Liver membrane antibodies in alcoholic liver disease:

1. prevalence and immunoglobulin class

A D BURT, R S ANTHONY, W S HISLOP, I A D, BOUCHIER, AND R N M MACSWEEN

From the University Department of Pathology, Western Infirmary, Glasgow, and Department of Medicine, University of Dundee and Ninewells Hospital, Dundee

SUMMARY Using an indirect immunofluorescence technique liver membrane antibodies of IgG and IgA class have been demonstrated in a statistically significant proportion of sera from patients with alcoholic hepatitis and alcoholic cirrhosis. IgG and IgA class antibodies were found respectively in 23 and 25% of 48 patients with alcoholic hepatitis, in 27 and 33% of 84 with active cirrhosis, and 67 and 58% of 12 with inactive cirrhosis. These results provide evidence of a humoral immune response in alcoholic liver disease which is directed against, as yet undefined, liver-cell membrane antigens.

Whereas the metabolic pathways associated with the hepatic metabolism of alcohol have been well defined,¹ the precise mechanisms by which alcohol produces hepatocellular injury are less clearly understood. In particular, the factors responsible for the development of alcoholic hepatitis and the progression to cirrhosis have not been fully elucidated. Host factors must be of importance, because it has been estimated that alcoholic hepatitis develops in approximately only 30% of chronic alcoholics, and only a third of these progress to cirrhosis.² The host susceptibility or resistance factors, however, are far from clear.

There has been considerable recent interest in the possibility that immunological mechanisms may be involved in the pathogenesis of alcohol-induced hepatocellular necrosis and in perpetuating the liver injury, thus contributing to the development of cirrhosis.^{3,4} In the present study we have sought for and demonstrated an increased prevalence of liver membrane antibodies (LMAs) of both IgG and IgA class in the sera of a large group of patients with histologically defined alcoholic liver disease.

Methods

PATIENTS

Sera were obtained from 210 patients with alcoholic liver disease and in whom a percutaneous liver biopsy had been performed. The histological diagnosis of alcohol-induced liver disease⁵ was established using routine staining methods. A distinction was made

between active and inactive cirrhosis; in the former, features of alcoholic hepatitis were additionally present, whereas, in the latter, these were absent and evidence of liver cell necrosis was minimal. The histological classification was carried out independently and without prior knowledge of the serological findings. Fifty control sera were collected from hospital patients or laboratory staff, approximately matched for age and sex, with no history of excessive alcohol intake, and in whom standard liver function tests were normal.

DETECTION OF LIVER MEMBRANE ANTIBODY

Liver membrane antibody was demonstrated by an indirect immunofluorescence technique, with slight modifications of the method described by Hopf *et al.*⁶ Rabbit (New Zealand White) hepatocytes, isolated by a modified non-enzymatic technique,⁷ were used as substrate. The cells were suspended at a concentration of 5×10^6 cells/ml in RPMI 1640 (Gibco Laboratories) containing 2.5% bovine serum albumin. Using 0.05% trypan blue in Hank's balanced salt solution, the viability of each cell preparation used was $>95\%$. Fifty microlitres of the hepatocyte suspension were incubated for 30 minutes at 37°C with $200\ \mu\text{l}$ of test or control sera diluted 1:4 in saline. With each batch of test sera, positive controls (initially comprising sera from hepatitis B surface antigen (HBsAg) negative chronic active hepatitis, and latterly sera from alcoholic liver disease patients previously found to be liver membrane antibody positive) and negative controls (comprising normal human serum and saline) were included. After washing, the hepatocytes were incubated for 30 minutes at 4°C with $100\ \mu\text{l}$ fluorescein-isothiocyanate (FITC) conjugated sheep anti-human IgG, IgA, or IgM (Wellcome Laboratories), diluted 1:10 in saline.

* Address for reprint requests: Professor R N M MacSween, Pathology Dept., Western Infirmary, Glasgow G11 6NT.

Received for publication 17 August 1981

The cells were then washed, mounted in PBS glycerol, and examined in a Leitz UV microscope using a Ploem illuminator. The monospecificity of each commercial antiserum was confirmed by gel diffusion against human IgG, IgA, and IgM, and by immunoelectrophoresis against normal human serum.

