Relationship between endotoxaemia and protein concentration of ascites in cirrhotic patients

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SUMMARY Endotoxaemia was investigated by the Limulus assay in 42 cirrhotic patients with ascites and in 33 without ascites. The incidence of endotoxaemia in the former group (59.5%) was significantly ($P < 0.05$) higher than in the latter (36.4%). Correlation between endotoxaemia and specific gravity and concentrations of total protein, albumin, and globulin in ascitic fluid was studied in the group with ascites. The specific gravity of ascites in 25 patients with endotoxaemia was significantly greater than that in 17 patients without endotoxaemia ($P < 0.01$). The concentration of total protein in patients with endotoxaemia (13.95 ± 7.18 g/l, mean $\pm$ SD) was nearly twice as high ($P < 0.01$) as in patients without endotoxaemia (7.49 ± 3.60 g/l). The protein content of those who showed reactions greater or equal to 2(+) in the Limulus assay (16.78 ± 7.14 g/l) was significantly ($P < 0.05$) higher than in those with 1(+) reaction (11.26 ± 6.33 g/l). Moreover, the concentration of albumin in patients with endotoxaemia (7.68 ± 4.60 g/l) was more than twice that of the patients without endotoxaemia (3.39 ± 1.58 g/l, $P < 0.01$). On the other hand, globulin concentration in patients with endotoxaemia was 1.6 times that of patients without endotoxaemia ($P < 0.01$). Similar differences were noted between endotoxaemic and non-endotoxaemic patients in the ascites-to-serum ratio in protein, albumin, and globulin. These results suggest that in liver cirrhosis endotoxaemia may cause an increase in protein concentrations in ascitic fluid, and that it may be a precipitating factor in the formation of ascites.

Among the various biological actions of endotoxin, an accelerating effect on capillary permeability to macromolecules was first demonstrated by Chien *et al.* (1963) in experimental animals. Endotoxin was also shown experimentally to cause portal hypertension by constriction of hepatic venules (MacLean *et al.*, 1956; Weil *et al.*, 1956; Chien *et al.*, 1964). Moreover, with regard to hepatic venous outflow block, it is known clinically that ascitic fluid resulting from hepatic venous obstruction such as is found in the Budd-Chiari syndrome is characterised by a high protein content (Gibson, 1960). It is also well established that endotoxaemia is encountered frequently in cirrhotic patients with ascites (Liehr *et al.*, 1975; Clemente *et al.*, 1977; Tarao *et al.*, 1977). If the above mentioned effects of endotoxin are present in cirrhotic patients with

ascites, the ascitic fluid of patients with endotoxaemia should contain more protein than in those without endotoxaemia. The present study was designed to investigate the relationship between endotoxaemia and the specific gravity—protein content as well as content of albumin—of the ascitic fluid of cirrhotic patients.

Methods

Subjects investigated comprised 75 cirrhotic patients, 42 (36 males, six females) with ascites and 33 (23 males, 10 females), without ascites. They were inpatients at the Yokohama City University Hospital between 8 September 1976 and 3 March 1978. Diagnosis was made by biopsy histology characterised by complete bridging of the portal areas with fibrous bands and disorganisation of the lobules. Their ages ranged from 37 to 68 years with a mean of 51 years in the former group and from 35 to 70 years with a mean of 53 years in the latter group. The cirrhotic group with ascites comprised alcoholic

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cirrhosis (20 patients), post-hepatic cirrhosis (eight), cryptogenic cirrhosis (13), and primary biliary cirrhosis (one). The cirrhotic group without ascites consisted of alcoholic cirrhosis (17), post-hepatic cirrhosis (five) and cryptogenic cirrhosis (11). HBsAg was detectable in the serum of 10 patients in the former and six in the latter group by radioimmunoassay. At the time of investigation, 21 of 42 in the former group and seven of 33 in the latter group were on diuretics. As to the other medications, no other drugs which would influence the study were administered.

