The Role of Intestinal Endotoxin in Liver Injury: A Long and Evolving History

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From the mid-1950s, it was observed that liver injury by a variety of toxins greatly sensitized the host to the effects of administered lipopolysaccharide. In the nutritional cirrhosis of choline deficiency, and in acute toxic injury as well, the need for the presence of enteric endotoxin was demonstrated. The universality of this association was striking for almost all agents associated with liver injury. In addition, the presence of endotoxemia in human liver disease was documented in the 1970s, when the hypothesis was first proposed, and correlated with the severity of the disease. Despite imposing evidence of the critical role of enteric endotoxin in liver injury, it did not excite much interest in investigators until the 1980s. With the ability to study effects of alcohol in newer delivery systems, and an increased understanding of the role of Kupffer cells in the process, the original hypothesis has been accepted. This historical review details the progress of this novel concept of disease initiation and suggests future directions to bring potential therapies to the bedside. (HEPATOLOGY **2010;52:1829-1835**)

Continuing work over the past several decades has further solidified the importance of intestinal endotoxins as critical cofactors in toxic liver injury by a number of agents. The evidence for the importance of this association goes back almost half a century, and although it initially met with considerable skepticism, this association has now become an accepted area of investigation.

The forward progress of the association reflects the newer methods of modeling, a deeper understanding of mediators involved in the association, a heightened knowledge of the role of hepatic macrophages in the process, and the further development of potential modifiers of endotoxin injury. Although endotoxin is the cell wall of gram-negative bacteria, and the core lipid A is the toxic moiety, the terms lipopolysaccharide (LPS) and endotoxin will be used interchangeably for this toxic material.

Based on our work and that of other investigators, who demonstrated a marked increase in sensitivity to LPS in livers impaired by hepatotoxins, the hypothesis of the importance of intestinal endotoxins in the resulting damage was first published in 1975.[1] Subsequently, the topic was presented as the Merrill Lecture at the American Association for the Study of Liver Diseases in 1980 and published in Hepatology.[2] A diagram of the hypothesis from the 1975 article is shown in Fig. 1. It was summarized as follows: (A) Portal vein endotoxemia of gut origin represents a normal physiological state. (B) The hepatic sinusoidal cells, particularly fixed macrophages (Kupffer cells), are critical to normal endotoxin detoxification. (C) The initial damage in a number of injuries is to sinusoidal cells, which seriously impacts the ability of the liver to handle the ordinarily innocuous amounts of LPS coming from the gut. (D) This marked increase in sensitivity to LPS, which may be of a magnitude of 10-fold to 1000-fold, leads to further hepatocytic damage and spillover of the endotoxins into the systemic circulation, resulting in the extrahepatic manifestations associated with liver injury. In the 1960s and 1970s, the hypothesis was not considered attractive, and the idea of "autointoxication" from intestinal sources was considered an outmoded concept.

Historical evidence of a synergism between bacteria in the gut and other toxins goes back to 1941 when sulfonamides protected against carbon tetrachloride (CCl_4) injury in animals.[3] In 1957, nonabsorbable antibiotics were found to prevent death in rats on the necrogenic diet of choline deficiency, and neomycin was superior in its effect compared to absorbable

Abbreviations: IL, interleukin; LPS, lipopolysaccharide; PTX, pentoxifylline; TNF, tumor necrosis factor.

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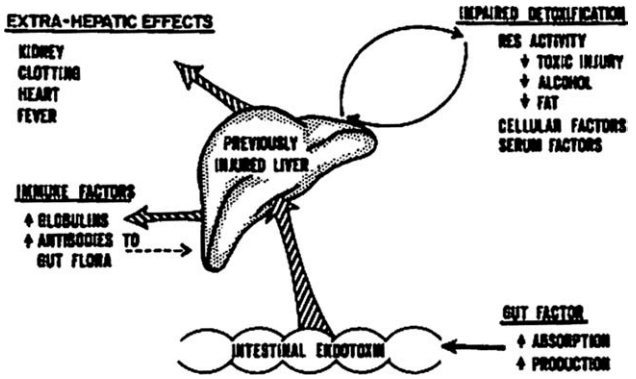

