Alpha-1-antitrypsin bodies in the liver

W. K. BLENKINSOPP AND G. P. HAFFENDEN

From the Department of Histopathology, St. Mary's Hospital, London W2

SUMMARY The cytoplasmic bodies in hepatocytes thought to indicate possession of the Z allele for α_1 -antitrypsin deficiency were found at necropsy in 10 of 64 adults with cirrhosis, four of nine with hepatic fibrosis, and four of 15 with hepatocellular carcinoma. They were also found in six of 76 adults with severe panacinar emphysema, and in four of a control series of 110 adults with neither emphysema nor liver disease. The association of the bodies with each of the three liver diseases was statistically significant, but the association of the bodies with emphysema was not. It is considered probable that heterozygous (PiMZ) α_1 -antitrypsin deficiency is associated with an increased incidence of cirrhosis, hepatic fibrosis, and hepatocellular carcinoma.

Alpha-1-antitrypsin deficiency is associated with chronic liver disease in both children and adults, and with emphysema in adults. The deficiency can be identified by estimation of serum antitryptic activity, although antitrypsin is an acute phase reactant, and levels within the normal range occur in both heterozygous and homozygous deficiency (Triger *et al.*, 1976), or by serum analysis of phenotype, although this is technically difficult. The normal phenotype is designated Pi (protease inhibitor) MM, and the commonest allele associated with deficiency is Z. Subjects homozygous or heterozygous for the Z allele have characteristic globules (α_1 -antitrypsin bodies) in their hepatocytes and can thereby be identified (Martin *et al.*, 1976). These globules are periodic acid Schiff positive and diastase resistant, and can also be shown by immunofluorescence or immunoperoxidase techniques on formalin-fixed, paraffin-embedded sections using an antibody to α_1 -antitrypsin.

This paper describes the frequency of occurrence of α_1 -antitrypsin bodies in the liver in three groups of adults at necropsy: those with severe emphysema, those with chronic liver disease (cirrhosis, fibrosis or hepatoma), and those with neither (controls).

Material and methods

Necropsy records for the 15-year period 1961-75 (total 4895) were searched for cases of severe panacinar emphysema: 98 were found, and in 76 cases a block of liver tissue was available. The same

records were examined for cases of hepatic fibrosis, hepatoma, and cirrhosis other than that due to large duct obstruction: 64 cirrhotic livers (11 with hepatocellular carcinoma), nine fibrotic livers (1 with hepatocellular carcinoma), and three livers with hepatocellular carcinoma but neither cirrhosis nor fibrosis were found; blocks of non-neoplastic liver were available from all cases (76), and blocks of hepatocellular carcinoma from the 15 cases with hepatocellular carcinoma. The control series consisted of blocks of liver from 110 consecutive adult necropsies in which there was neither clinical nor pathological evidence of liver disease or emphysema.

All the liver blocks were formalin-fixed and paraffin-embedded. Sections were cut at 4 μ m: one section from each case was stained with haematoxylin and eosin, one for reticulin, and one with periodic acid Schiff after diastase. The diagnosis on the liver was checked and confirmed in each case. The characteristic α_1 -antitrypsin bodies were not stained in the haematoxylin and eosin preparation but were seen as rounded, sharply-defined, solid bodies staining strongly with periodic acid Schiff after diastase, in the cytoplasm of hepatocytes, predominantly in the periportal cells. Bodies up to at least 3 μ m diameter were found in each positive case, and frequently bodies up to 15 μ m diameter were present. In addition, sections were cut at 4 μ m from the liver blocks of all cases with cirrhosis, hepatic fibrosis or hepatocellular carcinoma, of all cases showing α_1 -antitrypsin bodies on periodic acid Schiff staining, and of 27 cases of emphysema randomly selected from those without α_1 -antitrypsin bodies on periodic acid Schiff staining: these were mounted on albumin-

ised slides and immunofluorescence for α_1 -antitrypsin was done on at least one slide from each case.

IMMUNOFLUORESCENCE

Sections were dewaxed and pre-washed in phosphate buffered saline pH 7.2 (PBS). Using an indirect sandwich technique the sections were first covered with rabbit anti-human α_1 -antitrypsin (Behringwerke) optimally diluted in PBS, then washed in three changes of PBS over 30 minutes. The sections were then covered with fluorescein-conjugated goat anti-rabbit IgG (Behringwerke) optimally diluted in PBS, and washed in three changes of PBS over 30 minutes, after which they were mounted in phosphate buffered glycerol. The slides were examined using a Leitz Dialux microscope with Ploem incident illumination from a 50-watt ultra-high-pressure mercury lamp. The filter system comprised: exciter $2 \times$ KP 490, dichroic mirror TK 510, suppression K 515, and edge filter GG 455.

