

Detection of immunoglobulins G and A on the cell membrane of hepatocytes from patients with alcoholic liver disease

A TREVISAN, R CAVIGLI, R MELICONI, GF STEFANINI, S ZOTTI, M RUGGE, F NOVENTA, C BETTERLE, G REALDI

From the Istituto di Medicina Clinica, Cattedra di Patologia Medica dell'Università di Padova, Istituto di Patologia Medica, III, Università di Bologna, Divisione Medica III, Ospedale Civile di Padova, Istituto di Anatomia Patologica dell'Università di Padova and the Istituto di Semeiotica Medica dell'Università di Padova, Padova, Italy

SUMMARY The presence of immunoglobulins (Ig) G, A, and M and of complement fractions (C3-C4) on the liver cell surface was investigated by direct immunofluorescence in 40 patients with alcoholic liver disease.

IgG was detected on the liver cell membrane with a linear staining pattern in 29 patients. The percentage of IgG-positive hepatocytes correlated with transaminase activities, independently of the histological findings. IgA was demonstrable with a coarse granular staining pattern in 11 of the 14 cases with established cirrhosis. The finding of IgG bound to the hepatocyte surface in patients with alcohol-induced liver damage suggests that alcohol could be responsible for antigenic modifications of hepatocyte membrane with consequent triggering of a humoral immune response.

The abuse of alcohol is considered one of the most frequent causes of chronic liver disease. Following intake of an excess of alcohol, a number of hepatic functions may be altered, due in part to changes in the hepatic redox state, and in part to adaptive metabolic changes, resulting in activation of hepatotoxic compounds with production of new proteins.¹⁻³

Although metabolic and structural alterations associated with the hepatic metabolism of alcohol have been extensively delineated, the mechanisms responsible for alcohol-induced liver cell injury and for its progression to cirrhosis have not yet been clarified.⁴ Recently genetic and constitutional factors, including the immune system have been considered of importance.^{5,6} Indeed there are phenomena that indicate immune participation in the pathogenesis of liver disease due to alcohol.⁴ Immune response to liver specific autoantigens, such as liver membrane antigen (LMAg) and liver specific protein (LSP) and neoantigens, such as alcoholic hyalin, or other autoimmune phenomena are compatible with an immunological

hypothesis.⁷⁻¹⁰ However it must be noted that, at present, no definite data exist on the nature of the immune effector mechanism that might explain the pathogenesis of alcoholic hepatitis.

The aim of this study was to investigate the *in vivo* binding of immunoglobulins and complement to isolated hepatocytes obtained from liver biopsies of patients with histologically proven alcoholic hepatitis.

Patients and methods

PATIENTS

This study was carried out over a period of five months on a consecutive series of alcoholics admitted to hospital for detoxication and assessment of any associated liver disease. The group of patients consisted of 40 subjects (27 men and 13 women) aged between 32 and 66 yr (mean \pm SD = 45 \pm 9.8 yr) who were admitted with a history of high alcoholic intake (100 to 220 g of alcohol per day) for periods that ranged from 3 to 12 yr. All patients gave informed consent to diagnostic laparoscopy and liver biopsy. None of the patients was on therapy before and during the study.

LABORATORY INVESTIGATION

Laboratory investigation included routine tests as well as more specific analyses. Each serum was tested for hepatitis B surface antigen (HBsAg), hepatitis Be antigen (HBeAg), antibody to HBsAg, to HBeAg and to hepatitis B core antigen (anti-HBs, anti-HBe, anti-HBc) by commercial radioimmunoassay kits (RIA, Abbott laboratories). Antibody to liver specific protein (LSP) was tested on serum samples (diluted 1/20) by RIA according to Jensen *et al.*¹¹ Sera diluted 1/4 were also tested for liver membrane antibody (LMA) by indirect immunofluorescence on isolated rabbit hepatocytes after the method of Hopf *et al.*¹² Non-organ-specific autoantibodies, antinuclear antibody (ANA), anti-smooth muscle antibody (SMA) and antimitochondrial antibody (AMA), were studied by indirect immunofluorescence on sera diluted 1/10.

LIVER BIOPSY

Specimens obtained by Trucut needle (Stryl-lab, Milan) during laparoscopy, were divided into two parts: one was processed for histological diagnosis, while the other was employed for immunological studies on isolated liver cell suspensions.

For histological examination the liver tissue was fixed in 5% neutral formalin. Paraffin-embedded sections were stained with haematoxylin and eosin, van Gieson's, Gomori's, and Perls' stain and periodic acid-Schiff after diastase digestion.

