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Hepatotoxicity of ethanol in mice

R.D. Goldin and S.N. Wickramasinghe
Departments of Experimental Pathology and Haematology, St Mary's Hospital Medical School, University of London, UK

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Summary. Mice continuously exposed to ethanol vapour (for up to 19 days) developed fatty change in the liver (from 2 days onwards) and lesions resembling those of alcoholic hepatitis in man (from 5 days onwards). They also showed biochemical evidence of liver cell damage. Sera from ethanol-treated animals contained immunoglobulins that bound to the hepatocytes of ethanol-treated but not of control animals suggesting that exposure to ethanol was followed by an immunological response to a hepatocyte neo-antigen. In addition, such sera were cytotoxic in an in-vitro assay, and on Sephacryl S-300 gel filtration the cytotoxic activity eluted together with albumin molecules.

Keywords: alcoholic hepatitis, ethanol, fatty change, mice

Alcohol-induced liver disease is an increasingly important cause of morbidity and mortality in the Western world. Despite a considerable amount of work much remains to be learnt about the pathogenesis of the liver injury observed. One of the reasons for this is that the animal models, such as the alcohol-fed baboon, in which significant liver damage has been reported are not suitable for routine laboratory use (Lieber *et al.* 1975). Comparatively recently, exposure of mice to ethanol vapour has been used to study the effect of alcohol on a number of organ systems such as the central nervous system (Griffiths *et al.* 1974) and the bone marrow (Malik & Wickramasinghe 1987). In this paper we describe the effects of the inhalation of ethanol vapour on the structure and function of the mouse liver. In addition, we present evidence that two previously-discussed potential mechanisms of alcohol-induced liver damage in man may also operate in the mouse.

Materials and methods

Animals. C57BL female mice aged between 10 and 25 weeks were used.

Administration of ethanol. Ethanol was administered to mice housed in conventional cages by placing the latter within specially constructed perspex inhalation chambers through which a mixture of air and ethanol was pumped continuously as described by Griffiths *et al.* (1974). Further details of how the animals were maintained are provided by Malik and Wickramasinghe (1987). The mice were exposed to ethanol for up to 19 days. The ethanol concentration in the inhaled air was maintained between 12.6 mg/l and 15.2 mg/l. The blood alcohol levels were between 40 mg/dl and 160 mg/dl.

Collection of blood and tissue samples. Female mice exposed to ethanol vapour for various periods and age-matched control female

mice, kept in ethanol-free but otherwise similar conditions, were killed with ether. Blood was obtained by cardiac puncture; 100 μl was placed in a tightly-closed container for the determination of blood alcohol levels and the remainder allowed to clot. The serum was separated from the clotted blood and stored at −20°C. Several organs (liver, lung, kidney, spleen, heart and pancreas) were removed and their histology studied as described below.

Histological studies. Tissues were fixed in buffered formol saline. They were then processed to paraffin wax and sectioned. The sections of liver were stained with iron haematoxylin and eosin (H & E), periodic-acid-Schiff (PAS), iron-van-Gieson and Gordon and Sweet reticulin impregnation. Those of other tissues were stained only with H & E. In addition, samples of liver were snap frozen and stored in liquid nitrogen. Frozen sections were cut and either stained for fat with Oil Red O or used for immunochemical studies.

Immunohistochemical studies. Serum, from control or test animals, was applied to cryostat sections of snap frozen liver, from control and test animals, to see if there would be binding of immunoglobulin. Binding was investigated using peroxidase-labelled swine anti-mouse immunoglobulin. The sections were incubated with 1/20 mouse serum (diluted in normal swine serum) for 30 min. They were then washed for 15 min in Tris-buffered saline (TBS) which was made up by diluting a stock solution of Tris (1000 ml 0.1 M Tris and 800 ml 0.1 M hydrochloric acid with the pH adjusted to 7.6) 1/10 with 0.9% sodium chloride. The sections were then incubated with 1/40 peroxidase-labelled swine anti-mouse immunoglobulin for 30 min, washed in TBS for 15 min and developed with diaminobenzidine (DAB). The DAB reagent was made up fresh: DAB was added to TBS until the pH was 5.5, and a drop of 100 volumes hydrogen peroxide added. The DAB-treated sections were washed in tap water for 5 min, counter-stained with haematoxylin, dehydrated, cleared and mounted.

