

Aetiology of cirrhosis, hepatic fibrosis, and hepatocellular carcinoma¹

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SUMMARY Histological study of 69 cases of cirrhosis, 9 of severe generalised hepatic fibrosis, and 19 of hepatocellular carcinoma showed an association with alcohol, hepatitis B surface antigen (HBsAg) or α_1 -antitrypsin bodies in, respectively, 41 (cirrhosis), 5 (fibrosis), and 9 (carcinoma). Eight of the cirrhotic cases and two of the carcinoma cases had double associations, HBsAg being present in all. Orcein and aldehyde fuchsin staining gave both false positive and false negative results when compared with immunofluorescence and immunoperoxidase methods for HBsAg. Large amounts of copper were found in four cirrhotic livers, and moderate amounts in 13: the diagnostic value of copper staining is questioned.

In 1973 MacSween and Scott found that 62% of 520 cases of cirrhosis in Glasgow were cryptogenic. Since that time methods for the identification of hepatitis B infection and α_1 -antitrypsin deficiency have been developed, permitting the demonstration of aetiological factors in a larger proportion of cases. Because of the probability of sampling errors in needle biopsy specimens of liver, we decided to use postmortem material to investigate the contribution of histology in the diagnosis of the aetiology of cirrhosis, hepatic fibrosis, and hepatocellular carcinoma.

Material and methods

The hospital necropsy records for the 15 years ending August 1976 were searched for cases of cirrhosis, severe generalised hepatic fibrosis, and hepatocellular carcinoma. Sections were cut at 4μ from the paraffin blocks of the livers of these cases and stained by the following methods.

Fibrosis or cirrhosis secondary to large duct obstruction or to cardiac failure was excluded. Examination of sections stained by haematoxylin and eosin and for reticulin yielded 69 cases of cirrhosis, 9 of severe generalised hepatic fibrosis, and 19 hepatocellular carcinomas. The presence or absence of activity was noted, using the customary criteria for distinguishing between chronic persistent

and chronic aggressive hepatitis. The amount of large vacuole fat present was assessed and graded as little/none, moderate, or much, and the presence or absence of acute alcoholic hepatitis was noted. The presence of acute alcoholic hepatitis, with or without excess fat, was taken as the sole criterion of an alcoholic aetiology.

Sections stained by the combined Perls' reaction for iron and van Gieson stain for collagen were examined for iron and for bile thrombi.

Copper was demonstrated by a rubeanic acid technique (Cook, 1974a), and the density of positive staining was assessed and graded as little/none, moderate, or much.

Sections from each case were stained by Shikata's orcein method (Shikata *et al.*, 1974), using the Mallory bleach modification, and by Gomori's aldehyde fuchsin technique (Shikata *et al.*, 1974) for the identification of HBsAg. HBsAg was also identified in sections by indirect immunofluorescence using anti-HBsAg (Wellcome), fluorescein conjugate (Wellcome), and Ploem incident illumination (Leitz) after comparison of Wellcome with Hoechst antisera and conjugates which showed that both gave similar results although Hoechst was weaker. HBsAg was also identified in sections by the indirect immunoperoxidase method (Burns, 1975) using anti-HBsAg (Hoechst) and peroxidase conjugate (Nordic), and by the peroxidase-antiperoxidase method (Burns, 1975) using anti-HBsAg (Hoechst), anti-rabbit immunoglobulin (Dakopatts), and rabbit peroxidase-antiperoxidase (Dakopatts); for both peroxidase methods endogenous peroxidase was blocked by treatment of the sections with hydrogen peroxide

¹This report includes the cases of liver disease which formed part of a previous publication (Blenkinsopp and Haffenden, 1977).

in methanol, and the peroxidase was demonstrated by the diaminobenzidine reaction. After trials to establish that all three immunological methods gave satisfactory results, one or more sections of liver from each case were examined by one of the three techniques. Positive identification of HBsAg by immunofluorescence or immunoperoxidase methods was taken as the sole criterion of a hepatitis B viral aetiology.

Sections from each case were stained by periodic acid Schiff (PAS) after diastase digestion, and examined for typical α_1 -antitrypsin bodies in the periportal hepatocytes. Sections from each case were also examined by indirect immunofluorescence using anti- α_1 -antitrypsin (Behringwerke), fluorescein conjugate (Behringwerke), and Ploem incident illumination (Leitz) for the identification of α_1 -antitrypsin bodies. In addition, sections from 15 cases were examined by the peroxidase-antiperoxidase technique, using Behringwerke and Dakopatts sera (Blenkinsopp and Haffenden, 1977). Positive identification by immunofluorescence or immunoperoxidase methods was taken as the sole criterion of α_1 -antitrypsin deficiency.