ISOLATION OF AND IMMUNOFLUORESCENCE

TESTING OF IgG F(ab)₂ FRAGMENTS

IgG was purified from the sera of six patients with alcoholic liver disease and three controls by DEAE-cellulose chromatography.⁸ F(ab)₂ fragments were prepared from the isolated IgG by pepsin (Sigma) digestion;⁹ the fragments were separated by gel filtration on Sephadex G100, the first peak being additionally purified using Sephadex G200 to remove undigested IgG, Fab, and other small fragments.¹⁰ The purity of the F(ab)₂ fragments was established by Ouchterlony agarose-gel immunodiffusion tests:¹¹ no precipitin lines were seen when the fragments were tested against goat anti-human γ -chain (Dynatech), but with goat anti-human L-chain (Dynatech) a line of complete identity was present between F(ab)₂ and intact human IgG (Nordic). Immunoelectrophoresis of the F(ab)₂ against rabbit anti-human whole serum (Behring) produced a single precipitin line.

The sensitivity of the immunodiffusion test was determined using two-fold serial dilutions of the intact human IgG over a range from 2 mg/ml to 0.0076 mg/ml; precipitin lines were seen with all dilutions to 0.0156 mg/ml (= 15 μ g IgG/ml), whereas the F(ab)₂ isolates were consistently negative, indicating that they contained at least less than 0.1% (w/w) intact IgG.

Isolated F(ab)₂ fragments were then used in the immunofluorescence test already described. In addition to the use of FITC conjugated goat anti-human IgG F(ab)₂ (Dynatech) at a 1:10 dilution, the following controls were also set up: (1) F(ab)₂ fragment preparation + FITC conjugated swine anti-human IgG Fc (Nordic); (2) undigested IgG + FITC conjugated goat anti-human IgG F(ab)₂; (3) undigested IgG + FITC conjugated swine anti-human IgG Fc; (4) normal human serum + FITC conjugated goat anti-human IgG F(ab)₂.

ABSORPTION STUDIES

In an attempt to partially characterise the antigen(s) with which the liver membrane antibody was reactive, absorption studies were carried out on 15 IgG and 15 IgA liver membrane antibody-positive sera using the following preparations: (1) soluble human liver, rabbit liver, and rabbit kidney extracts, these being prepared from 0.25 M sucrose (pH 8.0) extracts of liver and kidney homogenates using the supernatants obtained after centrifugation at 100 000 g for 60 minutes; (2) rabbit liver specific protein (LSP) and LP-2,¹² and the corre-

sponding protein preparations from kidney, so-called rabbit kidney specific protein (KSP) and KP-2;¹³ (3) rabbit liver plasma membranes, prepared using the aqueous two phase polymer system of Lesko *et al.*¹⁴

The protein concentration of each of the above fractions was measured with the Folin phenol reagent,¹⁵ and adjusted to give a final concentration of 1 mg/ml in saline. Two hundred microlitres of the various liver and kidney fractions were incubated with 50 μ l aliquots of the test sera for one hour at 25°C and for another 18 hours at 4°C. As a control, 50 μ l aliquots of sera were incubated with 200 μ l saline. The control and absorbed sera were then centrifuged at 30 000 g for 10 minutes and the supernatants tested for liver membrane antibody activity as previously described.

Results

LIVER MEMBRANE ANTIBODIES

Sera which were positive for liver membrane antibody reacted with all the hepatocytes in suspension giving a continuous fluorescence staining pattern (Fig. 1a) with maximal intensity around the periphery of the cells. This continuous pattern of positive staining was similar to that obtained by Hopf and his colleagues using sera from patients with HBsAg-negative chronic active hepatitis.⁶

Using FITC-conjugated sheep anti-human IgM, binding of serum IgM to rabbit hepatocytes was found in 40% of both test and control sera. These IgM class antibodies, unlike IgG and IgA LMA, were absorbed by rabbit erythrocytes (50 μ l serum with 200 μ l packed washed red cells) and were considered to represent 'natural antibody' to rabbit antigens.

The results for antibodies of IgG and IgA class are summarised in Table 1. These are statistically signifi-

Fig. 1 (a) Positive immunofluorescence staining of rabbit hepatocyte by a serum containing liver membrane antibody (LMA) of IgG class; note the continuous staining pattern with maximal intensity at the periphery of the cell. (b) Staining pattern after absorption of an IgG-LMA positive serum with rabbit LSP; note the less intense staining and with granular positivity at the cell margin.

cant for alcoholic hepatitis and for both active and inactive cirrhosis when compared with the controls.

IMMUNOFLOUORESCENCE STUDIES WITH ISOLATED IgG F(ab)₂ FRAGMENTS

Isolated F(ab)₂ fragments from the six sera tested produced a similar staining pattern to that obtained with the whole serum or the isolated undigested IgG from the same specimens. Similar positive staining was obtained when the hepatocytes were reacted with undigested IgG followed by FITC-conjugated swine anti-human IgG Fc; all the other controls were negative.