Biochemical studies. The concentration of ethanol in the chamber was determined as described by Griffiths *et al.* (1974). The blood alcohol levels were measured using the alcohol dehydrogenase-based kit produced by Sigma Diagnostics (St Louis, MO, USA; Procedure No 332-UV).

Five biochemical measurements were made on the sera of some of the control and ethanol-treated mice using a 'Genesis 21' autoanalyser (Instrumentation Laboratory). The bilirubin level was measured by a modification of the method of Jendrassik and Roth, the total protein by the biuret method and the albumin by a method based on the binding of bromocresol green. The activities of the two enzymes alkaline phosphatase and aspartate aminotransferase (AST) were determined by the modified Bessey–Lowry method and the SCE method, respectively.

Cytotoxicity of sera. The cytotoxic activity of sera from ethanol-treated mice against A9 mouse tumour cells was investigated as described by Wickramasinghe (1986). The wells of tissue culture plates containing adherent A9 cells were filled with 0.5 ml undialysed serum from control or ethanol-treated mice and 1.5 ml tissue culture medium (Eagle's MEM with Earle's salts and L-glutamine, 200 u penicillin/ml and 100 μg streptomycin/ml). The plates were incubated at 37°C (5% CO_2) for 16 h and the number of residual adherent cells determined. Cytotoxic activity was considered to be inversely proportional to the number of residual adherent cells.

Some serum samples were dialysed at 4°C against 500 ml of tissue culture medium (with antibiotics) over a 24 h period and the cytotoxicity of the dialysed and undialysed sera was compared.

Gel filtration of sera. One millilitre of serum from control animals or animals exposed to ethanol for 6 days was subjected to ascend-

