JOURNAL OF PATHOLOGY, VOL. 151: 47–53 (1987)

ENDOTOXIN INDUCED HEPATIC NECROSIS IN RATS ON AN ALCOHOL DIET

BRAMDUTT S. BHAGWANDEEN*, MINOTI APTE*, LYNETTE MANWARRING* AND JOHN DICKESON†

*Discipline of Histopathology, The University of Newcastle
and
†Department of Clinical Chemistry, Royal Newcastle Hospital, Newcastle, Australia

Received 21 November 1986
Accepted 27 January 1987

SUMMARY

The role of endotoxin in the pathogenesis of progressive liver disease is receiving increasing attention, but remains controversial. Similarly, although alcoholic hepatitis is now recognized as the transitional link between alcoholic fatty liver and advanced alcoholic liver disease, the aetiology of liver cell necrosis in alcoholic hepatitis is not known.

Rats fed a nutritionally adequate liquid alcohol diet according to the formula of Lieber and DeCarli developed fatty livers. Littermates fed an identical diet and challenged with small IV doses (1μg/g body weight) of *E. coli* lipopolysaccharide endotoxin (LPS) developed focal necrotizing hepatitis. Control littermates fed an identical calorie balanced but alcohol free diet and challenged with identical doses of LPS did not develop any liver lesions.

The hepatocyte necrosis with associated inflammatory changes induced by LPS in fatty livers has some features of early human alcoholic hepatitis and suggests that progressive alcohol induced damage may be multifactorial in origin.

KEY WORDS—Alcoholic hepatitis, hepatic necrosis, experimental hepatitis, endotoxin.

INTRODUCTION

The role of endotoxin (LPS) in the pathogenesis of progressive liver disease remains controversial.[1,2] There is however, considerable evidence[3,4,5] to suggest that endotoxin has an important role in both acute and chronic liver disease.

Animal experiments indicate that LPS is a hepatotoxin which may act directly[4,6,7] and/or indirectly[4,5,7,8,9,10] in promoting liver damage.

LPS is normally cleared by the Kupffer cells of the liver[7] and there is experimental evidence that the hepatotoxic action of endotoxin is potentiated by depression of the Kupffer cell system[4,5,7,9] Nolan and Camara[7] therefore postulated that because of Kupffer cell depression and the consequent enhancement of endotoxic activity, even minimal quantities of endotoxin might precipitate hepatic injury.

Kupffer cell dysfunction and blockade can be caused by particulate matter, various toxins and metabolites.[7] It has been shown that Kupffer cell function is also depressed in rats fed alcohol.[7,11] There is now also good evidence of depression of Kupffer cell function in human alcoholic liver disease.[10,12,13]

The possible hepatotoxic effects of LPS in animals given alcohol over a prolonged period have however not been studied. This study was designed to examine the effects of minimal doses of exogenous LPS administered to rats on an alcohol diet over a prolonged period.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats bred in the University animal house were used. Rats approximately eight weeks old and with an average weight of 175 g were individually housed in wire cages and fed a liquid diet according to the formula devised by Lieber and DeCarli.[14] This provided the only source of food and water.

Addressee for correspondence: Brumdutt S. Bhagwandeen, Discipline of Histopathology, David Maddison Clinical Services Building, Royal Newcastle Hospital, NSW, Australia 2300.

0022–3417/87/010047–07$05.00

Feeding regime

Rats were divided into four groups. Each group had ten rats with each rat in a group matched by weight with a littermate in each of the other three groups. Two groups of ten rats each (Group 3 and Group 4) had their diet supplemented with alcohol. The feeding schedule adhered strictly to that of Lieber and DeCarli.[15,16] In brief, increasing concentrations of alcohol in the form of ethanol were introduced over 5 days to reach 36 per cent of total calories by the 5th day. Group 1 and Group 2 rats were fed an alcohol-free diet with dextrin-maltose substituted isocalorically for alcohol. Rats were pair fed (Group 1 versus Group 3 and Group 2 versus Group 4) and the alcohol fed rats determined the calorie requirement of the non-alcohol fed littermates. Rats were weighed daily and all rats showed a steady weight gain (Fig. 1). The average daily intake of alcohol was 0·012 g/g body weight which is comparable to an alcohol intake of more than 80 g/day in a 70 kg man. On this diet blood alcohol levels in rats determined at the time of sacrifice ranged from 30 mg/100 ml to 200 mg/100 ml with an average of 92 mg/100 ml. These levels compare favourably with those reported by Lieber and DeCarli.[17]