Fig. 1. Endotoxin and liver disease. Possible mechanisms for the toxic hepatic and extrahepatic effects of endotoxin after liver injury (from Nolan JP. The role of endotoxin in liver injury. Gastroenterology 1975;69:1346-1356).

antimicrobials.[4] Broitman and his colleagues in 1964, using this model of nutritional cirrhosis, found that the protective effect of neomycin was eliminated if purified LPS was added to the drinking water, confirming that endotoxin, rather than intact bacteria, caused the lesion.[5] Because of its morphologic similarity to Laennec's cirrhosis, it was used as a surrogate model for that disease. Alcohol given by gavage or in the water to rats did not cause any visible alteration of the liver with chronic administration. Alcohol given to rats, however, resulted in depression of reticuloendothelial function as measured by the uptake of labeled microaggregated albumin.[6] Furthermore, serial assessment of reticuloendothelial system function in rats on a choline-deficient diet revealed depression of the uptake of the colloid that was progressive over time.[7]

A strong relationship of LPS and liver injury was also demonstrated with acute hepatotoxins. CCl_4 was used in many studies of acute liver injury and the relationship to absorbed LPS was firmly established.[8,9] Importantly, induced endotoxin tolerance in rats by a progressive increase in the dose of administered LPS protected against the necrosis induced by CCl_4.[10] Polymyxin B has the unique property of binding endotoxin, which prevents its translocation. This is in contrast to other antibiotics that may kill gram-negative bacteria but transiently increases LPS level in the portal vein. When administered to rats prior to CCl_4 exposure, hepatic necrosis was significantly ameliorated.[11] Another model widely used to induce hepatic necrosis is D-galactosamine and again, experiments in this model revealed a key role for enteric LPS in its pathogenesis.[12]

A major advance in establishing the clinical role of enteric LPS in liver injury in humans was the development of the *Limulus* lysate assay to detect endotoxin in sera and body fluids. A number of assays done in the 1970s and 1980s revealed significant amounts of LPS in the sera of patients with cirrhosis and those with acute hepatic necrosis.[13,14] This assay also confirmed that endotoxins present in the portal vein from normal individuals was increased in those with liver disease.[15] Correlations of *Limulus* lysate assay activity with extrahepatic manifestations of alcoholic cirrhosis, such as the hepatorenal syndrome and clotting abnormalities, was also demonstrated.[16]

Thus, the critical role of gut-derived endotoxin as a cofactor in acute and chronic liver disease, both experimental and clinical, was already established more than 30-40 years ago. Advances since that time in solidifying the significance of the relationship mirrored major advances in animal models, our understanding of the role of hepatic macrophages as mediators and detoxifiers of endotoxin, and the increase of our knowledge of the mechanisms of injury by the cell wall of gram-negative bacteria.

Studies Since the Mid-1980s

Research over the past 25 years supports the original hypothesis that enteric LPS is a key factor in both acute and chronic liver injury. Select studies over this time will be cited.

Animal Models. Because alcoholic liver disease is the most common chronic liver injury, a major advance was made with the development of a technique that allowed continuous and high-dose administration of alcohol to rodents. Prior to the mid-1980s, alcohol was given by gavage or in the drinking water to rats. Although this method of administration significantly depressed the ability of Kupffer cells to remove labeled colloid and to prevent LPS from reaching the systemic circulation, it did not result in any histologic change in the liver. With the new technique, delivery was accomplished by direct administration into the stomach by a catheter funneled subcutaneously to the outside.[17] With such a method of high intensity delivery, the development of the disease could be duplicated and studied longitudinally.

