Liver membrane antibodies in alcoholic liver disease

II. Antibodies to ethanol-altered hepatocytes

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SUMMARY Using an indirect immunofluorescence technique, circulating liver membrane antibodies against normal rabbit hepatocytes and ethanol-altered rabbit hepatocytes have been sought in a series of patients with histologically confirmed alcoholic liver disease. Liver membrane antibodies against normal hepatocytes were found in 18 (28%) of the 65 sera examined, but with ethanol-altered hepatocytes as substrate liver membrane antibodies were found in 48 (74%) of the sera. Isolation of F(ab')₂ fragments confirmed that the positive results were due to antibody binding. Liver membrane antibodies against ethanol-altered hepatocytes are peculiar to alcoholic liver disease, and there is a similar incidence in the various histological types of alcoholic liver disease. Absorption studies suggest that the liver membrane antibodies are directed against new or altered antigens which are not present in normal hepatocytes. These new or altered antigens may also appear after pretreatment with other primary alcohols and seem likely to be induced by a haptenic effect of the alcohol or a metabolic break-down product. These studies represent a novel approach to the further investigation of the possible role of immunological mechanisms in alcohol-induced liver injury.

In the past few years there has been considerable interest in the possibility that immunological mechanisms may be involved in the pathogenesis of alcoholic liver disease, and in the perpetuation of the liver injury with ultimate progression to cirrhosis.¹⁻³ In previous studies, using normal rabbit hepatocytes as substrate in an indirect immunofluorescence test, we have found liver membrane antibodies of both IgG and IgA class in a statistically significant proportion of patients with alcoholic hepatitis and alcoholic cirrhosis.^{4,5} In the present study, a preliminary report of which has already been published,⁶ we have used hepatocytes from rabbits pretreated with daily doses of ethanol and have found liver membrane antibodies of IgG class in the majority of sera from patients with various histologically defined forms of alcoholic liver disease. Liver membrane antibodies against such ethanol-altered hepatocytes appear to be peculiar to alcoholic liver disease. This finding provides evidence that immune mechanisms may have a role in alcohol associated liver disease.

Material and methods

PATIENTS AND CONTROLS

Sera, which were stored at -70°C, were obtained from:

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(a) 65 patients (49 men, 16 women, mean age 50 ± 26 yr) with a documented history of alcohol abuse, clinical and biochemical evidence of alcoholic liver disease and in all of whom a histological diagnosis of alcoholic liver disease⁷ was established on percutaneous liver biopsy using routine staining methods.

(b) 40 patients (10 men, 30 women, mean age 47 ± 29 yr) with various other acute and chronic liver diseases; the diagnoses in these patients were established on clinical, biochemical and immunological (serum autoantibody profile) grounds, and were biopsy proven as indicated in Table 1.

(c) A control group of 30 individuals (19 men, 11 women, mean age 33 ± 23 yr) of whom 20 were members of laboratory staff with no history of excessive alcohol intake and 10 were patients with a known history of alcohol abuse, and receiving psychiatric help; in all members of the control group standard liver function tests were normal.

DETECTION OF LIVER MEMBRANE ANTIBODY *Isolation of rabbit hepatocytes*

New Zealand white rabbits of approximately 2.5 kg in weight were used. Hepatocytes were isolated by a modification of the method outlined by Jeejeebhoy and his colleagues.⁸ Rabbits were anaesthetised with sodium pentobarbitone and given 3000 units of sodium heparin to prevent blood coagulation during

Table 1 Liver membrane antibodies in alcoholic and other liver diseases using normal and ethanol-altered hepatocytes as substrate

