Hepatic and extrahepatic factors critical for liver injury during lipopolysaccharide exposure

FREDERIC MOULIN, BRYAN L. COPPLE, PATRICIA E. GANEY, AND ROBERT A. ROTH
Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824

Received 16 December 2000; accepted in final form 9 August 2001

Platelets accumulate in the liver after LPS administration in vivo and are a critical component of the mechanism of liver injury (45). Thrombin, which is also essential for LPS-induced liver injury (43), is the most potent stimulator of platelets (15, 50), causing them to spread, release the contents of their alpha and dense granules, and aggregate (31, 54). Stimulated platelets present a procoagulant surface that promotes assembly of the prothrombinase complex and subsequent formation of active thrombin (6). In addition, activated platelets express receptors that promote platelet adhesion to endothelium and to the subendothelial matrix as well as to other platelets and fibrin in the process of forming a platelet thrombus (41, 56). The components released from granules of activated platelets promote further platelet aggregation (39). Accordingly, it seemed conceivable that thrombin might play a role in LPS-induced liver injury through an effect on platelets. If so, thrombin might promote the sequestration of platelets in the liver and/or activate them.

PMNs are phagocytic cells that play an essential role in the defense against microorganisms. On activation, these cells undergo a respiratory burst and generate free radicals. They also release into the extracellular space the contents of their cytosolic granules, and the proteases contained within those granules contribute to the destruction of pathogens. Although PMNs are undoubtedly critical components of the host immune defense, their ability to become activated and release cell-damaging intermediates has been implicated in host tissue injuries (51). Several models of neutrophil-dependent injury have been described, including endotoxin-induced lung (9) and liver damage (29, 33).

Increasing evidence suggests that there may be a link between PMNs and thrombin in the pathogenesis of LPS-induced liver injury. For example, thrombin can stimulate degranulation (4) and the release of tissue kallikrein (11) and thromboxane B2 (17) from human PMNs. Thrombin also induces chemotaxis of PMNs (8), an important step in neutrophil-induced hepatocellular killing in vivo (35).

Platelets, PMNs, and thrombin are required in vivo for LPS-induced liver injury (29, 33, 46), but interac-
tions among these mediators and the roles of other extrahepatic factors that present in response to LPS remain poorly understood. Experiments in the isolated rat liver model have shown that perfusion of livers from LPS-treated donors with buffer containing thrombin results in hepatocellular injury; by contrast, no injury results when livers from naive donors are similarly exposed to thrombin (43). This result indicated that factors evoked by LPS in vivo are required for the hepatic injury precipitated by thrombin. Among the potential contributors are platelets and PMNs, the accumulation of which in the liver occurs soon after LPS exposure.

In this study, we investigated the requirement for these blood cells in causing hepatocellular injury in livers isolated from LPS-treated rats and perfused with thrombin. The results raised the possibility of interdependence between thrombin and PMNs in the genesis of hepatocellular injury. To explore this, we evaluated the influence of thrombin on PMN functions involved in hepatocellular killing in vitro. To determine whether PMNs, thrombin, and LPS were sufficient to produce hepatic damage, we perfused livers from naive donors with various combinations of these three factors.

**MATERIALS AND METHODS**

**Materials.** LPS (Escherichia coli, serotype 0128:B12, 24×10^6 EU/mg), BSA, glycogen (type II from Oyster), Kit 59 for determination of alanineaminotransferase (ALT) activity, and Kit 510-A for the determination of glucose concentration were purchased from Sigma Chemical (St. Louis, MO). The specific activity of the LPS was confirmed using a kinetic chromogenic modification of the limulus amebocyte lysate assay from BioWhittaker (Walkersville, MD). Human α-thrombin (3,048 NIH U/mg) was purchased from Enzyme Research Laboratories (South Bend, IN), and its activity was confirmed by measuring the conversion of fibrinogen to fibrin using a fibrrometer. Collagenase type B was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Williams’ medium E and gentamicin were purchased from GIBCO (Grand Island, NY), and fetal calf serum was purchased from Intergen (Purchase, NY). Triton X-100 was purchased from Research Products International (Mount Prospect, IL). Tissues were fixed using neutral buffered formalin (Surgipath Medical Industries, Richmond, IL).

