A. Bosma

Clinical and experimental studies of alcoholic liver disease and liver fibrosis

CLINICAL AND EXPERIMENTAL STUDIES OF ALCOHOLIC LIVER DISEASE AND LIVER FIBROSIS

1991

Pasmans Offsetdrukkerij bv

Cover Design: "Vineyard, Toscane"

This work represents a thesis for a doctoral degree at the State University of Leiden (Promotores: Prof.Dr. D.L. Knook and Prof.Dr. J.H.P. Wilson).

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Bosma, Anne

Clinical and experimental studies of alcoholic liver disease and liver fibrosis / Anne Bosma. - Rijswijk : TNO Institute for Experimental Gerontology. - Ill.
Also publ. as thesis Leiden, 1991. - With ref. - With summary in Dutch.
ISBN 90-70638-09-6
Subject headings: alcoholic liver disease / liver fibrosis.

CLINICAL AND EXPERIMENTAL STUDIES OF ALCOHOLIC LIVER DISEASE AND LIVER FIBROSIS

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1991

Publication of the Institute for Experimental Gerontology

IVVO-TNO, Leiden, The Netherlands

Aan mijn ouders

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CHAPTER I

GENERAL INTRODUCTION

1.1 Purpose of this thesis

This thesis deals with some aspects of clinical and experimental alcoholic liver disease and liver fibrosis. Alcohol abuse is one of the main causes of morbidity in Western society which interferes with mental, physical and social health by affecting virtually all organ systems. The organ systems that are affected in particular by alcohol abuse are the nervous system, the striated musculature - especially the heart - the digestive tract and - last but, as may be evidenced from this thesis, probably often least - the liver.
In human alcoholic liver disease (ALD), liver fibrosis (LF) is the most important and determinant pathological lesion. The pathogenetic mechanisms of ALD and LF are still poorly understood. In this thesis, the present literature on ALD, LF in general and, more specifically, in relation to nutritional factors, such as vitamin A, will be reviewed and compared with results obtained in own experiments.

1.2 Experimental animals

All experiments were carried out in the Institute for Experimental Gerontology (IVEG) TNO, Rijswijk, with rats of strains bred in the Institute's colonies. Wide experience has been gained with these strains and the pathology of aging of the strains has been well defined (Burek 1978).
Specific reason for choosing the rat as a model for the study of experimental ALD was the existence of literature data on the induction of LF by alcohol administration in rats when alcohol was incorporated in a diet of specific composition (Leo & Lieber 1983).

1.3 Motivation of the experimental approach

The liver research of the IVEG has generally been focused on the various roles of the different types of liver cells in physiological and pathological conditions. Much of this research involved methods for the isolation, purification and characterisation of liver cell types (Brouwer et al 1988). In addition, (electron-) microscopical techniques were used to study specific cell types *in situ* (De Leeuw 1985).
Because the mechanisms leading to LF in particular and in ALD in general are still largely unknown, we chose the rat as model for experiments with alcohol in order to try to elucidate some pathogenetic aspects of ALD, of which fibrosis is the central lesion.
We have primarily focused on the influence of nutritonal and other parameters, that are considered important determinants for the effects of alcohol (and other fibrosing agents) *in vivo* on the liver.

1.4 Introduction to the chapters

In Chapter II the torrent of often conflicting literature on alcohol and the liver will be reviewed, and current issues for debate are formulated.
In Chapter III current animal models of ALD and LF, and the cell types and processes possibly involved, will be discussed.
Chapter IV reports about the histopathological (IV a.) and the biochemical (IV b.) findings of a long-term experiment in rats, which were chronically fed an alcohol-containing liquid diet.
Although liver abnormalities developed, and could be explained as being indirectly related to the effect of alcohol, no LF was found. This is in contrast with the findings reported by the group of Lieber who, in an essentially similar experimental set-up but in rats of a different strain and age, could induce LF (Leo & Lieber 1983). The differences in findings of our and Lieber's group are extensively discussed, and the value of this experimental animal model for ALD is questioned.
Chapter V reports on the enhancement of LF by ethanol in livers, which were pre-treated by administration of carbon tetrachloride (CCl_4).

Chapter VI reports about the dual effect of vitamin A administration on the development of LF induced by CCl_4-administration. It shows that vitamin A does enhance or decrease the CCl_4-induced liver damage, dependent on the moment of administration of this vitamin relative to the treatment with CCl_4.
Chapter VII reports about a case history of (human) progressive LF which remained unexplained. Although, theoretically, various factors could have contributed to the pericellular parenchymal LF, no single factor *per se* could be incriminated as the cause, and the pathogenesis remained fully speculative.
Finally, in Chapter VIII a summary of the current, rather scarce and speculative knowledge about the pathogenesis of LF and of ALD in particular is presented. Some remarks and recommendations for research in the future are expressed.
On the basis of our results, it is possible to more clearly define under what circumstances the known factors leading to LF and ALD may become active.

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CHAPTER II

ALCOHOLIC LIVER DISEASE

2.1 Alcohol abuse

2.1.1 Introduction

Alcohol is the commonest addictive substance used by man and accepted as a legal psychotropic drug by most (Western) societies (Denney & Johnson 1984). It is reported that alcohol consumption has retained a crucial role in the socio- economic order of the USA during their history, in which the actual drinking behaviour (or temporarily absence by prohibition) remains an important factor in cultural *mores* and traditions (Watson et al 1986).

This wide-spread social acceptance of alcohol consumption is an important factor for explaining that society - including the medical profession and other health care workers - have a problem to recognise the turning-point of alcohol consumption into alcohol abuse with dependency. It estimated that three-quarters of all alcoholics are able to hold their jobs because of this tolerant and protective attitude of society to alcohol-dependent people (Denney & Johnson 1984).

Alcohol abuse has become one of the main causes of disease and death in the Western world. In the USA, it ranks as the fourth most common cause of death in adults (U.S. Department of Health and Human Services 1983), and the annual cost of alcoholism, due to health expenses and lost productivity, is estimated to be $ 117 billion (U.S. Department of Health and Human Services publication 1987-1519).
The noxious effects of alcohol may lead to systemic disease, and on chronic abuse only a few organ systems are spared from pathological changes (Edmondson 1980).

2.1.2 Systemic disease by alcohol abuse

The most important clinical effects of chronic alcohol abuse are on the nervous system, the striated musculature, the digestive tract, and on the liver.

2.1.2.1 Nervous system

The effects of alcoholism on the nervous system will characteristically lead to Wernicke's encephalopathy, dementia, cerebellar degeneration and peripheral neuropathy. Genetic and nutritional (deficiency) factors play an important role in the pathogenesis of these disorders (Charness et al 1989).
Recently, autonomic nervous dysfunction in alcoholism has been reviewed (Editorial Lancet 1989), and found to be an important cause of morbidity and mortality (Johnson & Robinson 1988). In one study (Thuluvath & Triger 1989) both peripheral and autonomic (parasympathetic) neuropathy were detected in 45% of patients with ALD. These percentages were 22 and 43, respectively, for patients with non-alcoholic liver disease. Sympathetic dysfunction was considerably less prominent than parasympathetic dysfunction in both groups, 11% for ALD and 12% for non-alcoholic liver disease, respectively. No clear correlation could be found between severity of liver damage and both neuropathies, and it appeared from this study that the high frequency of both peripheral and autonomic neuropathy are associated with (chronic) liver disease in general, and not merely with ALD and /or the toxic effect of alcohol on these nervous systems.

2.1.2.2 Musculature

In the striated (cardiac and skeletal) musculature, chronic alcohol abuse results in myopathy (Petersson 1988; Urbano-Marqez et al 1989; Charness et al 1989). Cardiac and skeletal myopathies are seriously underrecognised complications of alcohol abuse, and are probably directly related to the effect of alcohol itself and not by accompanying malnutrition, which may potentiate the adverse effects of alcohol (Diamond 1989). When peripheral muscle biopsy was performed, myopathy could be diagnosed in 46% (Urbano-Marquez et al 1989) and 60% (Martin et al 1985) of

ambulatory and hospitalised patients, respectively. A direct correlation was found for both myopathies of the prevalence and the severity of myopathy and the estimated lifetime large amount of ingested alcohol (Diamond 1989). Peripheral muscle damage was found when the total dose of alcohol exceeded 13 kg per kg body weight, which is equivalent to a man of 70 kg drinking over 120 gr ethanol a day for 20 years (Urbano-Marquez et al 1989).

2.1.2.3 Digestive tract and liver

The most important effects of alcohol abuse on the digestive tract are gastro- enteritis and pancreatitis. Both disorders cause malabsorption that may lead to malnutrition. Pancreatitis itself is associated with high mortality.
In the liver, alcohol abuse may lead to alcoholic liver disease (ALD), the main issue of this chapter.

2.1.2.4 Fetal and post-natal alcohol syndrome

In the last decades, the damaging effects of alcohol abuse on the outcome of pregnancy have been clearly defined. These effects are increased rates of abortion and still-birth, of fetal alcohol syndrome composed of anatomical and functional defects, notably of the central nervous system, and of facial abnormalities. Also functional (neuropsychological) deficits in early childhood have been recognised (Murray-Lyon 1985; Abel 1985).
The fetal alcohol syndrome is reported to occur in a third of infants born to mothers who drink more than 150 g of ethanol daily during pregnancy; another third becomes mentally retarded (West et al 1984).
Because of the potentially harmful early and late effects of alcohol ingestion on the developing fetus, even of such small dose as 3 drinks a day, the American Medical Association has recommended that pregnant women or women attempting to be become pregnant should refrain from drinking alcohol (Charness et al 1989).

2.2 Alcoholic liver disease: the problem

2.2.1 General introduction

In spite of the fact that alcoholic liver disease (ALD) is a very common disease with serious consequences for the individual involved and for society as a whole, the pathogenesis is still mysterious to a large extent. For that reason, no adequate therapeutic measures can be taken otherwise than giving the advise to further refrain from alcohol consumption.

Alcohol is removed from the body mainly by the liver through metabolism. Stomach, lungs and kidneys play only a minor role in the elimination of alcohol. In the liver, three metabolic pathways for alcohol clearance are recognised. They are located in different compartments of the parenchymal cell (hepatocyte) and are in order of significance: the alcohol dehydrogenase (ADH-) pathway, the microsomal ethanol-oxidising system (MEOS) and the catalase-pathway (see 2.2.5.1).

As already mentioned, alcohol abuse and alcohol related problems and diseases, included those of the liver, may be difficult to detect because a great proportion is not as easily visible as in excessive and addicted alcohol consumers with very high disease risk. This largely unknown category of alcohol abusers goes often hidden in the unsuspected majority of the so-called moderate (social and home-) consumers of alcohol (Brunt 1988). Other more basic and unsolved problems of alcohol consumption are specifically related to the liver.

Although the quantitative risk of ALD in the population rises parallel to increased alcohol consumption (Lelbach 1975), not all drinkers develop ALD, and it is still not known what the necessary daily quantum and the duration of alcohol intake are to incur ALD and - probably most importantly - which individuals have a predisposition to susceptibility for ALD (Bosma et al 1989a). Moreover, exogenous (co-) factors, such as other hepatotoxins (drugs) or concomitant (viral) liver disease may play an additional modulating or even determinant effect on the pathology of ALD (Johnson et al 1985; Brunt 1988). These factors all together may aggravate the difficulty to recognise alcohol related liver disease.

The diagnosis of ALD as such is often difficult because of the lack of specific symptoms and signs, and will be usually based on the combination of clinical,

laboratory and histopathological findings. Of all diagnostic tools, the histopathological evaluation of the liver biopsy is essential to make a definite diagnosis of ALD (Desmet 1985).

2.2.2 Histopathology

2.2.2.1 Introduction

ALD is remarkably diverse in its histopathological expression. Virtually all forms of liver pathology have been found in relation to alcohol, including the lesions chronic active hepatitis and cholestasis (Brunt 1988).
Moreover, in heavy drinkers an incidence as high as 20% of non-alcohol related liver diseases have been reported (Hall 1985), and this concomitant liver disease may modify the histopathological expression of ALD.
The three main liver lesions of ALD - which may occur together - are (Scheuer 1988): fatty liver, alcoholic hepatitis with fibrosis, and cirrhosis.
Other liver lesions and syndromes related to chronic alcohol abuse are chronic pancreatitis, siderosis, fetal alcohol syndrome with fatty liver and fibrosis as found in adult alcoholics (Lefkowitch et al 1983) and hepatocellular carcinoma.

2.2.2.2 Fatty liver

Fatty liver, or steatosis, caused by alcohol is of the macro-vesicular type. Some alcoholics develop, usually in addition, acute fatty liver of the micro-vesicular type, this so-called alcoholic foamy degeneration (Scheuer 1988) may be associated with a poor prognosis (Morgan et al 1978a).
Fatty liver starts in the perivenular area around the central vein of the liver lobule. Fat accumulation in the parenchymal cells gives rise to gross enlargement of these cells and, consequently, to compression of the sinusoids. Resulting obstruction to the sinusoidal blood flow has been suggested to be the cause of portal hypertension that may be present in cases of merely fatty liver without accompanying fibrosis or cirrhosis (Blendis 1982).

Fatty liver, as single lesion of ALD, is in general a benign condition which is rapidly - in a few weeks (Desmet 1985) - reversible following alcohol withdrawal. It can probably be induced in all individuals (Rubin & Lieber 1968).
Fatty liver is a very common, and for that reason non-specific, finding in liver biopsies. It is associated with a great variety of non-alcohol related diseases and disorders of the liver and other organs such as toxic and drug-induced liver disease, obesity but also malnutrition, metabolic disease notably diabetes mellitus, and inflammatory disease.
In a small proportion of alcoholic patients with the early lesion of fatty liver, acute cholestasis develops. It simulates large bile duct obstruction and may end in liver failure in a few cases (Morgan et al 1978b).

2.2.2.3 Alcoholic hepatitis and fibrosis

Alcoholic hepatitis, also called sclerosing hyaline necrosis (Edmondson et al 1963), is a highly characteristic histological lesion (fig.1). It is generally considered as the hallmark of ALD (MacSween & Anthony 1985) and severe alcoholic hepatitis has been established as a precursor lesion to the development of cirrhosis (Sorensen et al 1984; Marbet et al 1984; Maddrey 1988). It predominates in zone 3. Fatty liver is usually present, depending on recent alcohol intake. Bridging necrosis may be seen in florid cases and may be accompanied, in advanced cases, by formation of fibrous septa.
The three essential features of alcoholic hepatitis are: spotty parenchymal cell damage, inflammation, and fibrosis (Review by an International Group 1981; Scheuer 1988).

