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Bacterial translocation and peptidoglycan translocation by acute ethanol administration

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Background. We examined bacterial translocation (BT) by acute ethanol administration, using a peptidoglycan detecting system. ***Methods.*** Rats were given 20% (v/w) ethanol (10 ml/kg body weight), and heparinized samples of portal blood were collected at specific time points after administration. Plasma peptidoglycan, β-glucan, and endotoxin concentrations of portal blood were measured. The rats were divided into three groups: a 20% ethanol group (20ET group), a 5% ethanol group (5ET group), and a control group. The groups were given 10 ml/kg body weight of either 20% (v/w) ethanol (20ET group), 5% (v/w) ethanol (5ET group), or distilled water (control group). Femoral arterial blood, portal blood, mesenteric lymph nodes (MLNs), spleen, and liver were cultured to assess BT. Plasma peptidoglycan, β-glucan, and endotoxin concentrations of femoral arterial blood and portal blood were measured. ***Results.*** The plasma peptidoglycan concentration of portal blood was significantly increased 24 h after the administration of 20% ethanol. There was no significant difference in the incidence and magnitude of viable BT to the MLNs, spleen, and liver among any of the groups at this time point. The rate of plasma peptidoglycan positivity and the plasma peptidoglycan concentration were increased significantly in the portal blood of the 20ET group 24 h after administration. ***Conclusions.*** Peptidoglycan was translocated into the portal blood by acute administration of 20% ethanol. Our findings suggest that viable bacterial flora may translocate from the intestine under the influence of ethanol, and BT may be one of the causes of chronic alcoholic liver disease.

Key words: ethanol, bacterial translocation, SLP test, peptidoglycan

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Introduction

Recently, the causes of alcoholic liver disease were proposed to be oxidant stress, activation of Kupffer cells, and factors that control the activation of hepatic stellate cells which underlie liver fibrosis and cirrhosis.[1] Oxidant stress is known to involve both microsomal and mitochondrial systems. Stimulation of Kupffer cells causes the release of cytokines and chemokines, hepatocyte hypermetabolism, and the activation of hepatic stellate cells.

Endotoxemia is often observed in patients with alcoholic liver disease.[2,3] In the French-Tsukamoto rat model, a model of chronic alcoholic liver disease, it was reported that plasma endotoxin levels were high.[4] From these results, it was concluded that chronic ethanol feeding resulted in increased intestinal permeability to endotoxin and decreased liver detoxification of endotoxin. Orally administered antibiotics significantly suppressed plasma endotoxin levels and alcoholic liver damage in the French-Tsukamoto rat model;[5,6] therefore, it was concluded that intestinal bacteria may influence alcoholic liver disease. It was reported that alcohol ingestion increased intestinal permeability to endotoxin and that this may enhance its translocation from the intestine.[7] Thus, orally administered antibiotics may induce a decrease in endotoxin in the intestine and significantly suppress endotoxin levels in plasma.

It has also been reported that components of intestinal microorganisms which stimulate Kupffer cells may influence alcoholic liver disease.[8] Although bacterial translocation (BT) has the possibility of translocating the microorganism contents under the influence of alcohol administration, BT was only observed in an acute alcohol administration model that used large amounts of ethanol and in chronic ethanol administration models by using a bacterial toxin, endotoxin. However, endotoxin is not always detected in patients with Gram-negative bacterial infections, even though Gram-

negative bacteria are detected in their blood. In addition, endotoxin is produced only by Gram-negative bacteria. Plasma endotoxin cannot be detected in Gram-positive bacterial infections or in fungal infections. Therefore, we cannot accurately evaluate BT with endotoxin; thus, the mechanisms and influences of BT under conditions of acute and chronic alcohol administration are not clear. One method of evaluating BT is by measurement of intestinal permeability.[9–11] It was reported that acute alcohol feeding resulted in increased intestinal permeability,[12–15] but this is not direct evidence that BT might occur as a result of acute ethanol administration.