**Animals.** Male Sprague-Dawley rats (Crl:CD BR(SD) VAF/plus, Charles River, Portage, MI) weighing 250–350 g were used as hepatocyte and liver donors. Male retired breeder rats were used for PMN collection. The animals were maintained on a 12:12-h light-dark cycle under controlled temperature (18–21°C) and humidity (55 ± 5%). Food (Rat chow, Teklad, Madison, WI) and tap water were allowed ad libitum. All procedures on animals were carried out according to the guidelines of the American Association of Laboratory Animal Sciences and the University Laboratory Animal Research Unit at Michigan State University.

**Isolation of rat PMNs.** PMNs were isolated following a method previously described (21). Retired breeder rats received 35 ml of a 1% glycogen solution in sterile saline intraperitoneally. Four hours later, animals were anesthetized and killed by exsanguination. Thirty milliliters of heparinized PBS solution (pH 7.4) were injected into the peritoneum. The contents of the abdominal cavity were transferred into a tube and spun in a centrifuge at 500 g for 7 min. The pellet was resuspended in 15 ml of NH4Cl (0.15 M) to lyse red blood cells. After 2 min, 35 ml of PBS were added and the combined contents were spun for 7 min at 320 g. The pellet was resuspended in 50 ml PBS and spun again for 7 min at 320 g. The number of PMNs per milliliter was determined using a hemacytometer and adjusted to a final concentration of 3×10^6 PMNs/ml.

**Isolation and perfusion of rat livers.** The recirculating perfusion system used in these experiments was described in detail previously (43). Under pentobarbital sodium anesthesia (50 mg/kg ip), the abdominal cavity of the rat was opened and the portal vein was cannulated. Perfusion was started immediately so that the period of ischemia was <15 s. The perfusion medium comprised Krebs-Henseleit-bicarbonate buffer containing 2% BSA and saturated with 95% O2 and 5% CO2. Flow was constant at 35 ml/min. Livers were perfused inside a temperature-controlled cabinet maintained at 37°C. At the end of a 15-min stabilization period, a sample of the perfusion solution was taken from the reservoir before passage through the liver (time 0), and the system was switched to recirculating perfusion. The total volume of solution in the recirculating system was 50 ml. The temperature of the perfusion solution was maintained at 38.5°C, and the pH was monitored (7.4) by an in-line electrode connected to a pH controller (Chentrix, Hillsboro, OR). Experiments were performed using two identical systems, allowing simultaneous perfusion of treated and control livers.

**Perfusion of livers from platelet-depleted rats with thrombin.** Liver donors received anti-platelet serum (APS) or control serum (0.5 ml ip) 22 h before LPS (96×10^6 EU/kg iv) administration. APS was prepared from rabbits as described previously (45). APS activity in these sera was <0.1 EU/ml. A sample of blood was collected into 0.38% sodium citrate at the time of death for measurement of circulating platelet number. Platelets were counted using a system 9000 cell counter (Serono Baker Diagnostics, Allentown, PA). APS reduced the number of platelets in blood from 442 ± 43 × 10^3/mm^3 to 34.5 ± 7 × 10^3/mm^3. This degree of platelet depletion is associated with complete protection from LPS-induced liver injury in vivo (45). Livers were isolated for perfusion 2 h after LPS administration. Perfusion medium comprised Krebs-Henseleit buffer with 2% BSA and 10 nM thrombin. This concentration of thrombin activates protease-activated receptors on cells in vitro, has been estimated to occur in vivo during inflammatory responses, and was shown in preliminary studies to be a dose that produces maximal injury to perfused livers from LPS-treated animals (43, 55, 57). Plasma ALT activity was measured in the recirculating medium over time.