2.2.2.3.1 Parenchymal cell damage

The damage by alcohol is composed of swelling of the cytoplasm, also called ballooning degeneration, and necrosis of hepatocytes. In haematoxylin and eosin stained liver sections, ballooning cells stain pale and may contain large, mega-mitochondria and Mallory bodies.
Mega-mitochondria are easily seen as regular rounded, briskly red structures. Their

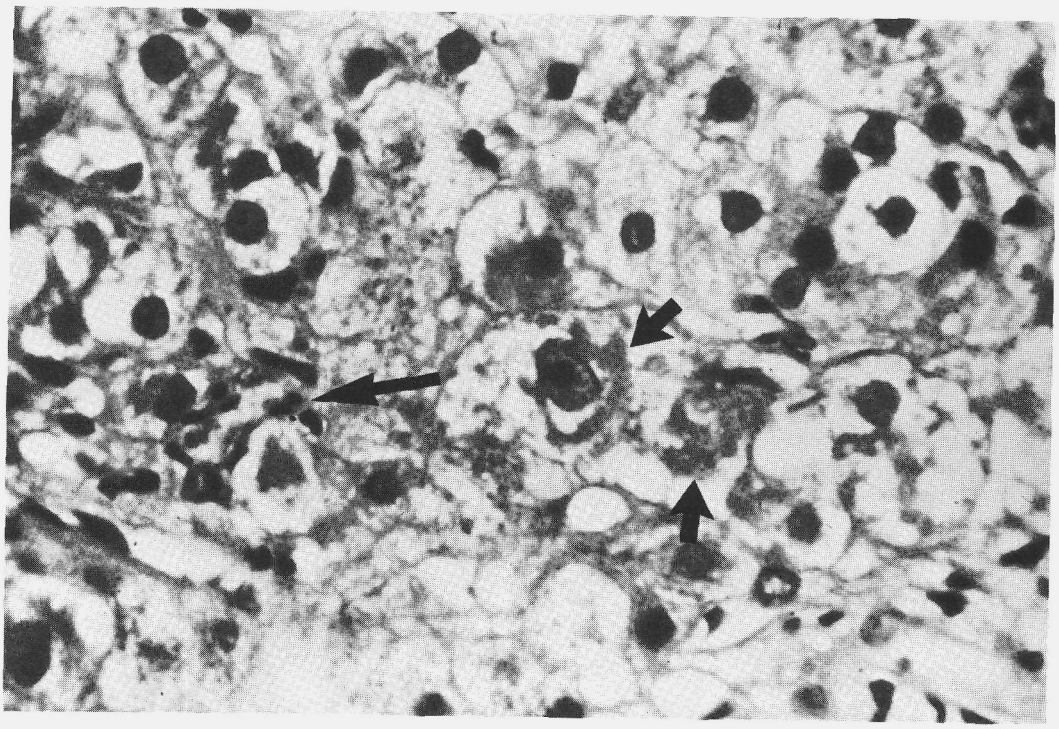

Fig. 2.1: Liver biopsy with severe alcoholic hepatitis. Hepatocytes are swollen and pale ("ballooning"), they contain Mallory bodies (short arrows) and are accompanied by (scarce) polymorphonuclear leucocytes (long arrow). Haematoxylin and eosin. Large magnification.

significance in ALD is not established. Their presence has been claimed to be a marker of recent heavy alcohol consumption (Bruguera et al 1977), but also to be related to a better prognosis of ALD (Chedid 1986).

Megamitochondria are, however, not specific for ALD and may also be found in non-alcohol related liver diseases and occasionally in normal liver (Stewart et al 1982).

Mallory bodies (Mb) or so-called alcoholic hyalin, which is a misnomer because Mb are also regularly found in non-alcohol related liver diseases, particularly in those associated with cholestasis and metabolic disorders. Mb can be experimentally produced in mouse liver by longterm treatment with the antibiotic drug griseofulvin (Denk et al 1975).

In haematoxylin and eosin stained liver sections, Mb are cloudy or compact, ill-

defined strands or clumps of pink-red cytoplasmatic material (fig. 1). Immunohistochemical and electron-microscopical studies have revealed that they are aggregates of altered intermediate filaments of cytoskeleton (Denk et al 1981; Katsuma et al 1987). This may suggest that liver diseases, associated with Mb-formation, are cytoskeletal disorders. However, typical MB can also be found electronmicroscopically in hepatocytes without apparent cellular damage (Feldmann 1989).

2.2.2.3.2 Inflammation and fibrosis

In the *inflammatory response,* usually polymorphonuclear leucocytes predominate. The inflammatory cells are found around or in damaged hepatocytes, so-called satellitosis. In some cases, inflammatory cells may be scarce or even absent, probably reflecting a late stage of alcoholic hepatitis (Scheuer 1988).

Fibrosis is always present in alcoholic hepatitis, and may remain as its only evidence after disappearance of cell damage and inflammation. This parenchymal type of fibrosis is called pericellular or perisinusoidal fibrosis (fig. 2). It is highly characteristic for alcoholic hepatitis but sometimes it is inconspicuous. Therefore, connective tissue stains should always be made in order to detect it as evidence of recent alcoholic liver damage.

Apart from this main type of pericellular fibrosis (a), four other types of fibrosis may be encountered in ALD (Fleming & McGee 1984; Desmet 1985) : round lipogranulomas developed from groups of ruptured and coalesced steatotic hepatocytes (b); (peri-) portal fibrosis (c) being suggestive of chronic fibrosing alcoholic pancreatitis and stricture of the pancreatic and large bile ducts (Petrozza et al 1984); perivenular fibrosis or sclerosis, resulting in thickening and narrowing of the central vein (PVS) (d); and central sclerosing hyaline necrosis (Edmondson et al 1963) (e). The last type of fibrosis (e) shows in acinar zone 3 an increasingly dense collagenisation that is associated with loss of hepatocytes and that entraps the surviving hepatocytes. It may represent a severe form of pericellular fibrosis (Desmet 1985).

Perivenular fibrosis or sclerosis

Lesions of the central vein (or terminal hepatic vein) in ALD have drawn much

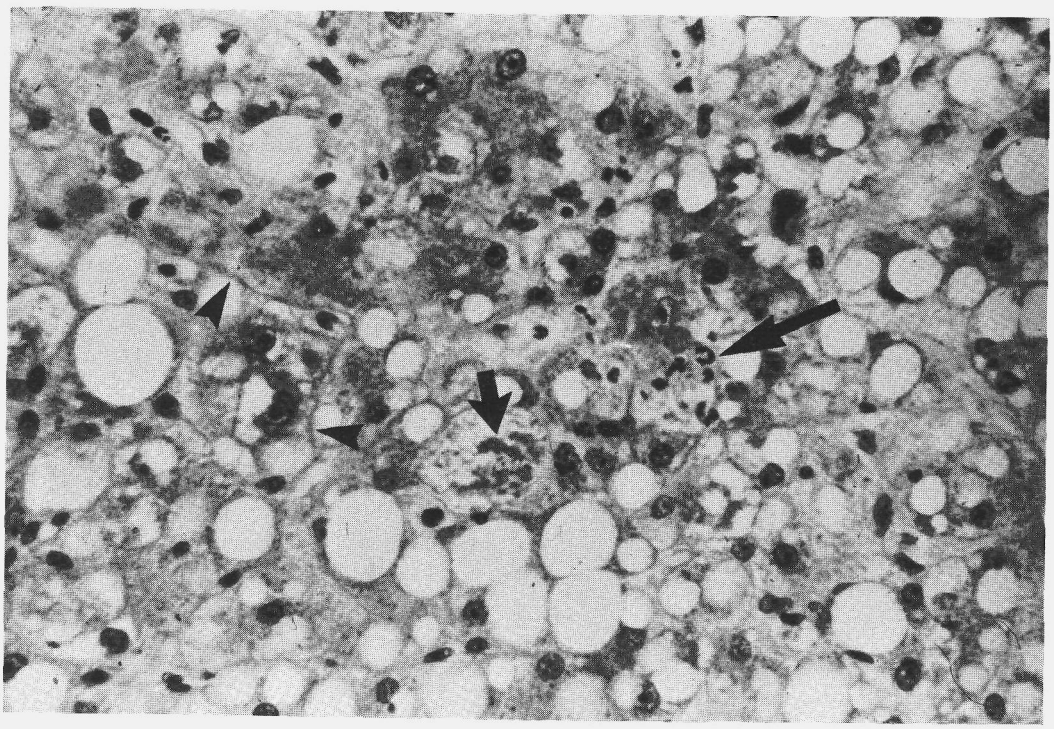

Fig. 2.2: The same liver biopsy of fig. 1 stained for collagen.
It shows macrovesicular steatosis and (in the center) alcoholic hepatitis composed of enlarged hepatocytes with Mallory bodies (short arrow), inflammatory response of several polymorpho-nuclear leucocytes (long arrow) and inconspicuous but clearly present pericellular fibrosis (arrow heads). Elastica van Gieson.

attention during the last decade. In a large study of ALD, Goodman and Ishak (1982) recognised three types of vascular lesions: lymphocytic phlebitis, phlebosclerosis (or perivenular fibrosis and or sclerosis: PVS), and veno-occlusive lesions. Lymphocytic phlebitis, composed of mononuclear inflammatory cell infiltrate of the wall of the terminal hepatic (central) and larger (sublobular) veins, was found in 16% of alcoholic hepatitis and in 4% of cirrhosis patients. PVS, showing scarring of the wall of the terminal hepatic and occasionally of sublobular veins with gradual narrowing of their lumina, was present in all patients with alcoholic hepatitis and cirrhosis. Veno-occlusive lesions, showing intima proliferation with fibrosis and narrowing of the lumen of terminal hepatic (central), sublobular and occasionally portal (lobular) veins,

were present in 74% of the patients, in 46% with total lumen obstruction. Corresponding with the degree of veno-occlusive disease and PVS, portal hypertension was found in 47% of cases with alcoholic hepatitis. However, in an also large retrospective biopsy and post mortem study, Burt and MacSween (1986) were not able to confirm this high prevalence of phlebitis (found in 4%) and veno-occlusive disease (in 22%) in alcoholic hepatitis or cirrhosis. These authors did confirm the high incidence of PVS: it was present in all cases of alcoholic hepatitis and cirrhosis. They suggested a different pathogenetic mechanism for PVS than for the other two vascular lesions, and that this particular lesion is an important contributory factor in the development of progressive liver injury.

PVS and fatty liver

In several other studies of both human and experimental (baboons) ALD, PVS has been found to occur in combination with fatty liver without alcoholic hepatitis.

This combination of lesions has been considered to be an index of prolonged heavy drinking and of susceptibility for progressive liver injury to develop cirrhosis (Van Waes & Lieber 1977b; Popper & Lieber 1980; Nakano et al 1982; Worner & Lieber 1985; Marbet et al 1987).

More in general: although alcoholic hepatitis is still considered to be the main pathway to progressive ALD, the concept has arisen that PVS accompanying fatty liver in absence of alcoholic hepatitis, may represent a second, 'alternative', pathway to cirrhosis in ALD (Lieber 1987).

Specificity of alcoholic hepatitis

The highly characteristic histopathological features of alcoholic hepatitis are not specific for ALD. They may be found in such different liver diseases as chronic cholestatic conditions and Wilson's disease (Scheuer 1988). Pericellular fibrosis, usually in combination with fatty liver and inflammation with polymorphs, may be found in a scala of disorders such as obesity, diabetes mellitus, gastro-intestinal by-pass surgery, and as the effect of drugs such as amiodarone and oestrogens. This histopathological similarity of alcoholic hepatitis with some liver diseases of various non-alcoholic etiology may suggest a (partly) common mechanistic pathway.

After jejuno-ileal bypass surgery, occurrence of an identical liver lesion may be explained (Lauterburg et al 1988) by the intestinal overgrowth of bacteria and associated production of acetaldehyde, the breakdown product of ethanol generated in

normal conditions in only neglectable amount.
Pericellular fibrosis, *per se* or in combination with fatty liver, may also be associated with a great variety of non-alcohol related conditions such as hypervitaminosis A (Russell et al 1974), chemotherapy (notably methotrexate), haematological disorders with increased platelet destruction (Lafon et al 1987), or may remain unexplained (Bosma et al 1989b, see chapter VII).

2.2.2.4 Cirrhosis

Cirrhosis is defined as transformation of the normal lobular parenchymal architecture into regeneration nodules surrounded by fibrous septa. It is considered as an irreversible end-stage liver disease.
The other main features of ALD, steatosis and alcoholic hepatitis, may be present in the cirrhotic stage dependent on continuation of alcohol intake.
Regeneration nodules originate from outgrowing parenchymal cells which survived preceding alcoholic hepatitis. Alcoholic cirrhosis is initially micro-nodular (fig.3),

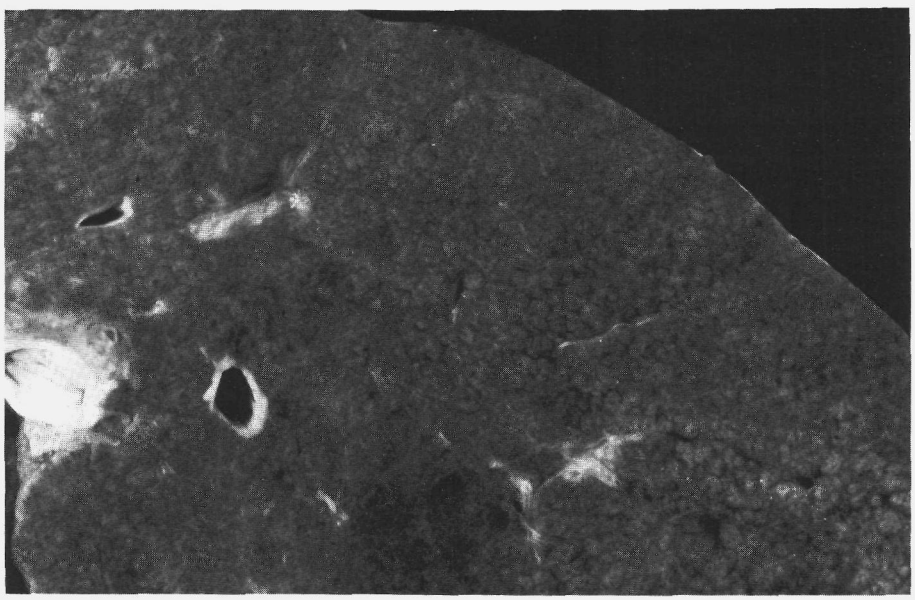

Fig. 2.3: Post mortem liver with micronodular cirrhosis due to chronic alcohol abuse. The cut surface shows diffuse nodularity of the parenchyma by regeneration. Virtually all nodules are small, of about the size of a normal liver lobule.

meaning that the maximal diameter of most parenchymal nodules does not exceed about three mm (the size of the normal liver lobule). The nodules may enlarge by compensatory hyperplasia (Anthony et al 1978), and hyperplastic nodules may develop, the rate and prevalence of which are related to the amount of continued alcohol consumption (Gluud et al 1987).
It is generally accepted, that, eventually micronodular cirrhosis will convert into cirrhosis of macronodular type (Fauerholdt et al 1983). In this late stage of macronodular cirrhosis, liver cell dysplasia and thereupon hepatocellular carcinoma may develop in patients who escaped, or survived, earlier life-threatening complications of cirrhosis, mainly bleeding from ruptured oesophageal varices and liver failure.
About 15% of alcoholic cirrhotics develop hepatocellular carcinoma (Fleming & McGee 1984).

2.2.2.5 Immunopathology

Immunoglobulin A deposition can be found in all stages of ALD in a continuous pattern along the sinusoids. This has been considered by some authors (Kater et al 1979; Swerdlow et al 1982; Goldin et al 1986; Van der Wiel et al 1987) as a reliable (immuno-) histological marker of ALD that combines high sensitivity with high specificity. The potential role of IgA and of other (auto-)immune phenomena in the pathogenesis of ALD will be reviewed later (see 2.2.5.2).
Several immunohistochemical changes of the cytoskeleton of hepatocytes in ALD consisted of alterations in cytokeratin expression on parenchymal cells normally found on bile duct epithelial cells (Burt et al 1987; Ray 1987; Katsuma et al 1987; Van Eyken et al 1988), but the diagnostic value and the significance of these findings are unclear as yet.