Liver function study included: total protein (estimated by the method of Gornall *et al.* (1949), normal 68 to 80 g/l), serum alkaline phosphatase (INU), serum glutamic oxalacetic transaminase (INU), thymol turbidity test (TTT, Japanese standard method, 0 to 5.5 Kunel U), total and prompt-reacting bilirubin (estimated by the modified method of Michaëlsson *et al.* (1965), total bilirubin 5 to 17 $\mu\text{mol/l}$), and bromsulphthalein retention (BSP, less than 5% in 45 minutes). Alpha-fetoprotein was determined in all patients by radioimmunoassay and those whose condition was complicated by hepatoma were excluded. Total protein in the ascitic fluid was determined by the biuret method. Disc electrophoresis (Ornstein, 1964) was used for electrophoretic analysis of ascites.

Blood-samples were obtained under aseptic conditions with pyrogen-free syringes. Endotoxin-like activity was measured immediately after sampling by the Limulus assay as described by Levin *et al.* (1970). All glassware was rendered pyrogen-free by heating at 175°C for three hours (Yin *et al.*, 1972). All procedures were carried out under sterile conditions. The amoebocyte lysate used in Limulus assay was provided by Difco Laboratories. Heparinised plasma was shaken vigorously with chloroform (1:0.25) for four hours, and the emulsion produced was centrifuged at 2500 rpm for 10 minutes; 0.1 ml of the middle layer was taken and mixed with 0.1 ml of pyrogen-free distilled water containing amoebocyte lysate. The mixture was incubated at 37°C for 24 hours and

examined after one, four, and 24 hours. The test was considered positive when a definite gelation occurred. The results were scored as follows: (3+) complete gelation—that is, shape of the gel did not change even if the tube were laid in a horizontal position; (2+) incomplete gelation—that is, shape of the gel changed slightly but did not flow out of the tube in the horizontal position; (+) definite increase in viscosity and turbidity; and (–) slight flocculation only. This last (–) score was interpreted as a negative result. Titration of the concentration of purified *E. coli* endotoxin (Difco Laboratories) dissolved in normal plasma from the batch used in the initial tests showed: (3+) ≥ 5 ng/ml, 5 ng/ml > (2+) ≥ 1 ng/ml, 1 ng/ml > (1+) ≥ 0.5 ng/ml. The result of each test was read by two independent observers. The sensitivity of the lysate preparation that was used allowed endotoxin concentrations above 0.5 ng/ml to be detected. In all patients cultures of blood and ascites were examined on the day of the endotoxin assay.

COAGULATION STUDIES

Plasma fibrinogen was estimated with a biuret method, and fibrinogen degradation products (FDP) by staphylococcal clumping test; platelets were counted by phase microscopy; prothrombin time and activated partial thromboplastin time were also measured. All of the coagulation studies were made within two days of the Limulus assay.

The statistical significance of differences was calculated by Student's *t* test.

Results

Of the 42 plasma specimens of cirrhotic patients with ascites, 25 (59.5%) showed positive (group A), and 17 showed negative (group B) results in Limulus assay. In more detail, 12 of group A showed reactions greater or equal to 2+ (group A₁), while the remaining 13 showed 1+ reactions (group A₂). Comparison of group A and group B revealed no significant differences in liver function tests (Table),

Table Biochemical laboratory tests*

Patients	No. of patients	Age (yr)	BP (mmHg)	TP (g/l)	Albumin (g/l)	Al-ph. (U/l)	S-GOT (U/l)	TTT	Total bilirubin ($\mu\text{mol/l}$)	BSP (45')
Cases with ascites										
With endotoxaemia	25	50.8 $\pm$ 10.1	131.2 $\pm$ 21.8/80.2 $\pm$ 11.9	62 $\pm$ 9	28 $\pm$ 6	150 $\pm$ 60	153 $\pm$ 113	7.4 $\pm$ 5.0	39 $\pm$ 34	22.4 $\pm$ 3.8
Without endotoxaemia	17	51.6 $\pm$ 8.9	130.7 $\pm$ 16.9/79.6 $\pm$ 12.1	63 $\pm$ 8	26 $\pm$ 4	133 $\pm$ 48	141 $\pm$ 108	8.1 $\pm$ 6.2	36 $\pm$ 31	20.9 $\pm$ 4.1
Cases without ascites										
With endotoxaemia	12	53.8 $\pm$ 12.3	138.8 $\pm$ 20.1/82.6 $\pm$ 13.1	66 $\pm$ 8	34 $\pm$ 5	142 $\pm$ 56	148 $\pm$ 104	7.8 $\pm$ 4.9	37 $\pm$ 23	20.2 $\pm$ 4.9
Without endotoxaemia	21	52.1 $\pm$ 9.2	140.2 $\pm$ 17.3/83.8 $\pm$ 15.6	68 $\pm$ 10	36 $\pm$ 6	121 $\pm$ 52	128 $\pm$ 96	6.9 $\pm$ 3.8	32 $\pm$ 29	18.8 $\pm$ 5.3