Reticuloendothelial System–Kupffer Cell Function. The interaction between Kupffer cells, endotoxins, and hepatic injury remains a major area for productive investigation. It has long been known that liver endocytosis by Kupffer cells is a major phagocytic activity that removes many antigens from the portal and general circulation, including foreign particulate matter, immune complexes, and gut-derived endotoxin.[18] Thus, the unhampered ability to remove LPS from the

portal circulation remains critical to protection from a variety of liver injuries. However, the release of mediators from these cells is also of major importance in endotoxin injury. It has been proposed by one group that LPS, alcohol, and Kupffer cells are critically involved in the disease process. The importance of these cells in producing the injury is illustrated by the researchers' work with gadolinium chloride ($GdCl_3$). This compound selectively injures Kupffer cells, destroying their normal function. When $GdCl_3$ is administered, it almost completely protects rats from alcohol-induced liver injury, showing that Kupffer cells do indeed participate in the early phase of this injury.[19] This work also illustrates the paradoxical role of these cells in response to endotoxin. They are usually protective in removing LPS from the portal system, but also critical to the damage itself by the release of destructive mediators.

The production of alcoholic hepatitis in experimental models[20] permitted a clinically important source of hepatic injury to be evaluated. The results of these investigations paralleled the findings used with administration of other hepatotoxins such as CCl_4 and galactosamine.

LPS: Mechanism of Action (Clinical and Experimental)

Since 1980, there has been steady progress on understanding the mechanisms underlying the many biological effects of endotoxin in experimental animals and humans. In liver transplant patients in 1989, Dr. Starzl and his group in Pittsburgh found a striking correlation between the perioperative serum endotoxin levels, the difficulty in convalescing from the surgery, and the ultimate outcome.[21]

As noted, the induction of endotoxin tolerance has been shown to protect rats from the liver necrosis resulting from CCl_4 administration.[10] More recently, it was established that endotoxin-tolerant mice produce an inhibitor of the synthesis of tumor necrosis factor (TNF),[22] which possibly explains the acute protection noted against the effects of CCl_4. When the original hypothesis of the relationship between hepatic injury and intestinal endotoxins was postulated, the phagocytic role of the Kupffer cells in ingesting and clearing gut-derived LPS was felt to be paramount. Although the release of macrophage mediators was emphasized, the magnitude of cytokine release was not appreciated until later. In 1988, we listed known mediators involved in the process (Table 1), and since that time, other mediators have been described that both cause and ameliorate the hepatic injury (Table 2).

Table 1. Macrophage Products Implicated in Liver Injury

Superoxides
Lysosomal enzymes
Protein synthesis-inhibitory factor
Procoagulants
Leukotrienes
Interleukins
Tumor necrosis factor
Platelet-activating factor

Nolan JP. Intestinal endotoxins as mediators of hepatic injury–an idea whose time has come again. Hepatology 1989;10:887-891.

In 1994, the role of TNFα in acute endotoxin-induced hepatotoxicity in rats fed alcohol was described.[23] On the basis of their work, the authors concluded that long-term alcohol administration sensitized Kupffer cells to secrete high levels of TNF after injection of LPS. Another important cytokine in liver injury is interleukin-10 (IL-10). In 1998, a study of IL-10 expression and function in experimental murine inflammation induced by CCl_4 was conducted.[24] The studies confirmed that IL-10 is expressed in liver injury and down-regulates various aspects of proinflammatory macrophage function, is expressed during CCl_4 liver injury, and offers some protection against inflammation and fibrosis

In an impressive clinical study from Japan in 2005, a retrospective analysis was done on 105 patients with severe alcoholic liver disease.[25] Plasma endotoxin levels increased as the severity increased and decreased as recovery occurred. Endotoxin-binding proteins were found to be protective in the course of the disease. TNFα, IL-6, and IL-8 levels were high in severe alcoholic liver injury. This study is important because it examines serial cytokine values in patients with this disease, and correlates them with the presence of endotoxemia.