Serum	No tested	Positive immunofluorescence staining when reacted with:			
		Normal hepatocytes	%	Ethanol-altered hepatocytes	%
Alcoholic liver disease	65	18	28	48	74
Fatty liver	23	5	22	17	74
Alcoholic hepatitis	15	4	27	12	80
Alcoholic cirrhosis	27	9	33	19	70
Other liver diseases	40	10	25	10	25
Chronic active hepatitis	8 (8)*	4	50	4	50
Acute hepatitis	14	3	21	3	21
viral	8 (3)*	1	12	1	12
drugs	6 (4)*	2	33	2	33
Extra-hepatic obstruction	8 (4)*	0		0	
Sclerosing cholangitis	4 (4)*	0		0	
Cryptogenic cirrhosis	6 (5)*	0		0	
Controls	30	2	7	2	6
No history of alcohol abuse	20	1	5	1	5
History of alcohol abuse	10	1	10	1	10

*The figure in parentheses indicate the numbers in whom a liver biopsy was performed. All the alcoholic liver disease patients were biopsied.

cannulation. The portal vein was cannulated and the liver perfused with Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS—Gibco) supplemented with 2.5% bovine serum albumin (BSA—Sigma) at pH 7.2. During the perfusion the HBSS was kept at a constant 39°C and oxygenated with a mixture of O_2 and CO_2 (95:5); 800–1000 ml were perfused through the liver at a rate of approximately 30 ml/min. After perfusion the liver was removed and was gently disrupted in approximately 250 ml of the HBSS using a scalpel and forceps. The suspension was then filtered through nylon wool and incubated with gentle shaking at 37°C for 15 min. The isolated cells were allowed to sediment by gravity for 15 min and were then resuspended in RPMI 1640 (Gibco) containing 2.5% BSA, pH 7.2, at a concentration of 5×10^6 cells/ml. No attempt was made to eliminate non-parenchymal cells; the preparations comprised approximately 90% hepatocytes and in the subsequent immunofluorescence tests they were, on the basis of size, easily distinguished from contaminating non-parenchymal cells. The viability of each cell preparation used, as assessed by a trypan blue exclusion test, was greater than 95%; in some experiments the viability of the hepatocytes was checked during and after the immunofluorescence staining procedures, but no significant reduction in viability was noted.

Hepatocytes were isolated as outlined above from:

(a) Normal rabbits.

(b) Rabbits pretreated with four daily intravenous doses of purified ethanol, 1 g per kg body weight diluted 1:1 in sterile saline. Preliminary experiment established that this dose schedule was optimal for detecting liver membrane antibodies to ethanol-altered hepatocytes. In all the routine immunofluorescence and absorption studies

ethanol-altered hepatocytes were isolated and used on the day after and therefore within 24 hours of the fourth injection of ethanol. In the serial studies ethanol-altered hepatocytes were isolated at 4, 8, 12 and 30 days after the fourth injection of ethanol.

(c) Rabbits pretreated with four daily intravenous doses, 1 g per kg body weight of methanol (methyl alcohol), propan-1-ol (isopropyl alcohol) or propan-2-ol (*n*-propyl alcohol). The hepatocytes were isolated on the day following the fourth dose of alcohol.

(d) Rabbits pretreated with four daily inhalational anaesthetics with ether (diethyl ether), anaesthesia being maintained for a period of 15 min on each day. The hepatocytes were isolated on the day following the fourth ether anaesthetic.

Immunofluorescence studies on isolated rabbit hepatocytes

Liver membrane antibody was demonstrated by an indirect immunofluorescence technique with slight modifications of the method described by Hopf and his colleagues.⁹ Fifty microlitres of the hepatocyte suspensions were incubated for 30 min at 37°C with 200 μl of serum diluted 1/4 in saline. All sera were heat inactivated (56°C for 30 min) prior to testing. In each test run a liver membrane antibody-positive and a liver membrane antibody-negative serum control and saline control were included. After washing three times in RPMI 1640, the hepatocytes were incubated for 30 min at 4°C with 100 μl of fluorescein-isothiocyanate conjugated sheep anti-human IgG (Wellcome Laboratories) diluted 1/10 in saline. The cells were washed a further three times, mounted in PBS glycerol and examined in a Leitz UV microscope using a Ploem illuminator. The monospecificity of the conjugated antiserum (and of

all conjugates used) was confirmed by immunoelectrophoresis against normal human serum prior to use in the immunofluorescence test.

