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The Contribution of Gut-Derived Endotoxins to Liver Injury

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The liver serves as the key organ for the removal and detoxification of bacterial endotoxins that are continously absorbed in small amounts from the gastrointestinal tract. This paper postulates that liver injury impairs this detoxification process leading to further liver damage and systemic effects as well. Evidence is reviewed to support the contention that endotoxin may be a major common pathway for liver injury by a variety of agents, and methods of reducing endotoxicity of gut origin are proposed. Finally, a new solid phase radioimmunometric assay for E. coli 026 is described and its usefulness as a gut marker suggested.

It is an honor to participate in this Festshcrift for Gerald Klatskin. In addition to providing his trainees with an unsurpassed experience in the clinical aspects of liver disease, he began many of us in directions we were to follow in the laboratory in later years. My own interest in Kupffer cell function, and the detoxification of endotoxins from the gut were stimulated by Dr. Klatskin's insistence in the early 1960s that fatty infiltration alone would not explain the extent of damage and fibrosis resulting after certain liver injuries. Since the liver stands as an effective filter of bacterial products arising in the gastrointestinal tract, the possibility that an impairment of the ability to perform this function might initiate or perpetuate hepatic injury has interested our laboratory over the years. To this end, we have sought evidence for a relationship between liver injury and altered endotoxin detoxification. Additionally, we have examined whether methods of modifying endotoxin toxicity modify acute hepatic injury by other toxins. While the role of endotoxin in liver disease has never been in the mainstream of liver research in this country, much recent work would tend to support the importance of such a relationship [1].

HYPOTHESIS

The postulates that form the basis of the hypothesis relating enteric endotoxin to liver injury are as follows:

a. Portal vein endotoxemia is a normal state; *b.* The liver prevents the lipopolysaccharide (LPS) of gram-negative bacteria from becoming systemic; *c.* Liver injury impairs endotoxin detoxification leading to further liver injury, and entry into the

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systemic circulation; and *d.* Modification of endotoxicity should lessen liver injury from other agents.

Figure 1 depicts the hypothesis and some of its implications. Essentially, endotoxin from the intestine is constantly being absorbed and carried to the liver for detoxification. If the detoxification mechanisms are impaired, endotoxin may reach concentrations in the liver that cause further injury which serves, in a circular fashion, to impair detoxification further. In addition, endotoxin may spill over into the systemic circulation and may contribute to such extra-hepatic effects of liver disease as fever, clotting abnormalities, and kidney failure. A key function of the liver is the sequestration of bacterial and dietary antigens entering the portal circulation, thus preventing them from reaching antibody forming tissue such as the spleen. Endotoxin that spills over into the systemic circulation in liver injury may stimulate such antibody formation and has been felt by some observers to be responsible for the hyperglobulinemia that accompanies chronic liver disease [2]. Since endotoxins have known stimulatory effects on lymphocytes as well, their role in observed T and B-cell abnormalities in hepatic disease needs further definition. While tissue and serum factors are important in endotoxin clearance, the key to the impairment of hepatic endotoxin detoxification seems to be the reticuloendothelial system activity which is depressed after toxic injury, alcohol administration, and fatty infiltration [3,4].

EVIDENCE OF SYNERGISM BETWEEN ENDOTOXIN AND OTHER HEPATOTOXINS

Historically, a postulated relationship between intestinal flora and dietary liver injury goes back many years. In 1954, Luckey demonstrated that conventional rats fed a necrogenic diet died of massive liver necrosis in 33 days, while germ-free animals suffered no hepatic damage over the same period [5]. Rutenberg and his associates showed significant modification of fibrosis and cirrhosis by the non-absorbable antibiotic neomycin, suggesting that systemic absorption was not important in the antibiotic effect [6]. In an elegant and important study in 1964, Broitman and his co-workers demonstrated that it was the presence of intra-intestinal endo-