The importance of nitric oxide (NO) in the hemodynamic disturbance of cirrhosis is a relatively new observation. It is known that endotoxin enhances the expression of inducible NO synthase, and it was postulated that the vasodilatation seen in cirrhosis might be related to the production of NO in the peripheral circulation. NO is an unstable molecule quickly

Table 2. Additional Mediators, Circa 2009

Intercellular adhesion molecule (ICAM)
Nitric oxide
Thromboxane
Tumor necrosis factor α
Prostaglandin D-2
IL-1
IL-10
Interferon-gamma
Lymphotoxin beta
Macrophage migration inhibiting factors

Table 3. Modification of Endotoxin Toxicity in Liver Disease

A. Increasing resistance
1. Development of tolerance
2. Specific immunization (core antigens)
3. Lysosomal stabilization
B. Decreasing gut absorption
1. Immunization (oral)
2. Antibiotics (paromomycin)
3. Cholestyramine
4. Lactulose
5. ? Bile salts
6. ? Cimetidine
C. Removal of circulating LPS
1. Activated charcoal
2. "Anti-endotoxins"

Nolan JP. Endotoxin, reticuloendothelial function, and liver injury. Hepatology 1981;1:458-465.

converted *in vivo* and *in vitro* to nitrite and nitrate ions (NO_2 and NO_3) which have been used to measure nitric acid levels. In 1993, in a study of 51 patients with cirrhosis, raised serum levels of endotoxin, nitrites, and nitrates were observed. These values were most elevated in decompensated cirrhosis with ascites.[26] Of further interest in this study, the oral administration of the antibiotic Colistin to 15 patients significantly reduced the blood levels of all these entities. Colistin is a polymyxin B which disrupts LPS in the gut.

Modification of Endotoxicity. Because enterically absorbed endotoxin is critical in the pathogenesis of liver injury by hepatotoxins, efforts to prevent, or modify, the effects of LPS have been central therapeutic goals. We summarized the then-documented and proposed approaches to lessen endotoxin toxicity in liver disease in our 1981 article (Table 3). Since that time, a variety of clinical and animal studies have advanced our knowledge in modification strategies. These advances have accompanied our new knowledge of mechanisms involved in the action of LPS. Unfortunately, whereas protective studies by a number of agents in animals have been impressive, translation to protection in human disease has been limited.

Because the activation of Kupffer cells seems critical to the development of liver injury due to alcohol, agents aimed at preventing the activation have been studied. Because calcium is essential to activation, a calcium channel blocker was administered to rats given high enteral doses of alcohol. Nimodipine significantly reduced both biochemical abnormalities and histologic changes in these alcohol-fed rats.[27] Another approach was to stimulate phagocytic activity of murine Kupffer cells. Tuftsin, a natural immunomodulator peptide, was shown to stimulate phagocytosis.[28] In still another approach with some promise, LPS-neutralizing antibody was found to ameliorate acute hepatocyte injury produced by galactosamine.[29]

Consistent with the fact that TNF is a major mediator of LPS injury, soluble TNF receptor was demonstrated to provide protection against CCl_4 liver injury in rats.[30] Although oral antibiotics had been shown in the 1960s and 1970s to protect against acute liver injury and the cirrhosis of choline deficiency, investigators more recently demonstrated that polymyxin and neomycin offered the same protection to the high-dose alcohol-fed rat model.[31] IL-10, which is an anti-inflammatory cytokine that inhibits TNFα production, prevented lethality from endotoxin in galactosamine-sensitized mice, offering another possible modifier of toxic liver injury.[32] Another protector against galactosamine lethality is high-dose alanine, which confers protection even up to 12 hours after toxic challenge. It resulted in increased hepatic adenosine triphosphate content probably due to high-dose alanine's promotion of ATP synthesis. It was felt that this impressive protection and low toxicity might be an effective therapy in humans.[33]

An important contribution to our knowledge of the mechanism of alcohol-induced liver injury in rats resulted from studies of dietary intervention. It was shown that the feeding of medium-chain triglycerides inhibited both free radical production and TNFα production in the ethanol-treated animals.[34] Another study investigated dietary saturated fatty acids in the ethanol rat model and found that this dietary intervention reversed the inflammatory and fibrotic changes despite continued alcohol administration.[35] Both these studies would seem to open exciting possibilities of a nutritional approach to the problem of alcohol-induced damage.