The histological diagnosis of alcoholic liver disease was based on the suggestions of an International Group.¹³ The following groups were defined: fatty infiltration alone or with minimal alcoholic hepatitis; fully or advanced alcoholic hepatitis; liver cirrhosis; cirrhosis with superimposed alcoholic hepatitis. Furthermore, several specific histological features were evaluated on each biopsy: steatosis, ballooning degeneration, Mallory bodies, spotty necrosis, intralobular inflammation with an infiltrate of polymorphonuclear leucocytes, intralobular inflammation with an infiltrate of mononuclear cells, portal inflammation, piecemeal necrosis, hepatic-vein fibrosis and portal fibrosis. The individual histological changes were graded as absent, minimal, moderate or marked.

IMMUNOFLUORESCENCE ON ISOLATED LIVER CELLS

Liver cells were mechanically isolated according to Hopf *et al.*¹⁴ with some minor modifications.¹⁵ Briefly, the tissue was gently fragmented in a Petri dish using two injection needles. The resulting cell suspension was filtered through a small nylon wool column to remove residual debris and macrophages. All treatments were done under a plastic chamber

with continuous oxygen supply (95%) at a temperature of 22°C. Hepatocytes in suspension were counted and the total number of liver cells obtained from each case ranged from 10⁵ to 2 × 10⁵. The viability of the cell population was assessed by phase contrast microscopy. The hepatocyte suspension contained numerous parenchymal cells which appeared round and refractile. Cellular debris was always scanty. Erythrocytes and lymphocytes were also observed and could be easily differentiated from parenchymal cells by their size and specific refractiveness.

The cells were incubated for 10 min at 22°C with FITC-conjugated antisera, washed and mounted in a liquid medium and examined by fluorescence microscopy (Leitz Orthoplan, Ploempak 2) using a K 490 interference filter.

ANTISERA

Rabbit antihuman IgG, rabbit antihuman IgA and rabbit antihuman IgM sera were purchased from Behringwerke (Marburg), while goat antihuman complement components sera (anti-C3 and anti-C4) were purchased from Meloy Laboratories. In all the direct immunofluorescence studies we used F(ab)₂ fragments prepared by hydrolysis with pepsin according to Winchester,¹⁶ since the use of whole antibody could result in non-specific binding to immunoglobulin-Fc receptors on liver cells.¹⁷ Briefly, the IgG fraction was purified from each commercial preparation by ammonium sulphate precipitation and DEAE-cellulose chromatography. The IgG solutions were then equilibrated with 0.1 M and 0.2 M sodium acetate buffer (for rabbit and goat IgG respectively) prior to pepsin treatment. After digestion, rabbit F(ab)₂ fragments were purified on a 1 × 10 cm bed of DEAE cellulose, while goat F(ab)₂ fragments were purified using gel filtration through Sephadex G-150. The purity of F(ab)₂ fragments was assessed by Ouchterlony agarose gel immunodiffusion after their concentration at one mg/ml: no precipitation line was observed with anti-rabbit and antigoat gamma-chain (Cappell), while a precipitation line was obtained with antirabbit and antigoat lambda-chain (Cappell). Immunoelectrophoresis of F(ab)₂ preparations against serial dilutions of human whole serum (dilution 1/4; 1/8; 1/16) confirmed the monospecificity of the antibodies employed in this study.

Antisera were then conjugated with fluorescein isothiocyanate (FITC) after the method of Arnold and Mayersbach.¹⁸ Labelled antisera had a molar FITC/protein ratio ranging from 2.5 to 3.9 and were employed at a protein concentration of 0.1 mg/ml.

Prior to immunological studies on isolated hepatocytes, rabbit antihuman IgG and IgA F(ab)₂

Hepatocyte membrane-bound IgG and IgA and serum antibody to liver specific protein (LSP), antibody to liver membrane antigen (LMA), and antinuclear antibody (ANA) in relation to liver histology in 40 patients with alcoholic liver disease.