Endotoxin challenge

At the end of the 10 weeks on the above dietary regime, rats of Groups 2 and 4 were challenged with intravenous LPS. LPS was stored as a freeze dried purified lipopolysaccharide, *E. coli* 026:B6 (Difco Laboratories), and was freshly reconstituted in sterile pyrogen-free water at the time of injections. Intravenous injections were given through the tail veins. The dose of LPS was calibrated to provide 1·0 μg/g body weight in 1 ml of diluent. Rats in Groups 1 and 3 received IV injections of 1 ml sterile pyrogen-free water only.

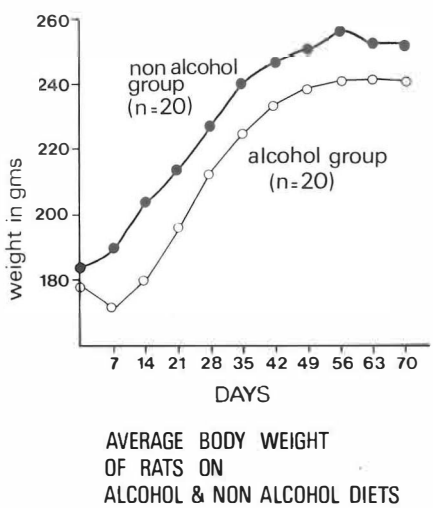

Fig. 1—Growth curve of rats on liquid alcohol diet and alcohol-free, liquid diet

Dietary regime and LPS challenge

The dietary regime and LPS challenge for all the rats are given in Table I.

Specimen Collection

All rats were killed by bleeding under ether anaesthesia 24 h after the IV injections. Blood was collected for liver enzyme analysis and blood alcohol levels. All organs were examined and tissue samples collected from heart, kidney, lung, large and small bowel. Tissue samples were fixed in 10 per cent buffered formalin and 3–4 μm paraffin embedded sections were prepared and stained with haematoxylin and eosin for light microscopy.

Liver

The liver was removed and after weighing, specimens were collected from each lobe with a minimum of five samples taken from each rat. Specimens were fixed and prepared for light microscopy. Sections (3–4 μm) were stained with haematoxylin and eosin, Masson's trichrome, Gordon & Sweets' silver stain and diastase–PAS. In every case fresh frozen sections were stained by oil Red 0 for assessment of fatty change.

RESULTS

Liver Histology

Both macroscopic and microscopic examination revealed no pathological lesions in any organ except the liver. Only the liver changes will therefore be described.

Group 1: (Alcohol free diet and no LPS)
The liver histology was normal in this group.

Table I

Group	1	2	3	4
$n=$	10	10	10	10
Diet	alcohol free	alcohol free	alcohol	alcohol
IV challenge	diluent	LPS	diluent	LPS

Group 2: (Alcohol free diet challenged with LPS) The liver histology differed only marginally from Group 1 rats. There was some prominence of Kupffer cells and occasional neutrophils were seen in sinusoids. There was no evidence of hepatocyte degeneration or necrosis. The minimal doses of LPS in these rats therefore produced no significant hepatocyte damage.

Group 3: (Alcohol diet but no LPS)
All the rats in Group 3 had fatty livers of varying degrees of severity. (Table II) Fatty change was graded as mild when fat was present in less than a third of the hepatocytes of each lobule, moderate when nearly 2/3 of the lobule was affected and severe when all the lobule was affected. Fatty change commenced in the perivenular area (Zone 3 of Rappaport). Occasional lipogranulomata were noted but there was no evidence of hepatocyte necrosis, inflammation or scarring (Fig. 2). These findings are similar to those described previously by Lieber and DeCarli.[14,15,16]

Group 4: (Alcohol diet with LPS challenge)
The rats in this group all had hepatic lesions different from those of the other three groups. In addition to fatty change, all had evidence of hepatocyte degeneration and necrosis associated with inflammation. These 'hepatitic' lesions were graded as mild, moderate or severe based on the extent of hepatocyte necrosis. In mild lesions (three rats) there was focal ballooning degeneration with occasional individual cell necrosis associated with neutrophil infiltrate. Both microvesicular and macrovesicular fatty change was prominent.

