Lactobacillus Feeding Reduces Endotoxemia and Severity of Experimental Alcoholic Liver (Disease) (43703)

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Abstract. **We have previously shown a relationship between plasma endotoxin levels and severity of alcoholic liver injury in the intragastric feeding rat model. We attempted to reduce both circulating endotoxin and liver injury in this model by administering a lactobacillus strain (species GG) which survives for prolonged periods in the gastrointestinal tract. Male Wistar rats were fed ethanol and liquid diet containing corn oil (CO + E). Another group of animals (CO + E + L) received the diet containing ethanol plus a daily bolus of lactobacilli GG concentrate (10^{10} CFU). All animals were sacrificed after one month. All animals had plasma endotoxin measurements and evaluation of severity of pathologic changes in the liver. The weight gain and blood alcohol levels were similar in both groups. The mean ± SE of the pathology score was significantly higher (3.4 ± 0.85) in the CO + E group compared to the CO + E + L group (0.5 ± 0.3, $P < 0.01$). The virtual absence of pathologic changes in the latter group was accompanied by significantly lower endotoxin levels (8.4 ± 2.9 pg/ml in CO + E + L group vs 48.3 ± 7.8 pg/ml in CO + E group, $P < 0.01$). Feeding of strains of lactobacilli that survive in the gastrointestinal tract reduces endotoxemia and alcohol-induced liver injury in the rat. Lactobacillus species GG provides a potential nontoxic form of therapy for both endotoxemia and alcoholic liver disease.** [P.S.E.B.M. 1994, Vol 205]

Our previous studies in the intragastric feeding rat model for alcoholic liver injury showed that the presence of liver injury occurred only in animals fed corn oil and ethanol; animals fed saturated fat and ethanol were protected from liver injury (1, 2). Recently, we also showed that when animals are fed saturated fat and ethanol (where no liver injury is seen), the plasma endotoxin levels were much lower than the levels seen in corn oil-ethanol fed animals (3). Furthermore, the levels of the proinflammatory eicosanoids, leukostriene B_4, and thromboxane B_2 were much higher in the latter group. We further extended our study to examine the relationship between plasma endotoxin levels and severity of pathologic injury in the corn-oil ethanol fed rats. A strong significant correlation between liver injury and plasma endotoxin suggested an important role for endotoxin in alcoholic liver injury (4).

The importance of endotoxin in other forms of liver injury has also been shown with galactosamine and carbon tetrachloride (5, 6). Although the exact mechanism by which endotoxins enhance liver injury is unknown, release of potent macrophage mediators such as tumor necrosis factor, platelet activating factor, and other inflammatory cytokines is believed to be important (7). Elimination of bacteria as a source of endotoxin has been used as one way of preventing these various types of toxin-induced liver injury. Colectomy done in rats prior to administration of galactosamine protected against liver injury in both rats (8) and rabbits (9); treatment with antibiotics such as polymyxin B and neomycin also protected against the effects of several hepatotoxins (9).

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Disadvantages associated with the above forms of treatment are their invasiveness, trauma, and potential serious side effects. It would appear that alteration of the intestinal flora by a more "benign" means might be beneficial in reducing the severity of endotoxin-related organ damage. One of the ways to alter the intestinal flora is to introduce lactobacilli into the gut. However, most lactobacilli strains survive in the gut for only short periods of time (10). One of the lactobacilli strains, Lactobacillus GG, in contrast to others, has been shown to survive in the gastrointestinal tract for several days (11). Furthermore, this strain has been shown to produce a bacterial inhibitory substance (12) which could potentially suppress the growth of endotoxin-producing bacteria. Lactobacillus GG is not known to produce endotoxin (12). Because of the observed synergism between alcohol and endotoxin in promoting liver injury (13), our study attempted to determine whether lactobacilli feeding could reduce both circulating endotoxin levels and the severity of liver injury in experimental alcoholic liver injury.

Materials and Methods

Animal Model. Male Wistar rats between 225 and 250 g were fed by continuous infusion of liquid diet through permanently implanted gastric tubes as described previously (14, 15). The use of spring coils and swivels allowed for the protection of cannulas and free movement of animals within the cage. The rats received their total nutrient intake by intragastric infusion. The average caloric distribution for each nutrient when the animals were receiving maximal amounts of ethanol was 25% of total calories as fat (corn oil), 21% as protein, 12% as carbohydrate, and 42% as ethanol. Two groups of animals were studied. One group (n = 6) was given the diet as above, i.e., corn oil as source of fat and ethanol. The amount of ethanol given was modified to maintain high levels of blood alcohol (150 to 300 mg/dl). This amount was initially 8 g/kg/day and increased up to 17 g/kg/day as tolerance developed. Animals were closely observed twice a day for the degree of alcohol intoxication which was assessed by loss of motor coordination and diminution of sensory reflexes. The animals received their highest ethanol intakes within two to two and a half weeks after start of feeding. Each ethanol-fed rat had at least two measurements of blood alcohol using tail vein blood. All rats had free access to water and non-nutrient fiber. The second group was pair-fed the same diet as the first group but in addition was given, every morning, via the same intragastric tube delivering ethanol and diet, a bolus of Lactobacillus species strain GG (Lactobacillus GG) concentrate. This treatment with Lactobacillus GG was begun when feeding was initially started after the operation. The concentrates had 2×10^{10} CFU/ml and were prepared as previously described (11). Immediately prior to use, the concentrates were defrosted and mixed with sterile water. A daily dose of 10^{10} CFU/rat was given. This dose was chosen on the basis of previous studies evaluating the survival and gastrointestinal tract colonization by Lactobacillus GG (11). No side effects such as diarrhea are seen at this dose. All animals were sacrificed at one month after the start of feeding. This time interval was chosen because in the intragastric feeding model, pathologic changes are evident by this time (1, 2). We did not test the viability and presence of lactobacilli in the gut contents or stool since this has been adequately tested and described in both humans and experimental animals (11). The studies were conducted according to the guidelines on care and use of laboratory animals (NIH).