	Liver histology				Total (40)
	Fatty liver alone or with minimal alcoholic hepatitis (11)	Alcoholic hepatitis (15)	Cirrhosis (12)	Cirrhosis with alcoholic hepatitis (2)	
Hepatocyte membrane-bound IgG	9	11	7	2	29
IgA	—	2	9	2	13
Autoantibodies in serum					
anti-LSP	—	1	1	1	3
ANA	2	—	—	—	2
LMA	3	9	7	—	19
Simultaneous presence of LMA and membrane-bound IgG	1	6	4	—	11

No of cases is shown in parentheses.

fragments FITC-conjugated were further purified by immunoabsorption on human IgG and IgA respectively and immobilised by glutaraldehyde after the method of Avrameas and Ternynck.¹⁹

CONTROLS

Blocking of anti-IgG, IgA and IgM/FITC staining was obtained by previous incubation with unlabelled antihuman antibodies. Monospecific antisera were also tested on isolated hepatocytes obtained during cholecystectomy in five patients with no history of alcohol intake.

Results

HISTOLOGY

The results of the histological examination are summarised in the Table. Eleven patients had minor abnormalities with increased fat content or minimal alcoholic hepatitis or both; 15 had alcoholic hepatitis, and cirrhosis was observed in 14 (two with concomitant hepatitis).

AUTOANTIBODIES

Antibodies to liver specific antigens, (anti-LSP) were detected by radioimmunoassay in three cases while antibody to liver membrane antigen (LMAg) were found in 19 cases (see Table). ANAs were detected in two cases, but AMA and SMA were found in none.

HEPATITIS B VIRAL ANTIGENS

Only one patient was found positive for HBsAg and for anti-HBc and anti-HBe. (not shown in Table). Five other cases had circulating anti-HBs, while six cases (two positive for anti-HBs) were positive for anti-HBc.

IMMUNOFLUORESCENCE ON ISOLATED HEPATOCYTES

When hepatocytes from alcoholic patients were incubated with fluoresceinated antisera, IgG, deposited in a linear fashion on the liver cell membrane (Fig. 1), was detected in more than half of the cases studied (Table). The linear pattern of antigen-antibody complex on the liver cell surface was not modified by different times or temperatures of incubation.

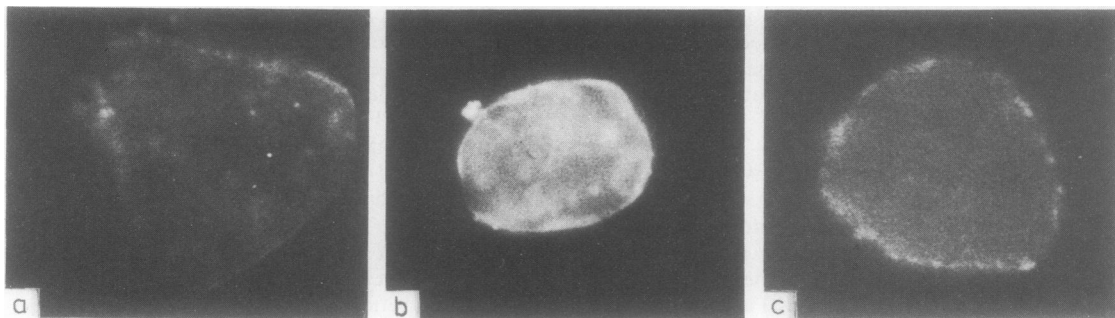


Fig 1 Linear immunofluorescence staining for IgG on the liver cell membrane in (a) a case with fatty liver and (b) alcoholic hepatitis; (c) coarse granular staining pattern of membrane IgA in a case with inactive cirrhosis. Original magnification $\times 250$.

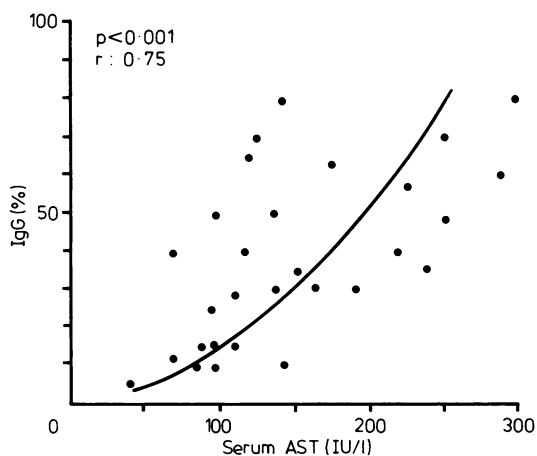


Fig. 2. Correlation between the percentage of IgG-positive hepatocytes and serum AST activities.

