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Endotoxin and liver diseases

High titres of enterobacterial common antigen antibodies in patients with alcoholic cirrhosis

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SUMMARY We have measured antibodies to the enterobacterial common antigen (ECA) in sera of 86 patients with various liver diseases. ECA is a component of the cell wall of all enteric bacteria, and ECA antibodies are a specific indication of the presence of enterobacterial components. Patients with alcoholic cirrhosis with or without signs of alcoholic hepatitis had significantly raised anti-ECA titres compared with healthy control subjects. Other groups of patients (alcoholic hepatitis and/or fatty liver, primary biliary cirrhosis, chronic active hepatitis, or liver metastases) did not differ significantly from controls in the height of their anti-ECA titres. The results support the concept that Gram-negative bacterial components may have some role in the pathophysiology of alcoholic cirrhosis.

In cirrhotic patients systemic endotoxaemia has been observed to be much more common than in patients without liver disease.[1] Endotoxin derived from intestinal bacteria has been found to be a normal constituent of portal venous blood.[2] The Kupffer cells of the liver normally protect systemic circulation from endotoxaemia and endotoxin is expected to be present in peripheral blood only during Gram-negative bacteraemia. Reduced clearance due to circulatory changes and decreased reticuloendothelial function capacity have been suggested as the mechanism of endotoxaemia. Other possible routes for escape of endotoxin into the systemic circulation are intestinal lymphatics and transmural passage from the gut into ascitic fluid.[3] Liver injury caused by other hepatotoxic factors may be enhanced by endotoxin.[4] It has been suggested that endotoxins play some role in the pathophysiology of liver diseases.[5] In particular, such complications of liver cirrhosis as coagulopathy, haemolysis, hypotension, and renal failure have been ascribed to endotoxins.[6 7] Anti-endotoxin treatment has, in fact, been used with promising results in patients with these complications.[8] However, the lack of reliable assays for endotoxin has retarded progress in this area. The only assay sensitive enough for endotoxin activity so far is the limulus lysate test. It is not, however, entirely specific for endotoxin, particularly not if applied to serum or body fluids.[9]

The enterobacterial 'common antigen' (ECA) is common to all enteric bacteria (family *Enterobacteriaceae*) and, like the endotoxic lipopolysaccharide (LPS), is part of the outer membrane of the cell wall of Gram-negative bacteria.[10 11] Anti-ECA antibodies can be serologically measured and have been found to be raised in infections caused by enteric bacteria.[11 12] We reasoned that anti-ECA antibodies might also be raised in cirrhotic patients as an indication of the escape of enterobacterial components into the systemic circulation, and thereby give further support to the presence of endotoxemia in cirrhosis. To test this, we have now assayed anti-ECA in patients with cirrhosis, as well as in patients with other liver diseases, and compared both groups with healthy persons.

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Methods

PATIENTS

Eighty-six patients with liver disease were studied. In each case the diagnosis was based on accepted histological criteria and supported by typical clinical and immunological findings. All patients were hospitalised at the time of the study. Liver biopsy was obtained at laparoscopy and cultures for bacteria and fungi were taken from liver tissue and ascitic fluid, if present. Urinary tract infections were routinely looked for and blood cultures were taken if the patient had fever. Patients with evidence of Gram-negative infection were not included in the study. Liver biopsies were fixed immediately in 10% formalin and stained with van Gieson's stain, haemotoxylin and eosin, and Wilder's stain for reticulin. Biopsies were examined and classified without knowledge of the serological data. The patients with alcoholic liver disease were divided into subgroups according to the severity of the liver injury. The division was based on established peritoneoscopical and histological findings.[13] Portal hypertension was assessed by the appearance of dilated peritoneal veins at laparoscopy and/or the presence of oesophageal varices. Serum albumin and plasma prothrombin were measured by standard laboratory methods. Clinical details of the groups of patients are presented in Table 1.