In nine cases the immunofluorescence technique gave equivocal results, and sections from these cases, from four known positive cases, and from two known negative cases were examined by the peroxidase-antiperoxidase technique using a modification of the method of Burns (1975).

IMMUNOPEROXIDASE

Dewaxed sections were treated with 10% hydrogen peroxide in absolute methanol for 30 minutes to block endogenous peroxidase staining, then washed in PBS for 30 minutes. Sections were then covered in turn with rabbit anti-human α_1 -antitrypsin (Behringwerke) optimally diluted in PBS, swine anti-rabbit IgG (Dako), and rabbit peroxidase-antiperoxidase (Dako). After each stage the sections were washed in three changes of PBS over 15 minutes. The peroxidase was demonstrated with 4-chlor-1-naphthol in a modified method of Nakane (1968). The substrate was freshly prepared by dissolving 20 mg 4-chlor-1-naphthol in 0.3 ml absolute ethanol, mixing with 50 ml PBS, filtering off the white precipitate formed, and adding 0.05 ml 30% hydrogen peroxide to the filtrate. The sections were stained in this solution for 10-20 minutes, when the peroxidase positive structures were a distinct blue-grey colour. After being washed in distilled water for

5 minutes the sections were mounted in von Apathy's aqueous mounting medium.

Results

Twenty livers showed α_1 -antitrypsin bodies on periodic acid Schiff staining, and two were equivocal (scanty bodies up to 3 μ m diameter in cirrhotic livers with marked cholestasis): all 22 were positive on immunofluorescence or immunoperoxidase. Of the 15 cases examined by immunoperoxidase, the four known positives were positive by this technique, the two known negatives were negative, and two of the nine equivocal cases were positive. Immunofluorescence and immunoperoxidase techniques produced no positive results in livers which were negative on periodic acid Schiff staining. The results are shown in the Table.

In the 110 control cases α_1 -antitrypsin bodies were found in four.

In the 76 cases of emphysema six showed α_1 -antitrypsin bodies, including two of four cases with emphysema and cirrhosis and one of four cases with emphysema and fibrosis. Comparison of the frequency of bodies in emphysema (6/76) with that in controls (4/110) gave $0.30 > P > 0.20$ (χ^2 test). When the emphysema cases with cirrhosis or fibrosis were excluded, the comparison gave $0.50 > P > 0.40$.

In the 64 cases of cirrhosis, including four with emphysema, 10 had α_1 -antitrypsin bodies. Comparison of the frequency of bodies in cirrhosis (10/64) with that in controls (4/110) gave $0.01 > P > 0.005$. The activity of the cirrhosis was assessed using the customary criteria for chronic active and chronic persistent hepatitis: three of 10 cases with bodies were active, compared with 15 of 54 without bodies (no significant difference). The pattern of cirrhosis was determined using the 1 mm diameter criterion (Scheuer, 1973): seven of the 10 cases with bodies were macronodular, compared with 38 of the 54 without bodies (no significant difference); the remaining cases were of mixed type, except for one micronodular. The hospital records were examined to identify which of the cases of cirrhosis had clinical evidence of chronic severe liver disease: seven of the 10 cases with bodies had clinical cirrhosis, compared

76 abnormal liver (15 + ve):	64 cirrhosis (10 + ve) 9 fibrosis (4 + ve) 3 hepatocellular carcinoma without cirrhosis or fibrosis (1 + ve)	11 with hepatocellular carcinoma (3 + ve) 1 with hepatocellular carcinoma (0 + ve)
76 emphysema (6 + ve): including 4 with cirrhosis (2 + ve) 4 with hepatic fibrosis (1 + ve)		
110 controls (4 + ve)		

Table Number of cases in each group, and frequency of positive finding of α_1 antitrypsin bodies

with 41 of the 54 without bodies (no significant difference).