In summary:
The histopathological presentation of ALD is very diverse, as yet for unknown reasons. The essential feature is alcoholic hepatitis; this and sclerotic lesions in and round the central (terminal hepatic) vein are associated with progressive liver disease and therefore with a poor prognosis.

2.2.3 Clinical and biochemical aspects

2.2.3.1 General remarks

The clinico-pathological spectrum of ALD with its main three components: fatty liver, alcoholic hepatitis and cirrhosis is dependent on the population which is studied (see also 2.2.4).
The prevalence of ALD in the general population is unknown (Mackinnon 1985), but in an Australian (Bhatal et al 1973) and British (Krasner et al 1977) study, 75% of alcoholic patients who presented clinical features had alcoholic hepatitis and/or cirrhosis.
The early recognition of alcohol abuse and associated liver disease depends on a high index of suspicion on the part of the physician (Sherlock 1985), because at presentation the patient's physical symptoms and complaints may be vague and non-specific or even misleading and only related to social and psychic dysfunctioning. Moreover, in ALD - as in liver disease in general - no good correlation exists between clinical-biochemical features and histopathological findings.
Alcohol abuse, arbitrarily defined as at least 100 g ethanol consumption per day, and ALD are generally underdiagnosed by the medical profession and health workers (Van der Wiel 1986). This underdiagnosis will be aggravated by the disparity of the clinical and histopathological features of ALD.

2.2.3.2 Fatty liver and alcoholic hepatitis

Fatty liver, the hallmark of continued alcohol ingestion (Mackinnon 1985), usually presents with hepatomegaly by fat accumulation in the hepatocytes. The alterations of serum lipoprotein fractions which accompany ALD, are not diagnostic.
Alcoholic hepatitis is reflected by aspecifically raised levels of serum liver enzymes, notably transaminases, released from damaged hepatocytes into the circulation. A wide range of severity is typical for alcoholic hepatitis, but usually serum transaminases levels are only modestly elevated and the SGOT/SGPT ratio is generally over 2 (Sherlock 1985; Cohen et al 1979).
Alcoholic hepatitis is potentially reversible (Williams et al 1984), but overall mortality

of severe cases is reported to be as high as 57% (Theodossi et al 1982). *Serum amino glutamyl transpeptidase (gamma-GT)* activity has been reported to be of diagnostic value in the early alcoholic patient with hepatitis (Van Waes & Lieber 1977a). This has been disputed by others because of a level of this enzyme only reflects induction of hepatocytic microsomes by ethanol (Ishi et al 1976). Therefore, it may be used as screening test for alcohol abuse (Nishimura et al 1983; Sherlock 1985) and not necessarily for the presence of ALD.
Another useful screening test for alcohol abuse is macrocytosis of the erythrocytes. A mean corpuscle volume of more than 95 μ is of diagnostic value (Sherlock 1985).
The liver plays an unique but still poorly understood role in mucosal immunity in relation to *IgA* and in the physiology of IgA in health and disease states (Brown & Kloppel 1989). Serum IgA-levels are elevated in liver disease of different etiology, such as primary biliary cirrhosis and cirrhosis of unknown etiology, called cryptogenic cirrhosis (Sherlock 1985). Among liver diseases, ALD is most closely linked to alterations in IgA metabolism (Brown & Kloppel 1989).
Considerable serum IgA-elevation is found in all stages of ALD. Although it has been found to correlate with the degree of liver damage (Itturiaga et al 1977), in general serum IgA appears to be related to alcohol abuse rather than to the resulting liver damage (Editorial Lancet 1983).
IgA-elevation in ALD is part of polyclonal hypergammaglobulinaemia. This feature will be discussed later (see 2.2.5.2).

2.2.3.3 Cirrhosis

In the cirrhotic stage of ALD, the dominant clinical signs are related to portal hypertension and to liver cell failure. Patients present with upper gastro-intestinal bleeding from ruptured oesophageal varices, liver failure with encephalopathy, and jaundice and ascites.

Bacterial infection
Increased incidence of bacterial infections is a well recognised complication of ALD (Rajkovic et al 1984). Endotoxaemia of enteric origin is common (Nolan 1975; 1989).

So-called spontaneous bacterial peritonitis is a common complication of cirrhosis (Frank et al 1977) and is often recurrent (Tito et al 1988). It is a cause of accelerated death (Mackinnon 1985) by its usually fatal outcome. It results from spontaneous bacteremia with seeding of "susceptible" ascites and is caused by impaired defence, mainly due to failing of the complement system, the neutrophil function and the mononuclear phagocytic system (Runyon 1988)(see 2.2.5.2.3).

Haemosiderosis

Haemosiderosis (iron accumulation) of the liver is common in ALD, found in up to 29% of cases (Chapman et al 1982). It reflects a derangement of iron metabolism associated with alcohol intake and is not attributable to an increased iron absorption from the gut (Chapman et al 1983). Haemosiderosis is not considered to be a factor in the pathogenesis of ALD (Aisen 1985).

Endocrine alterations

Signs consistent with a hyperoestrogenic state such as erythema palmare, spider nevi, testicular atrophy and gynaecomastia are often present in male cirrhotics. This hyperoestrogenic state is probably not caused by impaired hepatic metabolism of oestradiol alone. A hypothalamic-pituitary defect may also play a role (Mackinnon 1985).

These factors may contribute to the toxic effects of alcohol on the liver and to ethanol-induced gonadal injury and dysfunction (Galvao-Teles et al 1986). The gonadal effects are less prominent in females than in males (Van Thiel & Lester 1979).

Viral liver disease

Concomitant viral liver disease, notably hepatitis B and C virus infection, is a (co-) factor of controversial importance for the pathogenesis of ALD. Clinical and serological features of viral disease are often found in patients with ALD, and viral liver disease may even be the primary liver event in alcoholics in view of the findings at liver biopsy (Sherlock 1985) (see further 2.2.4.5.2).

In summary:

There is no reliable clinical or biochemical guide to trace ALD, the best is as yet (Sherlock 1985) the suspicion of alcohol abuse. Combined clinical and laboratory

investigations are helpful in trying to establish the diagnosis ALD, but they are poor indicators of subsequent performance (Brunt 1986).

2.2.4 Epidemiology

2.2.4.1 Introduction

In theory, epidemiology should greatly contribute to unravel factors related to etiology and pathogenesis of ALD by its capacity to quantify and evaluate the relationships between the many variables thought to influence the occurrence of ALD. In this way epidemiology should bridge the gap between multifactorial concepts and theories about etiology (Grant et al 1988).
However, in the epidemiological analysis of ALD many methodologic problems are encountered. These problems are related to variables such as: alcohol consumption with respect to quantity, duration and pattern of consumption; diet; the officially reported statistical data on per capita alcohol consumption, on the nature of the liver disease and on the mortality.
When interpreting the results of epidemiological investigations on ALD, the composition of the population which is studied should include age, sex, race, severity of liver disease, concomitant (liver) disease of other etiology, and environmental factors. Heterogeneity and inexact definition of many of these categories often preclude comparability of epidemiologic reports on ALD from different countries (Grant et al 1988).
The prevalence of ALD in different countries depends largely on religious and other customs, and on the cost of alcohol and on earnings (Sherlock 1985).
The incidence of ALD is usually calculated from the prevalence of liver cirrhosis among chronic alcoholics at autopsy. This incidence varies from 10-20% (Marbet et al 1987). Thus, it is clear that the majority of chronic alcohol abusers will escape end stage liver damage.

2.2.4.2 Recognised risk factors for ALD

2.2.4.2.1 Drinking habits

Alcohol quantum

Data of ALD in relation to drinking habits are drawn from statistics of various countries, which report death from cirrhosis in relation to the litres of pure alcohol consumed per caput per year (Johnson & Williams 1986). From these statistics it can be calculated that the average danger dose for all these (European) countries is larger than 80 g alcohol per day (Sherlock 1985), although the maximal amount of pure ethanol that can be metabolised by the liver per day without harm is generally considered to be 60 g for men and 40 g for women (Achord 1988).

Duration of alcohol consumption

The time span of consumption is important. In a study of a large group of alcoholics with an average daily alcohol consumption of 160 g for less than 5 years, neither cirrhosis nor alcoholic hepatitis were found, whereas 50% of 50 alcoholics who had been drinking for an average period of 21 years developed cirrhosis (Lelbach 1975).

Alcoholic beverage

The type of alcoholic drink is reported to be unimportant and liver injury to be related only to its alcohol content (Sherlock 1985; MacSween & Anthony 1985).

Drinking pattern

Continued daily drinking is thought to be more dangerous than intermittent drinking, the latter giving the liver a chance to recover (Sherlock 1985; Grant et al 1988).

Alcohol dependence

A study from London found that individuals who develop ALD are usually only mildly dependent on alcohol (Wodak et al 1983). This intriguing finding could have been due to a selection bias in the population studied, but the lack of overt signs of alcohol dependence increases the chances of an individual maintaining high alcohol consumption for many years, thereby increasing the risk of developing ALD.

2.2.4.2.2 Gender

Alcohol abuse is increasing in women due to changing social circumstances and by ready availability of alcohol in supermarkets (Sherlock 1985). Women are more susceptible to liver damage by alcohol than men (Sherlock 1985; Johnson & Williams 1985; Grant et al 1988), and they are more likely to relapse after treatment (Morgan & Sherlock 1977).

Women are, compared to men, more likely to develop progressive liver disease after consumption of less alcohol over a shorter period of time. Some have rapid progression, histologically reflected by chronic active hepatitis, suggesting that immunological factors might be responsible for this difference (Marbet et al 1987).

Even after stopping drinking, women may be at greater risk of developing cirrhosis (Pares et al 1986; Maddrey 1988).

The reason why alcohol affects the female liver differently is unclear. An contributing factor could be the smaller body water compartment of women over which alcohol is distributed.

This can explain why in women higher ethanol blood levels are reached than in men after ingestion of the same alcohol dose (Marshall et al 1983). A second factor is impaired oxidation of alcohol in the stomach. This has been shown to be present after chronic alcohol consumption (Di Padova et al 1987). Recently, Frezza et al (1990) showed that gastric alcohol dehydrogenase activity and alcohol oxidation in the stomach were lower in women than in men. This may contribute to higher blood levels and, in this way, to increased susceptibility to ethanol in women (Frezza et al 1990; Schenker & Speeg 1990).

2.2.4.3 Genetic and immunological factors

Genetics

Patterns of alcohol drinking behaviour are reported to be inherited, but no single gene has been shown to be associated with susceptibility to develop ALD (Sherlock 1985; Goodwin 1986).

Alcohol metabolism

Present knowledge is controversial whether genetic variation in the availability and activity of the alcohol metabolising enzymes: alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH), can explain individual susceptibility to develop ALD (Grant et al 1988).

HLA-system

Conflicting data regarding an association of particular phenotypes of the human leucocyte antigen (HLA) system with ALD have been reported. HLA-status is probably important (Johnson & Williams 1985; Doffoel et al 1986). In Britain, an increased prevalence of HLA-B8 was shown in several studies (Saunders et al 1982), while in Norway an association of ALD with B40 and in Chile with B13 was found (Sherlock 1985). However, in a previous review only a weak association between these HLA-antigens and alcoholic cirrhosis remained (Eddleston & Davis 1982).

Race

In a recent American epidemiological multicenter study of ALD among several ethnic groups, survival differed significantly, indicating that ethnic origin was clearly associated with the outcome of ALD (Mendenhall et al 1989). However, this study did not include several factors related to environment, socio-economic and dietary habits, and these factors may have contributed to the difference in prognosis.

2.2.4.4 Environmental factors

2.2.4.4.1 Nutrition

Good nutrition is considered not to protect the liver against the (potentially) toxic effects of alcohol (Achord 1988) and the influence of malnutrition on the course of ALD is controversial. In ALD, malnutrition is, however, undeniably as a co-factor (Sherlock 1985) greatly dependent on the population studied, especially when low socio-economic status is associated with malnutrition (Mendenhall et al 1984). Malnutrition can be considered as a complication of alcoholism that precedes liver

injury by alcohol (Achord 1988; Mendenhall et al 1984).
Although a difference was found in the nutritional state of alcoholic patients with and without cirrhosis, it is uncertain whether a bad nutritional state plays the role of making the individual more susceptible for ALD (Grant et al 1988).
However, adverse effect of malnutrition on rapid repair of alcohol induced liver damage has been suggested (Achord 1988), and nutritional deficiencies may determine the long-term prognosis of patients with ALD by predisposition to infection through impairment of the immune system by malnourishment (Maddrey 1988). The interaction of nutrition with alcohol in ALD will be dealt with in the next paragraph (see 2.2.5) and in chapter III (see 3.7) and chapter VIII.

2.2.4.4.2 Viral liver disease

It is not certain if concomitant viral liver disease will accelerate ALD.
Most studies on the relation of ALD with viral liver disease have been of hepatitis B virus (HBV) infection, the markers of which are often increased in patients with ALD. HBV infection did not influence ALD with cirrhosis (Chevillotte et al 1983) nor did it induce the development of chronic liver disease in alcoholics, except in the small number of cases with persistent infection who remained HBV surface antigen positive (Villa et al 1982; Saunders et al 1983).
Hepatocellular carcinoma
Although in general the weight of evidence is that markers of HBV are acquired late in the natural history of ALD, and that HBV infection is usually quickly resolved in ALD (Bird & Williams 1988), it may still play a role in the development of hepatocellular carcinoma in ALD. In one study, patients with ALD were screened for markers of HBV infection (Brechot et al 1982). All 20 cases who developed hepatocellular carcinoma in end-stage liver disease (cirrhosis) appeared to have integrated HBV DNA in the genome of the tumour cells, irrespective of the presence or absence of serum markers (all were HB surface antigen negative).
Hepatoxic drugs
Regarding the effects of potentially hepatotoxic drugs on the course of (human) ALD, little research has been conducted (Grant et al 1988).

In summary:

Epidemiologic studies definitely have shown, both at the aggregate and at the individual level, a positive correlation between total consumed alcohol and the risk to develop ALD. However, only a proportion of heavy drinkers will develop ALD and the specific factors which determine individual vulnerability for ALD remain unclear. Female sex is a risk factor, but the role of genetic HLA-related factors, of nutrition and of other environmental factors such as viral liver disease, is not obvious to play a determinant role in the development of ALD.

2.2.5 Pathobiology

2.2.5.1 Alcohol metabolism

The metabolism of alcohol will be reviewed only with regard to its potential implications for the pathogenesis of ALD.

2.2.5.1.1 General introduction

After ingestion and rapid absorption from the gut, alcohol has to be removed from the body because it can not be stored in tissues. Removal is mainly by the liver. Only

2-10% (Lieber 1985) will be eliminated by the kidneys and lungs. Recently, the importance of the stomach in first-pass ethanol elimination through oxidation by gastric dehydrogenase has drawn attention (Di Padova et al 1987). The stomach may trap a substantial percentage of the ingested ethanol and prevent this portion from entering the systemic circulation (Lieber 1988a).
The parenchymal liver cells are by far the most important ethanol metabolising cells of the body, but in man (Wickramasinghe 1985;1986) and in an experimental animal (mouse) (Wickramasinghe et al 1987) blood monocyte-derived tissue macrophages have been shown to have a considerable capacity, at least in vitro, to metabolise ethanol. These macrophages, isolated from various organs, generated ethanol-

dependent cytotoxic activity which was suggested to contribute to, or even to be fully responsible for, the organ damage as found in vivo as the effect of ethanol (Wickramasinghe et al 1987).
Alcohol is metabolised in the liver by oxidation. All known oxidative pathways of ethanol metabolism in the hepatocytes result in the production of two products: acetaldehyde and hydrogen. Acetaldehyde will be converted to acetate by aldehyde dehydrogenase.
Three main metabolic pathways for alcohol elimination by the liver exist, located in three different compartment of the hepatocyte: the alcohol dehydrogenase (ADH) pathway in the cytosol, the microsomal ethanol oxidising system (MEOS), and the catalase pathway in the peroxisomes.