A study in 2002 on the effect of LPS-binding protein in early alcohol liver injury in mice showed significant modification of the injury.[36] The investigators concluded that the LPS-binding protein enhanced LPS-induced signal transduction, most likely in Kupffer cells. Another protective agent described in 2003 was edaravone, which prevented liver injury and mortality in endotoxin-treated rats. It is another potent and novel free radical scavenger that might be used in treating alcoholic hepatitis.[37]

Clinical studies of various modifiers in alcoholic liver disease are relatively few. In the treatment of the hepatorenal syndrome, many strategies have been used, with liver transplantation often the only viable alternative. Pentoxifylline (PTX), which inhibits TNF production, has been suggested as an adjunct in the treatment of these patients,[38] and an important clinical study was done in 2000 by the University of Southern

California Liver Unit, using PTX to treat patients with alcoholic hepatitis.[39] The results demonstrated a short-term survival improvement in the PTX group, felt to be related to a significant decrease in the risk of developing the hepatorenal syndrome.

An interesting and well-designed clinical study on the effect of probiotics was recently published.[40] It included a controlled study of gut flora, endotoxin levels, and Child-Pugh severity score in patients with cirrhosis. Using *Escherichia coli* Nissle strain or a placebo, the *E. coli* Nissle seemed to be effective in the restoration of normal colonic colonization and can probably lower endotoxemia in patients with cirrhosis.

With the presumed role of endotoxin in the hyperdynamic circulatory state in cirrhosis, selective intestinal decontamination was studied using oral norfloxacin in 14 patients with alcohol-related cirrhosis and 14 controls.[41] This 4-week regimen of the antibiotic partially reversed the hyperdynamic circulatory state, further supporting the role of intestinal endotoxin in its pathogenesis.

However, in contrast to the above studies was a randomized, double-blinded, placebo-controlled study of etanercept, in which the TNF-lowering receptor binding compound was used to lower TNFα in the treatment of alcoholic hepatitis.[42] Unfortunately, despite lowering of TNF levels, there was a significantly higher mortality in the etanercept group. Rates of infection were significantly higher in the treated group, indicating it to be an ineffective therapy in acute alcoholic hepatitis. Thus, even though TNF is established as a major agent in causing liver damage, it also has an important role in immune protection. Because patients with alcoholic liver disease are more susceptible to serious infection, the whole concept of therapy to lower TNF levels may not be feasible.

Table 4 lists potential additional strategies developed thus far in attempts to lessen the damage from enteric LPS in toxic liver injury, and can be compared to the list of potential modifiers in Table 3 from 1981.

Table 4. Modification of Endotoxin Toxicity in Liver Disease, Circa 2009

LPS-binding proteins	Tuftsin (reticuloendothelial system stimulator)
LPS-neutralizing antibody	Alanine (high dose)
Hepatocyte growth factor	Thromboxane inhibitors
Nimodipine (Ca channel blocker)	Lactulose
Nitric oxide	Pioglitazone
IL-10	Edaravone
TNFα antibodies	Pentoxifylline
Soluble TNF receptors	Entanercept
Antibiotics (polymyxin, neomycin)	
Dietary intervention	
Medium chain triglycerides	
Saturated fatty acids	
Probiotics (*E. coli* Nissle)	

Discussion

Few investigators have the privilege to contribute to and then to follow a novel idea in disease causation through some 35 years of halting but substantial progress. In 1975, on the basis of our studies and those of other investigators, we postulated a key role for enteric endotoxin in injury from a variety of toxins. It was also postulated that, in chronic liver disease, the spillover of LPS into the systemic circulation resulted in many of the extrahepatic manifestations observed. Although interest was expressed in this concept, it did not receive early acceptance nor did it engage many investigators until later. Few if any symposia on the role of endotoxins in liver injury were held in the United States in that period. In the late 1970s, most work was presented in Europe in conferences sponsored by investigators interested in Kupffer cells and other sinusoidal lining cells. A number of these investigators also had an interest in the interaction of endotoxin with these cells. All these observations were noted with some interest, but it was not until 1980 that a major presentation on this subject was given nationally before academic and practicing hepatologists. By the late 1970s, it was well established that hepatotoxins such as CCl_4 and galactosamine required intestinal endotoxins to cause the biochemical and histologic injury observed. Furthermore, in other studies testing the hypothesis, it had been established that the cirrhosis of chronic choline deficiency was prevented by disruption of the enteric endotoxin pool and that a depression of macrophage function occurred in the development of the injury. Other interventions were explored to reduce the toxicity and availability of LPS as a means to protect this chronic lesion or against acute hepatotoxin injury. These publications received scant attention.

