysed with 1% Triton X-100 and sonication. Both the medium and the cell lysates from the plates were spun in a centrifuge at 600 × *g* for 10 minutes. The activity of ALT in the cell-free supernatant fluid was determined using Sigma kit No. 59-UV. The ALT activity in the medium was expressed as a percentage of the total activity (activity in the medium plus activity in cell lysates). At a PMN/HC ratio of 10:1, the total activity of ALT in PMNs was less than 10% of total activity in HCs, and ALT activity in the medium of wells containing only stimulated PMNs was below the limit of detection. Thus, ALT activity in PMNs is relatively low, and ALT activity released into the medium was taken as an index of injury to HCs. Neither PMNs nor PMN lysates interfered with the ability to measure ALT activity.

Determination of Viability of PMNs

Viability of PMNs in culture was determined from release of LDH into the medium. The activity of LDH in the cell-free supernatant fluid was measured spectrophotometrically from the disappearance of NADH using pyruvate as substrate (32). Total LDH activity was also determined in PMNs lysed as described above for HCs, and activity of LDH in the medium was expressed as a percentage of the total releasable LDH.

Examination of Cell Cultures by Scanning Electron Microscopy

For examination by scanning electron microsocopy, HCs were plated in the absence or presence of PMNs on glass coverslips coated with fibronectin (Sigma Chemical Company, St. Louis, Missouri) and inserted in 12-well plates. The ratio of PMNs to HCs was 10 to 1. Cocultures were treated with FMLP, PMA, or medium and 16 hours later, were fixed on ice in 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Cells were washed, and then dehydrated using a graded ethanol series (33). Samples were dried in a Balzers critical point dryer using liquid carbon dioxide as the transitional fluid and were coated with gold (20 nm thickness) in an Emscope Sputter Coater model SC 500 purged with argon gas. Samples were examined in a JEOL JSM-35CF SEM (Japan Electron Optics Limited, Tokyo, Japan). Neither fibronectin nor the presence of glass coverslips altered the cytotoxic effect of PMNs on HCs as determined by release of ALT into the medium.

Measurement of Oxygen Radical Production by PMNs

O_2^- generation by PMNs in the absence and presence of HCs or HC-conditioned medium was measured spectrophotometrically as the SOD-sensitive reduction of ferricytochrome C (34). Experiments were performed using minimum essential medium, and for every sample, two wells were incubated: one to which SOD (85 units/ml) was added before incubation and one to which SOD was added after incubation. The difference in absorbance (550 nm) of the cell-free supernatant from these two wells was used to estimate the amount of cytochrome *c* reduced, using an extinction coefficient of 18.5 $cm^{-1}mM^{-1}$.

Intracellular hydrogen peroxide (H_2O_2) was measured using the fluorescent indicator DCF (35, 36) in PMNs plated in the absence and presence of HCs. PMNs and HCs were incubated separately with DCF-diacetate (100 μM) at 37° C for 15 minutes. DCF-diacetate accumulates in cells, is deacetylated to a form that is trapped inside the cell (*i.e.*, DCF) and is oxidized in the presence of H_2O_2 to a fluorescent moiety. Changes in fluorescence in response to addition of PMA were monitored using the ACAS 570 laser cytometer (Meridian Instruments, Inc., Okemos, Michigan) at an excitation wavelength of 488 nm and emission wavelength of 520 nm.

Acknowledgements: The authors acknowledge Dr. James Klaunig for training in isolation of HCs, Dr. Karen Klomperens and Shirley Owens for their help with scanning electron microscopic analysis, and Beth Lockwood for excellent technical assistance.

Date of acceptance: July 8, 1993.

This study was supported by NIH Grants ES04911 and ES05722.

M. B. B. was supported by National Research Service Award ES05527.

R. A.R. was supported in part by a Burroughs Wellcome Toxicology Scholar Award.

Address reprint requests to: Dr. Patricia Ganey, Department of Pharmacology and Toxicology, Life Sciences Building, Michigan State University, East Lansing, MI 48824

REFERENCES

1. Ward PA, Till GO, Kunkel R, Beauchamp C. Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. J Clin Invest 1983;72:789–801.