2.2.5.1.2 The ADH-pathway

This pathway is the main ethanol metabolising route under 'normal' circumstances. The involved enzyme, ADH, has a broad specificity (Lieber 1985). It plays a physiological role in the removal of the small amount of ethanol generated by bacterial fermentation in the gut (Krebs et al 1970) and is involved in the metabolism of steroids and fatty acids in the liver.
Many forms of ADH exist with different frequencies in different populations, but the composition and genetic determination are not yet fully elucidated (Lieber 1985).
In ethanol oxidation, ADH mediates the transfer of hydrogen to the co-factor nicotinamide adenine dinucleotide (NAD) and reduces this to NADH. This generates an excess of reducing equivalents - as free NADH - in the cytosol, primarily because the metabolic systems involved in the removal of NADH are not able to offset its accumulation completely (Lieber 1988a). In this reaction acetaldehyde is produced that is converted to acetate by acetaldehyde dehydrogenase; this latter reaction is linked with the conversion of the oxidised form of NAD to its reduced form NADH. An enhanced NADH/NAD-ratio results, and this implies an altered redox state.
Altered redox state
Several hepatic impairments have been attributed to the altered redox state of the cytosol (Lieber 1982; Lauterburg et al 1988). Hyperlactacidaemia may develop

because both decreased utilisation and enhanced production of lactate by the liver occur.
The altered redox state may also lead to ketoacidosis, may impair gluconeogenesis from amino-acids, and may facilitate hypoglycaemia which is a common feature of alcohol consumption.
Instead of utilising fatty acids, which are normally the main energy supply source of the liver, the mitochondria will use the hydrogen equivalents originating from the ethanol oxidation. The decreased oxidation of fatty acids results in fat storage in the liver. This explains the first histological stage of ALD: fatty liver.
Also protein metabolism may be affected by the abnormal redox state because an inhibitory effect on protein synthesis in vitro has been shown (Baraona et al 1980). This has, however, not been clearly established to occur in vivo (Lieber 1985).
The ADH pathway has also been associated with altered hepatic steroid and testicular sex steroid metabolism in alcohol abuse (Lieber 1985).

2.2.5.1.3 The MEOS-pathway

Non-ADH mediated ethanol oxidation by MEOS is reported to account for 20- 50% (Lieber 1985) of total ethanol metabolism. The percentage is related to blood ethanol concentration. MEOS thus plays a significant role in chronic alcohol abuse when blood ethanol concentrations are increased.
The ethanol oxidation by MEOS is largely independent of the third pathway of ethanol oxidation by catalase (Lieber 1985).
MEOS activity is dependent on the reduced form of nicotinamide adenine phosphate (NADPH) which generates H_2O_2 for ethanol oxidation. Increased activity is morphologically reflected by proliferation of the smooth endoplasmic reticulum.
This proliferation is also observed after the ingestion of many xenobiotics (Maldolesi et al 1976) which points to a common metabolic pathway with ethanol.
MEOS and P 450IIE1
Recently the importance of an ethanol-inducible cytochrome P450 subfraction of MEOS, named P 450IIE1 (Nebert et al 1987), was discovered.
Before the human equivalent was purified and characterised (Lasker et al 1987), P 450IIE1 had been detected in the livers of rabbits (Koop et al 1982) and rats (Ryan et

al 1986). In animal livers, it showed to have a high capacity not only to metabolise ethanol but also agents like carbon tetrachloride (CCl_4), acetaminophen (paracetamol) and acetone.
Lieber (1988a) has focused the attention upon the clinical significance of this enzyme P 450IIE1 for the metabolism and tolerance to ethanol, and upon its unique capacity to activate hepatic metabolism of several xenobiotic agents and drugs (such as CCl_4 and acetaminophen) in chronic alcohol consumption. It explains the (metabolic) drug tolerance to many drugs commonly found in chronic alcoholics (Lieber 1985). This is in contrast with inhibited xenobiotic metabolism as observed in short-term alcohol consumption (Lieber 1982), which is partly explained by competition for common microsomal detoxification (Lieber 1988a).

2.2.5.1.4 The catalase-pathway

The enzyme catalase is located in the peroxisomes and mitochondria of hepatocytes. In spite of the capacity of catalase to oxidise ethanol in the presence of an H_2O_2 generating system in vitro (Keilin et al 1972), it is not generally believed that this pathway plays an important role in vivo (Lieber 1985) because its activity is limited by the rate of H_2O_2 generation and not by the amount of catalase itself.

2.2.5.1.5 Alterations in ethanol metabolism

It is often difficult to explain why *in vivo* a changed rate of alcohol metabolism is found (Lieber 1985), because many factors, such as concomitant liver disease and drugs, can influence it.
However, in general the effects of drugs and other agents on ethanol metabolism are not very pronounced (Lieber 1985), in severe contrast with the effect of acute and chronic ethanol consumption on drug metabolism.
Ethnic and genetic variation in the rate of ethanol metabolism and in the physiological response to alcohol exist but are often not clearly defined in view of other factors, such as drinking patterns and nutrition. Furthermore, present knowledge does not explain individual susceptibility to develop ALD (Grant et al 1988).
Chronic ethanol consumption leads to alcohol tolerance, mainly by adaptation of the

central nervous system but also by increased blood ethanol clearance or metabolic tolerance (Ugarte et al 1972).
The nature of this metabolic tolerance is still unclear (Lieber 1985). ADH-related increased metabolic activity is not likely to be the cause but increased activity of MEOS found to occur in chronic alcohol consumption of both experimental animal and man (Mezey et al 1971) and reversible on alcohol withdrawal.
Accelerated ethanol metabolism results in enhanced production of acetaldehyde and its toxic effects. This explains two early conspicuous features of ALD (Lieber 1985), namely fatty liver and hepatomegaly.

Hepatomegaly and portal hypertension
Fat accumulation is not the only explanation of liver enlargement. It is responsible for only half of the liver's dry weight increase. The other half is due to increase in protein content of the hepatocyte, with water retention in proportion to protein retention (Baraona et al 1975; 1977), as a secondary effect of aldehyde-induced impairment of protein secretion (Lieber 1985).
Both fat and protein accumulation in parenchymal cells lead to hepatomegaly and to associated portal hypertension by reduction of the sinusoidal space between the enlarged parenchymal cells (Orrego et al 1981; Vidins et al 1985). Sinusoidal narrowing, due to hepatocyte expansion and correlating with portal pressure, was found to occur only in ALD, and not in non-alcoholic liver disease (Vidins et al 1985). However, in studies of other research groups on human liver (Krogsgaard et al 1984) and on alcohol-fed baboon liver (Miyakawa et al 1985), a significant correlation between hepatocyte volume and portal pressure could not be demonstrated. Portal hypertension can therefore not be explained by structural changes of hepatocytes alone.
Ultrastructural changes
The swelling with distortion of hepatocytes in chronic alcohol consumption may result in severe impairment of important cell functions and could promote progression of liver injury (Lieber 1985). Additional structural subcellular changes are swollen and abnormal mitochondria found both in experimental animal and man (Lane & Lieber 1966; Rubin & Lieber 1967;)(see also 2.2.2.3.1).
These morphological abnormalities, which are not necessarily related to progressive

liver disease by alcohol (French et al 1983), are considered to be related to the effect of ethanol itself (Lieber 1988) and not to other, for instance nutritional factors. They are associated, as already mentioned, with important functional impairment such as decreased oxidation of fatty acids and many other substrates including acetaldehyde (Lieber 1988).

MEOS activation and hypermetabolic state

Increased microsomal activity in chronic alcohol abuse may also enhance oxygen requirements (Lieber 1988). This rather hypothetical, so-called hypermetabolic state - because increased oxygen consumption was found in rats after chronic ethanol administration (Videla et al 1970) - may aggravate already present liver hypoxia. Oxygen tension is lowest in zone 3 (perivenular area) of the liver lobule, and this may explain the predominance in this zone of morphological lesions caused by ALD and by various agents, such as CCl_4 and acetaminophen, which also need metabolisation or inactivation by microsomal enzymes of the cytochrome fraction.

This shared microsomal metabolic pathway of cytochrome P 450 of MEOS with spill-over to various other drug metabolising microsomal systems, may also at least partly explain (Lieber 1985) the enhanced hepatotoxicity of CCl_4 (Hasumura et al 1974; see also Chapter V) and of acetaminophen (Sato et al 1981) in chronic ethanol consumption.

However, the hypoxia-hypermetabolic state theory to explain relatively selective perivenular hepatoxicity by ethanol and agents as mentioned, is still hypothetical (Lieber 1988a). A better explanation might be that the redox shift produced by alcohol aggravates the perivenular hypoxia (Jauhonen et al 1983). Also zonal distribution of liver metabolic enzymes might contribute to this selective perivenular damage of ALD because MEOS and P 450 IIE1 are maximally induced in zone 3 after long term ethanol consumption (Lane & Lieber 1966).

Vitamin A

Nutritional factors too may interact with enhanced microsomal activity. For instance, altered vitamin A level, which is commonly depressed in alcoholics, may contribute to liver lesions after chronic ethanol consumption (Leo et al 1983; see also Chapters IV and VI) as vitamin A status has clearly been shown to influence, or even to

determine, liver lesions experimentally induced by CCl_4 administration (see Chapter VI).

2.2.5.1.6 Acetaldehyde toxicity

Acetaldehyde (and/or its metabolites) is the most likely candidate to play a key role in mediating, directly or indirectly, alcohol related-damage to the liver and other organ systems. This product of ethanol oxidation is a highly reactive agent, reacting with amino- groups of proteins, peptides, nucleotides, phospholipids and aminoacids. Possible toxic biochemical effects include (Peters & Ward 1988): direct toxic effect on smooth muscle, inhibition of protein synthesis, impairment of mitochondrial oxidative phosphorylation, inhibition of biogenic amine metabolism -including that of neuropeptides - and formation of condensation products with amines and neuropeptides, direct inhibition of enzyme activity, formation of protein adducts, reaction with nucleotides and phospholipids, and free radical activity.

Free radicals

Some evidence has been given from experimental animal studies that free radical activity is mediating liver injury (Peters et al 1986). In vitro, acetaldehyde elicited lipid peroxidation in isolated perfused rat liver (Mueller & Sies 1983). This may be enhanced, additionally, by glutathion depletion of the liver by binding to acetaldehyde (Shaw et al 1981), thereby reducing the capacity of this peptide to scavenge toxic free radicals (Lieber 1988).

In man, however, the evidence of enhanced free radical activity in chronic alcohol abuse is still limited (Peters & Ward 1988).

Collagen synthesis

In vitro cultures of fibroblasts and myo-fibroblasts - potential precursor cells of collagen production - derived from human (Holt et al 1984) and baboon (Savolainen et al 1984) livers are stimulated by acetaldehyde to produce increased levels of messenger RNA for collagen and collagen protein (Brenner & Chojkier 1987).

In summary:

Alcohol has to be removed after ingestion mainly by oxidative metabolisation in the liver. Acetaldehyde is the product of all three metabolic liver pathways. Only two are directly important: the ADH-pathway, predominant in normal alcohol use, and MEOS being of great clinical significance by its activation in chronic alcohol abuse. MEOS is largely mediated by a specific cytochrome P450 subfraction (P 450IIE1), but is also shared with many other potentially hepatotoxic agents and drugs commonly in use in alcoholics. This may explain enhanced toxic liver damage by these agents due to activated MEOS by chronic alcohol abuse.
The metabolite of alcohol, acetaldehyde, is a very reactive product which has shown to impair, at least in vitro, many cellular synthetic and catabolic functions of the liver (and other organs), and to form protein adducts and aggregates of several kinds. Acetaldehyde undoubtedly must play a key role in the pathogenesis of alcohol-related damage of the liver and other tissues, but the relative importance of all *in vitro* observed effects and events for their contribution to the *in vivo* established alcohol-associated organ damage is undetermined.

2.2.5.2 Immunological aspects

2.2.5.2.1 Introduction

Numerous immune disturbances have been reported in ALD, and immune mechanisms have been claimed to be relevant for the pathogenesis and progression of ALD (Poralla et al 1987; Marbet et 1987). There are, however, no prospective studies on a general alcohol-consuming population, but all present data concern results of *in vitro* tests of immunity of alcoholics who have already ALD (Eddleston & Vento 1988).
Immune disturbances in ALD seem at first sight remarkable in view of the small molecular size of both ethanol and its main metabolite acetaldehyde which excludes immunogenicity (Editorial Lancet 1983; Johnson & Williams 1985; MacSween 1985). Moreover, the usual clinical and histopathological findings of liver diseases of

established (auto-) immune nature, such as chronic active hepatitis and primary biliary cirrhosis, are conspicuously absent in end-stage ALD (Editorial Lancet 1983).
However, it has been established that alcohol consumption may interfere with the immune surveillance by suppressing the systemic immune response sufficiently enough to be at least in part responsible for the increased susceptibility for bacterial infections (Glassman et al 1985).
Defects of both humoral and cellular (specific and non-specific) immune response have been found in ALD.
Genetic factors have already been discussed in the previous paragraph (see 2.2.4.4). Briefly summarised: in alcohol abuse, the female sex is clearly a risk factor and HLA-related factors only are potential risk factors for developing ALD.

2.2.5.2.2 Humoral factors

2.2.5.2.2.1 Hypergammaglobulinaemia

Hypergammaglobulinaemia (HGG) is a well-recognised feature of advanced ALD (Johnson & Williams 1985), but is also found in other chronic liver diseases of widely different etiology (MacSween & Anthony 1985). Reversibility of HGG was demonstrated after (orthotopic) liver transplantation, performed for end-stage liver disease of various aetiology including ALD (Yanaga et al 1989).
In ALD, HGG is polyclonal with a disproportionate increase of immunoglobulin (Ig) A, being mainly of the dimeric fraction (Sancho et al 1981), and of IgG (Itturiaga et al 1977).

Immunoglobulin A
Serum IgA levels were found to correlate with the degree of liver damage (Itturriaga et al 1977). In the liver, IgA is found in all stages of ALD and it is deposited in a continuous pattern along the sinusoids (Kater et al 1979; Swerdlow et al 1982; Goldin et al 1986; Van der Wiel et al 1987). Liver IgA is mainly of the IgA-1 subclass, in contrast to serum IgA of which the IgA-2 fraction is higher (Van der Wiel et al 1987). The reason for this discrepancy in IgA-characteristics of serum and liver is still poorly understood and is thought by some authors (Van der Wiel et al 1987) to represent a

specific effect of alcohol on the immune system, related to the role of the liver in IgA metabolism in ALD.

The liver plays an unique role in mucosal immunity and in the physiology of IgA in normal and in disease states, but the precise associations between the liver and IgA are largely unidentified (Brown & Kloppel 1989).

