Hepatic cellular interactions in endotoxaemia and sepsis

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Introduction

Interactions of various hepatic cell types, e.g. hepatocytes and hepatic non-parenchymal cells, in endotoxaemia are associated with modulation of host defence mechanisms. The hepatic environment is the site of an extensive cytokine network and a complex interplay of mediators in endotoxaemia and various inflammatory processes. The various cell types found in the liver, namely the hepatocytes, Kupffer cells, sinusoidal endothelial cells, Ito and pit cells, contribute in a number of ways to this scenario [1-3].

The early response to an endotoxic insult or indeed to any inflammatory stimulus is the recruitment of neutrophils to the site of injury or inflammation. The liver is a primary site for neutrophil recruitment in endotoxaemia. In this context, liver-sequestered neutrophils (polymorphonuclear leucocytes or PMNs) can be classified as hepatic non-parenchymal cells. These inflammatory cells can produce large amounts of oxidants including both reactive oxygen intermediates such as superoxide anion, hydrogen peroxide and hydroxyl radicals as well as reactive nitrogen intermediates, in particular NO. Neutrophils can be 'primed' for an enhanced respiratory burst by bacterial lipopolysaccharide in concentrations measurable in patients with septic shock [4, 5]. Short-term, i.e. 90 min or 3 h, endotoxin (ET) infusion results in significant priming of cells of the 45 ml/min elutriated fraction, containing Kupffer cells and neutrophils (polymorphonuclear leucocytes or PMNs) can be classified as hepatic non-parenchymal cells. These inflammatory cells can produce large amounts of oxidants including both reactive oxygen intermediates such as superoxide anion, hydrogen peroxide and hydroxyl radicals as well as reactive nitrogen intermediates, in particular NO. Neutrophils can be 'primed' for an enhanced respiratory burst by bacterial lipopolysaccharide in concentrations measurable in patients with septic shock [4, 5]. Short-term, i.e. 90 min or 3 h, endotoxin (ET) infusion results in significant priming of cells of the 45 ml/min elutriated fraction, containing Kupffer cells and neutrophils for subsequent superoxide release in response to stimulation in vitro by phorbol ester and, to a smaller extent, by opsonized zymosan [6,7]. Sinusoidal endothelial cells under the same conditions produce insignificant, barely measurable amounts of superoxide anion.

The ability to generate NO from arginine by way of the enzyme NO synthase is shared by a wide variety of cells including hepatocytes [8,9] and Kupffer cells. Hepatocytes in culture spontaneously produce NO [10]. Induction of NO generation in hepatocytes has been reported to require multiple cytokines [11]. However, hepatocytes isolated from rats that have been exposed to a 30-h continuous ET infusion are responsive to stimulation by single cytokines in culture [12]. Although NO synthase activity in both hepatocytes and Kupffer cells is considered inducible, these two cell types display differential responses of NO formation to ET and cytokine stimulation as well as quantitative differences. Spontaneous production of NO by Kupffer cells is much smaller than that by hepatocytes. Nitric oxide production by Kupffer cells is elevated in endotoxic rats treated either chronically [12] or acutely [13] with ET.

Liver endothelial cells represent the main component of the sinusoidal cells (44% by volume [14]), and may also act as effector cells for immune responses; they present different surface antigens [15]. The contribution of liver endothelial cells to hepatic superoxide production after ET exposure in vivo and in response to stimulation by various agents in vitro is much less than that of Kupffer cells or liver-recruited neutrophils [6,16]. Liver endothelial cells of ET-treated rats also form NO in smaller amounts than Kupffer cells of the same rats, although the response to stimulation by ET or cytokines in vitro displays a similar qualitative pattern [12].
Neutrophils recruited to sites of infection and inflammation provide the first line of host defence against invading micro-organisms. They accomplish this important task by phagocytosis and microbicidal activity through the generation of reactive oxygen metabolites, NO and the secretion of several granular enzymes. Activated neutrophils, however, are also causally related to immunologically induced inflammation and tissue injury. Liver-recruited neutrophils in endotoxic rats undergo functional alterations with respect to neutrophils circulating in the peripheral blood in the same animals. This functional difference is especially evident in terms of NO production both spontaneously and upon an ET challenge, and in the upregulation of the expression of CD11b/c adhesion molecules. Significant differences can also be observed in the eicosanoid profile of liver and circulating PMNs of endotoxic rats [17].