Preparation of and immunofluorescence testing with F(ab')₂

IgG was purified from ten alcoholic liver disease sera by DEAE-cellulose chromatography (Whatman DE52);¹⁰ six of these sera were liver membrane antibody positive with both normal and ethanol-altered hepatocytes and four were positive only with ethanol-altered hepatocytes. F(ab')₂ fragments were prepared from the isolated IgG by digestion with pepsin (Sigma) in 0.1 M acetate buffer, pH 4.5;¹¹ the fragments were separated by gel filtration on Sephadex G100 (Pharmacia), the first peak being further purified by gel filtration on Sephadex G200 (Pharmacia) to separate undigested IgG, F(ab) and other small fragments.¹²

Immunoelectrophoresis of F(ab')₂ preparations against rabbit antihuman whole serum (Behring) produced a single precipitin line. The purity of the F(ab')₂ fragments was further established by Ouchterlony agarose-gel immunodiffusion tests.¹³ A precipitin line which showed complete identity with intact human IgG (Nordic) was seen when the fragments were tested against goat antihuman L-chain (Dynatech). No precipitin lines were seen when the fragments were tested against goat antihuman γ -chain (Dynatech). The sensitivity of the immunodiffusion test for detection of intact IgG with the antihuman γ -chain serum was assessed using known concentrations of IgG in serial dilutions;¹³ at IgG concentrations of less than 15 μ g/ml no precipitin line was formed, and on the basis of this finding it was concluded that the isolated F(ab')₂ preparation contained less than 15 μ g/ml (0.1%) intact IgG.

The F(ab')₂ fragments, rendered equimolar in saline with the IgG concentration of the original serum, were then reacted in the immunofluorescence test with both normal and ethanol-altered hepatocytes as substrate using FITC-conjugated goat antihuman IgG F(ab')₂ (Dynatech) at a 1/10 saline dilution. Controls were set up as follows: (i) F(ab')₂ fragments and a 1/10 saline dilution of FITC-conjugated swine antihuman IgG Fc (Nordic); (ii) undigested IgG and FITC-conjugated goat antihuman IgG F(ab')₂; (iii) undigested IgG and FITC-conjugated swine antihuman IgG Fc; and (iv) normal human serum and FITC-conjugated goat antihuman IgG F(ab')₂.

Absorption studies

Soluble extracts from the liver, kidneys and heart of normal and ethanol pretreated rabbits were pre-

pared using 0.25 M sucrose (pH 8.0) homogenates, 50% wt/vol. The protein concentration of the supernatants, collected after centrifugation of the homogenates (100 000 g for 60 min), was measured with the Folin phenol reagent¹⁴ and adjusted, with saline, to give a final concentration of 1 mg/ml. Soluble extracts were similarly prepared from post-mortem samples, collected within six hours of death, of a normal human liver and of a liver from a patient who died with alcoholic hepatitis; histological examination of a frozen section from the latter liver confirmed the presence of an active alcoholic hepatitis.

Rabbit liver-cell plasma membranes were prepared from normal and ethanol pretreated animals using a discontinuous sucrose density gradient method.¹⁵ Electron-microscopic examination confirmed a high state of purity but with some contamination by intracellular organelles. The protein content was estimated after solubilisation in 1% sodium dodecyl sulphate, and that of the suspension was then adjusted, with saline, to give a final concentration of 1 mg/ml.

Absorptions were carried out by incubating 50 μ l of serum with 200 μ l of the fractions/membranes for one hour at 37°C and for a further 18 hours at 4°C. As a control 50 μ l of serum were incubated with 200 μ l saline. The absorbed and control sera were centrifuged at 30 000 g for 10 min and the supernatants tested for liver membrane antibody activity as previously described.

Results

A positive immunofluorescence test both with normal and ethanol-altered hepatocytes, was characterised by uniform staining of more than 90% of the cells in each test but with some marginal accentuation of the staining producing a linear pattern at the cell periphery (Fig. 1a and b). In repeated tests each serum produced consistent results, and no evidence of any variation in reactivity between different rabbits was noted. In serial twofold dilutions the majority of positive sera gave titres of 1/16 or 1/32, and the highest titres found were 1/64.