In moderately severe lesions (four rats), focal hepatocyte necrosis was prominent (Fig. 3). On the periphery of necrotic hepatocytes were degenerating hepatocytes, some showing ballooning degeneration. Neutrophilic infiltrate in necrotic foci was prominent. There was also associated severe fatty change and Kupffer cells were prominent and swollen (Fig. 3).

In severe lesions (three rats) there was confluent necrosis especially prominent in Zone 3 of Rappaport (Fig. 4). A severe neutrophilic infiltrate was present in all areas of necrosis. Adjacent hepatocytes showed varying degrees of ballooning degeneration and both macro and microvesicular fatty change was present (Fig. 4). There was no evidence of Mallory's hyaline in any of the cases. Portal tracts were not altered and there was no evidence of fibrosis.

LIVER ENZYMES

The AST (SGOT) values in the four groups of rats are summarized in Table III and Fig. 5.

The markedly raised AST levels in Group 4 rats are consistent with hepatocyte necrosis, seen only

Table II—Histological grading of fatty change in rat livers

Fatty change	Group 1 $n=10$	Group 2 $n=10$	Group 3 $n=10$	Group 4 $n=10$
Mild	—	—	2	3
Moderate	—	—	6	5
Severe	—	—	2	2

Group 1: alcohol free diet, no LPS challenge.
Group 2: alcohol free diet, challenged with IV LPS.
Group 3: alcohol diet, no LPS challenge.
Group 4: alcohol diet, challenged with LPS.

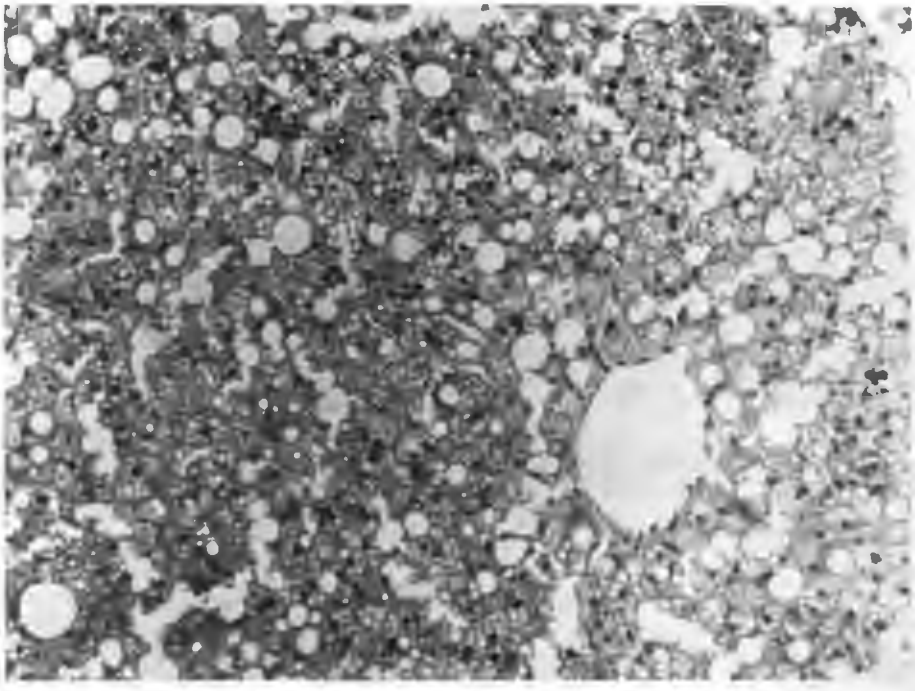

Fig. 2—Liver of Group 3 rat (alcohol diet) with severe fatty change of both macro and microvesicular type. There is no inflammation