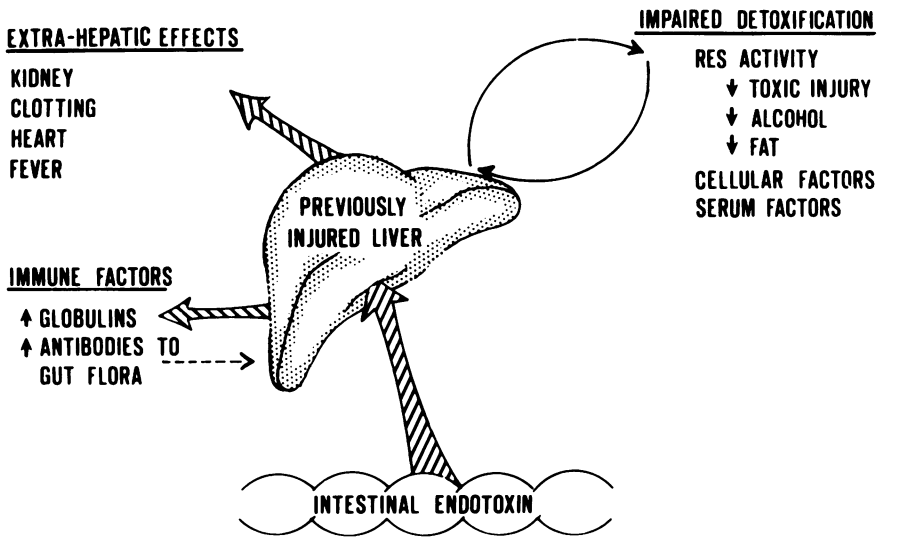

FIG. 1. Schematic representation of endotoxin effects in liver injury.

toxin rather than the intact bacteria that was crucial to the development of choline-deficiency cirrhosis [7]. These investigators confirmed the protective effect of neomycin on the development of the lesion, but showed that the simple addition of purified E. coli lipopolysaccharide to the drinking water of choline-deficient rats abolished this protection, and allowed fibrosis and subsequent cirrhosis to develop unimpeded.

Further, solid evidence exists that injured livers have a markedly increased sensitivity to the toxic effects of bacterial endotoxins. Rats receiving sublethal doses of carbon tetrachloride (CCL_4) showed a tenfold increase in lethality to E. coli endotoxin administration [8], and the significant effect of CCL_4 on reducing the endotoxin-detoxifying capacity of liver homogenate has been demonstrated as well [9]. In 1968, our laboratory studied the effects of endotoxin in rats on a choline-deficient diet and observed not only a reduction in the $L.D._{50}$ from 2.5 mg to 0.25 mg but also that transaminase elevations occurred in these animals at doses that were totally innocuous to animals on choline-supplemented diets [10]. The mechanisms by which endotoxin exerts its damaging effects are not altogether clear, but involve both direct and mediated mechanisms and have been previously summarized [1].