*Each value represents mean $\pm$ SD.

nor were there any significant differences in blood coagulation studies. Cultures of ascitic fluid and blood were all negative in the two groups.

Endotoxin was also demonstrated, although to a much lesser degree, in 12 of 33 (36.4%) of the control cirrhotics who had had no ascites (group C) as mentioned in our previous report (Tarao *et al.*, 1977). There was a significant difference ($P < 0.05$) between the incidence of endotoxaemia in cirrhotics with and without ascites.

The specific gravity of ascites in group A was 1.020 ± 0.005 (mean $\pm$ SD), which was significantly greater than that in group B (1.015 ± 0.003) ($P < 0.01$) (Fig. 1). The specific gravity in group A₁ (1.021 ± 0.005) was greater than that in group A₂ (1.018 ± 0.005), but this was not statistically significant. The protein concentration in group A was 13.95 ± 7.18 g/l, which was nearly twice as high as that in group B (7.49 ± 3.60 g/l) and this difference was highly significant ($P < 0.01$) (Fig. 2). Moreover, there was a significant difference ($P < 0.05$) between group A₁ (16.87 ± 7.14 g/l) and group A₂ (11.26 ± 6.33 g/l). The albumin concentrations in group A (7.68 ± 4.60 g/l) were more than twice as high as those in group B (3.39 ± 1.58 g/l) ($P < 0.01$) (Fig. 3). The content of albumin in group A₁ (9.06 ± 5.40 g/l) was higher than that in group A₂ (6.17 ± 3.12 g/l), but this again was not significant. The globulin concentration in group A

Fig. 1 Correlation between specific gravity of ascitic fluid and Limulus assay in plasma.

Fig. 2 Correlation between protein concentration of ascitic fluid and Limulus assay in plasma.

was 6.91 ± 3.21 g/l, which was approximately 1.6 times of that in group B (4.30 ± 1.75 g/l) ($P < 0.01$). The content of globulin in group A₁ (7.55 ± 2.50 g/l) was higher than that in group A₂ (5.94 ± 3.68 g/l), but this was also not significant.

The ascites-to-serum protein concentration ratio was 0.232 ± 0.129 in group A, which was significantly larger than that seen in group B (0.124 ± 0.059) ($P < 0.01$). The ratio in group A₁ (0.277 ± 0.129) was larger than that in group A₂ (0.190 ± 0.118), but this was not statistically significant. The ascites-to-serum albumin ratio was 0.266 ± 0.130 in group A, which was significantly larger than that in group B (0.134 ± 0.051) ($P < 0.01$). However, there was no significant difference between group A₁ (0.301 ± 0.129) and group A₂ (0.228 ± 0.127). Ascites-to-serum globulin ratio was 0.220 ± 0.132 in group A, which was significantly larger than that

Fig. 3 Correlation between albumin concentration of ascitic fluid and *Limulus* assay in plasma.

in group B (0.109 ± 0.045) ($P < 0.01$). Nevertheless, there was no significant difference between group A₁ (0.258 ± 0.131) and group A₂ (0.179 ± 0.126).