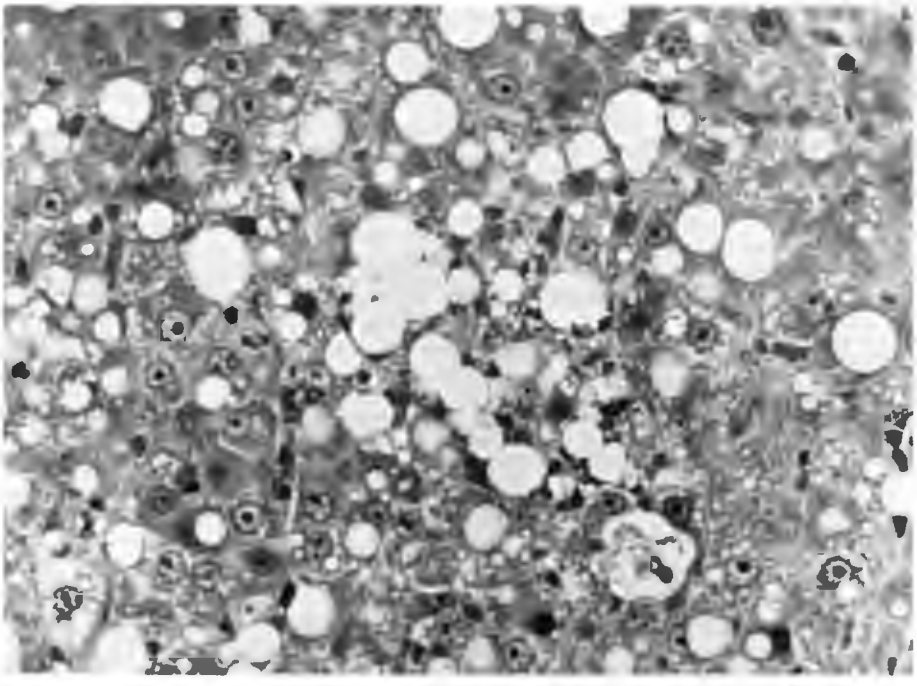

Fig. 3—Liver from Group 4 rat (alcohol diet + LPS). There is ballooning degeneration, focal hepatocyte necrosis, neutrophil infiltration and moderate fatty change

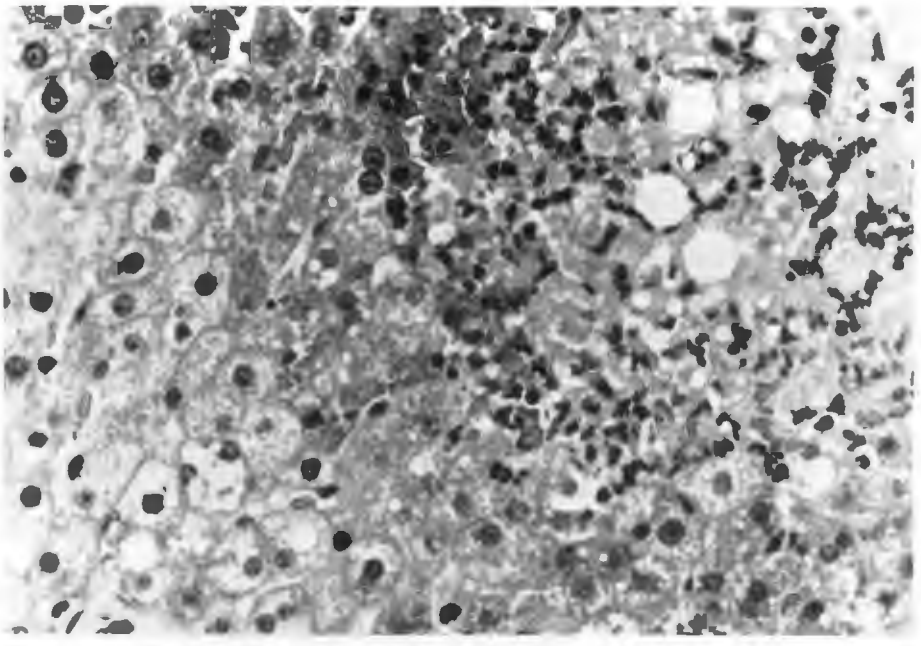

Fig. 4—Liver of rat from Group 4 (alcohol diet +LPS). There is confluent necrosis with associated severe neutrophil infiltration. Adjacent hepatocytes show ballooning degeneration and there is underlying fatty change