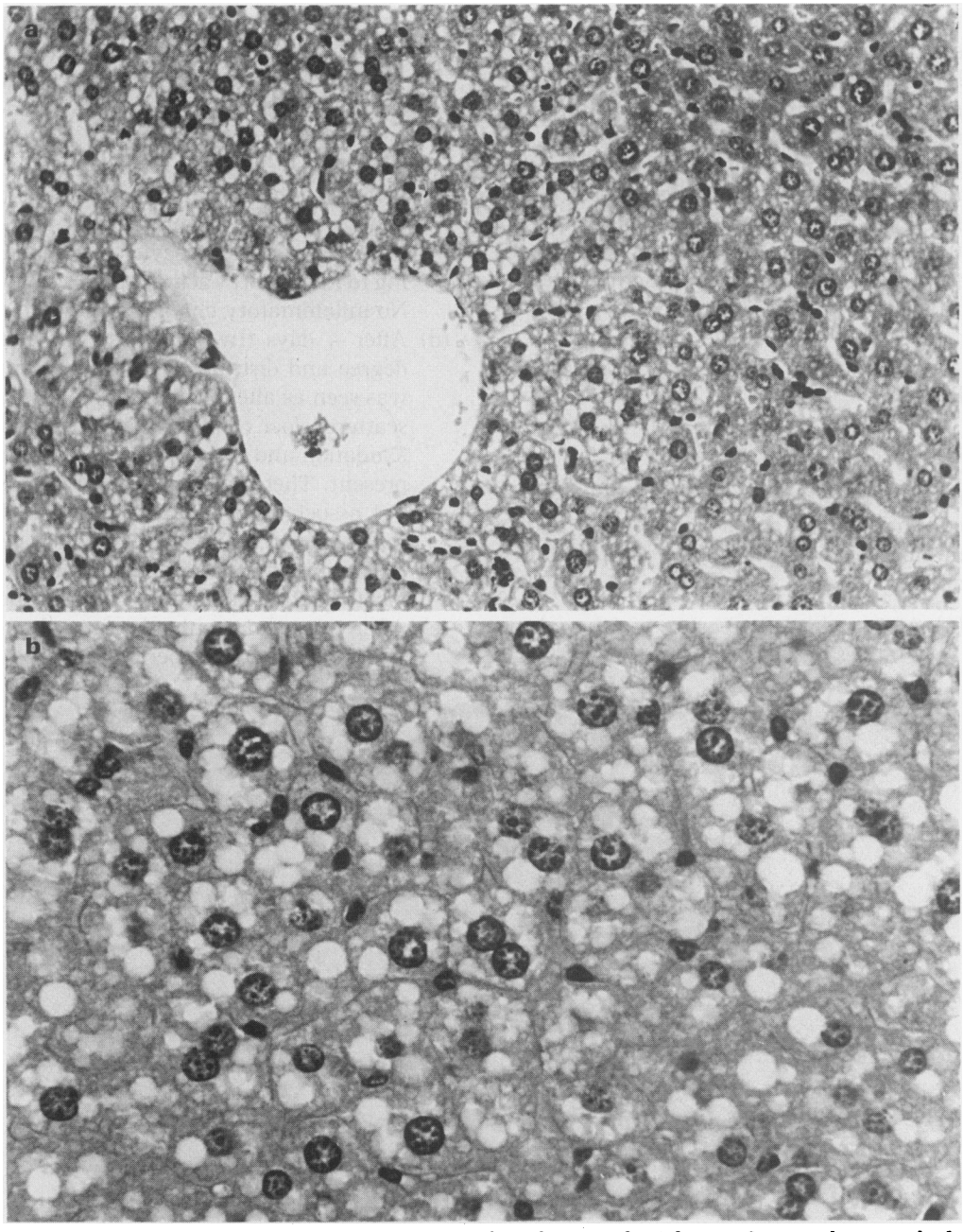

Fig. 1. Liver from a mouse continuously exposed to ethanol vapour for 4 days. *a*, Section photographed at low magnification showing fatty change which is most marked around the cenral vein. H & E, × 150. *b*, Detail of fatty change in pericentral hepatocytes. H & E, × 300.

ing gel filtration at room temperature on a 2.5 cm × 90 cm column packed with Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in phosphate-buffered saline (10 mM sodium phosphate, pH 7.2) containing 0.2% (w/v) sodium azide (PBS-A). After application of the serum, 0.5 ml PBS-A/min was passed through the column and 4 ml fractions collected. The eluted fractions were dialysed against 2 litres of RPMI-1640 at 4°C for 24 h and the dialysed fractions tested for cytotoxic activity against A9 cells as described earlier.

Results

Histological changes in the liver

Continuous exposure to alcohol. Animals were continuously exposed to alcohol and a number were killed at intervals, up to 19 days.

(a) After 1 day of exposure to ethanol, no changes were seen (two animals).

(b) After 2 days (eight animals), there was microvesiculation of hepatocytes predominantly in the pericentral zone (corresponding to Rappaport's acinar zone 3) (Fig. 1). Sections stained with Oil Red O confirmed that the vacuoles contained fat. No acute inflammatory changes were seen.

(c) After 4 days (two animals), the fatty change involved the midzonal as well as the pericentral hepatocytes (corresponding to Rappaport's acinar zones 2 and 3). No inflammatory changes were seen.

(d) After 5 days (two animals), the same degree and distribution of fatty change was seen as after 4 days and in addition scattered foci of a mainly neutrophilic exudation and hepatocyte necrosis were present. There were no foci of necrosis not associated with inflammation. The inflammatory exudate included a few eosinophils and macrophages and was located in the mid and pericentral zones.

(e) After 7 days (nine animals), fatty change was confined to some pericentral hepato-