The clinical records were examined for evidence of alcoholism or primary biliary cirrhosis, and laboratory records for results of serological testing for HBsAg and serum levels of α_1 -antitrypsin. The diagnosis of primary biliary cirrhosis was made on the history, supported by the presence of mitochondrial antibodies, appropriate results for serum alkaline phosphatase and serum bilirubin, and compatible liver biopsy.

Results

The results are shown in Tables 1-4. The single case of haemochromatosis was HBsAg positive and has been included in that category. Some cases gave more than one positive result, and these are shown in the tables.

The 20 cases of cirrhosis labelled alcoholic all showed acute alcoholic hepatitis, and 17 showed much fat; this degree of fatty change was not seen in the other cases of cirrhosis. Twenty-three cases of cirrhosis had a clinical diagnosis of an alcoholic aetiology: 14 of these were confirmed on histology, but nine had neither acute alcoholic hepatitis nor a marked degree of fatty change; one of the nine was HBsAg positive and the other eight gave no positive results.

Immunofluorescence for HBsAg was done on 54 cases, with 12 positive results, and immunoperoxidase (16 indirect, 12 peroxidase-antiperoxidase) was done on 28 cases, with six positive results. Orcein and aldehyde fuchsin stains were done on all cases:

Table 1 *Aetiology of cirrhosis*

	No. of cases	Alcohol	HBsAg	α_1 -antitrypsin	Primary biliary cirrhosis	No agent found
Total	69	20 (29%)	17 (25%)	11 (16%)	3 (4%)	25 (36%)
Male	49	13 (27%)	16 (33%)	11 (22%)	1 (2%)	15 (31%)
Female	20	7 (35%)	1 (5%)	0 (0%)	2 (10%)	10 (50%)

1 HBsAg case had haemochromatosis; 4 cases had both HBsAg and α_1 -antitrypsin bodies; 3 cases had both HBsAg and acute alcoholic hepatitis.

Table 2 *Aetiology of hepatic fibrosis: all cases male*

No. of cases	Alcohol	HBsAg	α_1 -antitrypsin	No agent found
9	0	1	4	4

Table 3 *Aetiology of hepatocellular carcinoma*

	No. of hepatomas	Alcohol	HBsAg	α_1 -antitrypsin	No agent found
With cirrhosis	14	2	4	4	6
With fibrosis	1	0	0	0	1
With neither	4	0	0	1	3

Two cases were female: one with α_1 -antitrypsin bodies and one cryptogenic. One male case was positive for both HBsAg and alcohol, and one for both HBsAg and α_1 -antitrypsin bodies.

Table 4 *Copper staining in cirrhosis*

	Alcohol	HBsAg	α_1 -antitrypsin	Primary biliary cirrhosis	No agent found
No copper	17	12	6	0	22
Moderate	3	4	4	3	1
Much	0	1	1	0	2

of the 18 cases positive on immunohistology, 10 were positive with both stains, three with orcein only, four with aldehyde fuchsin only, and one with neither. In five cases the orcein (3) and/or aldehyde fuchsin (4) was positive with the usual pattern of diffuse staining of part of the cell cytoplasm, but immunofluorescence was negative: the immunofluorescence was therefore repeated on new sections from these cases and all five were negative. None of these five cases had had serum tested for HBsAg. Sera from 10 of the cirrhosis cases had been tested for HBsAg, and all 10 were negative: these 10 were also negative on immunohistology for HBsAg. A membrane pattern of fluorescence was found in several cases: the intensity varied between barely perceptible and prominent, and invariably a control section from the same block stained in the same way except for the omission of anti-HBsAg showed the same pattern of fluorescence with the same intensity.

This membrane fluorescence was therefore classified as not being due to HBsAg. Membrane staining was not seen with orcein or aldehyde fuchsin.

As reported elsewhere (Blenkinsopp and Haffenden, 1977), the PAS proved satisfactory in identifying α_1 -antitrypsin bodies and gave essentially the same results as immunohistology. Serum levels of α_1 -antitrypsin were available on only two of these cases: there were no α_1 -antitrypsin bodies in one case with a normal level, and bodies were present in one case with a low level (1.7 g/l).

In 15 cases of hepatocellular carcinoma HBsAg was not found in either the neoplastic or the non-neoplastic liver; in two cases it was present in the neoplastic cells only, and in two cases in the non-neoplastic liver only; in no case was it found in both the neoplastic and non-neoplastic cells. The five cases of hepatocellular carcinoma associated with α_1 -antitrypsin bodies showed the bodies in the non-neoplastic hepatocytes only.

The four cases of cirrhosis with much copper showed cholestasis, which was severe in three. None of these was identified as primary biliary cirrhosis; there were no known cases of hepatolenticular degeneration in the series, and although the copper level approached that seen in hepatolenticular degeneration, review of these four cases provided no further evidence of this disease. The 13 cases of cirrhosis with moderate copper included the three cases of primary biliary cirrhosis: cholestasis was severe in seven, slight in four, and absent in two. There was no relationship between copper staining and aetiology (Table 4).