Until very recently, hepatocytes have been considered passive target cells for immunological attack by effector T-cells. This concept is reformulated due to evidence that activated hepatocytes can serve as immunomodulating cells, e.g. by actively synthesizing and secreting immunomodulatory factors involved in the acute phase of the inflammatory reaction [18]. Nitric oxide produced by hepatocytes upon stimulation by ET or cytokines may also contribute to a protective function that has been suggested for NO synthesis in vivo in ET-induced liver injury [19]. Increased formation of NO by hepatocytes in continuous endotoxaemia may modulate host defence mechanisms at two levels, by promoting microbicidal activity and preventing extensive damage due to its scavenger action for superoxide anion and as a putative endogenous inhibitor of leucocyte adhesion and peroxidative injury [12]. An immunomodulatory role for hepatocytes in endotoxaemia is also supported by our previous studies showing generation by acutely endotoxic hepatocytes of factor(s) responsible for CD11b/c upregulation and increased chemotaxis of naive circulating PMNs [20].

GSH is an important determinant for the reduction of oxidant stress generated by infection, inflammation or ischaemia reperfusion injury. In this study we explored the role of cytokine-induced neutrophil chemoattractant (CINC) in hepatic neutrophil influx in endotoxaemia and the influence of liver-recruited neutrophils and Kupffer cells of endotoxic and septic rats on hepatocyte GSH content.

**Materials and methods**

**Reagents**

Rabbit anti-rat CINC antiserum and CINC were purchased from Peptides International (Louisville, KY, U.S.A.). Anti-rabbit horseradish peroxidase was from Boehringer Mannheim Co. (Indianapolis, IN, U.S.A.). *Escherichia coli* endotoxin (026:B6 prepared by the Boivin method) was from Difco (Detroit, MI, U.S.A.). The affinity-purified goat anti-CINC antibody was prepared by Dr J. Zagorski of the Laboratory of Immunology, National Institute of Dental Research. All other enzymes, tissue culture reagents, density gradients and chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

**Animals**

Male Sprague–Dawley rats (323 ± 17 g, Hilltop Lab animals, Inc., Scottdale, PA, U.S.A.), maintained on a standard laboratory diet and housed in a controlled environment with a 12-h light/dark cycle, were used. On the day preceding each experiment, catheters were placed in the jugular vein and carotid artery using aseptic surgical techniques. Next morning the animals were infused intravenously with ET or sterile normal saline (Baxter Corp., Toronto, Canada) for 90 min. The total amount of ET infused over the 90 min period was 225 µg in a total volume of 2.5 ml of normal saline as reported previously [7]. Rats were rendered septic by caecal ligation and puncture (CLP) as described previously [17]. The use of animals conformed to National Institutes of health guidelines.

**Isolation of liver parenchymal cells, sequestered PMNs and Kupffer cells**

Isolation of hepatocytes, Kupffer cells and liver-sequestered neutrophils was performed as described previously [6,7]. Briefly, rats were anaesthetized with an intramuscular injection of ketamine (40 mg/rat). The portal vein and inferior vena cava were cannulated, and the liver was perfused in situ with continuously gassed (95% O₂/5% CO₂) Hank’s solution containing collagenase (2535 units/ml) and 2 mM CaCl₂. The cell suspension was centrifuged at 20 g for 2 min to pellet the parenchymal cells. Isolated hepatocytes were over 85% viable and their purity was 99%. Non-parenchymal cells were processed by centrifugal elutriation and the 45 ml/min fraction containing Kupffer cells and liver-sequestr
tered PMNs was collected as described previously [22]. The viability of the cells in this fraction was over 90%, assessed by Trypan Blue exclusion. Wright's stain was used to differentiate Kupffer cells and liver-sequestered neutrophils.

The 45 ml/min elutriation fraction was subjected to discontinuous Ficoll–Hypaque density-gradient sedimentation to separate Kupffer cells and neutrophils. The viability of the purified Kupffer cells and liver-sequestered PMNs was greater than 90% and the purity of the two types of cells was over 85%.

Cell culture
Isolated hepatocytes, Kupffer cells and liver-sequestered neutrophils were plated on 23-mm plastic 12-well tissue-culture plates (Costar, Cambridge, MA, U.S.A.) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as described previously [23]. Cell densities for hepatocytes were (0.25 x 10⁶) cells/0.85 ml well, and for Kupffer cells and liver-sequestered PMNs (1 x 10⁶) cells/0.85 ml well. Cells were maintained in primary culture for 3 h and 20 h. The supernatants of cell cultures were collected and kept at −70°C.

Measurement of CINC generation
CINC in the supernatants of cell cultures was measured by specific ELISA as previously described [24]. The concentration of goat anti-CINC antibody used to coat the plates was 14 µg/ml in 0.1 M NaHCO₃, and the dilution of rabbit anti-CINC antibody (second antibody) was 1:5000 in PBS-milk. Samples were measured in triplicate at various dilutions. The results of CINC generation are expressed as ng/10⁶ cells x hour of culture.

Measurement of GSH levels
After 20 h of culture, cells were denatured with 5% trichloroacetic acid containing 5 mM EDTA and homogenized by ultrasonication. After centrifugation at 10 000 g for 15 min, the supernatants were collected and GSH levels measured with Ellman's reagent as described previously [25].