We can only speculate on the reasons that the association failed to appeal to a larger number of investigators prior to the 1980s. A factor in the early lack of interest by major investigators was the feeling that the cause of liver injury was known by the effect of these agents on isolated and cultured hepatocytes. The microenvironment was felt to be more important than the macroenvironment. Although a great deal can be learned from isolated cells, their *in vivo* environment is far too complicated to allow sufficient understanding of their functions. The liver has a unique portal and systemic microcirculation. It is attached to the intestines

and has a complicated biliary excretion process. Liver injury occurs in this setting with many influences on the structure and function of parenchymal cells.

Although the 1980s saw much progress in defining the role and mechanism of damage in the endotoxin–liver cell relationship, it continued to be a low priority in many laboratories studying liver injury. Over the past 20 years, however, the development of newer techniques and studies with high-dose alcohol feeding in rodents has led to an explosion of knowledge documenting the importance of enteric endotoxin in alcoholic hepatitis and the mechanism of the interaction. The benchmark for general acceptance of the relationship can be found in the 2008 major National Institutes of Health Symposium titled "Alcohol, Intestinal Bacterial Growth: Intestinal Permeability to Endotoxin and Medical Consequences."[43] Basically, future trials based on current knowledge would fall into several categories. The most problematic approach presently is attempting to modify or neutralize systemic endotoxemia in liver failure. Lowering TNF levels has actually increased mortality, as described earlier. Safer strategies at present would involve trials of Kupffer cell stimulation with tuftsin, the use of potent radical scavengers as discussed, and the development of more potent LPS-binding proteins. Indeed, a combination of several approaches might be attempted. A much more attractive, feasible, and less potentially harmful approach involves the intestinal tract and the intestinal flora and the leaky gut syndrome in alcoholic liver disease. Such approaches are established to be effective in animal models. As noted previously, some of these approaches have been studied in clinical trials with some encouraging results. LPS can be effectively eliminated from the gut by polymyxin-like antibiotics, but these antibiotics need more clinical evaluations. Changing the gut flora is also an attractive, effective, and nontoxic approach. As discussed earlier, probiotics can be studied more rigorously perhaps combined with known effective dietary intervention including the use of medium chain triglycerides and saturated fat. Thus, multidimensional approaches in these areas might be most useful for future study. Lastly, the integrity of the intestinal mucosa to prevent large amounts of endotoxin absorption in alcoholic liver disease is a new and promising approach. MicroRNAs have been demonstrated to be overexpressed in animals treated with ethanol and contribute significantly to the leakiness of the gut to LPS. This hyperpermeability can potentially be approached by modifying the production of these microRNAs and may prove to be critical in maintaining the barrier function in alcoholic liver disease.[44]

The future of these clinical approaches is critical. The key role of endotoxin in alcoholic liver disease is now well established, and the development of an effective and accepted treatment remains the continuing challenge. The overarching concept is the universality of the role of enteric endotoxin in liver injury from toxic agents. Despite the varied structure of CCl_4, acetaminophen, galactosamine, and alcohol, eliminating or reducing the enteric endotoxin pool protects the liver from injury by all such agents. This is a powerful statement of the broad role of endotoxin in liver injury. The critical role of endotoxin in alcoholic liver disease is now well accepted. Application of this knowledge in the development of effective treatment is the continuing challenge, and identification of disease for earlier interventions continues to be difficult.

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