Table 1 *Number of patients and age and sex distribution in each group of liver diseases and healthy controls*

Group	Number of patients			Age (yr)	
	Total	M	F	Mean	Range
Healthy controls	97	43	54	43·2	22–67
Malignant disease	14	8	6	56·6	37–77
Chronic active hepatitis	18	5	13	45·6	23–74
Primary biliary cirrhosis	26	2	24	54·6	32–80
Alcoholic fatty liver	12	9	3	41·2	27–64
Alcoholic cirrhosis	16	12	4	47·7	31–65

ALCOHOLIC CIRRHOSIS

All sixteen patients had a clinical history of heavy alcohol consumption for years and the liver histology was consistent with micronodular alcoholic cirrhosis with regenerative nodules completely surrounded by connective tissue. Eleven of these 16 patients also showed histological evidence of alcoholic hepatitis and fatty infiltration.

ALCOHOLIC FATTY LIVER/ ALCOHOLIC HEPATITIS

Seven patients had a clinical history of heavy alcohol consumption and showed a histological picture of mild to severe fatty liver defined by the presence of fatty droplets in more than 25% of hepatocytes. Five patients had alcoholic hepatitis characterised by varying degrees of inflammation, necrosis, and/or presence of alcoholic hyalin with retention of normal liver architecture.

PRIMARY BILIARY CIRRHOSIS

Twenty-six patients had a histologically, immunologically, and clinically confirmed primary biliary cirrhosis. The clinical details of the material have been published elsewhere.[14] Fourteen patients had an advanced primary biliary cirrhosis with heavy deposits of orcein-positive copper protein complexes.[15] Their histological grading was III and IV.[16] Seventeen patients had been on penicillamine, corticosteroids, and/or azathioprine at some stage of the disease. However, in most cases the treatment had been discontinued at the time of the study.

CHRONIC ACTIVE HEPATITIS

Eleven of 18 patients with histologically proven chronic active hepatitis (portal inflammation with piecemeal necrosis and/or bridging portoportal or portocentral necrosis seen at least in initial biopsy[17]) were on immunosuppressive treatment. All patients were HB_sAg negative. Four patients had only fibrosis in liver biopsy at the time of the study, though the histology had initially been consistent with chronic active hepatitis.

METASTATIC LIVER DISEASE

Fourteen patients had a malignant disease with liver involvement. None of these patients received immunosuppressive treatment.

CONTROLS

Sera from 97 apparently healthy people were used as controls.[18] They were not screened for past or present Gram-negative infections.

ENTEROBACTERIAL COMMON ANTIGEN ANTIBODY ASSAY

Antibodies against ECA were titrated by the indirect haemagglutination method in microtitre plates.[18] To control the specificity of the results three kinds of human blood group O erythrocytes were used with each serum: pretreated ('coated') with (1) an extract from an ECA^+ *Salmonella typhimurium* strain, (2) a similar extract from its ECA^- congenic derivative, and (3) saline. In this series of patient sera, only the ECA^+-coated eryth-

rocytes were agglutinated at a serum dilution of 1 : 4 or more. The specificity of the anti-ECA was additionally controlled by haemagglutination inhibition experiments.[18] Twenty-five microlitres of the ECA^+ extract were added to 25 μl of the serially diluted sera and incubated together for 30 minutes before the addition of 50 μl of the pretreated erythrocytes. A positive control serum (from a rabbit hyperimmunised with *E. coli* 014) was included in the assay each day. Its titre varied to some extent from day to day, and therefore titres on each day were corrected to correspond to its mean titre. When the assay was standardised as described, its reproducibility was >95%, and, while titres in sera of 649 healthy persons ranged from less than 4 to 8192, only 4·2% were above 1024.[18]

STATISTICAL METHODS

Geometric mean titres and correlation coefficients were calculated and *t* tests and χ^2-tests performed with an SR52 calculator and its Statistics Library (Texas Instruments, Dallas, Texas).