in this group. The rise in AST values in Group 4 rats is highly significant (students t test) when compared with Group 3 rats ($p < 0{\cdot}0001$) and with Group 2 rats ($p < 0{\cdot}0005$).

The AST of rats in Group 2 (alcohol free diet challenged with LPS) revealed only minor elevations (Group 1 versus Group 2: $p = 0{\cdot}11$) indicating no significant hepatocyte injury. Similarly, AST values were not significantly raised in rats fed alcohol alone (Group 1 versus Group 3: $p = 0{\cdot}94$).

DISCUSSION

In this study rats fed a prolonged alcohol diet developed typical fatty livers of varying severity. Alcohol itself failed to induce hepatocyte necrosis even in the very severe cases of fatty liver. Our observations are similar to those reported previously by Lieber and his co-workers.[14,15,16,17,18]

In our study, littermate rats on an identical alcohol diet when challenged with small doses of IV LPS developed hepatocyte necrosis accompanied by neutrophil infiltrate on a background of severe fatty change. The extent of hepatocyte necrosis varied in different rats and in different areas of the liver of the same rat. The AST was correspondingly elevated in rats with hepatocyte necrosis. Littermate rats on an isocaloric but alcohol free diet, challenged with similar doses of LPS as the alcohol

Table III—AST levels in littermate rats on alcohol and alcohol free diets challenged with LPS

	Group 1 $n=10$	Group 2 $n=10$	Group 3 $n=10$	Group 4 $n=10$
Mean	84·50	106·10	85·00	287·00*
SD	16·18	35·50	15·00	129·90*
SEM	±5·12	±11·20	±4·75	±41·10*

Group 1: alcohol free diet, no LPS challenge.
Group 2: alcohol free diet challenged with IV LPS.
Group 3: alcohol diet, no LPS challenge.
Group 4: alcohol diet challenged with LPS.
*Statistically significant (see text).

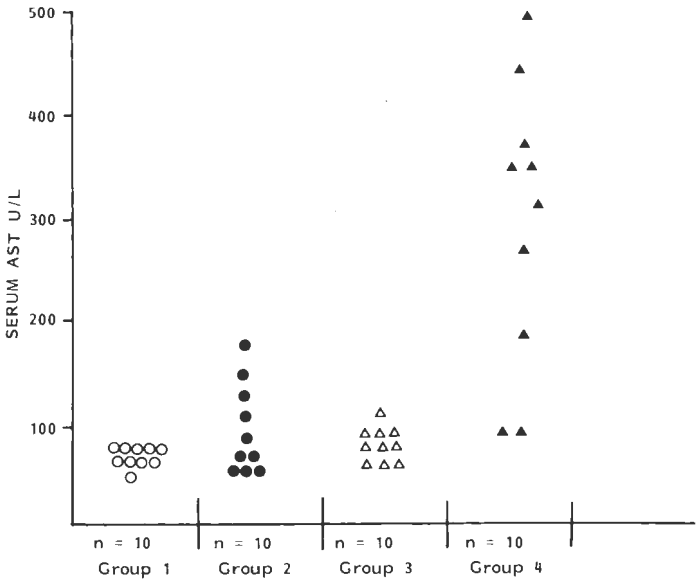

Fig. 5—AST levels in rats fed alcohol free diet (Group 1), alcohol-free diet and receiving IV LPS (Group 2), alcohol diet but no LPS (Group 3) and alcohol diet and receiving IV LPS (Group 4). The AST was significantly raised in Group 4 rats only (see text)

fed group, did not develop hepatocyte necrosis. In earlier studies (unpublished), rats on standard laboratory diet and rats on liquid alcohol free diet challenged with LPS in doses employed in this study did not have any evidence of hepatocyte necrosis. The raised AST levels only in rats of Group 4 are also consistent with the histological evidence of hepatic necrosis only in this group. The normal AST levels in the other three groups confirm the view that neither alcohol alone nor LPS alone was sufficiently hepatotoxic in this study.