ABSORPTION STUDIES

The results are summarised in Table 2. The saline controls all gave a positive staining pattern. Partial absorption comprised reduced fluorescence intensity and with a granular or particulate staining on the hepatocyte membrane (Fig. 1b). The data show that for both IgG and IgA class liver membrane antibody, total abrogation of staining resulted from absorption with human and rabbit liver homogenate and with rabbit liver-cell

plasma membranes. With the purified rabbit liver specific protein, LP-2 and kidney specific protein preparations partial or complete absorption of some of the IgG positive staining was obtained, whereas KP-2 was without effect; in contrast, IgA positive staining was not affected by any of these preparations.

Discussion

In the present study we have sought for liver membrane antibodies in 210 sera from patients with histologically defined alcoholic liver disease. These antibodies of IgG class were present in approximately one in four patients with alcoholic hepatitis and active cirrhosis, and in inactive cirrhosis they were found in two-thirds of the sera examined. Using F(ab)₂ fragments from a number of positive sera we have confirmed that the positive staining is due to antibody binding; it does not represent non-specific binding by IgG or IgG-containing complexes to IgG Fc receptors, previously demonstrated on rabbit hepatocyte membranes.¹⁶ As far as we are aware, this represents the first report of liver membrane antibodies of IgG class in alcoholic liver disease.

IgA binding to rabbit hepatocytes was also found in a significant proportion of patients with alcoholic hepatitis (one in four), with active cirrhosis (one in three) and with inactive cirrhosis (slightly more than one in two). We have not truly established that this represents antibody binding, due to the inherent difficulties in the preparation of F(ab)₂ fragments of IgA and because of the presence in sera of IgA in dimeric and polymeric form. However, strong circumstantial evidence in favour of the specificity of IgA binding is provided (1) by the absence of positive staining by normal sera, (2) by the absence of positive staining by many of the sera from alcoholic patients and in which the IgA levels were markedly raised, and (3) by the continuous fluorescence staining pattern, which contrasts with a granular staining pattern when immune complexes are involved.¹⁶ There are no reports, as far as we are aware, of IgA Fc receptors or of secretory piece receptors on rabbit hepatocyte membranes, but the possibility of

Table 1 Liver membrane antibodies in alcoholic liver disease

	No. tested	Liver membrane antibodies*			
		IgG class		IgG class	
		(No.)	(%)	(No.)	(%)
Fatty liver	66	7	10.6	6	9.1
Alcoholic hepatitis	48	11	22.9†	12	25‡
Active cirrhosis	84	23	27.4‡	28	33.3§
Inactive cirrhosis	12	8	66.7§	7	58.3§
Controls	50	2	4	1	2

* In 30 patients the serum contained antibodies of both IgG and IgA class—fatty liver (two) hepatitis (seven), active cirrhosis (17), and inactive cirrhosis (four).

† $P < 0.01$

‡ $P < 0.005$

§ $P < 0.005$

Comparison with controls χ^2 test with Yates correction factor.

Table 2 Absorption studies on sera positive for liver membrane antibodies of IgG class and IgA class

LMA (No. tested)	Result	Absorbed with								
		Human liver homogenate	Rabbit					Kidney homogenate	KSP	KP-2
			Liver homogenate	Liver cell membranes	LSP	LP-2				
IgG class (15)	No effect	—	—	—	7	10	11	10	15	
	Partial absorption	—	—	—	6	4	1	4	—	
	Complete absorption	15	15	15	2	1	3	1	—	
IgA class (15)	No effect	—	—	—	15	15	15	15	15	
	Complete absorption	15	15	15	—	—	—	—	—	

binding by an altered IgA in alcoholic liver disease must be considered. Confirmation of an IgA class liver membrane antibody in alcoholic liver disease would be of particular interest in view both of the documented increases in serum IgA levels characterising all histological forms of the disease,¹⁷ and of the important role which the liver is now thought to play in IgA metabolism.¹⁸

In their studies of liver membrane antibody of IgG class in patients with HBsAg-negative chronic active hepatitis Hopf and his colleagues^{6, 19} examined sera from 26 patients with alcoholic cirrhosis but with negative findings. In the present study we have slightly modified their immunofluorescence technique and have used a considerably reduced cell concentration in our substrate. This may explain the difference between our findings and theirs. It is worth emphasising that in our studies we also demonstrated liver membrane antibody in the sera of patients with HBsAg-negative chronic active hepatitis, producing a fluorescence staining pattern similar to that described by Hopf and his colleagues⁶ and indistinguishable from that produced by the sera from patients with alcoholic liver disease.