Results

The anti-ECA titres in the controls and various groups of patients are shown in the Figure. The geometric mean anti-ECA titre in the group of cirrhotic patients was 825, eight times the mean titre (104) in the control group; this difference is statistically highly significant ($t=5{\cdot}082$, $P<0{\cdot}001$). The group with cirrhosis also differed significantly ($t=5{\cdot}388$, $P<0{\cdot}001$) from the 70 patients with other forms of liver disease, whereas these as a whole did not differ from the controls ($t=0{\cdot}392$). The mean titre was 120 among the patients with alcoholic fatty liver and/or hepatitis and 74 in patients with primary biliary cirrhosis. The low titres (mean 65) among the patients with chronic active hepatitis could perhaps be related to the immunosuppressive treatment of 11 of these 18 patients; however, the titres of the seven not under immunosuppression were not clearly higher than the remaining ones. The 14 patients with malignant disease and liver metastases were not under immunosuppression therapy but had very low anti-ECA titres (mean 43); this was possibly related to their poor general condition.

We have previously set limit values for presumptive diagnostic anti-ECA titres on the basis of an analysis of 649 sera from apparently healthy persons.[18] In that analysis, 4·2% of the sera had titres higher than 1024, and 1·7% higher than 2048. As the presence of recent infections was not

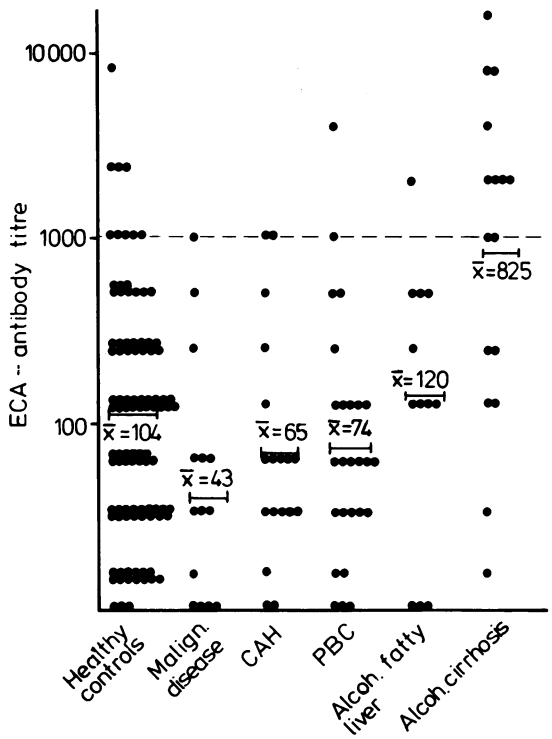

Figure *Distribution of enterobacterial common antigen antibody titres in each group of liver diseases and healthy controls. x̄: geometric mean of the titres.*

Table 2 *ECA antibody titre, serum albumin, prothrombin time, portal pressure, and presence of alcoholic hepatitis in each patient with alcoholic cirrhosis*

Patient	*ECA antibody titre*	*Serum albumin g/l (35–55 g/l)*	*Prothrombin and proconvertin (0·80–1·20)*	*Portal pressure*	*Alcoholic hepatitis*
I	1024	22·2	0·40	+	−
II	128	23·6	0·55	+	+
III	4096	30·8	0·61	++	+
IV	8192	30·3	0·80	++	+
V	1024	34·7	0·44	++	+
VI	256	34·2	0·59	++	−
VII	8192	27·2	0·37	++	+
VIII	2048	26·3	0·65	+	+
IX	16	34·2	0·33	+++	+
X	2048	30·5	1·08	+	−
XI	2048	25·4	0·42	+++	−
XII	32	30·3	0·64	++	+
XIII	16384	23·7	0·27	++	−
XIV	2048	30·5	0·44	++	+
XV	128	28·5	0·47	+++	+
XVI	256	27·6	0·68	+++	+