Recently, we developed a system for detecting peptidoglycan in human plasma, an alternative means of rapidly diagnosing bacterial infection by the silkworm larvae plasma (SLP) test.[16] This test can detect the whole-body invasion of Gram-positive bacteria, Gram-negative bacteria, and fungi, and Shimizu and colleagues[17] showed that it could reflect BT in a hemorrhagic shock model.

This study was designed to examine BT by acute alcoholic administration, using the SLP test.

Methods

Animals

All animal experiments were performed according to the guidelines of the Institute for Experimental Animals of Shiga University of Medical Science and were approved by the Institutional Review Board.

Male specific-pathogen-free Sprague-Dawley rats, weighing 300–400 g, were housed in a controlled environment with a 12-h light/dark cycle for at least 7 days before being studied, to allow the indigenous gastrointestinal flora to stabilize. All surgical procedures were performed using sterile techniques.

Ethanol administration and sample collection

Rats were given 20% (v/w) ethanol (10 ml/kg body weight) intragastrically with an intubation tube.[7,8,18,19] Rats were anesthetized with sodium penobarbital (30 mg/kg intraperitoneally), and heparinized samples of portal blood were collected in pyrogen-free polypropylene tubes at 0, 1, 2, 4, 6, 8, 12, 18, 24, 36, and 48 h after the administration of 20% ethanol. Platelet-rich plasma was obtained by centrifugation at 4°C, 150 *g* for 10 min, and stored at −80°C until analysis.

The rats were divided into three groups as follows: a 20% ethanol group (20ET group), a 5% ethanol group (5ET group), and a control group. The 20ET group was given 20% (v/w) ethanol (10 ml/kg body weight), the 5ET group was given 5% (v/w) ethanol (10 ml/kg body weight), and the control group was given distilled water (10 ml/kg body weight) via an intubation tube.[7,8] Twenty-four hours after the administration of ethanol or water, rats were anesthetized with sodium penobarbital (30 mg/kg intraperitoneally). The femoral artery of each animal was exposed, and the abdominal cavity was opened using an aseptic technique. Femoral arterial blood, portal blood, the mesenteric lymph nodes (MLNs), the spleen, and the liver were harvested for culture using sterile techniques. Samples of femoral arterial blood and portal blood were collected in heparinized, pyrogen-free, polypropylene tubes, and platelet-rich plasma was obtained by centrifugation at 4°C, 150 *g* for 10 min, and stored at −80°C until analysis. Twenty-four hours after the administration of ethanol or water, the cecal contents were weighed and diluted in pyrogen-free saline solution. Their supernatants were harvested after centrifugation and stored at −80°C until assay for peptidoglycan.

Detection of peptidoglycan, β-glucan, and endotoxin

Peptidoglycan is a component that makes up about 70% and 20% of Gram-positive and Gram-negative bacterial cell walls, respectively. The SLP test was prepared from the plasma fraction of the silkworm; this system exploits the prophenol oxidase cascade that the silkworm uses to defend itself against foreign matter. Small amounts of microorganism components, peptidoglycan and β-glucan, can be detected with this test. The concentration of the SLP activating substance (assayed peptidoglycan) was measured using a kinetic colorimetric method, as described in our previous reports.[16,20] This procedure was based on the SLP Test Wako (Wako Pure Chemical Industries, Osaka, Japan). Briefly, for sample pretreatment, 0.9 ml of pyrogen-free water and 0.1 ml of sample were mixed and agitated in a pyrogen-free tube. The mixture was incubated for 10 min at 80°C, then cooled on ice and used for the assay. The SLP solution and 0.1 ml of pretreated sample were mixed in a test tube at 30°C. The results of the kinetic colorimetric method were quantitated by means of a tube reader (Toxinometer MT251; Wako Pure Chemical Industries). The activation time (Ta) on the Toxinometer was defined as the reaction time required to obtain a 5% decrease in the amount of transmitted light. The concentration of the assayed peptidoglycan in the sample was obtained using the Ta of the sample in relation to the calibration curve. The detection limit of assayed peptidoglycan was 0.1 ng/ml.