**Perfusion of livers from PMN-depleted rats with thrombin.** Liver donors received immunoglobulin isolated from rabbits immunized with rat PMN (AN-Ig) or from untreated rabbits (C-Ig) as two separate injections (0.5 ml) in the tail vein at 18 and 6 h before LPS (96×10^6 EU/kg iv) administration. LPS activity in these Ig preparations was <0.1 EU/ml. A sample of blood was collected into 0.38% sodium citrate at the time of death. White blood cells were counted, and a differential count was made on blood smears to verify neutrophil depletion. AN-Ig administration reduced the number of circulating PMNs from 821 ± 200 to 49 ± 8 PMN/mm^3. This degree of PMN depletion completely eliminates LPS-induced liver injury in vivo (29). Livers were isolated for perfusion 2 h after LPS administration. Perfusion medium comprised Krebs-Henseleit buffer with 2% BSA and 10 nM thrombin. Samples (350 μl) of the recirculating solution were...
Liver perfusion with LPS, PMNs, and thrombin. Five hours before liver isolation, glycogen was injected into PMN donors. PMNs were isolated as described above and resuspended at a concentration of $3 \times 10^6$ PMN/ml in Krebs-Henseleit perfusion buffer containing 2% BSA. Liver donor rats were anesthetized, and the liver was removed and perfused for 4 h in a recirculating manner as described above with or without PMNs ($2 \times 10^6$ PMN/ml final concentration). LPS ($96 \times 10^6$ EU/kg of donor rat weight) or its saline vehicle was introduced into the perfusion buffer 10 min after the start of recirculation. Perfusate samples (350 μl) were taken every 30 min for the first 2 h. After 2 h of recirculating perfusion, either thrombin (10 nM final concentration) or an equal volume of saline vehicle was introduced into the perfusion buffer, and samples were collected every 15 min. All samples were used for determination of ALT activity.

Preparation and culture of isolated hepatocytes. Hepatocytes were isolated according to the method of Seglen (49) as modified by Klaunig et al. (56). Six-fused cultures of hepatocytes were treated with LPS ($96 \times 10^6$ EU/kg) 2 h before isolation. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip), and a cannula was inserted into the portal vein. The liver was then perfused with ~150 ml of Ca$^{2+}$-free, Ca$^{2+}$-free Hanks’ balanced salt solution followed by 250 ml of collagenase type B (0.5 mg/ml), and the liver digest was collected and filtered through gauze. The digestion product was subsequently centrifuged at 50 g for 2 min. Cells from the pellet fraction were resuspended in Williams’ medium E containing 10% fetal calf serum and 1% gentamicin and plated in six-well plates at a density of 5 × 10^6 cells per well. After a 3-h attachment period, the medium was removed, and fresh medium without fetal calf serum was added. With the use of this isolation procedure, 98% of the cells in the final preparation were hepatic parenchymal cells, and the viability of the hepatocytes was routinely >90%.

Hepatocyte/PMN cocultures. PMNs ($5 \times 10^6$/well) were added to adherent cultures of hepatocytes ($5 \times 10^5$/well). Cells were incubated as described above in the absence of fetal calf serum. PMNs were allowed to attach for 30 min, after which thrombin was added at concentrations of 0.04, 0.4, 4, or 40 nM. After a 3-, 6-, or 16-h incubation period, the medium was collected. The cells remaining on the plate were lysed with 1% Triton X-100 and sonication. Both medium and lysate from the plates were spun in a centrifuge at 600 g for 10 min. The activity of ALT in the cell-free supernatant fluids was determined. The ALT activity in the medium was expressed as a percentage of the total activity (activity in the medium plus activity in cell lysates). It was shown previously that at a PMN-hepatocyte ratio of 10:1, the total activity of ALT in PMNs is ~10% of the total activity in hepatocytes (21). Accordingly, ALT activity released into the medium was taken as an index of hepatocellular injury.