The percentage of positive cells ranged from 9% to 80%. This phenomenon correlated with biochemical signs of liver disease activity, as assessed by statistical analysis (Fig. 2), but it was independent of histological diagnosis (Table). In fact, when the patients were subdivided on the basis of their histology, membrane-bound IgG was detected in 81% of those with fatty liver or minimal alcoholic hepatitis, 73% of those with alcoholic hepatitis and 64% of those with cirrhosis. Moreover, no significant correlation was found between the presence or absence of membrane-bound IgG and any of the several specific histological features considered; nor was there any correlation with the presence of non-organ specific autoantibodies or antibodies to LSP and to LMAg in serum, or previous hepatitis B virus infection.

IgM and complement components were not detected on the liver cells, whereas IgA was demonstrable with a coarse granular pattern in the majority of the patients with liver cirrhosis (Fig. 1).

Isolated hepatocytes from control cases (surgical biopsies) produced negative results for the presence of immunoglobulins or complement on the cell surface.

Discussion

The mechanisms responsible for alcoholic hepatitis are still to be completely defined. Current hypotheses suggest the presence of toxic reactive metabolites as the basis of hepatocellular injury. The suggestion that immunological reactivity may play a role in the pathogenesis of alcoholic hepatitis is intriguing. Indeed, recent studies point to the

importance of immunological mechanisms in hepatitis due to alcohol ingestion.²⁰⁻²²

In the present study antibodies of IgG class, reacting *in vivo* with the membrane of hepatocytes, showed a linear pattern of distribution on the liver cell surface. This immunofluorescent pattern could be interpreted as the consequence of antibody reaction with an antigen homogeneously distributed on the liver cell membrane. Although the nature of such antigen and its relation with structural membrane proteins remains undefined, the lack of antibody-induced antigenic redistribution (with cap or patch formation) could be due to an alteration in microtubules induced by alcohol intake, as described by Baraona *et al*²³ in ethanol-fed rats.

As to the relation between ingestion of alcohol, hepatitis and presence of IgG on the hepatocyte surface, it may be suggested that alcohol itself, or its metabolites, could alter the antigenicity of liver cell membrane constituents, triggering a humoral immune response that appears to be distinct from autoantibodies directed against liver specific protein (LSP) or liver membrane antigen (LMAg). In this study the presence of membrane-bound IgG correlated with transaminase activities and this suggests that the antibody could be involved in the pathogenesis of liver cell damage. However, a cytolytic effect of antibodies demonstrable on the liver cell surfaces needs a more precise definition. Indeed, the absence of complement components on the hepatocyte membrane is against the hypothesis of a direct immune effect, even if it does not exclude the possibility of an antibody-dependent cytotoxicity system.⁷ Alternatively, the correlation between membrane-bound IgG and transaminase activities could be coincidental, with alcohol as the common factor in producing the two variables. At present the major question is whether such cytophilic antibody has to be considered as only a marker of alcohol-induced alterations on the liver cell structures rather than an important part in the cytolytic effector system.

The finding of IgA bound in a granular fashion to the hepatocyte membrane is another interesting aspect of alcohol-induced liver disease. Its restriction to active or inactive cirrhosis and the granular appearance of the antibody deposition represent an intriguing observation. The finding could be related to the increase of circulating IgA dimers complexed with intestinal antigens, previously reported in alcoholic cirrhosis.^{24,25}

The precise delineation of putative antigen(s) for the membrane-bound antibodies and the possible pathogenetic role for IgG and IgA in producing liver cell injury will be sought in further experiments involving elution of these antibodies.

This work was supported in part by a grant from the "Consiglio Nazionale delle Ricerche", Rome, Italy.