In the nine cases of fibrosis, including four with emphysema, four showed α_1 -antitrypsin bodies. Comparison of the frequency of bodies in fibrosis (4/9) with that in controls (4/110) gave $P < 0.001$. Two of the nine cases showed 'active chronic hepatitis'—both had α_1 -antitrypsin bodies. Two of the nine cases had clinical cirrhosis; both were 'inactive' and both showed no bodies.

In the 15 cases of hepatocellular carcinoma no bodies were found in neoplastic cells but the non-neoplastic liver contained α_1 -antitrypsin bodies in four cases, three of the 11 cases associated with cirrhosis and one of the three cases associated with neither cirrhosis nor fibrosis. Comparison of the frequency of bodies in livers with hepatocellular carcinoma (4/15) with that in controls (4/110) gave $P < 0.001$. Of the 11 hepatocellular carcinomas found in cirrhotic livers, three occurred in the 10 cases with bodies, and eight in the 54 cases without bodies: comparison of the frequency of hepatocellular carcinoma in cirrhotic livers with bodies (3/10) with that in cirrhotic livers without bodies (8/54) gave $0.30 > P > 0.20$.

Discussion

The frequency of Pi types in the population of England and Wales is MM 86%, MS 9%, MZ 3%, SS 0.25%, SZ 0.2%, and ZZ 0.029% (Cook, 1974); about 3.2% of the population therefore possess the Z allele. A similar frequency occurs in Sweden, and Eriksson *et al.* (1975) found periodic acid Schiff positive diastase resistant bodies in 3.7% of 700 consecutive necropsies. Alpha-1-antitrypsin bodies in the liver probably occur only in persons with the Z allele (Martin *et al.*, 1976), although one apparent exception is recorded in a patient with emphysema and the phenotype PiSS (Lieberman *et al.*, 1972). The bodies have been reported in PiMZ (Rawlings *et al.*, 1974), PiSZ (Campra *et al.*, 1973; Craig *et al.*, 1975), and PiPZ (Crawford *et al.*, 1974) phenotypes, and their absence has been noted in Pi-- (Feldmann *et al.*, 1975) and in S phenotypes without Z (Gordon *et al.*, 1972). The present observation of bodies in 3.6% of 110 controls supports the suggestion of Eriksson *et al.* (1975) that probably all persons with one or two Z alleles have the characteristic bodies in the liver.

There is general agreement that the PiZZ phenotype is associated with an increased risk of emphysema, but it is still uncertain whether other abnormal phenotypes—in particular PiMZ—carry an increased risk (British Medical Journal, 1973; Sharp, 1976). In patients with emphysema the frequency of inter-

mediate deficiency of α_1 -antitrypsin, ie, largely PiMZ phenotype, has been reported as normal (Talamo, 1972) and as increased (Kueppers *et al.*, 1969; Lieberman, 1973). Eriksson *et al.* (1975) found 13 cases of emphysema in 26 necropsies with α_1 -antitrypsin bodies in the liver, whereas in 100 necropsies without α_1 -antitrypsin bodies there were 18 cases of emphysema; these figures suggest that about 10% of cases of emphysema had α_1 -antitrypsin bodies. In the present study, 7.9% of emphysematous patients had α_1 -antitrypsin bodies compared with 3.6% of controls, but the difference was not statistically significant.

The phenotype PiZZ is associated with a high risk of adult liver disease (Berg and Eriksson, 1972) but there is no general agreement on whether phenotypes associated with intermediate deficiency carry a similar risk. Morin *et al.* (1975) determined the frequency of various alleles in 394 blood donors, 132 alcoholic cirrhotics, and 37 cryptogenic cirrhotics and found no difference between these groups in the frequency of heterozygous Z allele or in the frequency of the phenotype MZ. However, Eriksson *et al.* (1975) found three cases of cirrhosis and three of hepatic fibrosis in 26 necropsies with α_1 -antitrypsin bodies in the liver, compared with one cirrhosis and one fibrosis in 100 controls: the difference between the two groups was significant, and although the numbers are small the figures suggest the possibility that 30% of cases of cirrhosis and 30% of cases of fibrosis are associated with PiMZ phenotype. The authors considered that the bodies indicated PiMZ phenotype because this is the only phenotype containing a Z allele that occurs with a frequency of about 3.7% in the population (the frequency with which bodies were found in 700 consecutive necropsies); in particular, the 700 necropsies would be expected to include only 0.5 individuals with PiZZ phenotype. In nine of the 26 cases with bodies the heterozygous Z status was confirmed by phenotyping or by agarose gel electrophoresis.