2. Johnson KJ, Ward PA. Newer concepts in the pathogenesis of immune complex-induced tissue injury. Lab Invest 1982;47: 218–26.
3. Johnson KJ, Ward PA. Role of oxygen metabolites in immune complex injury of lung. J Immunol 1981;126:2365–9.
4. Elgebaly SA, Foronhar F, Gillies C, Williams S, O'Rourke J, Kreutzer DL. Leukocyte-mediated injury to corneal endothelial cells. A model of tissue injury. Am J Pathol 1984;116:407–16.
5. Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 1983;67:1016–23.
6. Brigham KL, Meyrick B. Interactions of granulocytes with the lungs. Circ Res 1984;54:623–35.
7. Dahm LJ, Schultze AE, Roth RA. An antibody to neutrophils attenuates alpha-naphthylisothiocyanate induced liver injury. J Pharmacol Exp Ther 1991;256:412–20.
8. Hewett JA, Schultze AE, VanCise S, Roth RA. Neutrophil depletion protects against liver injury from bacterial endotoxin. Lab Invest 1992;66:347–61.
9. Guigui B, Rosenbaum J, Preaux A-M, Martin N, Zafrani ES, Dhumeaux D, *et al.* Toxicity of phorbol myristate acetate-stimulated polymorphonuclear neutrophils against rat hepatocytes. Lab Invest 1988;59:831–7.
10. Loeb WF. Clinical biochemistry of laboratory rodents and rabbits. In: Kaneko JJ, editor. Clinical biochemistry of domestic animals. Ed. 4. San Diego; Academic Press, 1989:866.
11. Kodavanti PRS, Mehendale HM. Biochemical methods of studying hepatotoxicity. In: Meeks RG, Harrison SD, Bull RJ, editors. Hepatotoxicology. Boca Raton; CRC Press, 1991:241.
12. Miccadei S, Nakae D, Kyle ME, Gilfor D, Farber JL. Oxidative cell injury in the killing of cultured hepatocytes by allyl alcohol. Arch Biochem Biophys 1988;265:302–10.
13. Adams DO. Effector mechanisms of cytolytically activated macrophages. I. Secretion of neutral proteases and effect of protease inhibitors. J Immunol 1980;124:286–92.
14. Melloni E, Salamino F, Sparatore B, Michetti M, Pontremoli S, Horecker BL. Regulation of the Ca2+-dependent neutral proteinases from rabbit liver by an endogenous inhibitor. Arch Biochem Biophys 1984;232:513–9.
15. Takahashi-Nakamura M, Tsuji S, Suzuki K, Imahori K. Purification and characterization of an inhibitor of calcium-activated neutral protease from rabbit skeletal muscle. J Biochem 1981;90: 1583–9.
16. Roth RA, Hewett JA. The cholestatic agent, alpha-naphthylisothiocyanate, stimulates superoxide release by rat neutrophils *in vitro*. Lab Invest 1990;62:736–41.
17. Hewett JA, Kunkel SL, Roth RA. Antiserum to tumor necrosis factor protects against liver injury from bacterial endotoxin in the rat (abstr). FASEB J 1991;5:1629 .
18. Lemasters JJ, DiGuiseppi J, Neiminen A-L, Herman B. Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. Nature (London) 1987;325:78–81.
19. Orrenius S, McConkey DJ, Bellomo G, Nicotera P. Role of Ca^{2+} in toxic cell killing. TIPS 1989;10:281–5.
20. Tanner A, Keyhani A, Reiner R, Holdstock G, Wright R. Proteolytic enzymes released by liver macrophages may promote hepatic injury in a rat model of hepatic damage. Gastroenterology 1981;80:647–54.
21. Smolen JE. Characteristics and mechanisms of secretion by neutrophils. In: Hallett MB, editor. The neutrophil: cellular biochemistry and physiology. Boca Raton; CRC Press, 1989:23.
22. Billiar TR, Lysz TW, Curran RD, Bentz BG, Machiedo GW, Simmons RL. Hepatocyte modulation of Kupffer cell prostaglandin E_2 production *in vitro*. J Leuk Biol 1990;47:304–11.
23. Curran RD, Billiar TR, Kispert PH, Bentz BG, May MT, Simmons RL. Hepatocytes enhance Kupffer cell-mediated tumor cell cytostasis *in vitro*. Surgery 1989;106:126–32.
24. Carey D, Wu J, Sugiyama H, Wu T-W. Validation of the morphologic end point of necrosis in rat hepatocytes subjected to oxyradical damage. Biochem Cell Biol 1991;69:689–94.
25. Hewett JA, Roth RA. Dieldrin activates rat neutrophils *in vitro*. Toxicol Appl Pharmacol 1991;96:269–78.
26. Kuhns DB, Kaplan SS, Basford RE. Hexachlorocyclohexanes, potent stimuli of O_2^- production and calcium release in human polymorphonuclear leukocytes. Blood 1986;68:535–40.
27. English D, Schell M, Siakotos A, Gabig TG. Reversible activation of the neutrophil superoxide generating system by hexachlorocyclohexane: Correlation with effects on a subcellular superoxide-generating fraction. J Immunol 1986;137:283–90.
28. Ganey PE, Sirois JE, Denison M, Robinson JP, Roth RA. Neutrophil function after exposure to polychlorinated biphenyls *in vitro*. Environ Health Perspect 1993;101:430–4.
29. Seglen PO. Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. Exp Cell Res 1973;82:391–8.
30. Klaunig JE, Goldblatt PJ, Hinton DE, Lipsky MM, Chacko J, Trump BF. Mouse liver cell culture. I. Hepatocyte isolation. *In Vitro* 1981;17:913–25.
31. Roth RA, Ball TM. Technical grade but not recrystallized alpha-naphthylthiourea potentiates superoxide release by rat neutrophils stimulated *in vitro* by phorbol myristate acetate. Fund Appl Toxicol 1986;7:324–8.
32. Bergmeyer HV, Bernt E. Lactate dehydrogenase UV assay with pyruvate and NADH. In: Bergmeyer HV, editor. Methods of enzymatic analysis. New York; Academic Press, 1974:524.
33. Klomparens K, Flegler SL, Hooper GR. In: Procedures for transmission and scanning electron microscopy for biological and medical science. Burlington; Ladd Research Industries, 1986.
34. Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 1973;52:741–4.
35. Bass DA, Parce JW, DeChatelet LR, Szeda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 1983;130:1910–17.
36. Robinson JP, Bruner LH, Bassoe C-F, Hudson JL, Ward PA, Phan SH. Measurement of intracellular fluorescence of human monocytes indicative of oxidative metabolism. J Leuk Biol 1988;43: 304–10.