Results and discussion
CINC generation by hepatic parenchymal and non-parenchymal cells
Supernatants of hepatocytes, Kupffer cells and liver-sequestered PMNs were analysed for CINC generation using specific ELISA. As can be observed in Table 1, hepatocytes of endotoxic rats in 20 h culture produced significantly more CINC than did hepatocytes of saline-infused animals during the same time period. Overall, Kupffer cells generated significantly less (about one quarter as much) CINC than did hepatocytes under either experimental conditions. However, Kupffer cells also responded to ET treatment, with significantly enhanced CINC production over that of saline-infused animals.

CINC and hepatic neutrophil influx
Treatment of rats with anti-CINC antibody before ET infusion significantly reduced recruitment of PMNs into the liver. In ET-infused rats the total number of cells recovered in the 45 ml/min fraction significantly increased over that in saline-infused rats (14.02 ± 2.47 x 10⁷ and 5.13 ± 1.25 x 10⁷ respectively, P < 0.05). Pretreatment with anti-CINC antibody significantly reduced the total cell yield to 8.53 ± 2.27 x 10⁷, but this was still higher than the cell yield in saline-infused rats. In ET-infused rats, PMNs constituted 64.24% of the cells in the 45 ml/min fraction, which was reduced to 38.27% in the anti-CINC antibody-treated rats. In saline-infused

| Table I |
| CINC production (ng/10⁶ cells) by hepatocytes (H) and Kupffer cells (KC) of saline- and endotoxin (ET)-treated rats |
| All values are means ± SEM (n = 4). |
| H₄₇ | 24.54 ± 2.65** |
| H₄₉ | 7.10 ± 0.44 |
| KC₄₇ | 6.01 ± 0.91** |
| KC₄₉ | 1.78 ± 0.15## |

*P < 0.01 versus KC₄₉.  
**P < 0.001 versus H₄₉.  
##P < 0.001 versus hepatocytes of rats with the same treatment.
infused rats the 45 ml/min elutriated fraction contained 8.92% PMNs. There was no difference in the number of Kupffer cells recovered in each group. Injection of preimmune goat IgG into two rats in the same manner as the goat anti-CINC IgG did not influence PMN infiltration.

Cytokines generated at sites of inflammation may attract and/or activate neutrophils. CINC is a member of the chemokine supergene family of cytokines and, more specifically, of the chemokine-α subfamily. The amino acid sequence of CINC has significant homology to the mouse KC gene product (91%), to human melanoma growth stimulatory activity (MGSA/gro, 69%) and to human interleukin-8 (47%), the most widely known of the C-X-C family of chemoattractants. CINC can be induced in the rat by lipopolysaccharide and interleukin-1β [26]. CINC is chemotactic for rat neutrophils in vitro [26], causes dermal accumulation of neutrophils after intradermal injection [26] and an influx of neutrophils into bronchoalveolar lavage fluid [27]. CINC is also known to mediate neutrophilic alveolitis in rats [28].

As early as 3 h after plating the cells, analysis of the culture media revealed a significantly higher level of CINC produced by hepatocytes of endotoxic rats than that generated by hepatocytes of saline-infused controls (8.87 ± 0.59 ng/10⁶ cells versus 1.29 ± 0.45 ng/10⁶ cells, \( p < 0.01 \)). In similar fashion, Kupffer cells of endotoxic rats also generated more CINC at 3 h than their saline-infused controls (1.83 ± 0.97 ng/10⁶ cells versus 0.42 ± 0.21 ng/10⁶ cells, \( p < 0.05 \)). Liver-sequestered PMNs produced very little CINC at 3 h of culture, and even at 20 h of culture they still generated only a low level of CINC (1.61 ± 0.59 ng/10⁶ cells).

CINC expression in hepatocytes and Kupffer cells occurred rapidly after intravenous infusion of ET, resulting in significant influx of PMNs into the liver. Pretreatment with anti-CINC antibody inhibited PMN accumulation by 40%. These data demonstrate for the first time that CINC mediates ET-induced neutrophil accumulation in the liver, and are consistent with a potential role for CINC in the pathogenesis of hepatic neutrophilic inflammatory injury during sepsis.