Activity (piecemeal necrosis) was present in 20 of the 69 cases of cirrhosis and in two of the nine cases of fibrosis. In cirrhosis there was no significant difference in the proportion of cases which were active in each aetiological group (alcohol 3/20, HBsAg 7/17, α_1 -antitrypsin 4/11, primary biliary cirrhosis 1/3, and cryptogenic 7/25).

Discussion

Table 1 shows a sex difference in the aetiology of cirrhosis. In women, half the cases were cryptogenic, and 35% were alcoholic. In men, 30% were cryptogenic, 33% were associated with HBsAg, 26% with alcohol, and 22% with α_1 -antitrypsin bodies; 15% of the male cases had two aetiological factors identified.

Alcohol was the commonest association identified in this study—present in 29% of cirrhotics. This proportion is considerably less than that found in the most recent report from the London area (Hodgson and Thompson, 1976), in which 65% of 78 cases of cirrhosis were attributed to alcohol on

historical grounds. Unfortunately, consumption of alcohol over a five-year period is difficult to estimate with confidence, and alcoholics are notoriously unreliable historians. While the morphological criterion used in the present study to identify an alcoholic aetiology may underestimate the number of alcoholic cirrhotics, as Brunt *et al.* (1974) found alcoholic hepatitis in only 41 of 75 cases of alcoholic cirrhosis, we consider that the usual historical criterion is likely to overestimate that proportion of cirrhosis which is alcoholic. The histological diagnosis of acute alcoholic hepatitis was made on the finding of small foci of liver cell swelling and necrosis, usually with Mallory's hyaline in the damaged cells, with surrounding infiltration by neutrophils (Brunt *et al.*, 1974). This appearance is considered pathognomonic for alcohol (Rubin, 1973), provided Indian childhood cirrhosis (Nayak and Ramalingaswami, 1975) and jejunoileal bypass for obesity (Galambos, 1976) are excluded. The association between alcohol and fatty liver is well established, and in the series of Christoffersen and Nielsen (1971) 27 of 38 alcoholic cirrhotics had moderate or severe fatty change, compared with 17 of 20 in the present study. Severe fatty change was found only in alcoholic cirrhosis in this series.

HBsAg was identified in 25% of the 69 cases of cirrhosis. In London at a specialist centre 58% of 65 cases of cirrhosis had HBsAg in the serum (Portmann *et al.*, 1976), in Belgium 49% of 41 cases (van Waes *et al.*, 1974), and in Iraq 58% of 64 cases (Boxall *et al.*, 1976). In London HBsAg was identified in the serum of 0.3% of 1489 control hospital patients by the relatively insensitive immunodiffusion method (Medical Research Council, 1974). In most studies the identification of HBsAg by serology has produced more positive results than by histology, even after exclusion of cases of acute hepatitis, in which seropositive cases are rarely histologically positive. Portmann *et al.* (1976) found no positive results by orcein staining or immunofluorescence in 46 seronegative 'chronic' cases, and only 89% of 91 seropositive 'chronic' cases were histologically positive. Deodhar *et al.* (1975) found no orcein staining in 26 seronegative 'chronic' livers, and only 67% of 51 seropositive 'chronic' livers were orcein positive. Gerber *et al.* (1975) found that 41% of 74 seropositive 'chronic' livers were aldehyde fuchsin positive and immunofluorescence positive. Both Portmann *et al.* (1976) and Gerber *et al.* (1975) observed that seropositive carriers had a 100% histology positive rate, whereas seropositive cases with chronic hepatitis were histologically positive in 85% and 27% respectively. However, Ray *et al.* (1976b) have reported identification of HBsAg by immunofluorescence on frozen sections of liver not

only in all of 40 seropositive cases of chronic hepatitis but also in 12 of 36 seronegative cases.

Portmann *et al.* (1976) found that the number of positive identifications of HBsAg on sections of liver was the same by orcein staining as by immunofluorescence. On the other hand, Nayak and Sachdeva (1975) observed more positive results with orcein staining than with immunofluorescence or immunoperoxidase; their conclusion that the excess orcein positives were due to HBsAg may be questioned in the absence of serological identification of HBsAg. In the present study the two non-immunological staining methods—orcein and aldehyde fuchsin—gave both false positive and false negative results as compared with immunofluorescence and immunoperoxidase identification of HBsAg, and it is therefore difficult to support their continued use when the immunohistological methods are available.