ENDOTOXEMIA IN LIVER DISEASE

Key to the hypothesis suggesting a role for endotoxins in determining the magnitude of liver injury is the assumption that lipopolysaccharide is absorbed from the gut. Earlier experimental evidence was somewhat conflicting on the point of endotoxin absorption. In one study using Cr^{51} labelled lipopolysaccharide, no evidence of absorption was found [11], while using p^{32} labelled bacteria to produce the LPS, the endotoxin regularly appeared in the tissues [12]. Work from our laboratory using the isolated everted gut sac of the rat showed labelled LPS regularly passes transmurally in small amounts, and rather than being a passive diffusion, a saturable carrier mechanism appears to exist [13]. Furthermore, the material passing through the gut wall retains both its toxic and immunogenic properties. Using the limulus lysate gelation method of detecting minute quantities of circulating endotoxin, positive tests for endotoxin have been obtained in portal blood sampled at the time of surgery in up to 95 percent of patients tested [14]. Theoretically, in liver disease, endotoxin absorption and toxicity might be increased because of a relative decrease in the amount of bile salts produced. Sodium deoxycholate treatment diminishes the capacity of endotoxin to kill chick embryos [15], and it has recently been shown that deoxycholate is often absent in the bile of patients with alcoholic cirrhosis [16]. In the past few years, a number of studies have been reported correlating systemic endotoxemia in liver disease with extra-hepatic manifestations of liver failure. The most notable association has been with the functional renal failure associated with massive liver necrosis. Wilkinson and his colleagues showed an excellent correlation of limulus positivity with depression in parameters of renal function in patients with acute liver injury [17]. A similar correlation has been noted in patients with alcoholic cirrhosis [18]. Fever has been shown by Tisdale and Klatskin to be a significant feature of alcoholic liver disease [19] and limulus positivity can be shown to correlate with presence and duration of the febrile state in these patients [20]. Tarao and his Japanese colleagues have found high titers of limulus-positive material in the ascites of patients with alcoholic hepatitis and postulate that the ascites serves as a major source of endotoxemia in the peripheral circulation [21]. These investigators noted that death occurred within 6 months in 48 percent of those with lysate positivity as opposed to 16 percent in those without

detectable endotoxin. A provocative study of patients with Reyes Syndrome regularly found endotoxin in the peripheral circulation and the highest titers were associated with death [22]. Since endotoxin causes the same depression of mitochondrial function that is found in this disease [23], it is tempting to attach etiological significance to systemic endotoxemia in this syndrome.

SIGNIFICANCE AND NEW DIRECTIONS

The key question that arises in all of these descriptive clinical studies is the significance of lipopolysaccharide in the peripheral circulation in liver disease. Is endotoxemia causative in further decompensating a damaged liver, or is its presence simply another reflection of liver damage itself, and as a consequence, of little clinical significance? One approach to this problem would be to test whether measures aimed at modifying endotoxin toxicity would also modify liver injury by other hepatotoxic agents. If significant protection could be demonstrated with methods aimed at reducing endotoxicity alone, then the importance of lipopolysaccharide in promoting hepatic damage would be more likely. Table 1 summarizes the methods presently available that might modify endotoxicity in liver disease. While not all of these methods have been shown to modify hepatic damage, evidence now exists for the efficacy of several. The creation of the state of endotoxin tolerance through the administration of increasingly large doses of a single lipopolysaccharide over time protects against challenge with other endotoxins [24]. In rats made tolerant and then challenged acutely with carbon tetrachloride, the liver injury was significantly modified [25]. After a sublethal dose of CCL_4, the mean SGPT value in the tolerant animals at 24 hours was 57 compared with 241 in the non-tolerant pair-fed controls. Striking histological protection was noted at the same time in the livers of the endotoxin tolerant rats. With the advent of mutant, rough strains of gram-negative bacteria that contain common, core material, it may be possible to actively immunize against a broad spectrum of endotoxin strains [26]. Attempts to decrease endotoxin absorption from the gut offers another approach to modifying its hepatic toxicity. The intra-intestinal pool available for absorption could be decreased by antibiotic suppression of bacteria, disruption of the lipopolysaccharide with added bile salts [27], or the use of cholestyramine resin which tightly binds endotoxin and prevents its toxicity [28]. Once endotoxin gains entrance to the circulation, removal of the LPS might reduce its toxic effects. Hemoperfusion with activated charcoal columns has been shown to prolong life in dogs after hepatectomy [29]. Since charcoal efficiently removes labelled endotoxin from solutions, it is possible that this removal might explain the benefits of resin hemoperfusion. Lastly, polymyxin B has been shown to