In the present study α_1 -antitrypsin bodies were found in 10 of 64 cases of cirrhosis and in four of nine cases of hepatic fibrosis, both being significantly different from the control frequency. Neither phenotyping nor serum antitrypsin levels were available on these subjects, but as in the material of Eriksson *et al.* (1975) it seems probable that most if not all of the cases with α_1 -antitrypsin bodies were PiMZ; the frequency of bodies in the control series (4/110) is close to the frequency of PiMZ in the population (3%), and the 4895 necropsies from which the cases were selected would be expected to include only 1.5 individuals with PiZZ phenotype. The proportion of cases of cirrhosis with presumed PiMZ phenotype seems surprisingly high compared with liver biopsy

experience. It has been suggested that the adult type of liver damage associated with α_1 -antitrypsin deficiency is commonly not associated with clinical liver disease, but in this series the same proportion (7/10) of cases with bodies as of cases without bodies (41/54) had clinical cirrhosis. There are two other possible explanations: first, the bodies are not evenly distributed and needle biopsy produces a sampling error, and second, patients with cirrhosis and α_1 -antitrypsin deficiency may be less likely to have a liver biopsy. One possible reason for the second explanation would be that the deficient cirrhotics are older, but in this study the mean age (62.9 years) of the deficient cirrhotics at death was not significantly different from the mean age (61.7 years) of the non-deficient cirrhotics (Student *t* test). With regard to the sampling error, Palmer *et al.* (1973) examined three cases of α_1 -antitrypsin deficiency and found numerous bodies around every portal tract in one case, around most portal tracts in one case, and fewer bodies around about 10% of the portal tracts in the third case. The density and distribution of the bodies were assessed in the present series, and divided into three groups: four cases had numerous bodies in most periportal liver cells round most of the portal tracts, 13 cases had bodies in about half the periportal liver cells round about half the portal tracts, and five cases had bodies in less than one-quarter of the periportal liver cells round less than one-quarter of the portal tracts. These figures support the suggestion of an appreciable sampling error on needle biopsy sections.

The mechanism of production of liver disease in α_1 -antitrypsin deficiency is at present unknown, and no relationship to either alcohol or hepatitis B infection has been established (Sharp, 1976). In the 13 cases of Triger *et al.* (1976) there was no evidence of drug abuse. All were negative for hepatitis B surface antigen in the serum, and the 12 with liver tissue available were all negative on Shikata orcein staining for hepatitis B; four cases had a history of alcoholism, but none had histological evidence of alcoholic damage. In the present series of 15 cases with liver pathology, none had a history or histological evidence of alcohol; none had a recorded history of previous hepatitis, but four cases showed positive staining for hepatitis B surface antigen on immunofluorescence, Shikata orcein, and Gomori's aldehyde fuchsin staining. The details of these investigations will form part of a further report.

Hepatocellular carcinoma occurs commonly in patients with PiZZ phenotype and cirrhosis or hepatic fibrosis (Berg and Eriksson, 1972) and has been reported in patients with PiMZ phenotype (Rawlings *et al.*, 1974; Lieberman *et al.*, 1975). Norkin and Campagna-Pinto (1968) described globular hyaline

inclusions in eight of 81 cases of hepatoma; these were periodic acid Schiff positive, but they had two characteristics which α_1 -antitrypsin bodies do not: they were visible as hyaline masses in haematoxylin and eosin preparations, and they were extracellular as well as intracellular. They therefore cannot be regarded as α_1 -antitrypsin bodies. Berg and Eriksson (1972) mentioned that in a preliminary review of 78 cases of primary liver carcinoma seen at necropsy in Lund between 1952 and 1971 periodic acid Schiff positive material with the characteristic morphology of α_1 -antitrypsin bodies was seen in seven. Palmer and Wolfe (1976) examined 17 liver cell carcinomas for α_1 -antitrypsin bodies by periodic acid Schiff staining after diastase and by immunoperoxidase; in the non-neoplastic liver they found bodies by both methods in four cases, and by periodic acid Schiff only in one case. This incidence is similar to that found in the present study, in which α_1 -antitrypsin bodies were found in four of 15 cases of hepatocellular carcinoma compared with four of 110 controls, and this difference was significant ($P < 0.001$). The frequency of hepatocellular carcinoma in cirrhosis was examined; it was found in eight of 54 cases of cirrhosis without α_1 -antitrypsin bodies, compared with three of 10 cases of cirrhosis with bodies: the two frequencies are not significantly different, which suggests that the apparent increased liability of the α_1 -antitrypsin deficient phenotype to hepatoma may be mediated through an increased liability to cirrhosis rather than directly. The number of cases with hepatocellular carcinoma without cirrhosis is too small to be helpful in answering this question (the one in the nine cases of fibrosis was not associated with bodies, and one of the three with neither cirrhosis nor fibrosis was associated with bodies). In Palmer and Wolfe's (1976) 17 cases of liver cell carcinoma, cirrhosis was present in three of five cases with α_1 -antitrypsin bodies in the non-neoplastic liver, and in nine of 11 cases without bodies (no significant difference).