The β-glucan concentration was measured using limulus amebocyte lysate (LAL), as described in a previous report.[21] The procedure was based on the β-glucan Test Wako (Wako Pure Chemical Industries). Briefly, for sample pretreatment, 0.9 ml of sample pretreatment

solution (Wako Pure Chemical Industries) and 0.1 ml of sample were mixed and agitated. The mixture was incubated for 10 min at 70°C, then cooled on ice and used for the assay. A 0.2-ml quantity of the pretreated sample and LAL were mixed in a test tube and incubated at 37°C in the tube reader. Gelation time (Tg) was defined as the time needed for the transmittance of the reaction mixture to decrease by 8%. The β-glucan concentration was obtained using the Tg of the sample in relation to the calibration curve. The detection limit of this method was 11 pg/ml.

The endotoxin concentration was measured using LAL, as described in our previous report.[17] The procedure was based on the Endotoxin Test Wako (Wako Pure Chemical Industries). Briefly, for sample pretreatment, 0.9 ml of sample pretreatment solution (Wako Pure Chemical Industries) and 0.1 ml of sample were mixed and agitated. The mixture was incubated for 10 min at 70°C, then cooled on ice and used for the assay. A 0.2-ml quantity of the pretreated sample and LAL were mixed in a test tube and incubated at 37°C in the tube reader. Gelation time (Tg) was defined as the time needed for the transmittance of the reaction mixture to decrease by 8%. The endotoxin concentration was obtained using the Tg of the sample in relation to the calibration curve. The detection limit of this method was 5 pg/ml.

Testing for bacterial translocation

Two milliliters of femoral arterial blood and 2 ml of portal blood were cultured aerobically and anaerobically. The distal halves of the MLNs, the spleen, and the liver were harvested for bacterial culture essentially as described previously.[22] Briefly, they were removed using sterile techniques and placed in a preweighed grinding tube. The organs were weighed and diluted in brain-heart infusion broth and homogenized with Teflon grinders. Portions were cultured on MacConkey agar, chocolate agar, and blood agar. The plates were examined after 24–48 h of aerobic or anaerobic incubation at 37°C. Positive specimens from the anaerobic incubation were subcultured on the same agar aerobically at 37°C in order to confirm that they were anaerobic. The organisms were subcultured, and the bacteria were identified by Gram staining.

Statistical analyses

Values for all results were expressed as means ± standard error (SE). Discontinuous data on the incidence of translocation and positive assayed peptidoglycan, β-glucan, and endotoxin were evaluated by Fisher's exact test. Data on bacterial population levels and assayed peptidoglycan, β-glucan, and endotoxin concentrations were evaluated by the Mann-Whitney *U*-test. The correlations of parameters were evaluated by the Spearman rank order correlation. Probabilities less than 0.05 were considered significant.

Results

Detection of plasma assayed peptidoglycan, plasma β-glucan, and plasma endotoxin after acute administration of 20% ethanol

The rate of SLP test positivity in portal blood was significantly increased 24 h after acute administration of 20% ethanol (Fig. 1). In portal blood, no plasma β-glucan or plasma endotoxin was detected at any time.

Detection of plasma assayed peptidoglycan, plasma β-glucan, and plasma endotoxin 24 h after ethanol administration

In portal blood, the rate of SLP test positivity was significantly increased, to 41.7% (5/12) in the 20ET group (Table 1). There was no significant difference in the rate of positivity of the SLP test between the 5ET group and the control group in portal blood. The concentration of assayed peptidoglycan by the SLP test in portal blood was also significantly increased in the 20ET group (Fig. 2). There were no significant differences in either the positivity of assayed peptidoglycan or the concentration of assayed peptidoglycan in the femoral arterial blood among any of the groups. No plasma β-glucan or endotoxin was detected in portal blood or femoral arterial blood in any of the groups.