TABLE 1

1. Increasing Resistance
   a. Heterologous tolerance
   b. Specific immunization
2. Decreasing Gastrointestinal Absorption
   a. Non-absorbable antibiotics
   b. Cholestyramine resin
   c. Bile salts
3. Removal of Circulating Endotoxin
   a. Activated charcoal hemoperfusion
   b. Polymyxin B inactivation

have a protective effect against endotoxicity that is independent of its anti-bacterial action and that appears related to its ability to disrupt the LPS complex [30]. To test whether this anti-endotoxin protects against liver injury, rats were pre-treated with polymyxin B or gentamicin; an antibiotic with a similar antibacterial spectrum but without an effect on endotoxin itself. Striking biochemical and histological liver protection was noted in the animals given polymyxin B, and challenged with CCL_4 as compared to the control groups [31].

The biggest obstacle to further delineating the role of endotoxin in liver injury by more direct methods has been the lack of a suitable quantitative assay for gut derived LPS. The limulus assay is at best qualitative and its specificity still is questioned [32]. Other bioassays in animals, such as lead acetate enhancement of endotoxicity, are cumbersome and have the difficulties inherent in any such assay.

In our attempt to overcome these problems, we have developed an immunoradiometric method (IRMA) for the specific detection and quantitation of endotoxin from E. coli 026 [33]. The conventional radioimmunoassay involves labelling of an antigen with a marker, usually a radioactive isotope of iodine. The labelled antigen is then allowed to compete with an unknown amount of antigen for interaction with antibody. Iodination generally requires the presence of peptide moieties within the antigen molecule; and while protein is present in crude as well as commercial endotoxin, the pure antigen is a lipopolysaccharide containing no protein. Because of the difficulty in purifying and labelling this complex molecule, the antigen labelling RIA technique was abandoned in favor of using a labelled antibody in an immunoradiometric assay [34]. Since antibody may be separated and concentrated from the sera of animals and the peptide portions of globulin easily iodinated, a reproducible test for the detection of endotoxin could be developed. Because it seemed most desirable to measure a known endotoxin marker, E. coli 026 lipopolysaccharide was used as the test substance. In the procedure, antibody is first immobilized on the walls

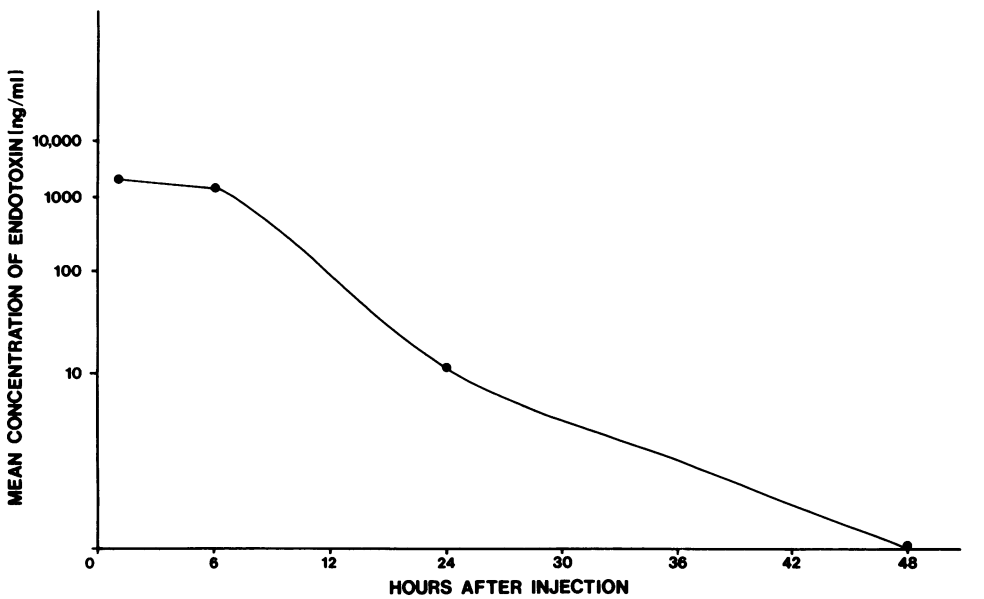

FIG. 2. Clearance of E. coli 026 endotoxin after intraperitoneal injection (0.1 mg). Concentration in ng/ml is plotted against time.