In subsequent experiments, the addition of PMNs to cultures of hepatocytes was followed by the addition of 40 nM thrombin in the presence or absence of cytocholasin B (5 μg/ml) and N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP; 100 nM). These concentrations of cytocholasin B and fMLP activate PMNs to damage hepatocytes in PMN/hepatocyte cocultures (21). ALT released into the medium was analyzed as described above.

Measurement of myeloperoxidase release from PMNs. Rat PMNs ($5 \times 10^6$ PMN/ml) in serum-free Williams’ medium E were incubated with 0, 4, or 40 nM thrombin in the presence or absence of 1, 10, or 100 nM fMLP with or without 5 μg/ml cytocholasin B. The cells were incubated in a shaking water bath at 37°C. After a 1-h incubation, the PMNs were removed from the samples by centrifugation at 400 g for 7 min. Myeloperoxidase (MPO) released into the medium was then measured according to the method of Henson (24). An additional sample of PMNs ($5 \times 10^6$ PMN/ml) was lysed by sonication. This sample was used to measure total MPO in the PMN samples. The percentage of total MPO released into the supernatant was calculated by dividing the value obtained for the supernatant by the value obtained for the PMN lysate and multiplying by 100.

Statistical analysis. Results are presented as the means ± SE. For all studies, n represents the number of repetitions of the experiment, each experiment using cells or a liver from a different rat. In cell culture experiments, data expressed as percentages of total enzyme release were subjected to angular transformation and analyzed by ANOVA. In the isolated liver studies, time-dependent changes in ALT activity were analyzed using repeated-measures ANOVA. Multiple comparisons were performed using the Games/Howell test. Data sets that did not meet the criterion of homogeneity of variance or normality were transformed before analysis. For all studies, the criterion for significance was P < 0.05.

RESULTS

Effect of platelet depletion on thrombin-induced damage to the isolated liver. LPS administration to rats results in evidence of thrombin activation 2.5–3 h later and the onset of hepatocellular injury at 3.5–4 h (45). Previous studies showed that platelets accumulate in the liver within minutes after LPS administration and are required for LPS-induced liver injury in vivo (45). Because thrombin is a potent stimulator of platelets (3, 18, 23), we investigated the requirement for platelets in the genesis of injury in livers isolated from LPS-treated rats and perfused with thrombin-containing buffer.

Circulating platelet numbers were reduced in rat liver donors by the administration of APS before LPS treatment. Two hours after APS administration, livers were removed and perfused with buffer containing thrombin. As before (43), perfusion of livers from LPS-treated animals with thrombin resulted in significant ALT release into the perfusion medium. Prior platelet depletion of liver donors failed to influence the injury (Fig. 1).

Effect of PMN depletion on thrombin-induced damage to the isolated liver. Similar to platelets, PMNs accumulate in the liver shortly after LPS injection and are necessary for LPS-induced liver injury in vivo (29). Thrombin damages perfused livers isolated from rats treated with LPS 2 h earlier but not livers from naive rats (43). To determine whether PMNs are required for thrombin to produce injury in isolated livers from LPS-treated rats, blood neutrophil numbers were reduced in rat liver donors using AN-Ig treatment. Two hours after LPS administration, livers were isolated and perfused with buffer containing thrombin. As previously reported (43), perfusion of livers from LPS-treated animals with thrombin resulted in pronounced ALT release into the perfusion medium when the donors had received the C-Ig (Fig. 2). This injury was markedly reduced by pretreatment with AN-Ig, decreasing the
time-dependent ALT release from these livers to the level that was observed in livers from LPS-treated donors perfused without thrombin (43).

Effect of thrombin on hepatocyte/PMN cocultures. In coculture with hepatocytes, activated PMNs damage hepatocytes through the release of toxic proteases (21, 30). Because several studies suggest that thrombin can directly stimulate PMNs (4), we investigated in a coculture system whether the damaging action of thrombin could be due to an ability to activate PMNs to kill hepatocytes directly. Thrombin, at concentrations up to 40 nM, did not activate PMNs to kill hepatocytes isolated from either LPS-treated donor rats (Fig. 3) or untreated rats (data not shown).