The overall results are summarised in Table 1. When ethanol-altered hepatocytes were used as substrate in place of normal hepatocytes the prevalence of liver membrane antibodies in alcoholic liver disease sera increased from 18/65 (28%) to 48/65 (74%). A similar increase in prevalence was noted in all three groups of alcoholic liver disease patients. All sera which were positive with normal hepatocytes were also positive with ethanol-altered hepatocytes. No increase in liver membrane antibody seropositivity was noted when the sera from

Fig. 1 Positive immunofluorescence staining patterns for liver membrane antibodies. (a) Normal rabbit hepatocytes as substrate and reacted with serum from a patient with alcoholic hepatitis; there is uniform staining of the hepatocyte with some marginal linear accentuation. (b) Ethanol-altered hepatocyte as substrate; the staining pattern is similar and the nucleus is also stained. (c) Ethanol-altered hepatocyte as substrate; serum previously absorbed with normal rabbit liver extract; the membrane staining is now discontinuous and granular, and there is also nuclear staining.

Fig. 2 Liver membrane antibodies using ethanol-altered rabbit hepatocytes as substrate. Serial studies on 40 sera which were tested 24 hours (day 0), 4, 8, 12 and 30 days after ethanol treatment of the rabbits. The interrupted line indicates the percentage of sera which were positive when normal hepatocytes were used as substrate.

other liver disease patients and from controls were tested with ethanol-altered hepatocytes. Immunofluorescent staining of nuclei, noted in 11/65 (17%) sera from patients with alcoholic liver disease when normal hepatocytes were used, was

present in 62/65 (95%) when ethanol-altered hepatocytes were used; the viability of these hepatocytes was checked on trypan blue exclusion testing performed at the end of the experiment. No increase in the prevalence of nuclear staining was seen when the control group of sera were tested with ethanol-altered hepatocytes.

In serial studies the prevalence of alcoholic liver disease seropositivity with ethanol-altered hepatocytes decreased as shown in Fig. 2; with hepatocytes isolated at 30 days after the last injection of ethanol 13/40 (33%) sera were positive compared with 11/40 (28%) with normal hepatocytes.

The results obtained following pretreatment with various alcohols and with ether are summarised in Table 2. With the two other primary alcohols, methanol and propan-1-ol, the results were closely similar to those with ethanol-altered hepatocytes; the same 18 sera reacted with the various alcohol pretreated hepatocytes. Pretreatment with the secondary alcohol (propan-2-ol) produced an increase in seropositivity of 8% (2/25); these two sera also gave positive staining with ethanol-altered hepatocytes. Ether pretreatment did not affect the prevalence of liver membrane antibody positivity. Haematoxylin and eosin stained sections from the livers of the various pretreated rabbits appeared normal with no evidence of liver cell damage.

F(ab')₂ fragments prepared from 10 sera which were liver membrane antibody-positive with normal and/or ethanol-altered hepatocytes produced a similar staining pattern to that obtained with the corresponding whole serum or the corresponding isolated undigested IgG. Of the controls included in these tests similar positive staining was obtained only

Table 2 Liver membrane antibodies in alcoholic liver disease; rabbit pretreatment with various alcohols and with ether

Rabbit pretreatment	Liver membrane antibody seropositivity
Nil	7/25 (28%)
Methanol (CH ₃ OH)	18/25 (72%)
Ethanol (CH ₃ CH ₂ OH)	18/25 (72%)
Propan-1-ol (CH ₃ CH ₂ CH ₂ OH)	18/25 (72%)
Propan-2-ol (CH ₃ CHOHCH ₃)	9/25 (36%)
Diethyl ether (C ₂ H ₅) ₂ O	7/25 (28%)

Table 3 Absorption studies on 20 alcoholic liver disease sera, positive for liver membrane antibodies with normal and/or ethanol-altered hepatocytes.