In many of the reported cases of hepatocellular carcinoma in α_1 -antitrypsin deficiency the presence or absence of bodies in the neoplastic cells has not been specified but in 10 cases (Eriksson and Hägerstrand, 1974; Rawlings *et al.*, 1974; present study) bodies were not found in the neoplastic cells. However, in two reports the bodies have been identified in the neoplastic cells of hepatocellular carcinomas—in a single case (Lieberman *et al.*, 1975), and in 10 of the 17 liver cell carcinomas described by Palmer and Wolfe (1976) (in eight cases by both periodic acid Schiff and immunoperoxidase, and in two cases by each method alone).

We consider that the periodic acid Schiff after diastase is an excellent screening method for identify-

ing α_1 -antitrypsin bodies, as we found neither false negative nor false positive results. Identification depends on typical morphology, as other periodic acid Schiff positive diastase resistant material is often present in livers. Residual glycogen due to inadequate diastase treatment can be distinguished by the smaller size (up to $1.5\ \mu\text{m}$ diameter) and poorer definition of the particles. Ceroid in macrophages lacks the typical morphology, and the pale cytoplasmic globules up to $15\ \mu\text{m}$ diameter commonly seen in dying liver cells are readily distinguished by their pale staining with both periodic acid Schiff and eosin and their predominantly centrilobular distribution. Mucin at the luminal end of cholangiolar cells can cause confusion if the lumen is not seen in the plane of the section, but the particles are less than $3\ \mu\text{m}$ diameter and the cells can be distinguished from hepatocytes by their smaller size and nuclear crowding. The main difficulty arises when there is periportal cholestasis, as the brown coloration of the rounded bodies formed may be so slight as to require close scrutiny for its detection. These bodies have been described in detail by Popper *et al.* (1960) and Biava (1965). Trigger *et al.* (1976) and Millward-Sadler *et al.* (1975) referred to two patients whose livers showed bodies with the typical morphology of α_1 -antitrypsin bodies, but giving negative results on immunological testing; both patients had normal serum levels of antitrypsin and normal phenotypes. We found no examples of this.

In order to select the most satisfactory immunological technique we carried out a trial of indirect immunofluorescence, indirect immunoperoxidase, and peroxidase-antiperoxidase methods. Unlike Trigger *et al.* (1976), we considered the indirect peroxidase method to be inferior to the others. Our trial also showed a difficulty apparently not experienced by others: abnormal livers contain a large amount of brown pigment (lipofuscin, ceroid, and bile pigments) and we found it difficult to distinguish with confidence between these and the brown colour produced by the diamino-benzidine customarily used to demonstrate the peroxidase. We therefore developed the 4-chlor-1-naphthol technique which produces a blue-grey colour. This reaction product is lost in the usual mounting media such as DPX but appears to be permanent in von Apathy's aqueous mountant. Although von Apathy's medium produces less satisfactory definition than DPX, the method does avoid confusion with endogenous brown pigments.

Morin *et al.* (1975) concluded that the association between heterozygous α_1 -antitrypsin deficiency and cirrhosis was fortuitous. The evidence presented here provides grounds for questioning that conclusion. The figures suggest that in 4895 necropsies

about 190 adults had heterozygous (PiMZ) α_1 -antitrypsin deficiency, and of these about 4% had severe emphysema, 5% had cirrhosis, 2% had hepatic fibrosis, and 2% had hepatocellular carcinoma. These figures are not compatible with the view that heterozygous α_1 -antitrypsin deficiency has little or no clinical significance.

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