of small volume plastic wells, and the unlabelled antigen is then allowed to react with the antibody coating the wells. After the unattached antibody is removed, labelled antibody is added and each well counted. Using known concentrations of the endotoxin, a curve of standard concentrations can be plotted. We have found that this method detects E. coli 026 in serum down to less than 1 nanogram per ml, and that no cross-reactivity occurs with other E. coli endotoxins, or LPS from S. typhosa 0901 or S. marceseus. Using this technique in groups of 5 rats each, the clearance of 0.1 mg of E. coli endotoxin given intraperitoneally is shown in Fig. 2. For the first hours, the rate of clearance was slow and by 6 hours, 1200 ng/ml was present. By 24 hours, 15 ng/ml remained and clearance was complete at 48 hours. When limulus lysate assays were performed on the same samples, it was found that the IRMA was more sensitive below 10 ng/ml. Since E. coli 026 is not a normal gut inhabitant, its placement in the gut of animals serves for the first time as a quantitative marker for the appearance, distribution, and possible modification of endotoxemia of gut origin.

Despite all the evidence supporting a role for gut derived endotoxin in the initiation and perpetuation of liver injury, certain theoretical difficulties remain to be answered. For example, if endotoxin is constantly entering into the circulation in liver disease, one might expect tolerance or immunity to develop and to be protective against further injury. Since LPS stimulates reticuloendothelial function, it is again uncertain why reticuloendothelial function is depressed in chronic liver disease. The new radioimmunoassay for E. coli 026 may allow for the first time a study of these perplexing but most important problems.

REFERENCES

1. Nolan JP: The role of endotoxin in liver injury. Gastroenterology 69:1346–1356, 1975
2. Triger DR, Alp MH, Wright R: Bacterial and dietary antibodies in liver disease. Lancet 1:60–63, 1972
3. Ali MV, Nolan JP: Alcohol-induced depression of reticuloendothelial function in the rat. J Lab Clin Med 70:295–301, 1967
4. Ali MV, Nolan JP: Serial assessment of reticuloendothelial function in experimentally induced nutritional cirrhosis. Lab Invest 20:184–189, 1969
5. Luckey TD, Reyniers JA, Gyorgy P, et al: Germ-free animals and liver necrosis. Ann NY Acad Sci 57:932–935, 1954
6. Rutenburg AM, Sonnenblick E, Koven I, et al: The role of intestinal bacteria in the development of dietary cirrhosis in rats. J Exp Med 106:1–13, 1957
7. Broitman SA, Gottlieb LS, Zamcheck N: Influence of neomycin and ingested endotoxin in the pathogenesis of choline deficiency cirrhosis in the adult rat. J Exp Med 119:633–641, 1964
8. Formal SB, Noyes HE, Schneider H: Experimental shigella infections. III. Sensitivity of normal, starved, and carbon tetrachloride treated guinea pigs to endotoxin. Proc Soc Exp Biol Med 103:415–418, 1960
9. Farrar WE, Edison M, Kant TH: Susceptibility of rabbits to pyrogenic and lethal effects of endotoxin after acute liver injury. Proc Soc Exp Biol Med 128:711–715, 1968
10. Nolan JP, Ali MV: Endotoxin and the liver. I. Toxicity in rats with choline deficient fatty livers. Proc Soc Exp Biol Med 129:29–31, 1968
11. Sanford JP, Noyes HE: Studies on the absorption of Escherichia coli endotoxin from the gastrointestinal tract of dogs in the pathogenesis of irreversible hemorrhagic shock. J Clin Invest 37:1425–1435, 1958
12. Ravin HA, Rowley P, Jenkins C, et al: On the absorption of bacterial endotoxin from the gastrointestinal tract of the normal and shocked animal. J Exp Med 112:783–792, 1960
13. Nolan JP, Hare DK, McDevitt JJ, et al: In vitro studies of intestinal endotoxin absorption. I. Kinetics of absorption in the isolated everted gut sac. Gastroenterology 72:434, 1977
14. Jacob AI, Goldberg PK, Bloom N, et al: Endotoxin and bacteria in portal blood. Gastroenterology 72:1268, 1977
15. Tarmina DF, Milner KC, Ribi E, et al: Modification of selected host-reactive properties of endotoxin by treatment with sodium deoxycholate. J Bacteriol 96:1611, 1968
16. Knodell R, Kinsey C, Boedeker EC, et al: Deoxycholate metabolism in alcoholic cirrhosis. Gastroenterology 71:196, 1976
17. Wilkinson SP, Arroyo V, Gazzard BG, et al: Relationship of renal impairment and hemorrhagic diathesis to endotoxemia in fulminant hepatic failure. Lancet 1:521–524, 1974