Several explanations are possible for increased serum IgA-levels in ALD (MacSween & Anthony 1985): it could reflect (the degree of) hepatocellular damage or - in view of the relatively greater increase of the polymeric fraction - a defect in the transport of IgA across the bile duct epithelium into the bile. A third (still hypothetical) explanation of HGG could be a defect in the regulatory function of serum concentration of Ig's due to impairment of the hepatocyte transport of Ig's in ALD.

The mechanisms responsible for HGG in ALD are not established. The number of B-cells, which secrete the Ig's after plasma cell differentiation, is not increased in ALD (MacSween & Anthony 1985). Decreased breakdown of Ig is an unlikely explanation because it is not a known feature of liver disease (Havens et al 1954).

Two main explanations of HGG (Johnson & Williams 1985; MacSween & Anthony 1985) have been presented: firstly, a normal immune response to increased presentation of gut- and food-derived antigens as a result of porto-systemic shunting and of reduced Kupffer cell phagocytic function and, secondly, an abnormal immune response due to impaired T-lymphocyte function, which fails to switch off in time the Ig-production of B-lymphocytes (Kawanishi et al 1981) and so results in an hyperactive immune response of B-cells to normal antigen stimulation. Such T-cell defect has been reported by some authors (Nouri-Aria et al 1986), but was not confirmed by others (Mc Keever et al 1985). Increased antigen availability is found in ALD (Triger et al 1972; Stann-Olsen et al 1983).

Excessive antigenic stimulation (MacSween & Anthony 1985) may arise from:

a. alcohol-induced changes of the gastro-intestinal tract, leading to increased mucosal permeability of antigens and explaining the high levels of antibodies to dietary and bacterial proteins found in ALD;

b. impaired sequestration of gut-derived antigens by the liver (Thomas et al 1973), as a direct effect of alcohol on the phagocytic function of Kc, or due to porto- caval shunting whose degree correlates with (highly) elevated serum IgM levels (Krasner et al 1976; Bailey et al 1976);

c. release into the circulation of hepato-biliary antigens due to liver cell damage or to alcohol altered tissue or of dietary proteins.

Endotoxaemia

It has been established that high titres of IgA-antibodies to lipid A, the main component of endotoxin, can be found in ALD (Nolan et al 1986). Titres of serum antibodies to Escherichia coli - which correlated well with serum Ig levels - were found higher in active than in inactive ALD (cirrhosis), and dropped likewise significantly after improvement of liver function on alcohol abstinence (Stann - Olsen et al 1983). This suggests that in ALD higher Ig-levels, in particular of IgA, are due to the presence of Escherichia coli (or endotoxin) in the systemic circulation and in the liver. Endotoxaemia has been demonstrated in the peripheral venous blood of patients with both alcoholic and non-alcoholic cirrhosis, but was also found, transiently in 50%, after acute alcohol abuse (Bode et al 1987).

In Chapter III, endotoxaemia will be reviewed in relation to factors involved in (alcoholic) liver fibrosis (see 3.3.3.2).

2.2.5.2.2.2 Immune complexes

Circulating immune complexes (IC), containing IgG and IgA, have been found in the sera of patients with active ALD (alcoholic hepatitis), and these IC disappeared after resolution of the hepatitis. However, the nature of the antigens in the complexes could not be established (MacSween & Anthony 1985) and it is possible that these complexes represent immunoglobulin aggregates which may have been formed in vivo (Stoltenberg and Soltis 1984). Moreover, circulating IC are found in a wide variety of diseases but their pathogenetic importance has been shown in only a small proportion of these diseases. Therefore, IC in ALD can be considered to be only an associated phenomenon: a result rather than the cause of local liver tissue injury (MacSween & Anthony 1985) as a reflection of Kupffer cell impairment (Lahnberg et al 1981) to adequate clearing of IC from the circulation.

Nevertheless, failed clearing of circulating IC may be important in ALD by modulating the immune response, because remaining IC may bind to Fc-receptors of lymphocytes and so interfere with cytotoxic and proliferative T-cell functions

(MacSween & Anthony 1985).

2.2.5.2.2.3 Auto-antibodies

The presence of auto-antibodies in ALD, usually present in low titres (Johnson & Williams 1985), is well established.
The reported prevalence of non-organ specific auto-antibodies, notably against nuclear and smooth muscle, varies considerably. This probably reflects differences in methodology (MacSween & Anthony 1985). Titres of auto-antibodies are largely influenced by sex, age, and hormonal (androgen) state, not only in patients with ALD (Gluud et al 1981) but also in the general population (Andersen 1977).
Also antibodies have been found against epitopes of protein-adducts, formed in vitro or in vivo (Lin & Lumeng 1989) due to modification by acetaldehyde (which per se, as mentioned before, is too small to be immunogenic). The triggering of the corresponding immune response has been suggested to be implicated in the pathogenesis of ALD by some authors (Israel et al 1986; Niemelae et al 1987). Other workers (Hoerner et al 1988), however, have suggested that it is the result, rather than the cause of alcoholic liver damage, because serum antibodies against acetaldehyde adducts did not correlate with liver enzyme activity and were highest in the more advanced stages of ALD.

2.2.5.2.2.4 Liver-specific antibodies

Liver-specific immune response has also been observed in ALD. Circulating IgA and IgG-antibodies against liver cell membrane (Burt et al 1982) have been reported to occur, and several papers reported liver cell membrane antibodies against ethanol-altered hepatocytes (Anthony et al 1983) or determinants of these cells (Neuberger et al 1984). Although alcohol or acetaldehyde are implicated as initiator, the exact nature of the antigen induced is not clear (Johnson & Williams 1985).
Also antigens of and antibodies to Mallory bodies occur, both in alcoholic and non-alcoholic liver disease (Fleming et al 1981). These findings were not consistent in all studies of ALD (Grant et al 1988) and therefore their significance is as yet unclear.

2.2.5.2.3 Cellular factors

2.2.5.2.3.1 Polymorphonuclear leucocytes

Several studies have shown defective leucocyte functions (Frank & Raicht 1985), including locomotion and aggregation capacity (Frank & Raicht 1985).
Leucocyte impairment has been explained as a non-serum mediated cellular defect, possibly in part of the chemotactic receptor function (Rajkovic et al 1984).

2.2.5.2.3.2 Mononuclear phagocytic system

The main function of the mononuclear phagocytic system (MPS) - previously called reticulo-endothelial system - is to remove micro-organisms, notably bacteria, from the blood circulation (Rimola et al 1984). The largest body compartment of the MPS is located in the liver, in Kupffer cells, which represents 80-90% of the total MPS functional capacity (Biozzi & Stiffeld 1965).
A depressed phagocytic capacity of this system is common in ALD and is probably a major factor in the increased predisposition to bacterial infection, especially in end-stage disease with decompensated cirrhosis (Rimola et al 1984).
In hepatology, recently both depressed MPS function, notably in relation to endotoxaemia (Nolan 1989), and activated MPS, whose cells subsequently release biologically very potent protein mediators (cytokines) such as TNF (Ziegler 1988), have been core issues in explaining developing liver lesions in liver diseases of various kinds and notably in alcohol related disease (Thiele 1989). They will be reviewed extensively later (see 3.3.3).

2.2.5.2.3.3 T-lymphocytes

The qualitative - no quantitative - alterations of B-lymphocytes which have been found in ALD have already been reviewed (see 2.2.5.2.2).
In T-lymphocytes, both qualitative and quantitative alterations have been found, especially in active ALD (alcoholic hepatitis).
The number of T-cells, mainly of the helper subset (Fernandez et al 1982), is reduced

in peripheral blood but increased in liver biopsies (MacSween & Anthony 1985). This suggests that homing of these cells in the liver contributes to the blood lymphocytopenia, commonly observed in ALD.
Immunohistochemical typing has shown that in ALD (cirrhosis) T-helper cells could be retrieved predominantly from the portal tracts, and T-suppressor cells from the parenchyma of the liver. This finding may suggest a role of T-suppressor cells in ALD in mediation of cytotoxic injury to hepatocytes (Si et al 1983).
Several in vitro studies have shown qualitative defects of cell-mediated immunity, such as delayed type of hypersensitivity and blast transformation response (Johnson & Williams 1985; MacSween & Anthony 1985). These abnormalities are usually present in active ALD and are reversible with improvement of liver damage on alcohol withdrawal, indicating that the effect is probably a direct one of alcohol on the lymphocyte immune response (MacSween & Anthony 1985).

In summary:

The numerous reported immune disturbances, with defects of both cellular and humoral immune response, give no evidence so far of a causal relation between (progressive) liver damage and immune mechanisms by alcohol. On the contrary, the immune phenomena appear to be epiphenomena, secondary to disturbance of normal immunoregulatory mechanisms induced by alcohol.
However, recently highlighted depressed function and activation of the mononuclear phagocytic system by alcohol can be of great interest for the pathogenesis of ALD.

2.3 Summary and conclusions of the chapter

The effects of alcohol on the human liver are harmful in only a minority of cases of alcohol abuse.
No clinical and biochemical parameters are specific for ALD, a high index of suspicion (Sherlock) combined with histopathological examination of the liver biopsy is the best guide to detect alcohol-related liver damage. Though virtually all forms of liver pathology may be encountered, the three main histopathological lesions of ALD

are: firstly, fatty liver, an early and predictable feature of heavy and chronic alcohol abuse and reversible on alcohol withdrawal; secondly, alcoholic hepatitis with pericellular fibrosis: the most characteristic lesion and considered the usual pathway to progressive liver disease; third, cirrhosis, as end-stage lesion.
Alcohol is harmful for the liver only in a minority of cases and the harm usually starts after several years of alcohol abuse. Furthermore, the occurrence of this damaging effect is unpredictable, the initial and reversible stage of fatty liver excluded.
ALD may result from interaction of various factors which per se are only potentially or doubtfully hepatotoxic. These factors include genetics, notably female sex; products of ethanol metabolism, mainly acetaldehyde; concomitant non- alcoholic liver disease (but probably viral liver disease excluded); and (other) environmental factors such as nutritional state, toxic agents and drugs; and, possibly, secondary effects of ethanol-induced immune disturbances.
However, the relative hepatotoxic capacity of these factors in combination with alcohol is not well defined as yet. From the literature it is doubtful whether nutritional factors play a role in influencing the course of human ALD. Malnutrition is definitively a feature of alcohol abuse which precedes the occurrence of ALD and does negatively interfere with the complications of mainly end-stage ALD.
The mechanisms which lead to alcohol-related liver damage, notably alcoholic hepatitis and fibrosis, are likewise poorly understood as yet. Acetaldehyde is scoring high as the main inducer of liver damage. Acute hepatoxicity of alcohol (or acetaldehyde) does not appear to be of major importance; what matters seems to be that liver damage develops after chronic alcohol consumption at a certain moment in a certain " susceptible" individual, but the determining factors for it are unknown.
To elucidate the mechanisms which effect ALD, and to determine the relative importance of the factors which are involved or contribute to liver damage by alcohol, well defined animal models may be expected to solve at least some of the enigma's of the complex interaction of alcohol and the liver, both on the level of the individual alcohol consumer and of the population as a whole.

2.4 References: see 3.10

CHAPTER III

SOME ASPECTS OF EXPERIMENTAL ANIMAL MODELS FOR ALCOHOLIC LIVER DISEASE AND LIVER FIBROSIS

3.1 General introduction

3.1.1 The structure of the normal liver

The structural unit of the liver is the lobule. It is composed of the liver parenchyma surrounding one central, or efferent, hepatic vein, and of several portal tracts at its periphery.
The architecture of the lobular parenchyma shows a radial arrangement of, in adult liver single, parenchymal cell plates at both sides lined by sinusoidal blood spaces draining in the central hepatic vein.
The blood supply of the lobule originates from the portal tract vessels: 80% from branches of the portal vein and 20% of the hepatic artery. Except blood vessels, portal tracts contain lymph vessels, nerves and bile ducts.
Five intrinsic liver cell types have been identified in the lobule outside the portal tracts: the parenchymal cell or hepatocyte, and four non-parenchymal or sinusoidal cells situated in proximity to the sinusoids. The four sinusoidal cell types are: the endothelial cell, the Kupffer cell, the Ito- or fat storing cell, and the pit cell.

3.1.2 Cells of the normal liver

3.1.2.1 Hepatocytes

The parenchymal cells of the liver are hexagonal, with one or two central chromatin-rich nuclei and ample cytoplasma which contains numerous organelles, reflecting the very active metabolic demands.
The liver is the main metabolic factory of the body, and its parenchymal cells have to deal with three different functions: first,. synthesis of many essential or important

proteins, carbohydrates and fats; second, break-down of components of the blood stream; third, excretion and bile production.

3.1.2.2 Endothelial cells

The endothelial cells form a fenestrated cytoplasmatic web-like wall of the sinusoid for filtering the blood and exchange of blood components and hepatic products.
Along the entire surface of the cell membrane are numerous bristle-coated invaginations, vesicles and lysosome-like vacuoles which are all indicative of the well-developed capacity of endocytosis of these cells (Brouwer et al 1988). In contrast to other organs, endothelial cells of the liver have no (or an incomplete) basement membrane.

3.1.2.3 Kupffer cells

The Kupffer cells are the resident macrophages of the liver. They constitute the main organ compartment of the mononuclear phagocytic system of the body, comprising 80-90% of the total activity of this system (Biozzi & Stiffeld 1965).
Kupffer cells are located with their cytoplasmatic processes "floating" in the sinusoidal bloodstream in order to clear it from foreign body material, notably microorganisms and (endo-) toxins originating from the gut.
This clearing of the blood - mainly of the portal vein - from bacteria and endotoxins, viruses, immune complexes and other molecules and particulate material, is achieved by endocytosis. In advanced liver disease, notably cirrhosis, the endocytic capacity to clear the bloodstream is impaired, which leads to an increased incidence of bacteraemia, endotoxaemia and eventually sepsis (Rimola et al 1984).
Kupffer cells may also play a role in destruction of tumour cells and thus in the defence against developing (liver) metastases (Bucana & Fidler 1984).
The possible role which Kupffer cells may play in fibrosis of the liver will be dealt with later in this chapter.

3.1.2.4 Fat-storing or Ito cells

Ito or fat-storing cells (FSC), (other synonyms are perisinusoidal or stellate-shaped cell), are located in the narrow space of Disse, between the sinusoid and adjoining liver cell plate.
One of the important functions is to store vitamin A, which is morphologically reflected by small cytoplasmic fat droplets.
The other main role of Ito- cells is to play as precursor cells of fibrosis. Both functions will be reviewed later in this Chapter.

3.1.2.5 Pit cells

The fourth sinusoidal cell type of the liver, discovered rather recently in rat liver (Wisse et al 1976) and later also described for human liver (Kaneda et al 1984), was called the pit cell because of its characteristic cytoplasmic electron- dense granules. This cell has natural killer cell capacity.

3.1.3 Collagen in the normal liver

In the normal human liver collagen is found in the surrounding capsule and in the portal tracts as supportive connective tissue around vessels and bile ducts, in the wall of the terminal hepatic (central) veins, and as rather sparse tiny fibres along the sinusoids.
The Ito cell or FSC has been generally recognised as the main collagen producing cell of the liver lobule (Roykind & Perez-Tamayo 1983). However, with the exception of the pit cell all other liver cell types, including the parenchymal cell (Clement et al 1984; Chojkier 1986), are able to produce collagen under certain conditions, at least *in vitro*. Consequently, collagen production in the liver can be considered as the resultant from close *in situ* interaction of several, perhaps all liver cells. In this respect the concept of the extracellular matrix (EM) has to be introduced.