Discussion

As far as the mechanism of endotoxaemia was concerned, as there was no evidence of overt gram-negative bacterial infection in any case in our study; flora of the gastrointestinal tract appeared to be the only source of endotoxin. In normal conditions, endotoxin is absorbed from the intestinal tract (Ravin *et al.*, 1960; Greene *et al.*, 1961; Nolan *et al.*, 1977), via the portal vein (Prytz *et al.*, 1976) and completely cleared from the blood in the liver (Braude *et al.*, 1955) by the Kupffer cells in terms of their detoxifying capacity. In support of the detoxifying capacity of the liver, Greene *et al.* (1961)

showed that, in normal rabbits, endotoxin was found in the portal vein blood in 50% of the animals, but in only 17% of inferior vena caval bloods. If Thorotrast were given intravenously together with oral endotoxin, endotoxin was found in the blood of the inferior vena cava in 89%, suggesting a reduction of the liver's capacity to extract endotoxin delivered to it from the intestine. Moreover, it is a clinically well-known fact that, in patients with a severely damaged liver, as in fulminant hepatic failure, endotoxaemia is frequently observed (Wilkinson *et al.*, 1974). On the other hand, experimental evidence indicates that, when a venous outflow block is produced in an intestinal loop, endotoxin rapidly diffuses through the intestinal wall (Nolan and Ali, 1972). On the above bases, we had expected that endotoxaemia would frequently be found in patients with liver cirrhosis and in our patients this was indeed the case.

In the present study, a close correlation between endotoxaemia and a high protein content in the ascitic fluid was reported in patients with cirrhosis and ascites. However, the nature of the present investigation did not allow us to draw definite conclusions about whether this was a casual relationship or whether they were parallel manifestations of the cirrhotic process. Nevertheless, there seem to be several possible explanations for this phenomenon. First, endotoxin may cause an increase in capillary permeability to the macromolecules in plasma. With regard to the effect of endotoxin on capillary permeability, Chien *et al.* (1963, 1964) found in dogs that intravenous injections of *Escherichia coli* endotoxin resulted in increases of both flow and concentration of intravenously administered macromolecules (^{131}I -albumin and dextran) in the thoracic duct. They found at the same time that endogenous protein was also increased in the thoracic lymph leading to the lymph-to-serum ratios approaching unity. This same mechanism may be acting in cirrhotic patients with ascites and endotoxaemia, for ascitic fluid can exchange with blood through an enormous capillary bed under the visceral peritoneum (Prentice *et al.*, 1952; Birkenfeld *et al.*, 1958).

Second, it is possible that endotoxin may cause constriction of hepatic venules or small veins which results in portal hypertension of the postsinusoidal type. MacLean *et al.* (1956) demonstrated a marked rise in portal vein pressure in the dog after *Escherichia coli* endotoxin had been administered, and Weil *et al.* (1956) attributed the rise in portal vein pressure to localised venous spasm in the hepatic venous system caused by endotoxin. Chien *et al.* (1964) found in a study using dogs that portal hypertension caused by endotoxin was accompanied

by an increase in the wedge hepatic venous pressure and a decrease in the pressure of large hepatic veins, central venous pressure, and hepatic blood flow, and concluded that constriction of hepatic venules or small veins was elicited by endotoxin. On the other hand, Witte *et al.* (1969) investigated lymph circulation in cirrhotic patients and demonstrated that, when postsinusoidal obstruction predominates, highly permeable liver sinusoids are the major source of increased thoracic duct lymph high in protein but, as presinusoidal block develops, liver lymph is diluted in the thoracic duct by increasing amounts of intestinal lymph low in protein, and protein in thoracic duct lymph fall. Moreover, hepatic lymph has been shown to be related closely to the formation of ascitic fluid especially when hepatic venous outflow block exists (Bolton and Barnard, 1931; Hyatt *et al.*, 1955), and, indeed, it is well known that ascitic fluid resulting from hepatic venous outflow block in man such as in the Budd-Chiari syndrome (Gibson, 1960; Sherlock, 1968), and in experimental animals (Hyatt and Smith, 1954; Losowsky and Davidson, 1962) is characterised by a high protein content.

These observations suggest that endotoxaemia in patients with cirrhosis and ascites would also exert similar effects and that the ascites produced might contain higher concentrations of protein and that the increased protein concentration and resulting high colloid osmotic pressure of the ascitic fluid would in turn accelerate ascites formation, according to Starling's principle (Starling, 1894; 1896).

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