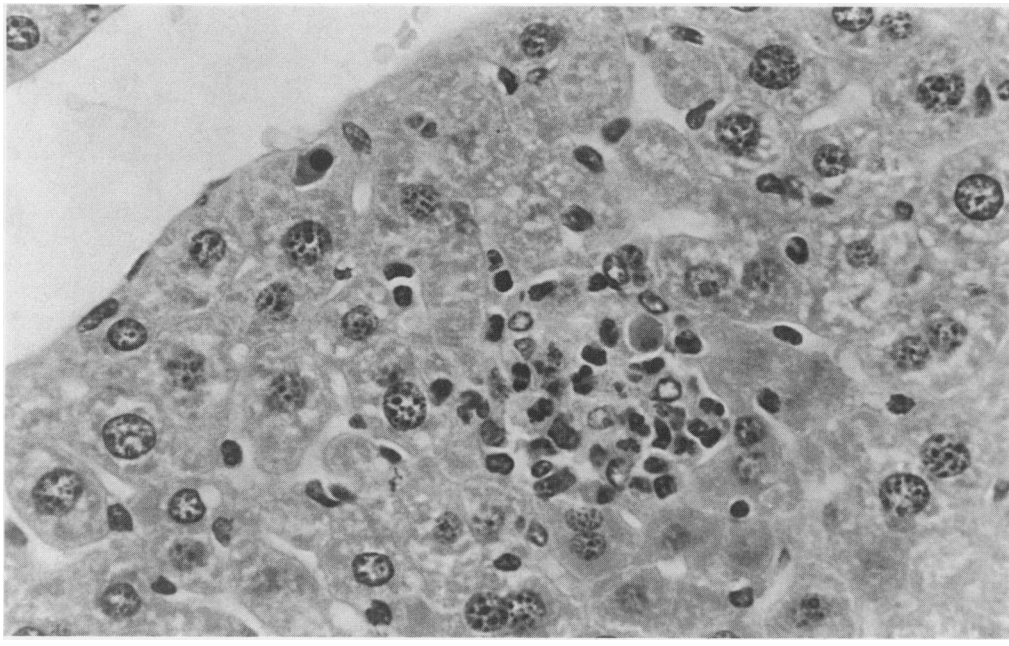

Fig. 2. Liver from a mouse exposed to ethanol for 10 days showing a focus of inflammation and hepatocyte necrosis. H & E, × 300.