We investigated the ability of thrombin to enhance hepatic parenchymal cell killing by PMNs activated with a primary stimulant. As previously reported (21), administration to hepatocyte/PMN cocultures of 100 nM fMLP in the presence of cytochalasin B resulted in a significant increase in ALT released into the medium (Fig. 4). However, thrombin at 40 nM did not enhance the hepatic parenchymal cell killing.

Effect of thrombin administration on PMN degranulation in culture. The lack of influence of thrombin on PMN-dependent hepatocyte killing was unexpected because human PMNs degranulate in response to throm-

---

Fig. 1. Thrombin-induced injury in isolated livers from platelet-depleted rats. Liver donors received antplatelet (APS) or control serum (CS) 22 h before lipopolysaccharide (LPS) administration (96 × 10⁶ EU/kg iv). Two hours later, livers were removed and perfused with buffer containing thrombin (10 nM). Alanine aminotransferase (ALT) activity was measured in the recirculating medium. Control represents livers from donors treated with CS and LPS and then perfused with buffer without thrombin. Results are expressed as means ± SE; n = 5. *Significantly different from livers perfused with thrombin-free medium.

Fig. 2. Thrombin-induced injury in isolated livers from PMN-depleted rats treated with LPS. Liver donors received antineutrophil immunoglobulin (AN-Ig) or control immunoglobulin (C-Ig) 18 and 6 h before LPS administration (96 × 10⁶ EU/kg iv). Two hours later, livers were removed and perfused with Krebs-Henseleit buffer containing thrombin (10 nM). ALT activity was measured in the recirculating medium. Results are expressed as means ± SE; n = 5. *Significantly different from livers from C-Ig-treated rats perfused with thrombin.

Fig. 3. Effect of thrombin on the viability of rat hepatocytes from LPS-treated donors in culture with polymorphonuclear leukocytes (PMNs). Hepatocyte donor rats received LPS (96 × 10⁶ EU/kg iv). Two hours later, hepatic parenchymal cells were isolated and plated (5 × 10⁶ well) as described in MATERIALS AND METHODS. The medium was replaced after 3 h, and rat PMNs (5 × 10⁶ /well) were added. PMNs were allowed to attach for 30 min, and human α-thrombin was added at concentrations of 0.04, 0.4, 4, or 40 nM. The activity of ALT was determined in the cell-free medium and the cell lysate 3, 6, or 16 h after thrombin addition. The percentage of total ALT released was calculated. Values represent means ± SE; n = 4 donors.
bin (4). To test whether rat PMNs responded similarly to thrombin, we measured the activity of MPO in the medium of PMNs in culture as an indicator of release of contents of azurophilic granules. Thrombin at concentrations up to 40 nM did not increase the release of MPO from PMNs (Fig. 5A).

fMLP is a stimulus for degranulation of lysosomes, and its action is greatly potentiated by the activity of cytochalasin B on the PMN actin filament system (5). Because thrombin has effects on the actin filament network of endothelial cells (38) and platelets (19), we investigated whether thrombin could replace cytochalasin B in promoting PMN degranulation. Consistent with previous reports (5), in the absence of cytochalasin B, fMLP did not increase MPO release from PMNs (Fig. 5A). MPO release was not increased by the combination of fMLP and thrombin.

As previously reported (5), degranulation of PMNs in response to fMLP is concentration dependent and facilitated by the presence of cytochalasin B. We examined whether thrombin could increase the sensitivity of PMNs to the combination of fMLP and cytochalasin B. At any concentration, thrombin failed to augment MPO release caused by fMLP in the presence of cytochalasin B (Fig. 5B).

Requirement for PMNs, LPS, and thrombin for injury to livers from naive donors. Livers from rat donors treated with LPS 2 h before isolation release ALT into the perfusion medium only when the medium contains thrombin (43). Within the 2-h time period between LPS injection and liver isolation, liver cells as well as PMNs in the sinusoids have been exposed to various soluble inflammatory mediators released during systemic LPS exposure. It is known from studies in vivo that the presence of PMNs (29, 33), active thrombin (43, 46), and exposure to LPS are necessary for the expression of endotoxic liver injury in the rat. However, other extrahepatic factors may be important determinants of the hepatocellular damage in vivo or in the isolated, perfused liver. To determine whether the presence of LPS, PMNs, and thrombin is sufficient to produce hepatocellular injury, livers isolated from naive donors were perfused with various combinations of these three factors.