3.2 The extracellular matrix of the liver

3.2.1 General introduction

The extracellular matrix (EM) of the liver is the tissue component between the constituting cells. It forms a dynamic and metabolically very active communication platform between these cells in order to maintain or adapt the milieu interior to the actual demands.
The EM is biochemically composed of three main groups of macromolecules derived from the various surrounding liver cells: collagens, glycoproteins and proteoglycogans.

3.2.2 Collagens

Collagens are the major EM-component. They compose of a group of structural proteins of at least 11 genetically and biochemically different types (Miller & Gay 1987), designated I to XI.
In normal human liver, about one-third of collagen is type I, one-third type III, 5-10% type IV, and 7-10% type V (Seyer et al 1977; Roykind et al 1979).
Type I collagen is mainly found in the wall of vessels and sinusoids, more specifically: in Disse's space (Martinez-Hernandez 1984). Type III is also found in Disse's space, in connection with the processes of fat-storing cells.
Type IV collagen forms only a small constituent of the liver. It is generally associated with basement membrane (-like) structures being present in the wall of vessels and bile ducts and -incomplete and discontinuously - along the sinusoids.
In normal liver, type V collagen has been found immunohistochemically in a well defined fibrous pattern in the portal tracts as interwoven fibrillar material and in the parenchyma as fine fibres between parenchymal cells and sinusoids; in cirrhotic liver, it is present as thick bundles in the fibrous septa (Schuppan et al 1986a).
Previously, type V has nearly exclusively been associated with basement membrane localisation by many authors. However, type V collagen is a common component of the interstitial tissue of many organs, and is increased in granulation tissue of wound healing (Hering et al 1983). In rat liver it is common in the early regeneration period after partial hepatectomy (Rojkind et al 1983).

The road from (pro-) collagen production to extracellular deposition is a complex biochemical process. In contrast to type IV collagen, type I, III and V need processing after the intracellular precursors have been synthesised in the rough endoplasmic reticulum (RER). Whether this processing of procollagens is the same in normal and in diseased liver is not established (Rojkind & Kershenobich 1987).

3.2.3 Glycoproteins

3.2.3.1 General remarks

Laminin and fibronectin are the main glycoproteins (GP) of the EM of the liver.
The function of GP in the EM is to serve as adhesion molecules between liver cells and collagen. Coupling is effected by use of specific binding sites. GP play an important role in (im-) mobilising tissue cells, directing and keeping them at a particular site.

Both laminin and fibronectin have a rather specific affinity to certain collagen types: laminin predominantly to type IV (Hahn et al 1980), and fibronectin for type I and III (Dardenne et al 1983). Laminin in normal liver is thus mainly found in the basement membranes of the portal blood vessels and of bile ductules. In the liver parenchyma, laminin is only very sparsely present in discontinuous sheets in basement membrane-like material in Disse's space because a real basement membrane is lacking. Fibronectin is also found in Disse's space.

3.2.3.2 Fibronectin and chronic liver disease

In chronic liver disease, fibronectin is present in areas of parenchymal (piecemeal) necrosis, in portal tracts and in fibrous septa. Laminin-except the usual basement membrane location of portal tract structures- is found in fibrous septa and in cirrhotic (regeneration) nodules in association with type IV collagen at the sinusoidal side of parenchymal cells. This polarized accumulation of laminin as basement membrane (like-) material can be used as a marker of hepatocyte regeneration following liver damage (Bianchi et al 1984).

The great biological relevance of fibronectin, especially in relation to liver diseases, has been stipulated by Mosher (1986). Present in insoluble form, fibronectin is not only an important component of fibrillar extracellular structures and basal membranes of all tissues (with possible exception of the nervous tissue), it occurs also in soluble form in body fluids.
The blood plasma of *soluble fibronectin* level is high, about 300 mg per litre, and most, if not all, circulating fibronectin is produced by hepatocytes (Mosher 1986). During blood coagulation, fibronectin is incorporated into the fibrin clot. It is considered to be the most important non-specific opsonin to clear debris particles from the circulation. Fibronectin is predominantly composed of three different types of homologous units and is coded for by only one gene (Hynes 1975). It has the property to bind to several substances of biological interest such as fibrin, heparin, collagen and staphylococcus aureus.
Plasma fibronectin concentration is decreased in cirrhosis and is a predictor of one year survival rate (Naveau et al 1985). It is very low - often 10% of normal - in fulminant hepatic failure (Almasio et al 1986), probably due to failure of synthesis and secretion by hepatocytes (Mosher et al 1986). Correction of low plasma levels has been reported (Moriyama et al 1986) to have a protective effect from experimentally (galactosamine-) induced liver failure in rats for as yet unexplained reasons.

3.2.4 Proteoglycans

Proteoglycans (PGly) are composed of a core-protein to which glycosaminoglycans are covalently bound. Glycosaminoglycans are sugar polymers which are alternatingly composed of glucuronic acid and sulphated hexosamine residues.
PGly are present in the liver in small amounts. The majority are heparane sulphate and heparan PGly (Kjellin et al 1980). They are connected with the plasma membrane of parenchymal cells and other cell types. They form complexes with laminin and fibronectin and are in this way associated with type III and IV collagen, respectively (Murata et al 1985).
Other PGly, such as dermatan- and chondroitin-sulphate, are mainly linked with fibronectin and type I collagen.
An important capacity of PGly is the modulation of the adhesive properties of the

EM and of the formation of collagen fibrils (Hayashi et al 1972). Therefore, PGly play a role in the formation and maintenance of the structure and function of both the EM and the cellular localization in the liver.

3.3 Liver fibrosis: Cells of origin and factors involved

3.3.1 General introduction

Liver fibrosis (LF) is defined as an absolute increase of intrahepatic connective tissue, and more specifically: of collagen. It is usually accompanied by loss of parenchymal cells of variable degree. More in general: LF is the hallmark of most clinically significant chronic liver diseases of very diverse etiology. Usually, the pattern of fibrosis will point to the etiology. For instance, ALD will result in parenchymal fibrosis (see 2.2.2), whereas bile duct disease results in portal fibrosis. In the course of many chronic liver diseases, fibrosis will be progressive and will result in dissection of the liver parenchyma by the development of fibrous septa, which interconnect portal and central areas of liver lobules. Eventually, cirrhosis may develop, which is the irreversible transformation of the lobular parenchymal architecture into regeneration nodules surrounded by fibrous septa. In cirrhosis, all types of collagen, present in normal liver, are increased. In (human) cirrhotic livers, which contain more than 20 mg per g liver tissue, type I is increased predominantly and, therefore, the ratio type I/type III collagen is found to be increased (Rojkind et al 1979; Seyer et al 1977). This is in contrast with (CCl_4-induced) cirrhotic rat liver in which the contents of 35% to 40% collagen type III to type I of normal liver (Seyer 1980) are maintained unless cirrhosis is far advanced (Rojkind & Perez-Tamayo 1983).
Along with progression of fibrosis, portal hypertension will develop, mainly by increase of the sinusoidal blood pressure (Rojkind & Perez-Tamayo 1983). On transition to cirrhosis, portal hypertension will increase by the distortion of the parenchymal architecture, resulting in further impairment of the blood flow through the liver. Advanced fibrosis and notably cirrhosis, as end-stage liver disease, are associated with a poor prognosis mainly caused by haemodynamic disturbances due to portal hypertension, liver failure, and by increased risk of infections (see previous chapter).

Fibrosis and cirrhosis of the liver are very common diseases. Their prevalence is high in the Western world, mainly due to alcohol abuse and viral liver disease, but still higher in the majority of so-called developing countries in the Tropics, due to viral and parasitic diseases of the liver. Notably hepatitis B infection and schistosomiasis are often endemic and affect the livers of many hundred millions of people of African and South American countries, resulting in extensive fibrosis and cirrhosis.

3.3.2 Cells of origin of liver fibrosis

3.3.2.1 General remarks

The cell types involved in, and/or responsible for the pathogenesis of LF are still the subject of much debate and some controversy (Davis 1987).
In most organs, fibrosis results from differentiated fibroblasts. Fibroblasts are elongated or spindle-shaped mesenchymal cells which synthesise collagen components intracellularly. After secretion, these collagen components are deposited extracellularly as morphologically distinct and characteristic fibres that run parallel to the cell body.
Since the parenchyma of normal liver contains no fibroblasts outside the portal tracts (Desmet 1985), (an-)other cell type(s) must be held responsible for parenchymal fibrosis.
Much information about cells and factors involved in LF has been collected during the last decade from human and experimental animal studies.
A great deal of experimental studies of liver fibrosis has made use of the carbon tetrachloride (CCl_4) model of induction of liver damage, mainly in rats. This results, on repeated doses of CCl_4 administration, in fibrosis and eventually true cirrhosis (Cameron & Karunaratne 1936). CCl_4 intoxication has been shown to be one of the more valuable experimental models to study LF, although it does not fully cover all aspects of LF in human liver (Perez-Tamayo 1983).
A host of data from experimental studies of LF in vitro and in vivo has become available. In these studies, factors related to the primary inducing agent(s) and experimental conditions, for instance nutrition, varied greatly and, therefore, comparison of results is difficult. This holds even more true for many experimental

studies of fibrosis of ALD in which, in the initial studies, nutritional factors have been generally considered to be more important for the pathogenesis than the noxious effect of ethanol itself (vide infra).

In many human and experimental studies of LF of the last decades, attention has been focused specifically on the role of the FSC in LF.

Along with development and application of new techniques, notably with respect to isolation, purification and culture of liver cells, and after discovery of factors derived from activated cells (cytokines), it became gradually clear that LF is not caused by the aberrant function of one single cell type. Studies of LF are presently centred around the concept of the liver as a small bio-ecological system, of which dynamic homeostatic and adaptive interactions between all cellular and EM components will determine growth and differentiation of these components in health and disease.

It has been a merit of Rojkind (see Liver Annual 1985, 1986 and 1987) to lead the way in this conceptual approach of the dynamic EM of the liver, which is needed to identify the pathogenetic factors of LF.

Most studies on the cellular origin of collagen and other EM-components in LF have been done in *in vitro* experiments, mostly in primary cultures of liver cells. However, what is going on *in vivo* is less known or still speculative, and it is hoped that extrapolation of *in vitro* events will reflect the reality *in vivo*.

Even in vivo animal studies, with their particular experimental conditions, can not be expected to necessarily reflect the human situation. This aspect of experimental animal studies will be discussed in this chapter in relation to ALD (sub 3.3.2).

The characteristic of fibrotic responses in general, and of ALD in particular, is the initial production and deposition of predominantly collagen type III which is, as the fibrous tissue matures, partly replaced by type I (Rojkind & Perez-Tamayo 1983;Fleming & McGee 1984; Desmet 1985).

Although the FSC is widely considered to be the main - and according to some authors (Ballardini et al 1989) the true - *in vivo* EM producing cell of the normal and the diseased liver, other liver cells too, are capable of producing EM components under particular experimental conditions.

3.3.2.2 Hepatocytes

Parenchymal liver cells have been shown to be able to produce collagen *in vitro* (Guzelian et al 1981; Tseng et al 1982; Tseng et al 1983; Diegelmann et al 1983; Clement et al 1984). In *in vivo* experiments in rats, hepatocytes of normal liver (Chojkier 1986) and of CCl_4-induced fibrotic liver (Chojkier et al 1988) even appeared to be the main collagen producing cells. This is in contrast with the results of an *in vitro* experiment of cultured cells, isolated from normal rat liver, in which FSC produced 25 times more collagens (I, III and IV) than hepatocytes and 3 times more than endothelial liver cells (Friedman et al 1985). Therefore, the importance and relative contribution of hepatocytes (and other liver cells) to collagen production and deposition in normal and diseased (fibrotic) liver is not really known (Rojkind & Kershenobich 1987).

3.3.2.3 Fat-storing cells

Despite increasing evidence of EM-production by other cells, fat-storing cells (FSC) are still almost generally believed to be the main EM-producing cell type of the liver.
A main function of FSC is uptake and storage of retinoids (Hendriks et al 1984), a quality which seems to be closely associated with the fibrogenetic capacity of these cells (see later this chapter and chapters VI and VIII).
In normal rat and human liver, FSC are rather inconspicuous on light microscopy.
After vitamin A supplementation to the diet, FSC of rat liver have a highly characteristic appearance. They show, in unfixed and unstained frozen slices of liver tissue, rapidly fading autofluorescence at 328 nm characteristic for vitamin A (De Leeuw et al 1984). In formalin fixed and conventionally processed haematoxylin & eosin stained liver sections, FSC show small fat-containing vacuoles in the cytoplasm whose number largely depends on the amount of stored vitamin A.
FSC can be clearly stained with the modified gold chloride method (Tozuka et al 1984). They then show their stellate shape by cytoplasmic processus which extend into the perisinusoidal space of Disse in close interaction with endothelial cells. Ultrastructurally these processus contain microfilament bundles running parallel to the plasma membrane. Desmin, the intermediate filament of the cytoplasm of muscle

cells, is also found in FSC. Both microfilaments and desmin are characteristic components of contractile cells (Yokoi et al 1983). It has been suggested that the presence of these cytoplasmatic structures indicative of a contractile function, is related to a specific function of the fenestrated endothelial cells of the liver, namely the regulation of the liver blood flow through the sinusoids (Wisse 1970).
The cytoplasm of FSC contains a variable amount of rough endoplasmic reticulum (RER), a relatively large Golgi apparatus and only few mitochondria.
FSC are not only inconspicuous in normal liver, but also look inactive, and a mitosis is seldomly found. Information on proliferation of FSC is scarce. Using desmin and 5-bromo-2-deoxyuridine as markers to detect proliferating FSC, the labelling index was found to be 3.7% in normal rat liver which increased up to 25.7% after 48 hours following hemihepatectomy (Tanaka & Lieber 1990). Increased mitotic activity of FSC in rat liver following partial hepatectomy had been previously noticed (Wisse et al 1974). This was also found in the acute phase of CCl_4-induced damage to rat liver (McGee & Patrick 1972; Kent et al 1977; Enzan 1985), and has also been observed in human liver with ALD adjacent to foci of parenchymal damage (Horn et al 1986).

Both in CCl_4-induced liver damage in rats (Senoo & Wake 1985) and in ALD in baboons (Mak et al 1984), the number of FSC decreased in the course of progressive fibrosis. In contradistinction to that, FSC showed marked increase in DNA synthesis at the time that, or just before, fibroblast-like cells did appear in necrotic areas of CCl_4-induced parenchymal damage in rat liver (Enzan 1985).
These data indicate that FSC should be considered as potential fibroblasts, or fibrogenetic cells, and that FSC, in these particular experimental conditions, could be mainly responsible for fibrosis of the liver parenchyma, in which normally no fibroblasts are present (Desmet 1985).