Portal pressure: + =normal peritoneal veins, ++ =dilated peritoneal veins, +++ =manifest oesophageal varices.

excluded in that study, from which the present control material was withdrawn, high anti-ECA titres in both cases very probably indicated responses to an enterobacterial infection. In the present study eight of the 16 patients with cirrhosis —that is, 50%—but only four (4%) of the healthy controls ($\chi^2=30{\cdot}45$, $P<0{\cdot}001$) and two (3%) of the other patients had high titres in excess of 1024. To confirm the specificity of the high titres, these sera were retitrated with the addition of ECA control antigen; in each case this resulted in a significant (eight- to 32-fold) reduction of titre.

Among the 16 patients with alcoholic cirrhosis, 11 also had signs of alcoholic hepatitis, whereas five did not (Table 2). High anti-ECA values were found among both (five and three patients, respectively). Extent of portal hypertension, serum albumin levels, and prothrombin/proconvertin values did not show significant correlation with the anti-ECA titres (Table 2).

Discussion

In this study the common enterobacterial cell wall component ECA was detected on the basis of the antibody response it had elicited. There is good reason to believe that it is an indicator showing the escape into circulation of other bacterial cell wall components, of which the highly reactive endotoxin (LPS) is a major one. The value of a direct demonstration of endotoxaemia in cirrhotic patients has recently been questioned on the ground that it may have been caused by specimen contamination.[19] As the limulus lysate test measures endotoxin activity and the ECA antibody test an immune response, they could, if used together, provide additional information.

There have been numerous reports of high antibody titres, specific or unspecific, to a variety of bacteria and viruses in liver diseases.[20,21] Antibodies to the O antigens of various strains of *Escherichia coli*, the most common member of *Enterobacteriaceae* in the intestine, have been found to be associated mainly with alcoholic liver disease,[22] which agrees well with our results. However, because different *E. coli* strains have a wide variety of O antigens, many separate strains had to be tested, and only suggestive estimates of the frequency of antibody responses could be obtained. Endotoxin activity resides in the lipid A region of the cell wall lipopolysaccharide of Gram-negative bacteria. Although this region is structurally similar in most Gram-negative bacteria, lipid A as part of LPS is poorly immunogenic, and anti-lipid A antibodies are only rarely detectable in human sera.[23] ECA is an acidic glycolipid without endotoxin activity.[11] It is a cell wall component common to all members of *Enterobacteriaceae*, and a good immunogen under certain conditions.[11] Thus, ECA antibody determination seems to offer at the present time the best method of measuring an immune response to members of *Enterobacteriaecae* in general. The specificity of our method of measuring anti-ECA antibodies has been amply confirmed both in this study and elsewhere.[18]

The findings presented indicate that advanced alcoholic liver injury is associated with an immune response to Gram-negative bacteria. The most likely explanation is the escape of components of intestinal bacteria into systemic circulation. An attempt was made to exclude patients with Gram-negative infections in this study. Though this may not have been altogether successful, it is unlikely that increased incidence of Gram-negative infections would explain the much higher incidence of high anti-ECA titres among the cirrhotic patients compared with the control subjects among whom no attempt was made to exclude recent infections.

In this study, the patients with alcoholic cirrhosis had the most advanced liver damage as measured by serum albumin level, by prothrombin and portal hypertension. Therefore it is not possible to decide whether the endotoxemia is due to the degree of liver damage alone or whether endotoxin and alcohol have had a synergistic effect in these patients. Experimentally, alcohol has been found to depress reticuloendothelial function.[24] However, patients with fatty liver and/or alcoholic hepatitis connected with heavy alcohol consumption did not have high ECA antibody levels.

ECA antibody assay may offer a new sensitive and generally applicable indication of the presence of enterobacterial components. Thus, it can be expected to help in the study of the pathophysiology and treatment of liver disease and especially such systemic manifestations of cirrhosis as haemolysis, hypotension, renal failure, and coagulopathy.

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