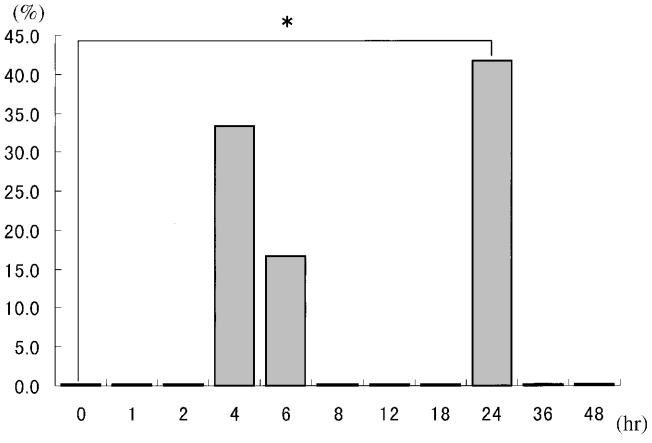

Fig. 1. Detection of assayed peptidoglycan in portal blood. Percentage of rats with portal plasma positive for assayed peptidoglycan by silkworm larvae plasma (*SLP*) test after the administration of 20% ethanol (10 ml/kg body weight). There were 6 rats at 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 18 h, 36 h, and 48 h, and 12 rats at 0 h and 24 h. *$P < 0.05$ vs 0 h

Table 1. Positivity of silkworm larvae plasma (SLP) test, beta-glucan test, and endotoxin test

	n	20ET	5ET	Control
SLP				
Femoral artery	12	1 (8.3)	0 (0)	0 (0)
Portal vein	12	5 (41.7)*	1 (8.3)	0 (0)
Beta-glucan				
Femoral artery	12	0 (0)	0 (0)	0 (0)
Portal vein	12	0 (0)	0 (0)	0 (0)
Endotoxin				
Femoral artery	12	0 (0)	0 (0)	0 (0)
Portal vein	12	0 (0)	0 (0)	0 (0)

*$P < 0.05$ vs control

n, Numbers of rats with a positive SLP test (percentages in parentheses), beta-glucan test (percentages in parentheses), and endotoxin test (percentages in parentheses); 20ET, 20% ethanol group (10 ml/kg body weight); 5ET, 5% ethanol group (10 ml/kg body weight); control, control group (10 ml/kg body weight of distilled water); 12 rats in each group

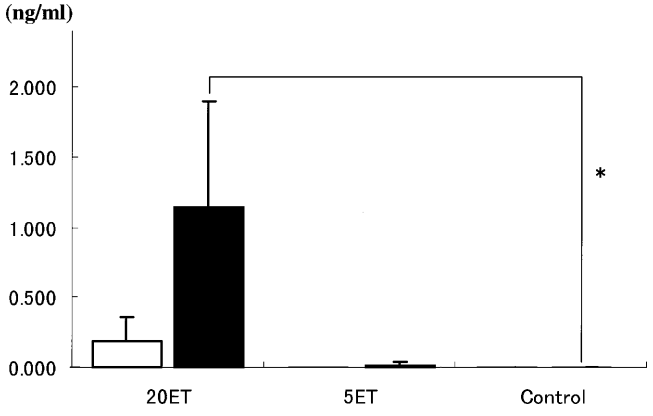

Fig. 2. Concentration of assayed peptidoglycan (ng/ml plasma). All data values are expressed as means ± SE. *20ET*, 20% Ethanol group (10 ml/kg body weight); *5ET*, 5% ethanol group (10 ml/kg body weight); *control*, control group (10 ml/kg body weight of distilled water); there were 12 rats in each group. *$P < 0.05$ vs control. *Open bar*, femoral artery; *closed bars*, portal vein

Detection of assayed peptidoglycan in cecal contents

The concentration of assayed peptidoglycan in the cecal contents was 23.7 ± 0.9 μg/g, 34.5 ± 4.8 μg/g, and 10.5 ± 2.3 μg/g in the 20ET group, the 5ET group, and the control group, respectively. The concentration of assayed peptidoglycan in the cecal contents was significantly increased in the 20ET group and the 5ET group.