3.3.2.4 Transitional cells

Experimental liver fibrosis (LF) can be induced by administration of CCl_4 (McGee & Patrick 1972), of heterologous serum (Ballardini et al 1983) and of alcohol in baboons (Mak et al 1984). These experimental studies but also studies in man (Minato et al 1983) demonstrated a close association between developing LF and the transfor-

mation of fat-storing cells to fibroblasts. This transformation occurs via an intermediate type of cell, called transitional cell (Kent et al 1976). Transitional cells show structural - and probably have functional - characteristics of both FSC and fibroblasts: they contain fat droplets in the cytoplasm, but less and smaller than FSC; microfilaments and desmin are present; RER is hypertrophied and contains flocculent and fibrillar (synthesised) material; and outside and parallel to the cell body, collagen fibres are found.
A study of fat-storing cells, which were isolated from CCl_4-treated rat liver and were stimulated by acetaldehyde to increased collagen synthesis *in vitro*, showed transformation of fat-storing cells with characteristics of transitional cells and of fibroblasts (Shiratori et al 1986a).

3.3.2.5 Myofibroblasts

Another potentially fibrogenic cell, called myofibroblast, has also been recognised in the parenchyma of the liver.
Myofibroblasts form a normal constituent of the adventitia of the terminal hepatic (or central) vein - the so-called perivenular sheath - of normal human (Nakano et al 1982a) and primate (Nakano & Lieber 1982b) liver. These cells are thought to produce various types of collagen (I,III, IV) and laminin (Nakano et al 1982 a).
Myofibroblasts are found in increased numbers in experimental ALD after chronic ethanol administration to baboons (Nakano & Lieber 1982b) and to rats, of which alcohol containing liquid diet was supplemented with a moderate amount of vitamin A (Leo & Lieber 1983). Myofibroblasts isolated from chronically (years) alcohol fed baboons, were stimulated to synthesize collagen by acetaldehyde (and not by ethanol) (Savolainen et al 1984).
Increased numbers of myofibroblasts are also found in human liver with alcoholic cirrhosis (Irle et al 1982) and with various other pathological conditions, for instance focal nodular hyperplasia (Callea et al 1982). It is possible that part of these increased myofibroblasts is derived from transformed FSC.
In ALD, myofibroblasts by proliferation and collagen synthesis may be responsible for the perivenular type of fibrosis, and (transformed) FSC - possibly together with endothelial and parenchymal liver cells - may be responsible for the pericellular and

other types of LF (Desmet 1985).
Fat-storing cells, transitional cells and myofibroblasts seem to belong to one family of cells, which modulate their structural characteristics and functional activities according to local, micro- environmental influence (Desmet 1985) and needs.

3.3.2.6 Endothelial cells

Only recently, attention has been paid to the endothelial cell as potential source of fibrogenesis. Several EM-components - collagens and fibronectin - were immunohistochemically found in the RER of endothelial cells of normal rat and human liver (Clement et al 1984), in endothelial cells of CCl_4-treated rat liver (Martinez- Hernandez 1985), and in endothelial cell cultures derived from normal rat (Irving et al 1984) and from human fibrotic liver (Voss et al 1982b).
The evidence of the association of EM-component production and endothelial cells is as yet rather circumstantial. In an electron microscopical study of human liver of patients with chronic active hepatitis (Bardadin & Desmet 1985), 'active' endothelial cells resembled fibroblastic reticulum cells of lymph nodes. These lymph node cells had already previously been suggested to be derived from endothelial cells (Tykocinski et al 1983). It was postulated from the findings of this ultrastructural study that these cells might play a role in protecting the hepatocytes against toxic or damaging substances, by assuring narrowing of the endothelial cell sieve plate fenestrae and by laying down of basement membrane-like material and reticulin fibres. In this way, these cells might initiate progressive intralobular fibrosis in the process of chronic inflammation of the liver (chronic active hepatitis) (Bardadin & Desmet 1985). However, the possible involvement of endothelium-derived "hyperplastic capillaries" in the formation of granulation tissue and scarring of tissues in general and of the liver in particular (Beranek et al 1986), has to be showed as yet by other studies.

3.3.2.7 Kupffer cells

Kupffer cells (Kc) of mouse liver have some capacity to produce EM- components *in vitro* (Voss et al 1982a), but production has not been established *in vivo* (Geerts et al 1987). However, Kc, as major part of the mononuclear phagocytic system (MPS), are

likely to have fibrogenic potential indirectly. They may effect induction of fat-storing or fibroblast-like cells by release of soluble collagen-stimulating factors, called cytokines, which are produced in response to liver damage (Geerts et al 1987).
In CCl_4-induced fibrosis of rat liver, increase of the Kc population precedes the increase of the FSC population (Geerts et al 1984), and isolated Kc from CCl_4 treated rat liver stimulated in vitro FSC to proliferate (Shiratori et al 1986b).
The potential role of Kc-related cytokines involved in LF, will be discussed in the next paragraph.
Kc also have the potential to produce and secrete collagenase (Fujiware et al 1982) which degrades hepatic matrix components.

3.3.3 Factors involved in liver fibrosis

Many cellular and humoral factors, with a confusingly rich spectrum of nomenclature, have been reported to be involved in the generation of LF. Most data, however, are gathered from very diverse *in vitro* studies on liver of experimental animals. The interpretation and comparison of the results are difficult and their value for *in vivo* events and for the human situation is still questionable.

3.3.3.1 Cytokines

Cytokines (Ctk) are cellular factors derived mainly from activated blood monocytes or tissue macrophages, but also from T and B lymphocytes, endothelial and others cell types. Ctk are elicited from these cells on tissue damage and thereafter effect biological responses by interaction with cell surface receptors which are specific for each Ctk and are widely distributed on various cell types within brain, liver, lymphoid organs and other tissues (Thiele 1989). The total of those biological responses of elicited Ctk after tissue damage is a common systemic response with many clinical manifestations, called acute phase response (APR).
The three main Ctk that are elicited in the APR are now known as: interleukin-1 (IL-1), tumour necrosis factor (TNF, previously cachectin), and interleukin-6 (IL-6).
The action of Ctk on the stroma (or EM) of tissues is important. This Ctk-EM axis is a dynamic forum with complex and very active effects in order to maintain or adapt

the homeostasis of the particular organ. An important initiator of the activation of the Ctk-EM axis is the APR (Dinarello 1984). Both Ctk and APR might be of great importance in the generation of LF following liver injury.

3.3.3.2 Acute phase response

3.3.3.2.1 General remarks

Acute phase response (APR) is the name given to a characteristic pattern of alteration in concentration of a number of plasma proteins, called acute phase proteins (APP), which follows a wide variety of different forms of infection, inflammation or tissue damage (Pepys & Baltz 1983). APR can be considered as the general response of the organism to local or generalized injury and is aimed to localize and eliminate it and to be of benefit to the host. It is induced by a variety of microbial agents, but also by other injuries or noxious conditions of various etiology such as trauma, chronic non-infectious inflammatory disease like rheumatoid arthritis and chronic idiopathic inflammatory bowel disease, and neoplasia (Thiele 1989). Although the stimuli which induce the APR are diverse, the common denominator is the occurrence of injury or death of cells or tissue (Pepys & Baltz 1983). It encompasses a constellation of systemic events which include several biochemical, cellular and metabolic changes such as fever, leucocytosis, depressed serum zinc and iron, and raised serum copper levels, increased protein catabolism and gluconeogenesis as well as increased synthesis of several hormones such as glucagon, insulin, ACTH, cortisol, catecholamines, growth hormone, TSH, T4, aldosterone and vasopressin (Kushner 1982).

APP represent a large and heterogeneous group of plasma proteins of which levels change considerably during most acute and chronic inflammatory processes (Kushner 1982). They serve as mediators, inhibitors, scavengers and immune regulators.

Most APP are synthesized by hepatocytes, but also other cells such as monocytes - macrophages, lymphocytes, polymorphonuclear leucocytes, endothelial cells and fibroblasts, may contribute to the synthesis.

Functionally, APP belong to the groups of proteinase inhibitors (e.g. alpha1- anti-(chymo-) trypsin), coagulation factors (e.g. fibrinogen, prothrombin, factor VIII,

plasminogen), complement factors, transport proteins (e.g. haptoglobulin, transferrin, ceruloplasmin), or to other groups such as alpha 2-macroglobulin, alpha 1-acid glycoprotein, serum amyloid A, C-reactive protein and fibronectin.

3.3.3.2.2 Acute phase response and cytokines in relation to (alcoholic) liver disease

Recent studies on the relation of Ctk to the APR have led to a better understanding of the ways in which diseases of very different etiology and organ specificity can induce a similar host response (Thiele 1989).
Liver diseases of various etiology are accompanied by APR. A general trend common to all liver diseases is not observed, but a consistent change of two acute phase proteins was reported, namely increase in plasma levels of alpha 2-macroglobulin and decrease of haptoglobin levels (Meliconi et al 1988).
Increased Ctk-production, notably of TNF, by peripheral blood monocytes was found in chronic liver disease and correlated with histologic activity (hepatitis)(Yoshioka et al 1989), but local TNF-production in inflammatory areas of the liver was not studied. In ALD, this TNF-response is found in association with the occurrence of alcoholic hepatitis. Monocytes from peripheral blood of alcoholic hepatitis patients show increased TNF-production. It has been suggested that this might contribute to the generation of the metabolic and histologic liver injury of alcoholic hepatitis (McClain & Cohen 1989).
The role of TNF - and of the other Ctk - could be of crucial importance for the pathogenesis of alcoholic hepatitis and the related bad prognosis (Thiele 1989).
The mechanism of TNF-release in ALD is as yet unclear. Alcohol itself does not elicit TNF response. Bacterial endotoxin might be the inducer (McClain & Cohen 1989), but also products of ethanol-induced or ethanol-injured hepatocytes are strong candidates to be the initial stimuli for the induction of the Ctk mediators in APR (Thiele 1989).

3.3.3.2.3 Endotoxin

Some noxious agents are particularly potent inducers of APR. Such an agent is endotoxin, the lipopolysaccharide (LPS) of the outer wall of Gram-negative bacteria.

Endotoxin is present in large quantities in the colon and rectum, released from dead or intact bacteria. In normal conditions, LPS can pass the barrier of the bowel wall in only small amounts. However, entrance of endotoxin into the portal circulation has been shown to be significantly increased by conditions which interfere with the integrity of this barrier such as ischaemia or shock, bowel inflammation (Van Deventer et al 1988) and alcohol ingestion (Bode et al 1987). In this way, the concept of gut associated endotoxinaemia not only has gained much support to explain the multiple organ failure associated with severe trauma (accompanied by shock) and with sepsis, but even has been considered to be a key factor in the development of the liver lesions (Nolan 1989).

Endotoxin in relation to liver disease

Low doses of endotoxin can provoke a strong APR, without causing other clinical evidence of toxicity. This is the result of the strong capacity of endotoxin to stimulate and activate macrophages c.q. Kupffer cells (Pepys & Baltz 1983), with subsequent release of Ctk.

The integrity of the mononuclear phagocytic system (MPS) is known to be critical to host sensitivity to LPS (Nolan 1989). With respect to endotoxin, it was hypothesised already more than a decade ago, based on human and experimental studies, that liver damage was largely indirectly dependent on the functional integrity of sinusoidal cells, notably Kc. These cells remove and detoxify harmful agents such as endotoxin to prevent parenchymal damage. If this damage occurs, it leads to further impairment of LPS detoxification, resulting in spill-over of toxins to the systemic circulation with consequently extrahepatic manifestations (Nolan 1975).

However, this early, rather simple view that focuses mainly on the functional integrity of sinusoidal liver cells to remove harmful molecules in explaining liver damage, has been modified by current knowledge about potentially harmful products released by activated macrophages. In relation to liver injury, it has become clear that Kc behave differently from other macrophages and appear to have a special capacity to remove endotoxin from the portal circulation (McCuskey et al 1987; Van Bossuyt & Wisse 1988). The release of a number of mediators and other products from Kc appears to be critical to the development of endotoxin-induced liver injury. Additionally, endotoxin may also directly damage hepatocytes after failed clearance by Kc, for instance the parenchymal cell membrane (Pagani et al 1988).

Many macrophage products have been implicated in the generation of the liver injury that is induced by endotoxin (Nolan 1989): superoxides, lysosomal enzymes, protein synthesis-inhibitory factor, procoagulants, leukotrienes, interleukins, TNF, platelet-activating factor (PAF). Of all these macrophage products released by LPS stimulation, TNF may be a major mediator of liver damage. Endotoxin is the most potent known stimulus for TNF production (Ziegler 1988). Intravenous injection of a single (low) dosis in human volunteers, evoking many of the signs of acute Gram-negative bacterial infections, was associated with a brief pulse of circulating TNF (Michie et al 1988). When LPS was added to peripheral blood monocytes derived from patients with alcoholic hepatitis, TNF response was significantly more increased as compared to healthy volunteers; basal TNF-release was also increased in the alcoholics (McClain & Cohen 1989). Injection of TNF can produce virtually all the effects of LPS, including the release of many other Ctk, such as IL-1 and IL-6, leukotrienes, and PAF (Nolan 1989).
Although the role of gut-derived LPS and the subsequent release of mediators, notably TNF, seem to be important in the generation of liver injury, as evidenced from mainly short-term experimental *in vivo* and *in vitro* studies, their relative contribution to more chronic liver damage still remains to be elucidated (Nolan 1989).

3.3.3.2.4 Eicosanoids and leukotrienes in relation to (alcoholic) liver disease

In liver diseases associated with cell damage, not only APR but also eicosanoid response is increasingly recognised to be important. Eicosanoid is a generic name encompassing compounds derived from C-20 polyunsaturated fatty acids, mainly arachidonic acid. The eicosanoid group includes widely distributed, biologically very active local tissue hormones such as prostaglandins, thromboxane and leukotrienes.
In the liver, eicosanoids are important signal molecules of intracellular communication (Decker 1985). Among eicosanoids, leukotrienes play a particularly important role in liver pathophysiology, and especially in the pathogenesis of inflammatory liver disease (Hagmann & Keppler 1988).
Leukotrienes are produced by almost all cells and tissues. Blood cells: granulocytes and monocytes, and tissue macrophages, including Kc, are particularly active producers of leukotrienes. The pattern of production and release differs for cell type

and stimulus involved (Hagman & Keppler 1988).
Cysteinyl leukotrienes LTC4 and LTD4 have recently found to play an important role in the very complex intercellular parenchymal non-parenchymal cell communication of the regulation of liver metabolism of rats (Wettstein et al 1989; Iwai & Jungermann 1989).
Leukotrienes mediate immediate hypersensitivity reactions and inflammation (Samuelsson 1983). Endotoxin is a potent trigger for cysteinyl leukotriene production in vivo (Hagmann et al 1984). Mainly from experimental rat studies, indirect evidence has been obtained that leukotrienes even may play a key mediating role in inflammatory liver disease. In experimental models of liver damage with inflammation (for instance by D-galactosamine), histological and biochemical evidence of liver injury were largely prevented by drugs interfering with the synthesis or action of leukotrienes (Keppler et al 1985).

3.4 Experimental models for liver fibrosis

Several experimental animal models have been employed to study developing liver fibrosis (LF). Mostly rodents, notably rats, rather than dogs, pigs or monkeys, have been used as experimental animals for obvious ethical, practical and, last but not least, economical reasons.
Four compounds have been commonly used to elicit LF: carbon tetrachloride (CCl_4), galactosamine, heterologous serum, and dimethylnitrosamine. Application of the first two toxins will effect chemically induced liver damage and, subsequently, fibrosis (scarring). The last two compounds elicit fibrosis presumably on basis of an immune-mediated mechanism. As representatives for these two groups of components the effect of CCl_4 and of dimethylnitrosamine will be discussed below.