Thus we have demonstrated that in the small doses used in this study, LPS alone could not by itself produce hepatic necrosis. Our findings are consistent with those of Nolan and his co-workers.[4,5] Other workers have shown similar features.[19] Using higher single doses of LPS some workers were able to show both structural and functional cellular derangement but not significant hepatic necrosis[6,20,21] LPS in sufficiently high doses does eventually produce marked liver damage,[20,21] and hepatotoxicity of LPS is therefore dose related.[4]

From our study, we can therefore conclude that hepatocyte necrosis was produced by LPS only in those rats on a chronic alcohol diet and therefore alcohol induced fatty livers are more susceptible to hepatotoxic effects of even minimal doses of LPS. This in effect demonstrates synergism between alcohol and LPS. Others have shown similar synergism between LPS and other hepatotoxins.[4,5,7,19,21,22]

It is also generally agreed that Kupffer cell dysfunction is particularly important in potentiating LPS toxicity.[7,9,10,12] There is now both clinical[10,12] and experimental evidence[11] of depression of Kupffer cell function in chronic alcoholism.

However chronic alcoholism produces not only supression of the Kupffer cells[11] but also significant metabolic derangements of hepatocytes accompanied by fatty change[18] and enhanced LPS activity.[4] Our experimental evidence supports this theory. The associated acute inflammatory cellular infiltrate is stimulated by tissue damage and also by LPS.[18]

Hepatocyte necrosis with associated inflammatory changes supervening on an alcoholic induced fatty liver in man is now recognized as an important marker of alcoholic hepatitis.[23,24,25] Alcoholic hepatitis is the transitional lesion between simple fatty liver and more advanced alcoholic cirrhosis. Although various theories have been proposed,[8,18,22,25-30] the pathogenesis of hepatocyte necrosis in alcoholic hepatitis is still unclear.

Our study suggests that the cause of hepatocyte necrosis in alcoholism may be multifactorial and not due to alcohol alone. Our study also suggests

that LPS might have an important role in promoting hepatocyte necrosis in advanced alcoholic liver disease.

ACKNOWLEDGEMENT

This work was supported by a Faculty of Medicine Research grant and by a grant from The Austalian Associated Brewers.