18. Wilkinson SP, Moodie H, Stammatakis JD, et al: Endotoxemia and renal failure in cirrhosis and obstructive jaundice. Brit Med J 2:1415, 1976
19. Tisdale WA, Klatskin G: The fever of Laennec's cirrhosis. Yale J Biol Med 33:94–106, 1960
20. Nolan JP, Ali MV: Endotoxemia in liver disease. Lancet 1:999, 1974
21. Tarao K, So K, Moroi T, et al: Detection of endotoxin in plasma and ascitic fluid of patients with cirrhosis: Its clinical significance. Gastroenterology 73:539, 1977
22. Cooperstock MS: Possible pathogenic role of endotoxin in Reye's Syndrome. Lancet 1:1272–1273, 1975
23. Schumer W, DasGupta TK, Moss GS, et al: Effect of endotoxemia on liver cell mitochondria in man. Ann Surg 171:875, 1970
24. Abernathy RS: Homologous and heterologous resistance of mice given bacterial endotoxins. J Immunol 78:387, 1957
25. Nolan JP, Ali MV: Endotoxin and the liver. II. Effect of tolerance on carbon tetrachloride induced injury. J Med 4:28, 1973
26. Bruins SC, Stumacher R, Johns MJ, et al: Immunization with R mutant of Salmonella minnesota. III. Comparison of the protective effect of immunization with Lipid A and the Re mutants. Infect Immun 17:16, 1977
27. Bailey M: Endotoxin, bile salts, and renal failure in obstructive jaundice. Br J Surg 63:774–778, 1976
28. Nolan JP, McDevitt JJ, Goldmann GS: Characteristics of endotoxin binding by charged and uncharged resins. Proc Soc Exp Biol Med 149:766, 1975
29. Wilson RA, Hofmann AF, Kuster GGR: Toward an artificial liver. II. Removal of cholephilic anions from dogs with biliary obstruction by hemoperfusion through charged and uncharged resins. Gastroenterology 66:95, 1974
30. Loges J, Inniss WE: Electron microscopic effect of polymyxin on Escherichia coli lipopolysaccharide. J Bact 100:1128, 1969
31. Leibowitz AI, Nolan JP: Endotoxin and the liver. III. Modification of acute carbon tetrachloride injury by polymyxin B—an anti-endotoxin. Gastroenterology 75:445–449, 1978
32. Martinez-G LA, Quintilian R, Tilton RC: Clinical experience on the detection of endotoxemia with the limulus test. J Infect Dis 127:102, 1973
33. Leibowitz AI, Vladutiu AO. Nolan JP: Quantitation of circulating endotoxin in rats by a solid phase radioimmunoassay. Clin Chem 25:68–70, 1979
34. Woodhead JS, Addison GM, Hales CN: The immunoradiometric assay and related techniques. Br Med Bull 30:44, 1974