Absorbed with	Positive immunofluorescence staining when reacted with:	
	Normal hepatocytes	Ethanol-altered hepatocytes
Saline (control)	6/20	20/20
Normal rabbit liver extract	0/20	20/20 (20)*
Normal rabbit liver-cell membranes	0/20	20/20 (10)*
Ethanol pretreated rabbit liver extract	0/20	0/20
Ethanol pretreated rabbit liver-cell membranes	0/20	0/20
Normal human liver extract	0/20	20/20 (5)*
"Alcoholic" human liver extract	0/20	6/20
Normal rabbit kidney extract	5/20†	20/20 (9)*
Ethanol pretreated rabbit kidney extract	5/20†	5/20†
Normal rabbit heart extract	6/20	20/20
Ethanol pretreated rabbit heart extract	6/20	20/20

() *Change to a granular staining pattern.

†These five were the same serum samples.

when normal or ethanol-altered hepatocytes were reacted with undigested IgG followed by FITC-conjugated swine antihuman IgG Fc.

The absorption studies are summarised in Table 3. A granular staining pattern was noted after some of the absorption treatments (Fig. 1c and see Table 3). Liver membrane antibody reacting with normal hepatocytes were absorbed by all of the rabbit and human liver preparations; the rabbit kidney preparations absorbed only one serum and rabbit heart preparations were without effect. In contrast, liver membrane antibody reacting with ethanol-altered hepatocytes was absorbed completely only by liver extract and membrane preparations of ethanol pretreated rabbits; a change to a granular staining pattern was produced in a number of sera, when absorbed with normal rabbit liver extract or membranes, normal human liver extract and normal rabbit kidney extract; absorption was complete in 14 of 20 and 15 of 20 sera absorbed with "alcoholic" human liver extract and ethanol pretreated kidney extract respectively; rabbit heart extracts were without effect.

Discussion

In the present study, using isolated normal rabbit hepatocytes, we have confirmed our earlier observations^{4, 5} by demonstrating liver membrane antibodies of IgG class in 18 (28%) of 65 patients with histologically defined alcoholic liver disease. Using hepatocytes isolated from rabbits which had been pretreated with four daily doses of ethanol, liver membrane antibodies were found in 48 (74%) of these patients; those 18 sera which were positive with normal hepatocytes also reacted with ethanol-altered hepatocytes, but a further 30 sera stained the latter. No such increase in prevalence of liver mem-

brane antibodies was found when sera from controls and from patients with other forms of acute and chronic liver disease were reacted with ethanol-altered hepatocytes. In respect of the liver membrane antibodies both to normal hepatocytes and to ethanol-altered hepatocytes we established, using isolated F(ab')₂ fragments, that the positive staining was indeed due to specific antibody binding and was not the result of non-specific binding of IgG or of Ig-containing complexes to Fc receptors which have been reported on rabbit hepatocytes.¹⁶ We have thus demonstrated that liver membrane antibodies against ethanol-altered hepatocytes are present in most patients with alcoholic liver disease, and furthermore that these antibodies are peculiar to alcoholic liver disease.

The absorption studies have demonstrated liver membrane antibodies directed against new or altered antigens of ethanol-altered hepatocytes and which are not apparently present in normal rabbit hepatocytes. These new or altered antigens are probably present on the cell membranes and the granular staining pattern noted after absorption suggests a different distribution pattern to those antigens which react with liver membrane antibody-positive sera using normal hepatocytes. Furthermore, the absorption results with human normal liver and "alcoholic" liver indicate that human hepatocytes may change their antigenicity as a result of alcohol consumption. The antibodies are thus certainly xenoantibodies and seem likely also to be autoantibodies. The absorption results obtained with rabbit kidney extracts indicate that the ethanol-induced changes in cell membranes may not be confined to liver cells. It is established that there are non-organ-specific antigens common to liver and kidney^{17, 18} and one or more of these may be modified by ethanol administration.