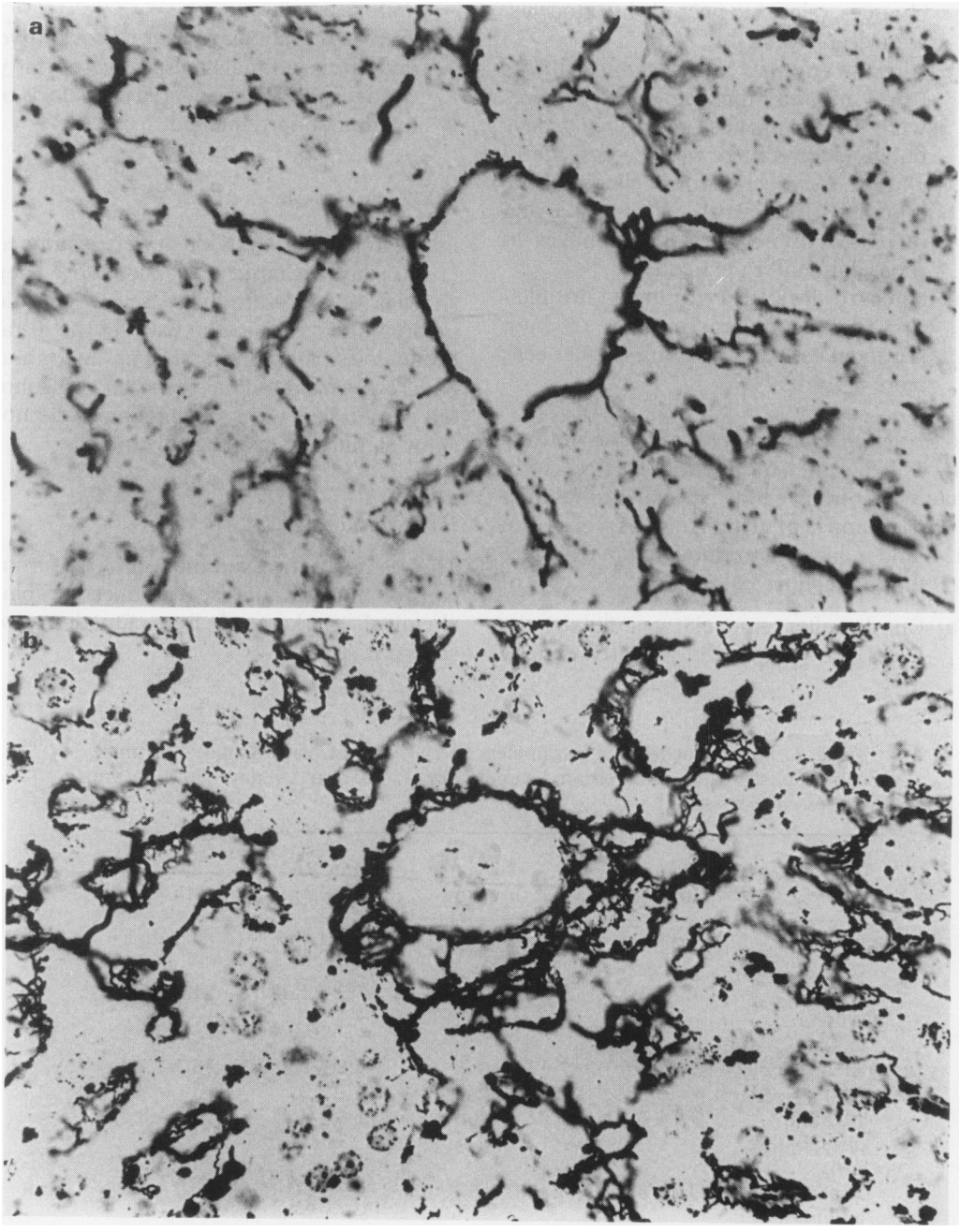

Fig. 3. Sections of liver stained for reticulin from a mouse which had been exposed to ethanol vapour for 10 days (*b*) and from a control mouse (*a*). *a*, Central vein showing no abnormality. *b*, Central vein with an adjacent focus of collapse of the reticulin framework. Gordon and Sweet reticulin impregnation, × 300.

cytes but increased acute inflammatory changes, related to necrotic hepatocytes, were seen (Fig. 2).

(f) After 10 days (six animals) and 14 days (three animals), the histological changes were similar to those seen after 7 days but in addition there was decreased fatty change, focal pericentral collapse of the reticulin framework (Fig. 3), and increased numbers of macrophages in the inflammatory exudates.

(g) After 19 days (three animals), in addition to the changes seen after 10 days, scattered ceroid-containing Kupffer cells were present.

Reversibility of histological changes. After 7 days exposure to ethanol, mice were removed from the special inhalation chamber and kept in air. Animals were killed 1, 2 and 4 days after removal from exposure to ethanol vapour.

(a) One day after removal (two animals), the livers showed pericentral fatty change and there was a reduction in the inflammatory exudate.

(b) Two days and 4 days after removal (two and three animals, respectively), fatty changes had disappeared but foci of inflammation remained.

Other tissues

Lungs from all animals killed were examined (1–19 days exposure to alcohol). They showed a moderate increase in chronic inflammatory cells in the walls of the distal bronchioles. The kidneys, spleens, hearts and pancreases from animals exposed to alcohol for 10–19 days were examined and did not show significant histological changes.

Biochemical changes

The results of the examination of sera from control and ethanol-treated mice for biochemical markers of liver function are shown in Table 1.

Table 1. Some biochemical parameters of the sera of control mice and mice continuously exposed to ethanol vapour for 7, 14 and 19 days. Results are expressed as mean ± 1 s.d.

	Control ($n=6$)	Day 7 ($n=4$)	Day 14 ($n=3$)	Day 19 ($n=3$)
Bilirubin (μmol/l)	3.0±0.3	4.5±0.7*	9.3±3.5*	18.3±5.1*
Total protein (g/l)	74.0±3.1	73.0±2.5	55.5±2.3*	54.2±1.7
Albumin (g/l)	50.0±1.9	47.0±1.8	33.8±1.9*	33.7±1.4
Alkaline phosphatase (u/l)	50.4±3.8	72.3±8.7*	111.1±8.2*	118.5±0.7
Aspartate amino transferase (u/l)	150.0±3.7	238.0±2.1*	374.2±70.3*	350.7±60.5

n Number of animals tested.
* Result significantly different from previous one (Student's *t*-test $P<0.01$).