Bacterial translocation

There was no significant difference in the incidence of BT to the MLNs, spleen, and liver among any of the groups (Table 2). The magnitude of BT to the MLNs was 42.9 ± 32.9 CFU/g (colony-forming units per grams of tissue), 14.4 ± 14.4 CFU/g, and 7.4 ± 7.4 CFU/g in the 20ET group, the 5ET group, and the control group, respectively (Fig. 3). Although the magnitude of BT to the MLNs showed a tendency to increase in a dose-dependent fashion, this trend was not significant. There was no significant difference in the magnitude of BT to spleen and liver among any of the groups. The identified organisms were Gram-positive rods, Gram-positive cocci, and Gram-negative cocci. No bacteria were cultured from the femoral arterial blood and portal blood in any of the groups.

Table 2. Incidence of bacterial translocation

	n	20ET	5ET	Control
MLNs	12	2 (16.7)	1 (8.3)	1 (8.3)
Spleen	12	0 (0)	0 (0)	0 (0)
Liver	12	1 (8.3)	1 (8.3)	0 (0)

n, Numbers of rats with positive bacterial cultures from mesenteric lymph nodes (MLNs; percentages in parentheses), spleen (percentages in parentheses), and liver (percentages in parentheses); 20ET, 20% ethanol group (10 ml/kg body weight); 5ET, 5% ethanol group (10 ml/kg body weight); control, control group (10 ml/kg body weight of distilled water); 12 rats in each group

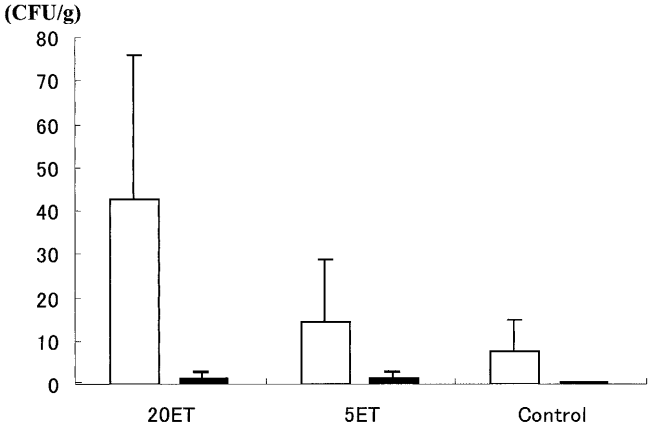

Fig. 3. Magnitude of bacterial translocation (CFU/g) to the mesenteric lymph nodes (MLNs; *open bars*) and liver (*black bars*). All data values are expressed as means ± SE. *20ET*, 20% Ethanol group (10 ml/kg body weight); *5ET*, 5% ethanol group (10 ml/kg body weight); *control*, control group (10 ml/kg body weight of distilled water). There were 12 rats in each group

Discussion

As one of the causes of chronic alcoholic liver disease, it was supposed that chronic ethanol feeding may result in

increased intestinal permeability, and that this induced the translocation of endotoxin or viable bacteria. However, the effects of moderate or small amounts of ethanol administered acutely on BT in animals have not been investigated.

Since Berg and Garlington[23] reported BT in animals, BT has been observed under various conditions. To detect it, the presence of bacteria is confirmed by culturing MLNs, liver, blood, and other organs,[23,24] or by microscopic examination of the intestinal wall and MLNs. Measurements of blood microbial DNA,[25] intestinal permeability,[9–11] and blood mediators[26,27] have also been used to detect BT. Measurement of the blood endotoxin level has been used, but it is not sufficient to detect BT, because endotoxin is produced only by Gram-negative bacteria and it is not always detectable in patients with Gram-negative bacterial infections.