Our findings are somewhat similar to those reported by Vergani and his colleagues¹⁹ who demonstrated circulating antibodies in 9 of 11 patients with halothane-associated hepatitis and reacting specifically with the cell membranes of hepatocytes isolated from halothane pretreated rabbits. In their experiments administration of halothane for one hour was sufficient to induce liver-cell membrane reactivity. We found that the ethanol-induced change in hepatocyte reactivity was first demonstrable after three daily doses of alcohol and was optimal after four doses—that is, further daily doses did not increase seropositivity against ethanol-altered hepatocytes.

The precise mechanisms whereby the alcohols induce the appearance of new or altered antigen on the liver cell membrane must remain speculative. Alcohol molecules are intercalated between the lipids of the bilayer cell membrane causing increased membrane fluidity and expansion,²⁰ a property which they share with other lipid-soluble agents.²¹ We have found that methanol and propan-1-ol (primary alcohols) produced changes similar to ethanol, whereas propan-2-ol (a secondary alcohol) was much less effective in changing liver-cell membrane reactivity and diethyl ether was without effect. Methanol, ethanol and propan-1-ol are metabolised in the liver to their corresponding aldehydes and propan-2-ol to the corresponding ketone, predominantly by the alcohol dehydrogenase pathway²² but also by the microsomal oxidising system.^{23 24} Aldehydes are reactive substances which can bind non-enzymatically to the amino groups of proteins and phospholipids.^{25 26} It is possible that it is they rather than the alcohols which are responsible for the change in antigenicity. In any event it would seem that the effect is likely to be due to a haptenic alteration in membrane protein. The delay in the appearance of the ethanol-altered antigen(s) would be consistent with the effect being due to some ethanol metabolite, but could also be explained on the basis of a cumulative effect exerted somewhere in the pathway of membrane-protein synthesis. The changes in nuclear antigenicity in the ethanol pretreated hepatocytes seems also likely to be due to a haptenic effect and exerted on protein constituents which may be common to nuclear and plasma membranes and which are probably synthesised on the endoplasmic reticulum and then inserted into the membranes. The persistence of the effect for up to 30 days may be explained on the basis of plasma membrane reutilisation, a concept for which there is some evidence.²⁷ Alternatively, as the synthesis of ethanol altered proteins ceases after treatment with alcohol, the concentration of these altered proteins at the cell surface will eventually decrease. The rate

of change is slow, however, and it is probable that many of these proteins exist in relatively large concentrations on intracellular membranes, including the nuclear membrane, and which may be reutilised to replace the cell's plasma membrane.²⁸

Our studies have demonstrated a circulating liver membrane antibody apparently peculiar to alcoholic liver disease and the reactive antigen(s) for which are inducible by ethanol and other primary alcohols. The liver membrane antibody was found in all forms of alcoholic liver disease including fatty liver in which, histologically, no evidence of significant inflammation or fibrosis was seen. However we were unable to show any clinical, biochemical or other serological index which distinguished liver membrane antibody-positive from liver membrane antibody-negative patients. The absence of liver membrane antibodies in our control group of alcoholic patients receiving psychiatric help does suggest, however, that the antibodies are not simply the result of alcohol abuse. Alcoholic patients with and without antibodies are now undergoing a prospective study.

In vivo binding of liver membrane antibodies to hepatocytes would afford a means whereby antibody-dependent or complement-dependent mechanisms could have a role in producing liver-cell injury. Neuberger and his colleagues²⁹ have reported that 25 of 45 sera from patients with alcoholic hepatitis induced significant cytotoxicity to normal lymphocytes in vitro when they were reacted with alcohol pretreated rabbit hepatocytes as compared with control hepatocytes. Using a migration-inhibition technique evidence of cellular sensitisation to ethanol-altered liver components in alcoholic liver disease has also been reported.³ The use of ethanol-altered hepatocytes represents a novel approach to the investigation of mechanisms in alcoholic liver disease, and should be of particular value in elucidating whether immunological sensitisation to ethanol-altered antigens is of significance in initiating and/or perpetuating the disease process.

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