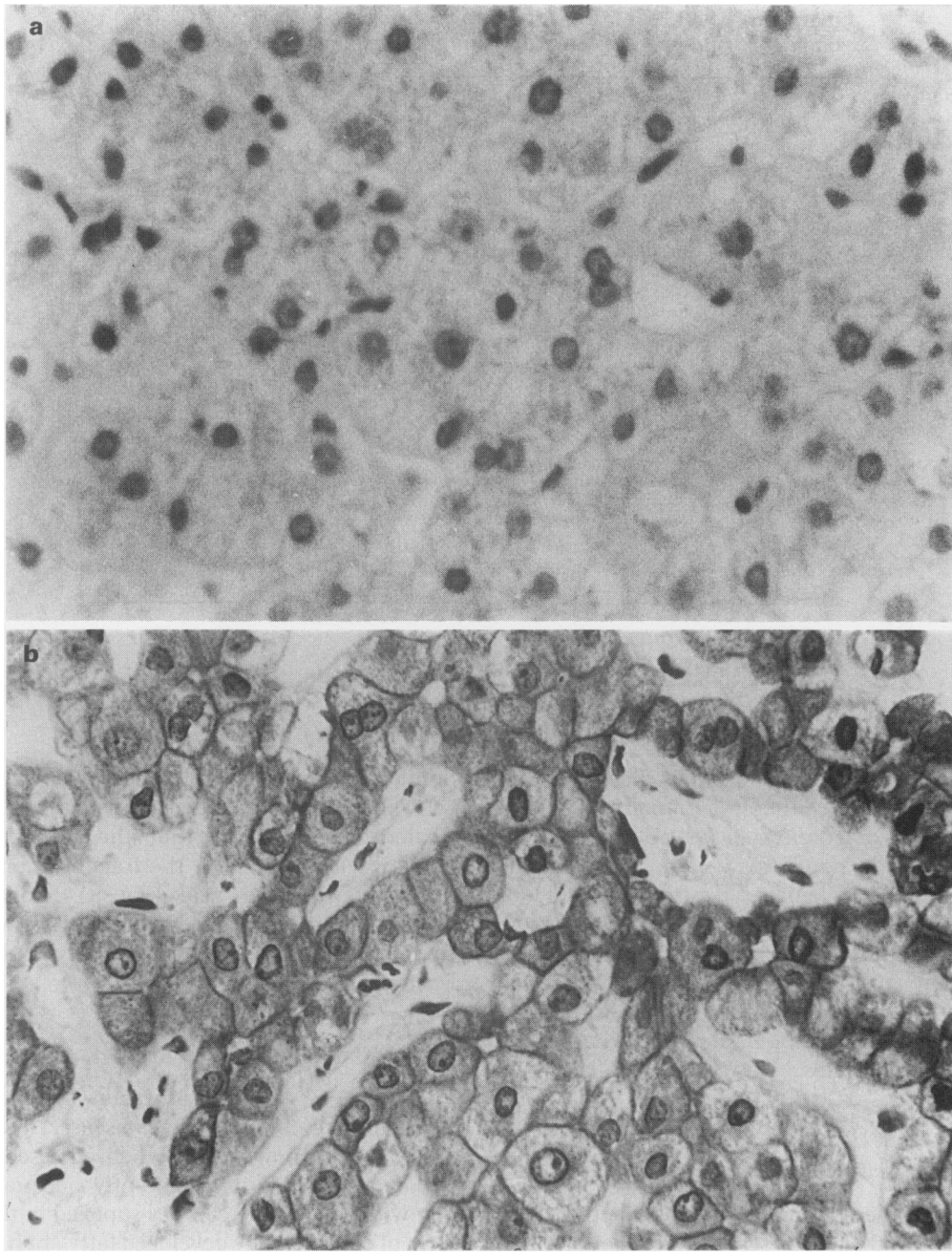

Fig. 4. Cryostat sections of the liver from a mouse exposed to ethanol for 10 days (*b*) and from a control mouse (*a*). The sections were treated with serum from a mouse exposed to ethanol and stained by an immunoperoxidase technique. The section from the ethanol-treated mouse but not from the control mouse shows binding of immunoglobulin to the cytoplasm of hepatocytes. × 300.

Table 2. The number of adherent cells remaining after incubation of a monolayer of A9 cells for 16 h with culture medium containing 25% (v/v) undialysed or dialysed serum from control and ethanol-treated mice

Duration of exposure (days)	Duration of recovery after exposure to ethanol (days)	No. of residual adherent A9 cells per well $\times 10^{-5}$				
		Expt. 1		Expt. 2		Expt. 3
		(a)	(b)	(a)	(b)	(a)
0 (control mice)	–	2.04	2.05	2.77	2.71	2.70
1	–	1.81	1.85	1.62	1.66	1.85
2	–	–	–	1.41	–	–
3	–	–	–	1.26	1.29	–
4	–	1.75	1.80	1.30	–	1.74
5	–	–	–	–	–	1.66
6	–	1.60	1.61	–	–	–
7	–	–	–	1.39	1.58	1.72
7	1	–	–	1.68	–	1.87
7	2	–	–	–	–	2.58
7	4	–	–	–	–	2.77
8	–	1.68	1.60	–	–	–
8	1	1.77	1.76	–	–	–

(a) Undialysed serum.
(b) Dialysed serum.

Immunohistochemical data

Serum from control animals did not 'stain' the livers from either test animals (exposed to alcohol for 10 days) or control animals. On the other hand, sera from test animals 'stained' the hepatocytes from test but not control animals and the staining was most intense in the pericentral region (Fig. 4). There was no staining of Kupffer cells, endothelial cells or bile ducts. The same sera did not 'stain' lung tissue from either test or control animals.