Perfusion of livers from naive donors with buffer resulted in a small increase in ALT activity in the recirculating medium, which resembled that seen previously in control livers (43). At 2 h, the ALT activity in the perfusion buffer of controls was 27 ± 4 U/l. The addition of PMNs to the perfusion buffer did not significantly change the release of ALT during the first 2 h.
Thrombin is formed during liver diseases, and an ac-
cumulation of fibrin deposits in necrotic areas has been
proven. Thrombin is also involved in the resolution of
inflammation processes (20, 52, 55). Thrombin plays an
important role in liver injury and repair, recent studies
suggest that this enzyme plays a causal role in inflam-
matory liver injury. For example, the observations that
thrombin inactivation completely blocked LPS-induced
hepatic necrosis in vivo after LPS exposure (40, 46) and
that thrombin injures isolated livers from LPS-treated
donor rats (43) point toward thrombin as one of the key
mediators of liver injury in response to LPS. Moreover,
previous results in buffer-perfused livers strongly sug-
 gest that thrombin contributes to injury independent of
its capacity to form fibrin clots (28, 43). This raises the
possibility that thrombin interacts with one or more
cells in the genesis of LPS-induced liver damage. In-
deed, preliminary evidence suggests that thrombin par-
icipates in liver injury by activating a protease-
activated receptor in livers of LPS-treated rats (13).
Platelet or PMN depletion protects against LPS-
induced hepatic necrosis (29, 45), demonstrating the
importance of these cells in the mechanism of tissue
damage in vivo. Platelet accumulation in the liver
begins shortly after LPS administration in vivo. Ac-
cordingly, it seemed conceivable that thrombin might
play a role in LPS-induced liver injury through an
interaction with platelets. Thrombin might promote
the sequestration of platelets in the liver and/or acti-
vate these cells. Results from previous studies suggest
that thrombin does not influence the sequestration of
platelets in the liver: hepatic platelet accumulation
begins within 15 min after intravenous injection of
LPS into rats (45), whereas activation of the coagula-
tion system does not occur until more than 2 h later.
Furthermore, hirudin, a selective inhibitor of throm-
bin, failed to affect hepatic platelet accumulation (46).
The possibility remained that thrombin acts through
activation of platelets already in the liver. However,
platelet depletion of rat liver donors to a degree that
afforded protection from LPS-induced liver injury in
vivo failed to alter the damaging effect of thrombin in
the isolated liver (Fig. 1). These results indicate that
thrombin does not act through platelet activation to
injure hepatocytes. Together, these results suggest
that the critical role of platelets may be to promote
formation of thrombin in the sinusoids, perhaps by
providing a surface for assembly of the prothrombinase
complex (6, 7, 44). This conclusion is supported by the
finding in vivo that platelet depletion prevented the
LPS-induced decrease in plasma fibrinogen (45). Thus
platelet accumulation or activation in LPS-induced
liver injury does not depend on thrombin, but these
cells may play a critical role in thrombin generation
during endotoxin-induced liver injury.

By contrast, depletion of PMNs from liver donors
before LPS injection provided protection from throm-
bin-induced hepatic injury in the isolated, perfused
liver (Fig. 2), reducing the release of ALT in the perfu-
sion medium to the level of livers never exposed to
thrombin (compare Fig. 1 controls with AN-Ig livers in
Fig. 2). The protection afforded by PMN depletion
suggests an interdependence between thrombin and
PMNs. Accordingly, additional studies were under-