Characterisation of the liver cell antigens against which liver membrane antibodies are directed presents considerable problems.²⁰ Meyer zum Buschenfelde and Miescher²¹ isolated two antigens, liver protein 1 and 2, which subsequently became known as liver specific protein and LP-2 respectively. These antigens have been shown to be plasma-membrane associated,^{13, 20} and, in particular, liver specific protein contains multiple antigenic specificities, some of which are species cross-reactive.^{12, 22} They also share antigenic determinants with the corresponding kidney antigens—kidney specific protein and KP-2—but there is some evidence that they may contain some liver-specific components.^{13, 23} Meyer zum Buschenfelde and his group characterised an antigen against which liver membrane antibody from HBsAg-negative sera reacted, and this they named liver membrane antigen (LM-Ag)²⁴. They found no correlation between anti-liver specific protein activity and liver membrane antibody in various liver diseases, and concluded that the antibody is directed against other liver membrane antigens.^{24, 25}

In our studies both the IgG and IgA class liver membrane antibodies were absorbed by human liver extracts, and this shows that they are isoantibodies and almost certainly autoantibodies. The absorption studies using liver specific protein, LP-2, kidney specific protein, and KP-2 indicate that, for the IgG class, the liver membrane antibody is probably directed against a heterogeneous group of antigens on the liver cell membrane, some of which cross-react with kidney cell antigens. In contrast, the IgA class liver membrane antibody could be absorbed only with rabbit and human

liver homogenates and liver-cell membrane preparations, suggesting that it reacts with membrane antigens other than liver specific protein and LP-2. Anti-LSP activity was measured by radio-immunoassay in 7 IgG-LMA and 4 IgA-LMA positive sera, but with negative results. Additional studies are being undertaken in an attempt better to delineate the antigen(s) against which the liver membrane antibodies are directed in alcoholic liver disease.

We are unable at present to assess the possible significance of liver membrane antibody in the pathogenesis of alcoholic liver disease. Isolated liver cells may express newly exposed or altered antigenic determinants, so that *in vitro* reactivity does not necessarily correlate with immune reactions *in vivo*. However, if liver membrane antibody can be shown to bind to the liver cells *in vivo*, this would afford a means whereby antibody-dependent cellular or complement-dependent mechanisms could produce liver cell injury.

ADDENDUM In preliminary studies, reported since acceptance of this manuscript, we have used hepatocytes isolated from rabbits pre-treated with daily doses of ethanol intravenously, and have found that the prevalence of IgG class liver membrane antibodies is considerably increased.²⁶ Thus natural and/or ethanol-altered liver membrane antigens may become target antigens in alcoholic liver disease.

We are grateful to Dr I G McFarlane and Dr A L W F Eddleston, of King's College Hospital, London, for carrying out radio-immunoassay estimations for anti-LSP antibody. We are grateful to our clinical colleagues who entered patients into a grant-aided study of alcoholic liver disease: the grants are provided by the Scottish Home and Health Department, Grant K/MRS/50/C14 to Professor I A D Bouchier, and Grant K/MRS/50/C161 to Professor R N M MacSween and Dr H M Dick.

References

- 1 Lieber CS. Alcohol, protein metabolism, and liver injury. *Gastroenterology* 1980; **79**:373–90.
- 2 Galambos JT. Alcoholic hepatitis. In: Schaffner F, Sherlock S, Leevy C, eds. *The liver and its disease*. New York: International Medical Book Corp, 1974: 255–67.
- 3 Leevy CM, Chen T, Luisada-Opper A, Kanasundaram N, Zetterman R. Liver disease of the alcoholic: role of immunologic abnormalities in pathogenesis recognition and treatment. In: Popper H, Schaffner F, eds. *Progress in liver diseases*, vol. 5. New York: Grune and Stratton, 1976: 516–30.
- 4 Fox RA. Immune mechanisms in alcoholic liver disease. In: Fisher MM, Rankin JG, eds. *Alcohol and the liver*. New York: Plenum Press, 1977: 309–20.
- 5 MacSween RNM. Alcoholic liver disease. In: Anthony