REFERENCES

1. Fulenwider, JT, Sibley C, Stein SF, Evatt B, Nordlinger M, Ivey GL. Endotoxemia of cirrhosis: an observation not substantiated. *Gastroenterology* 1980; **78:** 1001–1004.
2. Editorial Lancet. Endotoxin and Cirrhosis. *Lancet* 1982; **1:** 318–319.
3. Ali MV, Nolan JP. Serial assessment of reticuloendothelial function in experimentally induced nutritional cirrhosis. *Lab Invest* 1969; **20:** 184–189.
4. Nolan JP. The role of endotoxin in liver injury. *Gastroenterology* 1975; **69:** 1346–1356.
5. Nolan JP, Leibowitz AI. Endotoxin and the liver III. Modification of acute carbon tetrachloride injury by polymixin B—an antiendotoxin. *Gastroenterology* 1978; **75:** 445–449.
6. Levy E, Ruebner JS. Hepatic changes produced by a single dose of endotoxin in the mouse. Light microscopy and histochemistry. *Amer J Pathol* 1967; **51:** 269–285.
7. Nolan JP, Camara DS. Endotoxin, sinusoidal cells and liver injury. In: Popper, H, Schaffner, F, eds. Progress in Liver Diseases, Vol. 7, New York: Grune and Stratton, 1982: 361–376.
8. Barry RE. The pathogenesis of hepatitis in alcohol abuse and jejunoileal bypass. *Lancet* 1983; **ii:** 489–490.
9. Tanner AR, Arthur MJP, Wright R. Progress report: Macrophage activation, chronic inflammation and gastrointestinal disease. *Gut* 1984; **25:** 760–783.
10. Mills LR, Scheuer PJ. Hepatic sinusoidal macrophages in alcoholic liver disease. *J Pathol*, 1985; **147**: 127–132.
11. Ali MV, Nolan JP. Alcohol induced depression of reticulo endothelial function in the rat. *J Lab Clin Med* 1967; **70:** 295–301.
12. Lohnberg G, Friman L, Berghem L. Reticuloendothelial function in patients with alcoholic cirrhosis. *Scand J Gastroenterol* 1981; **16:** 481–489.
13. Turunen U, Malkamaki M, Valtonen V *et al.* Endotoxin and liver diseases; high titres of enterobacterial common antigen antibodies in patients with alcoholic cirrhosis. *Gut* 1981; **22:** 849–853.
14. Lieber CS, Decarli LM. Alcoholic fatty liver—model no. 30. Supplemental update, 1980. In: CC Capen, DB Hackel, JC Jones, G Migaki, eds. Handbook: Animal Models of Human Disease, Fasc. 2. Registry of Comparative Pathology, Armed Forces Institute of Pathology, Washington, D.C. 1980.
15. Lieber CS, Jones DP, Decarli LM. Effects of prolonged ethanol intake: production of fatty liver despite adequate diets. *J Clin Invest* 1965, **44:** 1009–1021.
16. Decarli LM, Lieber CS. Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet. *J Nutr* 1967; **91:** 331–336.
17. Lieber CS, Decarli LM. The feeding of alcohol in liquid diets. Two decades of applications and 1982 update. *Alcoholism Clin Exp Res* 1982; **6:** 523–531.
18. Lieber CS. Alcohol and the liver: metabolism of ethanol, metabolic effects and pathogenesis of injury. *Acta Med Scand* 1985, suppl. 703: 11–15.
19. Nolan JP, Ali MV. Endotoxin in the liver 1. Toxicity in rats with choline deficient fatty livers. *Proc Soc Exp Biol Med* 1968; **129**: 29–31.
20. Levy E, Slusser RJ, Ruebner BH. Hepatic changes produced by a single dose of endotoxin in the mouse. Electron microscopy. *Amer J Pathol* 1968; **52:** 477–502.
21. Ferluga J, Allison AC. Role of mononuclear infiltrating cells in pathogenesis of hepatitis. *Lancet* 1978; **ii:** 610–611.
22. Bradfield JWB, Wells M. Liver diseases caused by lysosomal enzymes released from kupffer cells. *Lancet* 1978; **ii:** 836.
23. Leevy CM, Popper H, Sherlock S. *et al.* In: Diseases of the Biliary Tract. Standardization of Nomenclature, Diagnostic Criteria and Diagnostic Methodology. Fogarty international centre proceeding no. 22. 1979, 6.
24. Baptista A, Bianchi L, de Groote J. *et al.* Alcoholic liver disease: morphological manifestations. Review by an international group. *Lancet* 1981, **i:** 707–711.
25. Desmet VJ. Alcoholic liver disease: histological features and evolution. *Acta Med Scand* 1985: suppl. 703: 111–121.
26. Lindros KO, Stowell L, Vaananen H. *et al.* Uninterrupted prolonged ethanol oxidation as a main pathogenetic factor of alcohol liver damage: evidence from a new liquid diet animal model. *Liver* 1983; **3:** 79–91.
27. Leevy CM, Chen T, Luisada-Opper A. 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; 526–530.
28. Lewis KO, Paton A. Could superoxide cause cirrhosis? *Lancet* 1982; **ii:** 188–189.
29. Fleming KA, McGee JO'D. Alcohol induced liver disease. *J Clin Pathol 1984;* **37:** 721–733.
30. Faber JL. Reactions of the liver in injury. Necrosis. In: Faber E, Fisher MM, eds. Toxic Injury of the Liver (in two parts) part A, New York, Marcel Dekker 1979: 215–241.