Histologic and Biochemical Analyses. A small sample of liver was obtained at sacrifice and formalin-fixed. Hematoxylin and eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat), 1+ = 0%–25% of cells containing fat, 2+ = 25%–50%, 3+ = 51%–75%, 4+ = >75%. Inflammation and necrosis were scored as one focus per low power field = 1+, two or more foci = 2+. At least three different sections and ten low power fields were examined per sample of liver. The pathology score was calculated by adding the individual parameter scores.

Blood was collected at sacrifice in appropriate tubes made pyrogen-free by heating at 180° for 24 hr. Plasma was immediately separated in a refrigerated centrifuge and stored at −70°C until analysis. Endotoxin in plasma was measured within two weeks of collection using a synthetic chromogenic assay (Whittaker Bioproducts Inc., Walkersville, MD). The glassware used in the assays was rendered endotoxin-free by heating at 180°C for 24 hr. Pyrogen-free water was supplied by the manufacturer. The procedures used in the testing for endotoxin conform with those described in the Food and Drug Administration guidelines. This assay provides better quantitation and is more sensitive and specific than previous gelation assays (16, 17). Blood alcohol was measured using the alcohol dehydrogenase assay (Sigma Chemical Co., St. Louis, MO).

Data are expressed as mean ± SE. Different groups were compared using the Student's t test. The accepted level of significance was $P < 0.05$.

Results

The weight gain and blood alcohol values (at least two measurements/rat) were not significantly different in the two groups. Although the weight gain (g) in the ethanol-fed group was lower (26.9 ± 3.1) than the lactobacillus fed group (29.2 ± 3.8), the difference was

not significant. Blood alcohol levels in the corn oil-ethanol group was 202 ± 31 mg/dl, levels in the lactobacillus-treated group were 212 ± 29 mg/dl. The details of pathologic severity and plasma endotoxin levels is shown in Table I. The mean ± SE of the pathologic score in the corn oil-ethanol group ($n = 6$) is significantly higher (3.4 ± 0.85) than in the lactobacilli group ($n = 6$) (0.5 ± 0.3) ($P < 0.01$). Figure 1 shows an example of representative pathologic changes seen in animals fed corn oil and ethanol. The protective effect of lactobacilli is reflected in Figure 2, which shows absence of any pathology in the liver of lactobacilli-fed rats. The less severe pathology in the lactobacilli group is accompanied by lower endotoxin levels (Table I). In one of the animals in the lactobacilli-fed group that developed a 2+ fatty change, the endotoxin level was about three times the mean level seen in the same group.

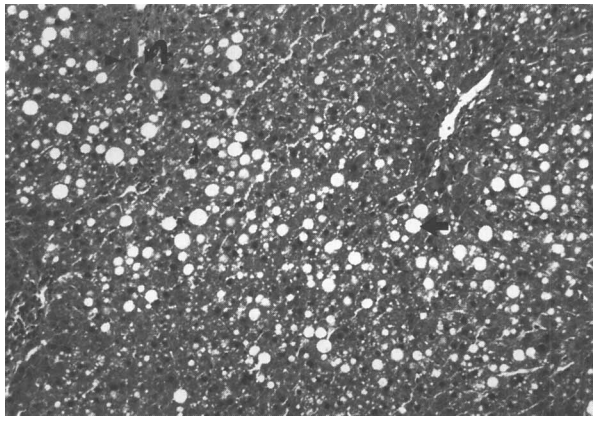

Figure 1. Liver from a rat fed alcohol and corn oil for one month shows the presence of macrovesicular fat (large arrow) and microvesicular fat (small arrow). Also shown is an area of hepatocyte necrosis (curved arrow) and inflammatory infiltrate (arrow head). Hematoxylin and eosin; magnification ×155.