On the other hand, the SLP test that we recently developed is a method for detecting peptidoglycan and β-glucan for the rapid diagnosis of bacterial infection.[16] Plasma β-glucan was not detected in this study; therefore, the plasma assayed peptidoglycan came only from plasma peptidoglycan. We reported that the SLP test could detect BT correctly and was suitable for detecting BT in the hemorrhagic shock model.[17] We also reported that the peptidoglycan measured by using the SLP test was correlated with BT to MLNs and to blood neutrophil activation in the hemorrhagic shock model, and the time course of peptidoglycan concentration was correlated to that of blood neutrophil activation.[28] The SLP test may also detect BT after acute ethanol administration.

In this study, the plasma peptidoglycan concentration of portal blood was significantly increased 24 h after the acute administration of 10 ml/kg of 20% (V/W) ethanol. Therefore, we examined BT at 24 h after moderate and small amounts of ethanol were administered acutely. There was no significant difference in the incidence and magnitude of viable BT to the MLNs among any of the groups 24 h after administration, as previously reported.[29] There was no significant difference in the incidence and magnitude of viable BT to the spleen and liver among any of the groups 24 h after administration. We demonstrated that the rate of plasma peptidoglycan positivity and the plasma peptidoglycan concentration were increased significantly in the portal blood in the 20ET group (moderate amount of ethanol) 24 h after administration. There was no significant difference in plasma peptidoglycan positivity and plasma peptidoglycan concentration in the femoral arterial blood 24 h after administration.

The mechanism of peptidoglycan presence in portal blood was not clarified in this study. Sartor and colleagues[30] reported that when radioisotope (RI)-labeled peptidoglycan polysaccharides were administered to rats, they were absorbed from the normal rat intestine into the blood. Lichtman and coworkers[31] showed small-bowel overgrowth to be associated with increased peptidoglycan absorption from the intestine. They suggested that peptidoglycans are directly absorbed from the intestine under conditions of peptidoglycan overload. Increasing intestinal permeability by ethanol could increase peptidoglycan absorption from the intestine. However, the ethanol levels in femoral arterial blood were 0 mg/ml within 12 h in the present study (data not shown); thus, it is not likely that increasing intestinal permeability by ethanol made the peptidoglycan translocate to the portal blood directly. Moreover, there was no overgrowth of intestinal bacteria in the present study (data not shown). The peptidoglycan concentration of the cecal contents was significantly increased in the 20ET group and the 5ET group, and the peptidoglycan concentration of the cecal contents in the 5ET group was higher than that in the 20ET group. It is not likely that the peptidoglycan in the portal blood was absorbed from the intestine under conditions of peptidoglycan overload.

Ethanol has been shown to cause hemorrhagic erosions of the proximal small intestine in rats and to increase the area of erosion in the proximal small intestine in a dose-dependent fashion.[32,33] Acute alcohol ingestion increased intestinal permeability, as measured by the administration of horseradish peroxidase, polyethylene glycol, and endotoxin in experimental animals.[12–15] Increased intestinal permeability was demonstrated in other conditions of BT.[9–12] It is feasible that these morphologic changes may cause direct intestinal BT. These bacteria, translocated from the intestine, might be eliminated by host defense mechanisms, and their degradation products may enter the bloodstream. But there was no significant difference in viable bacterial culture between the 20ET group, the 5ET group, and the control group in the present study. Alexander and colleagues[24] showed that the magnitude of BT was grossly underestimated when only viable bacteria were measured, using RI-labeled endotoxin and bacteria. Therefore, it was suggested that BT could not be evaluated correctly by the culture technique.

In conclusion, in the present study, we used the SLP test to demonstrate the translocation of peptidoglycan into the portal blood by acute administration of 20% (v/w) ethanol, although there was no significant difference in the incidence and magnitude of viable BT to the MLNs, spleen, and liver by 20% (v/w) ethanol and 5% (v/w) ethanol. These data suggest that viable bacterial flora may translocate from the intestine under the influence of 10 ml/kg of 20% (v/w) ethanol and be eliminated by host defense mechanisms, and peptidoglycan may influx into the portal bloodstream. These results suggest that BT with microorganism components, caused by

long-term and repeated ingestion of alcohol, is one of the causes of chronic alcoholic liver disease.

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