Figure 6. Release of ALT from isolated livers perfused with LPS,
PMNs, and thrombin. Livers from untreated donor rats were per-
fused with Krebs-Henseleit buffer containing 2% BSA. Rat PMNs
(final concentration 2 × 10^6 PMN/ml) were added to the perfusion
buffer at time 0 in the indicated treatment groups. After 10 min, LPS
(96 × 10^6 EU/kg of donor rat weight) or its saline vehicle was added
to the perfusion buffer. After 2 h, human α-thrombin (10 nM) or its
vehicle was added to the perfusion solution and livers were perfused
for another 2 h. Samples were taken every 15 min from 2 to 4 h. ALT
activity was determined in all samples as described in MATERIALS AND
METHODS. Results are expressed as mean of ALT activity. Average
SE = 31; n = 8 per group. *Time-dependent ALT release signifi-
cantly different from all other groups. †Time-dependent ALT release
significantly different from groups 1–3, 5, 6, and 8.
taken for the purpose of uncovering potential interactions between thrombin and PMNs. The ability of thrombin to influence PMN-induced hepatocyte killing was investigated. In the coculture system, concentrations of thrombin up to four times those that produced injury in the isolated liver had no effect on the viability of hepatocytes isolated either from naive or LPS-treated rats, confirming that thrombin was not directly toxic to hepatocytes and raising doubt about the ability of thrombin to stimulate PMNs to kill these cells. Furthermore, although addition of fMLP to a PMN/hepatocyte coculture system yielded significant hepatic parenchymal cell death, thrombin was unable to enhance the cytotoxic effect of PMNs activated by fMLP. Thus none of the conditions explored in cell cultures was consistent with the thrombin/PMN interdependence in hepatocellular killing suggested by experiments in vivo and in the isolated, perfused liver.

Because cytotoxicity under these conditions is dependent on PMN proteases, it seemed possible that thrombin might promote PMN degranulation and release of toxic proteases. Furthermore, because cytoskeletal changes are important for PMN degranulation in response to an inflammatory stimulus (5) and because thrombin affects the cytoskeleton of other cell types (19, 38), we hypothesized that thrombin might either replace or enhance the effects of the cytoskeleton-altering agent cytochalasin B in fMLP-stimulated PMNs. However, thrombin neither stimulated the degranulation of rat PMNs nor enhanced the degranulation of fMLP-stimulated PMNs in the presence or absence of cytochalasin B. These observations suggest that thrombin has no direct effect on the release of lysosomal contents from rat PMNs. This finding is in contrast with results in human PMNs, which can be activated directly by thrombin (4).

In these studies, we used PMNs isolated from rat peritoneum after glycogen elicitation. We considered the possibility that the lack of effect of thrombin on these cells (Figs. 3 and 4) might be explained by desensitization to thrombin in vivo as a result of the isolation procedure. However, cells isolated by this method do not differ in response to activating agents compared with PMNs isolated from blood (26). Moreover, limited studies on PMNs isolated from rat blood revealed a lack of responsiveness to thrombin similar to that found (Figs. 4 and 5) for elicited PMNs, i.e., no activation by fMLP and no influence on PMN-mediated hepatocellular killing (data not shown). In addition, PMNs isolated by this method were found to be critical in causing injury to thrombin-perfused livers (Fig. 6), a result that argues against prior desensitization to effects of thrombin during the elicitation procedure.

Results of studies with isolated cells suggested that the presence of PMNs and thrombin is not sufficient to reproduce hepatocellular killing observed in intact liver. Thus one or more additional factors may be necessary. To identify the minimum extrahepatic elements required for LPS-induced liver injury, livers from naive donors were perfused with buffer containing LPS, PMNs, and/or thrombin. Platelets were not included in this experiment because our earlier results (Fig. 1) suggested that the platelets' critical role was to generate thrombin, which we added directly to the perfusion medium. The LPS-perfused livers were damaged by inclusion of PMNs and thrombin as extrahepatic factors (Fig. 6). This result suggests that any additional cellular or soluble factors required for hepatic injury are either already present in the normal liver or can be synthesized by liver cells. The combination of all three elements (i.e., LPS, PMNs, and thrombin) produced the greatest degree of injury, supporting the idea of an interaction between thrombin and PMNs in response to LPS that maximizes hepatocellular killing.