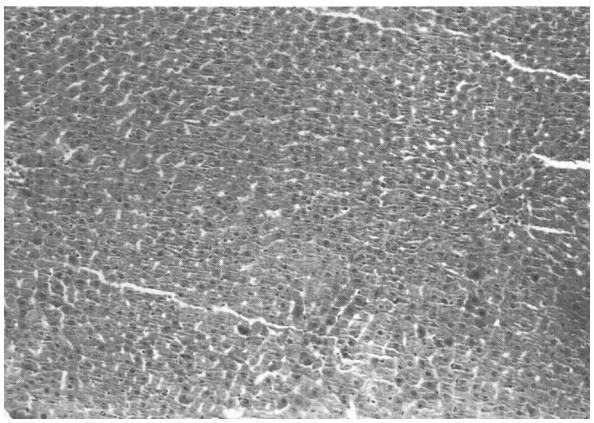

Figure 2. Liver from a rat fed alcohol, corn oil, and lactobacilli for one month. The liver shows normal histology with no fatty change, necrosis, or inflammation. Hematoxylin and eosin; magnification ×155.

Discussion

Historically, there has been a great deal of interest in evaluating the relationship between intestinal microflora and liver injury. In 1940, sulfonamide drugs were shown to be protective against carbon tetrachloride induced liver injury (18). In the 1950s and 1960s, several studies were carried out which demonstrated the relationship between intestinal bacteria and liver injury (19–21). For example, rats receiving a necrogenic diet developed severe hepatic necrosis while those in a germ free environment did not (22). There is also evidence that endotoxin potentiates alcohol-induced liver injury. Bhagwandeen *et al.* (23) showed that endotoxin promoted hepatic necrosis in rats receiving alcohol; Arai *et al.* showed that chronic ethanol ingestion consumption in rats potentiated endotoxin-induced organ injury (24). Our recent results (4, 5) also confirm the relationship between endotoxin and pathologic liver injury in the intragastric feeding rat model for alcoholic liver disease.

Removal of the source of endotoxin has been attempted as one way of reducing organ damage associated with endotoxemia (25). Colectomy done in rats prior to administration of galactosamine protects against liver injury (7); enterectomy protected against

Table I. Comparison of Severity of Pathologic Changes and Plasma Endotoxin Levels in the Corn Oil–Ethanol–Fed Group and Corn Oil–Ethanol–Lactobacilli Group

Corn oil + ethanol					Corn oil + ethanol + lactobacilli				
Animal no.	Pathology F	N	I	Endotoxin (pg/ml)	Animal no.	Pathology F	N	I	Endotoxin (pg/ml)
1	1+	1+	1+	60.6	1	2+	0	0	21.8
2	0	0	1+	33	2	0	1+	0	7.7
3	3+	2+	2+	62.5	3	0	0	0	3.3
4	3+	1+	0	52.6	4	0	0	0	8.3
5	1+	0	0	13.5	5	0	0	0	0.8
6	2+	1+	1+	67.5	6	0	0	0	4.6

F = fatty liver; N = necrosis; I = inflammation. The mean ± SE of the composite pathology score for the corn oil–ethanol animals (3.4 ± 0.85) is significantly different from the score in the lactobacilli group (0.5 ± 0.30, $P < 0.01$). Also, the endotoxin levels are lower in the lactobacilli group (8.4 ± 2.9 pg/ml) compared with the corn oil–ethanol group (48.3 ± 7.8 pg/ml) ($P < 0.01$).

the toxic effects of galactosamine in rabbits (8). Antibiotic treatment with neomycin or polymyxin B (26) or with a binding agent such as lactulose (27) also has the effect of protecting against toxic liver injury. Recently, Van Leuwen *et al.* (28) showed that hepatic failure and coma in rats after liver resection was reversed by pretreatment with agents that altered gut contents and reduced endotoxemia. These agents included neomycin, cefazolin, cholestyramine, and lactulose.

We are not aware of any studies evaluating endotoxin-reducing treatments in alcoholic liver injury. The use of lactobacilli provides a relatively nontoxic and noninvasive method of altering gut contents. Our study shows that reduction in plasma endotoxin levels in lactobacilli-fed animals is also accompanied by a reduction in the severity of liver injury. Lactobacillus GG has been shown to remain in the gastrointestinal tract for at least four days (11); furthermore the organism is relatively resistant to acid and bile, and elaborates an antimicrobial substance (12).

Although our study did not specifically address the suppression of endotoxin-producing bacteria in the gut, there is both *in vitro* and *in vivo* evidence that the Lactobacillus strain GG used in the present study is able to suppress the growth of a broad spectrum of gram-negative bacteria (11, 12). The lower levels of plasma endotoxin in the lactobacilli fed group supports these observations. This inhibitory activity is thought to be due to the elaboration of a low–molecular weight substance by Lactobacillus GG (12).

In conclusion, feeding strains of lactobacilli that survive in the intestinal tract may provide a potentially nontoxic form of therapy in order to reduce endotoxin levels. Since there is evidence of increased endotoxin absorption from the gut and increased plasma levels of endotoxin in human alcoholic liver disease (29), lactobacilli feeding is worthy of further study and would provide an additional therapeutic measure, especially in those patients with high levels of endotoxin.

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