References

- ¹ Lieber CS, De Carli LM, Rubin E. Sequential production of fatty liver, hepatitis, and cirrhosis in sub-human primates fed ethanol with adequate diets. *Proc Natl Acad Sci USA* 1975;**294**:437-41.
- ² Harinasuta U, Zimmerman HJ. Alcoholic steatonecrosis. 1-Relationship between severity of hepatic disease and presence of Mallory bodies in the liver. *Gastroenterology* 1971;**60**:1036-46.
- ³ Leibach WK. Cirrhosis in the alcoholic and its relation to the volume of alcohol abuse. *Ann NY Acad Sci* 1975;**252**:85-105.
- ⁴ Leevy CM, Chen T, Zetterman RK. Alcoholic hepatitis, cirrhosis and immunologic reactivity. *Ann NY Acad Sci* 1975;**255**:106-15.
- ⁵ Morgan MY, Ross MGR, CM NG, Adams DM, Thomas HC, Sherlock S. HLA-B8, immunoglobulins, and antibody response in alcohol related liver disease. *J Clin Pathol* 1980;**33**:488-92.
- ⁶ Krasner N, Davis M, Portmann B, Williams R. Changing pattern of alcoholic liver disease in Great Britain: relation to sex and signs of autoimmunity. *Br Med J* 1977;**i**:1497-1500.
- ⁷ Leevy CM, Kanagasundaram N, Matsumoto K, Chen T. Alcoholic hyalin and immunologic reactivity. In: Eddleston ALWF, Weber JCP, Williams R, eds. *Immune reactions in liver disease*. London: Pitman Medical, 1979:195-207.
- ⁸ Burt A, MacSween RNM, Galbraith I, Hislop WS. Liver membrane antibodies in alcoholic liver disease. *Scott Med J* 1980;**25**:163-4.
- ⁹ Kanagasundaram N, Kakumu S, Chen T, Leevy CM. Alcoholic hyalin antigen (AHAg) and antibody (AHAb) in alcoholic hepatitis. *Gastroenterology* 1977;**73**:1368-73.
- ¹⁰ Zetterman RK, Luisada-Opper A, Leevy CM. Alcoholic hepatitis: cell-mediated immunologic response to alcoholic hyalin. *Gastroenterology* 1976;**70**:382-4.
- ¹¹ Jensen DM, McFarlane IG, Portmann BS, Eddleston ALWF, Williams R. Detection of antibodies directed against a liver-specific membrane lipoprotein in patients with acute and chronic active hepatitis. *N Engl J Med* 1978;**299**:1-7.
- ¹² Hopf U, Meyer zum Buschenfelde KH, Arnold W. Detection of a liver membrane autoantibody in HBsAg-negative chronic active hepatitis. *N Engl J Med* 1976;**294**:578-82.
- ¹³ Review by an International Group. Alcoholic liver disease: morphological manifestations. *Lancet* 1981;**i**:707-11.
- ¹⁴ Hopf U, Arnold W, Meyer zum Buschenfelde KH, Forster E, Bolte JP. Studies on the pathogenesis of chronic inflammatory liver disease: membrane-fixed IgG on isolated hepatocytes from patients. *Clin Exp Immunol* 1975;**22**:1-8.
- ¹⁵ Trevisan A, Realdi G, Alberti A, Noventa F. Relationship between membrane-bound immunoglobulin and viral antigens in liver cells from patients with hepatitis B virus infection. *Gastroenterology* 1979;**77**:209-14.
- ¹⁶ Winchester RJ. Techniques of surface immune fluorescence applied to the analysis of the lymphocyte. In: Bloom BR, David JR, eds. *In vitro methods in cell-mediated and tumor immunity*. New York: Academic Press, 1977:171-86.
- ¹⁷ 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.
- ¹⁸ Arnold W, Mayersbach H. Changes in the solubility of immunoglobulins after fluorescent labelling and its influence on immunofluorescent techniques. *J Histochem Cytochem* 1972;**20**:975-85.
- ¹⁹ Avrameas S, Ternynck T. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry* 1969;**6**:53-66.
- ²⁰ Bailey RJ, Krasner N, Eddleston ALWF, Williams R, Tee DEH, Doniach D, Kennedy LA, Batchelor JR. Histocompatibility antigens, autoantibodies, and immunoglobulins in alcoholic liver disease. *Br Med J* 1976;**iii**:727-9.
- ²¹ Sorrell MF, Leevy CM. Lymphocyte transformation and alcoholic liver injury. *Gastroenterology* 1972;**63**:1020-5.
- ²² Burt AD, Anthony RS, Hislop WS, Bouchier IAD, MacSween RNM. Liver membrane antibodies in alcoholic liver disease. 1-Prevalence and immunoglobulin class. *Gut* 1982;**23**:221-5.
- ²³ Baraona E, Leo MA, Borowsky SA, Lieber CS. Pathogenesis of alcohol-induced accumulation of protein in the liver. *J Clin Invest* 1977;**60**:546-54.
- ²⁴ Penner E, Albini B, Milgrom F. Detection of circulating immune complexes in alcoholic liver disease. *Clin Exp Immunol* 1978;**34**:28-31.
- ²⁵ Hopf U, Brandtzaeg P, Hütteroth TH, Meyer zum Buschenfelde KH. In vivo and in vitro binding of IgA to the plasma membrane of hepatocytes. *Scand J Immunol* 1978;**8**:543-9.

Requests for reprints to: Dr Giuseppe Realdi, Istituto di Medicina Clinica, Patologia Medica dell'Università, Via Giustiniani 2, 35100 Padova, Italy.