Cytotoxicity of sera

It is evident from Table 2 that both undialysed and dialysed sera from ethanol-treated mice were cytotoxic to A9 cells as judged by their ability to cause a substantial reduction in the number of adherent cells. The blood alcohol levels in the ethanol-treated mice varied between 39 and 157 (mean, 79) mg/dl.

Gel filtration of sera

Studies of the fractions obtained by Sephacryl S-300 gel filtration of serum from alcohol-treated mice revealed that the cytotoxic activity was confined to the fractions comprising the albumin peak (Fig. 4). Similar studies on serum obtained from control mice revealed no cytotoxic activity in any of the fractions (Fig. 4).

Discussion

There is no entirely satisfactory animal model of alcohol-induced liver disease. Lieber *et al.* (1975) showed that when adolescent baboons were fed an apparently adequate diet with 50% of the calories supplied in the form of ethanol, they developed fatty change associated with mononuclear inflammation. Although they did not develop the histological features seen in human alcoholic hepatitis, one-third of the animals studied developed cirrhosis after 2 to 4 years. Some doubt

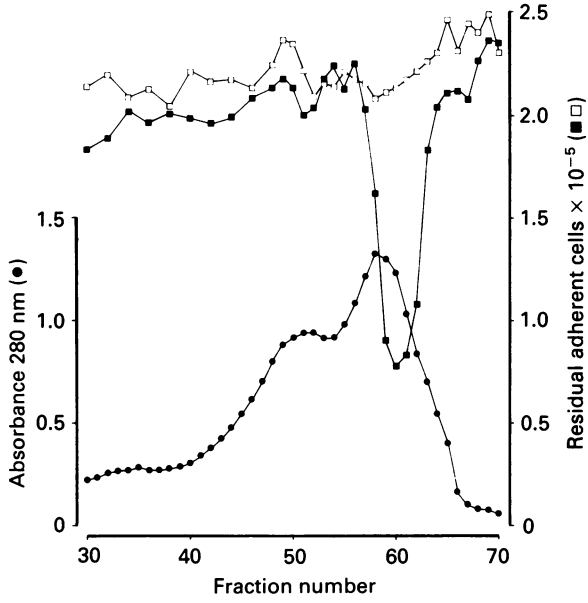

Fig. 5. Sephacryl S-300 gel filtration of serum from control (□) and ethanol-treated (■) mice; relationship between the absorbance at 280 nm of individual fractions and their cytotoxic activity. The latter was considered to be inversely proportional to the number of adherent A9 cells remaining per well after incubating a monolayer of adherent cells with 2 ml of each fraction at 37°C for 16 h. The absorbance curves given by sera from control (●) and ethanol-treated animals were similar.

has been thrown on the validity of this model by the work of Ainley *et al.* (1985; 1986) who fed baboons large amounts of ethanol for up to 60 months without producing fibrosis or cirrhosis. These workers suggested that there may have been nutritional differences between the two studies. Furthermore, the expense and inconvenience of this model precludes its widespread use. When rats are exposed to ethanol vapour (di Luzio & Stege 1979) they develop fatty change alone.

In the present study, fatty change developed in the livers of mice exposed to ethanol for 2 days. This abnormality was initially localized to pericentral hepatocytes, as is the case in the alcohol-induced fatty liver in man (Hall *et al.* 1980). Subsequently, inflammatory changes with collections of mainly neutrophils associated with hepatocyte necrosis became apparent, also mostly in the pericentral region. The overall appearance was similar to that seen in human alcoholic hepatitis, although some features, such as Mallory's hyaline, were absent. These histological abnormalities were accompanied by biochemical evidence of hepatocellular dysfunction with significantly raised levels of bilirubin, aspartate aminotransferase and alkaline phosphatase. The fatty change seen in the mice, but not the hepatitic changes, reversed rapidly after cessation of exposure to ethanol; this pattern of recovery parallels that in man (Lieber & Rubin 1968).

Despite continuing inflammatory changes no evidence of collagen deposition around terminal hepatic venules or peri-venular hepatocytes was seen up to the end of the study. In man these features are associated with irreversible liver injury. They were also seen in precirrhotic animals in the study of Lieber *et al.* (1975), but not that of Ainley *et al.* (1985; 1986) referred to above.