- PP, Woolf N, eds. *Recent advances in histopathology*. Edinburgh: Churchill Livingstone, 1978: 193–212.
6. Hopf U, Meyer zum Buschenfelde KH, Arnold W. Detection of a liver-membrane autoantibody in HBs Ag-negative chronic active hepatitis. *N Engl J Med* 1976; **294**:578–82.
 7. Jeejeebhoy KN, Ho J, Greenberg GR, Phillips MJ, Bruce-Robertson A, Sodtke U. Albumin, fibrinogen and transferrin synthesis in isolated rat hepatocyte suspensions. A model for the study of plasma protein synthesis. *Biochem J* 1975; **146**:141–55.
 8. Fahay JL, Terry EW. Ion exchange chromatography and gel filtration. In: Weir DM, ed. *Handbook of experimental immunology*, volume 1. *Immunochemistry*. Oxford: Blackwell, 1973: 7-1–7-16.
 9. Nisonoff A, Wissler FC, Lipman LN, Woernley DL. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulphide bonds. *Arch Biochem Biophys* 1960; **89**:230–44.
 10. Nasu H, Chia DS, Knutson DW, Barnett EV. Naturally occurring human antibodies to the F(ab')₂ portion of IgG. *Clin Exp Immunol* 1980; **42**:378–86.
 11. Steele EJ, Rowley D. The mechanism of complement fixation by rabbit F(ab')₂: properties of the bactericidal reaction. *Immunochemistry* 1977; **14**:319–24.
 12. McFarlane IG, Wojcicka BM, Zucker GM, Eddleston ALWF, Williams R. Purification and characterization of human liver-specific membrane lipoprotein (LSP). *Clin Exp Immunol* 1977; **27**:381–90.
 13. Behrens UJ, Paronetto F. Studies on 'liver specific' antigens. 1. Evaluation of the liver specificity of 'LSP' and 'LP-2'. *Gastroenterology* 1979; **77**:1045–52.
 14. Lesko L, Donlon M, Marinetti GV, Hare JD. A rapid method for the isolation of rat liver plasma membranes using an aqueous two-phase polymer system. *Biochim Biophys Acta* 1973; **311**:173–9.
 15. Lowry OH, Rosebrough MJ, Farr AL, Randall RI. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951; **193**:265–75.
 16. Hopf U, Meyer zum Buschenfelde KH, Dierich MP. Demonstration of binding sites for IgG Fc and the third complement component (C3) on isolated hepatocytes. *J Immunol* 1976; **117**:639–45.
 17. Morgan MY, Ross MGR, Ng CM, Adams DM, Thomas HC, Sherlock S. HLA-B8, immunoglobulins, and antibody responses in alcohol-related liver disease. *J Clin Pathol* 1980; **33**:488–92.
 18. Hsu S, Hsu P. Demonstration of IgA and secretory component in human hepatocytes. *Gut* 1980; **21**:985–9.
 19. Tage-Jenson U, Arnold W, Dietrichson O, Hardt F, Hopf U, Meyer zum Buschenfelde KH, Nielsen JO. Liver-cell-membrane autoantibody specific for inflammatory liver diseases. *Br Med J* 1977; **1**:206–8.
 20. McFarlane IG, Wojcicka BM, Williams R. Review. Antigens of the human liver. *Clin Exp Immunol* 1980; **40**:1–7.
 21. Meyer zum Buschenfelde KH, Miescher PA. Liver specific antigens. Purification and characterization. *Clin Exp Immunol* 1972; **10**:89–102.
 22. Manns M, Meyer zum Buschenfelde KH, Hutteroth T, Hopf U. The liver specific protein: evidence for species-specific and non-species-specific determinants. *J Clin Lab Immunol* 1980; **3**:9–13.
 23. Behrens UJ, Vernace S, Paronetto F. Studies on 'liver specific' antigens. II. Detection of serum antibodies to liver and kidney cell membrane antigens in patients with chronic liver disease. *Gastroenterology* 1979; **77**:1053–61.
 24. Meyer zum Buschenfelde KH, Manns M, Hutteroth TH, Hopf U, Arnold W. LM-Ag and LSP—two different target antigens involved in the immunopathogenesis of chronic active hepatitis. *Clin Exp Immunol* 1979; **37**:205–12.
 25. Manns M, Meyer zum Buschenfelde KH, Hess G. Auto-antibodies against liver-specific membrane lipoprotein in acute and chronic liver diseases: studies on organ-, species-, and disease-specificity. *Gut* 1980; **21**:955–61.
 26. MacSween RNM, Anthony RS, Farquharson M. Antibodies to alcohol-altered hepatocytes in patients with alcoholic liver disease. *Lancet* 1981; **2**:803.