Surprisingly, ANOVA revealed no direct interaction between LPS and PMNs in isolated perfused livers (Fig. 6). The mechanism by which PMNs accumulate and become activated in the liver in vivo in response to LPS is not entirely clear, but it appears to be related to the expression of cellular adhesion molecules (34, 35). However, this effect is dependent on the presence of LPS-binding protein or soluble CD14 (22), two elements that were not added to the perfusion buffer. The mere process of perfusing PMNs through the sinusoids of an isolated liver causes neutrophil adhesion, independent of the addition of LPS (14). Accordingly, it is possible that endothelial cell surface modification, due strictly to the procedure of liver isolation and perfusion, supplanted the need for LPS interaction with PMNs that is required in vivo.

The observation that thrombin and LPS in combination (i.e., without PMNs) led to modest but significant liver damage (Fig. 6) suggests either that thrombin has the ability to compromise the viability of hepatocytes directly after they are exposed to LPS or that a cell type constitutively present in the liver can become activated by thrombin to damage hepatocytes during LPS exposure. Thrombin is not directly toxic to cultured hepatocytes either in the presence of LPS or after 2 h of exposure to LPS in vivo (12). Therefore, it appears likely that a cell type other than the PMN can be activated by thrombin to injure hepatocytes after exposure to LPS. This cell type must be a component of the hepatic nonparenchymal cell population, because naive, buffer-perfused livers are virtually devoid of blood cells. Moreover, the response of these cells to thrombin requires prior LPS exposure, because perfusion with thrombin alone failed to injure naive isolated livers (Fig. 6). Finally, this cell type must possess the machinery to damage hepatic parenchymal cells. The observation that livers treated with the combination of all three factors experienced more damage than the ones treated either with thrombin plus PMNs or with thrombin plus LPS also suggests the possibility of an interaction between PMNs and this cell type that results in increased killing.

One candidate that fits these criteria is the Kupffer cell. Kupffer cells express toll-like receptor 4 and are activated by the binding of LPS to this receptor (53). They have been implicated in the mechanism of hepatic parenchymal cell death in various liver diseases.
(2, 48), including LPS-induced liver injury in rats (10). Furthermore, they can enhance the activation of neutrophils through the release of various proinflammatory cytokines (47, 57). Thrombin has been shown to stimulate macrophages (37), and our recent preliminary studies have identified thrombin receptor PAR-1 on Kupffer cells (13). Thus both LPS and thrombin might activate Kupffer cells during endotoxemia.

In summary, we have shown that in the isolated liver as in vivo PMN depletion prevents liver damage caused by intravenous injection of endotoxin. This confirms the critical role of PMNs in the pathogenesis and supports the usefulness of the isolated organ model in the exploration of LPS-induced hepatocellular injury. By contrast, platelets, which play a critical role in the mechanism of toxicity in vivo, appear to act proximal to thrombin’s action and most likely contribute by increasing local thrombin activation. It was possible to reproduce in naive livers the thrombin-mediated injury observed during perfusion of livers isolated from LPS-treated rats by adding LPS and PMNs to the perfusion buffer. This demonstrates that thrombin and PMNs are sufficient as extrahepatic factors to initiate liver damage during LPS exposure. Moreover, maximal injury occurred in the presence of PMNs, LPS, and thrombin, and ANOVA indicated significant interaction among these three factors. Thrombin did not stimulate or enhance degranulation of rat PMNs, and it was not directly toxic to isolated rat hepatocytes in the presence of PMNs in vitro even after LPS exposure. Accordingly, hepatocellular killing by the PMN/thrombin combination appears to require an additional factor(s) present in normal liver.

This research was supported by National Institutes of Health (NIH) Grant RO1-DK-50728. Drs. F. Moulin and B. L. Copple were supported in part by NIH training Grant T32-ES-07255.

REFERENCES