**GSH levels in hepatocytes and in hepatocyte/ non-parenchymal cell co-cultures**

At any inflammatory locus or in endotoxaemia or sepsis, the infiltration of phagocytes is associated with oxidant stress due to the release of reactive oxygen species. Because of the considerable damaging potential of reactive oxygen, the cells depend on several defence mechanisms to neutralize these toxic intermediates and to prevent significant free radical injury. The reduced form of glutathione plays an important role in such defence mechanisms by providing reducing equivalents for the metabolism of cytosolic and mitochondrial hydrogen peroxide by glutathione peroxidase. Glutathione peroxidases can also ameliorate superoxide-mediated lipid peroxidation by reducing lipid hydroperoxides to alcohols to prevent their reaction with reducing agents such as ferrous iron [29]. To assess liver injury due to the endotoxic insult and peritoneal sepsis, we measured GSH levels in cultured hepatocytes and in hepatocyte/PMN and hepatocyte/Kupffer cell co-cultures. As shown in Figure 1, the GSH levels of hepatocytes of endotoxic rats are significantly reduced compared with hepatocytes of naive control rats. Co-culture of hepatocytes of naive rats with liver-sequestered PMNs or Kupffer cells of endotoxic rats in a ratio of 1:3 resulted in essentially the same reduction in GSH levels as found in endotoxic hepatocytes. Co-culture of hepatocytes and non-parenchymal cells in the presence of superoxide dismutase (SOD) and catalase (CAT), two agents that can detoxify hydrogen peroxide, significantly reversed
the deleterious effect of both types of non-parenchymal cells. Similar results were observed when hepatocytes and liver-sequestered PMNs or Kupffer cells of septic rats were co-cultured in a ratio of 1:2, except that SOD and CAT addition to the co-culture was not able to cause significant reversion of the Kupffer cell effect on naive hepatocytes (Figure 2).

Thus, the results presented in Figures 1 and 2 clearly show significant decreases in GSH levels in hepatocytes of both endotoxic and CLP rats compared with naive controls. Furthermore, the GSH content of naive hepatocytes co-cultured with either liver PMNs or Kupffer cells of endotoxic or septic rats was significantly suppressed relative to naive hepatocytes and comparable to hepatocytes obtained from rats subjected to either infectious insult.

Neutrophil aggregation and adherence is considered to be an important mechanism in eliciting hepatic injury since adherence of neutrophils may localize and thereby intensify the effects of toxic oxygen species and proteases [30]. Due to the close anatomical association of Kupffer cells and parenchymal cells in the liver, it was postulated that particulate ingestion by Kupffer cells may elicit injury to nearby hepatic parenchymal cells [31]. However, studies in vitro demonstrated that activated PMNs may be more harmful mediators in hepatocyte injury during sepsis. Using a co-culture of hepatocytes and PMNs isolated from peritoneal fluid, the ability of activated PMNs to injure hepatocytes was demonstrated, whereas bacterial challenge to a co-culture of Kupffer cells and hepatocytes resulted in little parenchymal cell injury [32]. In our experimental models, both short-term infusion of ET- and CLP-induced sepsis were associated with mediation of hepatocyte injury (as assessed by reduced GSH content) to about the same extent by liver-sequestered PMNs and Kupffer cells.

Our findings extend support for an immunomodulatory role for hepatocytes in acute endotoxaemia. CINC production by endotoxic hepatocytes (and to a lesser extent by endotoxic Kupffer cells) is associated with hepatic neutrophil influx, an important functional consequence of CINC generation. In addition, our studies also point to a potentially harmful outcome of the interplay between hepatocytes and liver-sequestered PMNs, leading to reduced capability to overcome oxidant stress.

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Bacterial endotoxin effects on carbohydrate utilization and transport
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Introduction
After the early pioneering investigations of Berry [1], the results of extensive studies over the last two decades clearly identified some of the marked metabolic alterations that accompany endotoxaemia and sepsis. Although the intermediary metabolism of all three major substrates, carbohydrates, lipids and proteins, is affected, changes in carbohydrate metabolism are especially important in conditions of infection and inflammation, as glucose contributes to the maintenance of the intracellular balance between oxidants and antioxidants, in addition to serving as an oxidizable energy-yielding substrate. Although both aspects of carbohydrate metabolism, i.e. supply as well as utilization of metabolites, are altered in endotoxaemia or sepsis, this brief review will only concentrate on alterations in carbohydrate utilization. Furthermore, it will

primarily attempt to summarize some of the investigations carried out in our laboratories over the last 20 years.

Total body glucose uptake
Shortly after the administration of Escherichia coli endotoxin [lipopolysaccharide (LPS)], total body glucose uptake (Rd), as determined by the primed constant infusion of [6-3H]glucose, increases [2]. Elevated Rd is also observed in rats after non-lethal doses of LPS [3]. Similar changes are noted during acute, hypermetabolic septic state was induced in these animals by intra-peritoneal administration of a pooled faecal inoculum [4]. In experiments seeking to ascertain the involvement of cytokines in this effect, glucose kinetics were found to be elevated in rats by the administration in vivo of conditioned media from LPS-stimulated RAW 264.7 cells, as well as by the administration of human recombinant tumour necrosis factor-α (TNF-α) [5], indicating that cytokines may play a role either directly or indirectly in the LPS- or sepsis-