Serum from animals exposed to ethanol for 10 days reacted with frozen sections of livers from mice exposed to ethanol but not from control mice. This suggests that an

antibody is produced by mice exposed to ethanol which recognizes a hepatocyte neoantigen induced by ethanol or, more probably, its metabolite, acetaldehyde. These findings are of considerable interest as the involvement of immunological mechanisms in alcohol-induced liver damage in humans has been suggested previously (reviewed by MacSween & Anthony 1985). Our data are similar to those of MacSween *et al.* (1981) who have shown that antibody molecules in the sera of chronic alcoholics react with hepatocytes from alcohol-treated but not control rabbits.

Mice exposed to ethanol for 1–8 days developed a cytotoxic activity against A9 cells in their sera which persisted for more than 24 h after discontinuation of exposure to ethanol. This activity was largely non-dialysable and, therefore, neither ethanol nor free acetaldehyde could account for it. Gel filtration studies showed that the cytotoxic activity was confined to the fractions containing albumin molecules. Recently, a non-dialysable cytotoxic activity with a relatively long half-life has also been demonstrated in the sera of healthy volunteers after the consumption of 500–700 ml of wine over 20–35 min (Wickramasinghe *et al.* 1986). The cytotoxic molecules developing in these volunteers appeared to consist of acetaldehyde–albumin complexes. It has been suggested that the formation of such circulating cytotoxic complexes may constitute another potential mechanism of alcohol-induced tissue injury and, particularly, of extrahepatic tissue damage (Wickramasinghe 1987).

In conclusion, the data presented here indicate that mice chronically exposed to ethanol vapour provide a convenient and useful model for the study of the mechanisms underlying at least some aspects of ethanol-induced liver damage in man.

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References

Ainley C., Senapati A., Brown I.M.H., Iles C.A., Slavin B.M., Davies D.R., Keeling P.W.N. & Thompson R.P.H. (1985) Is alcohol hepatotoxic in the baboon. *J. Hepatol.* **Suppl. 1**, S2.

Ainley C., Senapati A., Brown I.M.H., Iles C.A., Slavin B.M., Davies D.R., Keeling P.W.N. & Thompson R.P.H. (1986) Studies with alcohol in the baboon. *Gut* **27**, A1248.

Di Luzio N.R. & Stege T.E. (1979) Influence of chronic ethanol vapour inhalation on hepatic parenchymal and Kupffer cell function. *Alcoholism: Clin. Exp. Res.* **3**, 240–247.

Griffiths P.J., Littleton J.M. & Oritz A. (1974) Changes in monoamine concentrations in mouse brain associated with ethanol dependence and withdrawal. *Br. J. Pharmacol.* **50**, 489–498.

Hall P. de la M., Gormley B.M., Jarvis L.R. & Smith R.D. (1980) A staining method for the detection and measurement of fat droplets in hepatic tissue. *Pathology* **12**, 605–608.

Lieber C.S. & Rubin E. (1968) Ethanol—an hepatotoxic drug. *Gastroenterology* **54**, 642–646.

Lieber C.S., De Carli I.M. & Rubin E. (1975) Sequential production of fatty liver, hepatitis and cirrhosis in sub-human primates fed ethanol with adequate diets. *Proc. Natl. Acad. Sci. USA.* **72**, 437–441.

MacSween R.N.M., Anthony R.S. & Farquarson M. (1981) Antibodies to alcohol-altered hepatocytes in patients with alcoholic liver disease. *Lancet* **ii**, 803–804.

MacSween R.N.M. & Anthony R.S. (1985) Immune mechanisms in alcoholic liver disease. In *Alcoholic Liver Disease.* Ed. P. Hall. London: Edward Arnold, pp. 69–89.

Malik F. & Wickramasinghe S.N. (1987) Haematological abnormalities in mice continuously exposed to ethanol vapour. *Br. J. exp. Path.* **67**, 831–838.

Wickramasinghe S.N. (1987) Role of macrophages in the pathogenesis of alcohol induced tissue damage. *Br. Med. J.* **294**, 1137–1140.

Wickramasinghe S.N., Gardner B. & Barden G. (1986) Cytotoxic protein molecules generated as a consequence of ethanol metabolism *in vitro* and *in vivo*. *Lancet* **ii**, 823–826.

Wickramasinghe S.N. (1986) Supernatants from ethanol-containing macrophage cultures have cytotoxic activity. *Alcohol Alcohol.* **21**, 263–268.