Cell membrane immunofluorescence identification of HBsAg has been described by Ray *et al.* (1976c) and Roos *et al.* (1976), in addition to the usual cytoplasmic fluorescence. In both these series membrane fluorescence alone made up a significant proportion of the positive cases. This membrane pattern was not noted by Portmann *et al.* (1976) or Gerber *et al.* (1975). In the present study no true membrane pattern of HBsAg was found. The reason for this discrepancy has not been established; the major difference in technique is the use of frozen sections in the three studies in which a membrane pattern has been reported. It is of interest that Ray *et al.* (1976a) have recently reported identification of hepatitis B core antigen in all of 38 biopsies with a membrane pattern of HBsAg.

The two common subtypes in Europe of HBsAg are *ad* and *ay*, and in chronic liver disease in England each of these subtypes is found in about half of the cases associated with HBsAg (Green and Turner, 1975). The manufacturers (Wellcome) of the anti-HBsAg used for immunofluorescence in the present study confirmed that this anti-HBsAg was produced by injection of both subtypes, and that antibodies to both subtypes were present in the antiserum.

Alpha-1-antitrypsin bodies in the hepatocytes probably occur almost exclusively in persons with the *Pi^z* allele, though one exception is recorded in a patient with the normal phenotype *Pi^M* (Bradfield and Blenkinsopp, 1977), and probably all persons with one or two *Pi^z* alleles have the characteristic bodies in the liver (Blenkinsopp and Haffenden, 1977). About 3.2% of the population possess the *Pi^z* allele in England and Wales (Cook, 1974b), and the frequency of occurrence of α_1 -antitrypsin bodies in cirrhosis in the present study (11/69) is significantly greater than in the population at large. Eriksson *et al.* (1975) also found an excess of cirrhotics

among patients with α_1 -antitrypsin bodies, although Morin *et al.* (1975) found no difference between 394 blood donors, 132 alcoholic cirrhotics, and 37 cryptogenic cirrhotics in the frequency of the *Pi^z* allele.

There were 49 cases of hepatocellular carcinoma among the 520 cirrhotics reported by MacSween and Scott (1973), and 11 cases in the 51 cirrhotics reported by Portmann *et al.* (1976). In the present study there were 14 cases in 69 cirrhotics; there was no significant difference in the frequency of carcinoma between the different aetiological groups (2/20 alcoholic, 4/17 HBsAg, 4/11 α_1 -antitrypsin, and 6/25 cryptogenic). The association of hepatocellular carcinoma with alcoholic cirrhosis (Levy *et al.*, 1964) and with HBsAg positive cirrhosis (Portmann *et al.*, 1976) is well known, and although we are not aware of any previous report of the frequency at necropsy of the carcinoma in cirrhosis associated with α_1 -antitrypsin deficiency, Berg and Eriksson (1972) reported finding the bodies in 7 of 78 cases of primary liver carcinoma, and Palmer and Wolfe (1976) found the bodies in 4 of 17 cases. HBsAg has been identified in both the neoplastic and non-neoplastic liver cells in cases of primary liver carcinoma (Nayak and Sachdeva, 1975) as in the present study.

High copper levels are found in the liver in hepatolenticular degeneration, primary biliary cirrhosis, and long-standing bile duct obstruction (Fleming *et al.*, 1974), and although the rubeanic acid technique is relatively insensitive it usually demonstrates high levels provided the copper is in lysosomes, as it is in the later stages (Sternlieb, 1972). About 6% of patients with hepatolenticular degeneration present with chronic hepatitis (Sternlieb and Scheinberg, 1972), and hepatolenticular degeneration has been found in 1 of 54 cases of active chronic hepatitis (Cook *et al.*, 1971) in one series and in 1 of 23 cases in another (Page *et al.*, 1969), but there was no evidence apart from the copper that the four cases with heavy copper staining in the present study were of hepatolenticular degeneration. The demonstration of copper in other cirrhotoses suggests that staining for copper is not of diagnostic value.

The overlap of positive associations found in the present series of cirrhotics (4 cases positive for both HBsAg and α_1 -antitrypsin bodies, and 3 cases positive for both HBsAg and alcohol) is not described in most other reports, perhaps because most recent studies have concentrated on one particular aetiological factor. Although the experimental production of cirrhosis in baboons by alcohol (Lieber, 1975) indicates that alcohol alone can produce cirrhosis, cirrhosis develops in only about

20% of alcoholics, and in Leibach's study (1975) only 14% of patients with a mean daily ethanol intake of 227 g (1 bottle of whisky) over a mean period of eight years had cirrhosis. The possibility that in some cases of human cirrhosis alcohol is a predisposing background on which other factors such as hepatitis -B infection produce cirrhosis receives some support from this overlap. Perhaps a similar situation might apply to α_1 -antitrypsin deficiency in adults. In this study the frequency of occurrence of HBsAg in cirrhotic livers did not differ significantly between the three groups—alcoholic (3/20), α_1 -antitrypsin (4/11), and the remainder excluding haemochromatosis and primary biliary cirrhosis (9/34).

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