3.4.1 Carbon tetrachloride

3.4.1.1 General remarks

Since its introduction in 1936 (Cameron & Karunaratne), the carbon tetrachloride (CCl_4)-model has been the most frequently applied tool in experimental liver research to study parenchymal damage that results in inflammation and fibrosis.
CCl_4 can be administered by several routes: via subcutaneous, intramuscular or intraperitoneal injections, via intragastric intubation, or via inhalation. Administration of a single sublethal dose results in acute parenchymal inflammation with necrosis of the perivenular zone of the liver lobule. Repeated administration with short intervals (to prevent recovery of damage), usually twice weekly, causes dissecting fibrosis with interconnecting centro-central and centro-portal collagen septa within 4 weeks. Transition to cirrhosis starts after 8 weeks. At progression of fibrosis, collagen content may increase 4-5 fold, with maintenance of type I/type III ratio of normal rat liver till advanced cirrhotic stage when this ratio is lost (Perez-Tamayo 1983).

3.4.1.2 Metabolic activation

Carbon tetrachloride (CCl_4) is metabolized in the microsomes of hepatocytes by the cytochrome-P450 system. Hepatotoxic reactive intermediates are formed, notably free radicals, which induce lipid peroxidation and covalent binding to intracellular proteins (Slater et al 1985). Free radical scavenging compounds, notably dietary agents, have been shown to be protective against the hepatotoxic effects of CCl_4 (Wang et al 1985, Shertzer et al 1987), but the specific molecular mechanisms involved in the parenchymal liver necrosis that is elicited by CCl_4-reactive metabolites, remain obscure in spite of extensive research efforts (Brattin et al 1985). Also early occurring and partly reversible direct CCl_4-toxicity to cell membranes including plasma, lysosomal and mitochondrial membranes due to its solvent properties, was found in isolated hepatocytes (Berger et al 1986) and this may contribute to over-all CCl_4 toxicity.

3.4.1.3 Cell kinetics

A detailed report on the kinetics of fat-storing cells (FSC) and Kupffer cells (Kc) during chronic CCl_4-intoxication was published by Wisse and coworkers (Geerts et al 1984, 1988):

Fat-storing cells

After a decrease during the first week, FSC start to increase in number by proliferation and their number is doubled by the fourth week; this process is accompanied by appearance of fibroblast-like cells in the central lobular areas of parenchymal damage. From the fourth week on, the number of FSC remains constant (about 50% of normal), and large numbers of fibroblasts appear. On electron-microscopy, transitional forms between FSC and fibroblasts are found.

Kupffer cells

Kc start to proliferate and infiltrate in the damaged pericentral areas from the first week on. Part of the early increase of the Kc population is derived from influx of mono-nuclear phagocytes into the sinusoids. Kc numbers are doubled after two weeks; no excess matrix is then found. Kc numbers increase further till a steady state is reached on prolonged CCl_4-treatment after 9 weeks. Kc are frequently found, but not exclusively so, in and around developed fibrotic septa. This contrasts with another CCl_4 rat study reporting depletion of Kc when cirrhosis, with regeneration of liver parenchyma and capillarization of sinusoids, was established (Lough et al 1987).

From one of the studies of Wisse's group (Geerts et al 1984) it was concluded that, in the chronic CCl_4-intoxication model, FSC are directly related to developing fibrosis, but that another cell type, possibly Kc, is reacting to the acute liver damage of the first week and is responsible for the stimulation to FSC prolife-ration.

In the single dose (acute) CCl-intoxication model, using desmin as immunohisto-chemical marker for FSC, Burt et al (1986) found a significant increase in FSC with five-fold peak increase at 72 hours.

All those studies are consistent with the hypothesis that FSC are involved in the fibrogenetic response to the acute CCl_4-induced liver damage.

3.4.2 Dimethylnitrosamine

Dimethylnitrosamine has been used to study the early events of developing LF and cirrhosis in rats. It provides a good reproducible model in which micronodular cirrhosis develops in 3 weeks on administration of a single low dosis (Jezequel et al 1987).
Evidence for an immune-mediated mechanism of the induced LF has been found by the cascade of cellular events involved: quantitative increase of expression on macrophages of Ia antigen (reflecting the antigen presenting function of these cells), lymphoid infiltration of the liver parenchyma by T- and B-cells, accompanying the development of dissecting LF along with activation of FSC and the presence of (increased) transitional cells, myofibroblasts and fibroblasts.
The findings in this model were considered to support the hypothesis about the relationship between inflammation and immune-mediated fibrogenesis of the liver (Jezequel et al 1989).

3.5 Biochemical markers of liver fibrosis

From the present knowledge of the composition of the extracellular matrix, several enzymes and metabolic products, notably of collagen, have been tried as biochemical blood and urine markers for liver fibrosis.
There are currently no generally accepted non-invasive methods to diagnose and monitor hepatic fibrogenesis (Davis & Madri 1987a), but of all such methods only the determination of blood levels of the aminoterminal procollagen type III peptide (PIIIP) is the best accepted blood test for collagen metabolism (Hahn & Schuppan 1985).

3.5.1 PIIIP

Procollagen peptides are terminal structures on both sides of the procollagen molecules which are particularly well characterised for types I and III (Timpl & Glanville 1981). PIIIP is the cleavage product released from the procollagen molecule

when a collagen type III fibril is produced and deposited in the EM. This split-off is mediated by special endopeptidases at the surface of thin collagen fibrils, allowing them to grow further (Fleischmajer et al 1983). The exact pathway of the liberation of procollagen peptides from the precursor molecule has still to be established (Hahn & Schuppan 1985).

PIIIP blood levels are usually determined using a radioimmunoassay which has become available since human PIIIP has been purified and characterised (Risteli & Risteli 1986).

Some uncertainty and controversy still exist about the significance of elevated PIIIP serum levels. PIIIP has been found immunohistochemically to persist in the extracellular matrix of the human liver (Davis & Madri 1987a). In the CCl_4 rat model, serum PIIIP is used to assess early hepatic fibrogenesis and it also reflects growth-related changes of collagen type III metabolism (Schuppan et al 1986b).

However, in another CCl_4 study serum PIIIP usefulness, as an unequivocal marker of active hepatic collagen deposition, appeared limited: liver matrix PIIIP declined much earlier than serum transaminases elevation which was paralleled by serum PIIIP levels and by histological inflammation (Davis & Madri 1987b). This suggested that the serum PIIIP-levels reflected extracellular collagen degradation as well as active collagen secretion. Moreover, it is not certain whether the processing of type III procollagen is similar in health and in diseases of various kinds (Rojkind & Kershenobich 1987).

PIIIP is probably a marker of liver fibrosis that is of little help in differential diagnosis of the etiology, but it may be useful as marker of progression of ongoing fibrosis and developing cirrhosis (Weigand et al 1984).

PIIIP in ALD

In several studies, serum PIII-radioimmunoassay has been used as screening test for ALD and to monitor liver fibrogenesis, notably when alcoholic hepatitis and cirrhosis are present. In addition, laminin serum levels may be used to detect alcoholic hepatitis in alcohol abusers (Annoni et al 1989).

PIIIP discriminates between alcoholic fatty liver and hepatitis with high efficacy (Niemela et al 1983; Hahn & Schuppan 1985). Fab radioimmunoassay of serum PIIIP showed discrimination between alcoholics with fatty liver and (perivenular) fibrosis (Sato et al 1986). The degree of activity of ALD and the rate of EM-deposition

appear to be closely correlated with PIIIP-levels (Niemela et al 1983). However, the clinical value in respect to prognosis of alcoholic patients with elevated serum PIIIP still awaits the results of multicenter prospective clinical studies. Several issues about collagen type III metabolism need to be addressed more precisely, notably whether PIIIP-elevation is related to progression of fibrosis and/or cirrhosis and whether PIIIP reflects more synthesis or more degradation due to matrix remodelling (Hahn & Schuppan 1985).

3.5.2 Hydroxyproline

Several collagen fractions can be extracted from wet liver tissue. Hydroxyproline is an aminoacid that is abundantly present in collagen and practically absent from other mammalian proteins. As a consequence, determination of hydroxyproline in hydrolysed tissue samples gives a reliable measure of their collagen content. In addition, hydroxyproline in serum and urine appears to originate primarily from the degradation of collagens in bone and other tissues. Increased hydroxyproline excretion in urine has been described during active LF, but also in several wholly unrelated conditions, including osteopenia. Collagen fractions are usually divided into neutral soluble, acid soluble and pepsine solubilized ones, and their sum expressed as the total content of hydroxyproline per wet gram tissue. The total collagen content, in this way determined by hydroxyproline, is reflecting *in vivo* collagen synthesis and deposition. It is enhanced in fibrotic and cirrhotic liver, but also in regenerating rat liver after partial hepatectomy (Rojkind et al 1983) due to collagen synthesis by actively regenerating hepatocytes and the endothelial cells, both participating in remodelling of the liver architecture (Guzelian et al 1981).
In the near future, large contributions to knowledge on protein synthesis in the liver, including fibrogenesis, can be expected from further development of molecular hybridization technology on liver tissue using mRNA and cDNA probes (Weiner et al 1987).

3.6 Collagen degradation

Knowledge on the mechanisms of matrix breakdown is even scarcer than that on matrix synthesis. However, the rate of collagen turnover is now known to occur faster than previously believed and, for that reason, knowledge of (altered) collagen degradation in matrix collagen homeostasis of normal tissues is important (Mcanulty & Laurent 1987).

Two main pathways of degradation of soft tissue collagen are recognised: an extracellular and an intracellular pathway. The extracellular pathway depends mainly on the activity of collagen degrading enzymes, including specific collagenases and several proteinase classes, notably serine-proteinases (viz. cathepsin G and elastase), cysteine-proteinases (cathepsin B, L, and N), aspartate-proteinase (cathepsin D), and metallo-proteinases. The collagenases have a high degree of selectivity and are generally accepted as the most important enzymes for collagen break down (Everts 1987).

Little is known about rates of collagen degradation, but from an *in vivo* study on normal rat tissues, it appeared that newly synthesized collagen was rapidly degradated, probably intracellularly (Mcanulty & Laurent 1987).

Studies on degradation have been particularly difficult because collagen has varying susceptibilities to degradation at different stages of post-translational and extracellular processing (Mcanulty & Laurent 1987).

Degradation in the liver

The net increase in liver collagen is the result of an imbalance between the amount of collagen produced and the capacity of the available collagenases to degrade collagen (Rojkind & Perez- Tamayo 1982).

In vivo collagen degradation has been investigated mainly in CCl_4- induced cirrhotic rat liver. Both hepatocytes (Nagai et al 1982) and Kupffer cells (Fujiwara et al 1973) isolated from rat liver produce and secrete collagenase.

Also schistosomiasis-induced portal fibrosis in mouse liver has been a much used model to study both collagen deposition and degradation. In actively fibrosing liver schistosomiasis collagenase activity was found to be low (Takahashi et al 1980), whereas fibrosis appeared to be reversible in all stages (Andrade & Grimaud 1988)

due to collagenase activity during specific treatment (Emonard & Grimaud 1989).

CCl_4-cirrhosis

From the CCl_4 rat model, it appeared that collagen degradation is an active process that occurs in normal liver but is greater in cirrhotic liver (Rojkind & Perez- Tamayo 1983), in which it is largely limited to the newly produced collagen fibrils of the septa (Rojkind et al 1982).

In the late (true) cirrhosis stage, collagenase activity is absent from the fibrous tissue (Perez-Tamayo 1978). Irreversibility of this end stage disease has been claimed to be due to decreased susceptibility of collagen to degradation by collagenase because of molecular change, such as increased cross-linking.

However, both in CCl_4-induced experimental and in human cirrhosis (Perez- Tamayo & Montfort 1980), it has been shown that all collagen of the liver is susceptible provided active collagenase is present in sufficient amounts.

3.7 Animal models for alcoholic liver disease

3.7.1 General introduction

It is evident that the pathogenesis of ALD is far from being elucidated. Although the relation of ALD with total amount and duration of alcohol ingestion has been established, alcohol-related liver damage will only be effectuated in a rather limited number (about 20-25%?) of alcohol abusers, and the reasons why individuals are prone to develop ALD are unknown. In this sensitivity, genetics is likely to play an role, as yet undefined (see 2.2.4.4).

Several other factors may contribute to or protect against sensitivity to develop alcohol-related liver damage.

Malnutrition, very frequently found in patients with ALD, seems to play only a modulating role and not a pathogenetic role in the course of ALD (Achord 1988; see 2.2.4.5.1.), liver fibrosis and cirrhosis in general (Nayak 1987). However, convincing studies, notably prospective ones, are lacking to rule out that nutritional factors can play a decisive pathogenetic role as a co-factor with alcohol in the development of ALD.

In this respect, animal models could be of potential help, provided that these models are sufficiently comparable with human conditions related to ALD. This raises the issue of comparative human and experimental animal pathology, for it is not realistic - apart from mere species differences - to expect full comparability of human alcohol consumption in all its diversity, related to drinking pattern and brands, to "artificial" well defined models of ethanol consuming animals.
It has been proposed (Lieber & DeCarli 1989) that animal models for ALD should satisfy at least two criteria in order to mimic prevailing human conditions: firstly, significant blood alcohol levels, over about 100 mg/dl, must be achieved and, secondly, alcohol consumption must be about 50% of total energy intake as is found on average in alcoholics (Patek 1979; Lieber 1988a).

3.7.2 Specific models

3.7.2.1 General remarks

The animal species most used to study the effect of alcohol on the liver are monkeys, sub- and non-human primates, and rodents, notably rats. An evident advantage of the use of monkeys over rats is the long life span of monkeys. Therefore, monkeys are par excellence suitable for long-term prospective studies which are requested for studying the lesions of human ALD. Important draw-backs of the monkey model are the difficulty to handle the animals and the very high cost of maintenance.
Because most animals have a natural aversion to alcohol, considerable quantum of alcohol intake and adequate ethanol blood levels to expect liver damage, are usually not achieved by voluntary alcohol drinking. Therefore, alcohol intake has been mostly effectuated as component of a diet of a particular composition (see later).

3.7.2.2 Sub-human primates and monkeys

3.7.2.2.1 Baboons

Conflicting results have been published on the effect of long-term ethanol

administration on the liver of sub-human primates. In 1973, Rubin and Lieber reported a new primate model for human ALD. In this model large, inebriating amounts of ethanol (36% up to 50% of calories intake) were ingested by incorporation in a liquid diet that was composed to be nutritionally adequate.
Given to young (adolescent) baboons, the treatment resulted in the production of the entire constellation of the characteristic histological liver features of human ALD, including alcoholic hepatitis with Mallory bodies and central hyaline sclerosis (Rubin & Lieber 1973).
Several reports of Lieber and coworkers followed. They showed that in this model the three main histological features of human ALD: steatosis, alcoholic hepatitis and cirrhosis, developed sequentially (Rubin & Lieber 1974; Lieber et al 1975).
Fatty liver was consistently elicited in all baboons within 1-2 months, fibrosis in all in 1-2 years, and progression to (incomplete) cirrhosis occurred in one third of the animals (Popper & Lieber 1980).
Alcoholic hepatitis was, in the initial reports, a considerable feature present in 4 of 13 baboons after nine months (Rubin & Lieber 1974). Five of 15 baboons with alcoholic hepatitis showed further progression to extensive fibrosis and cirrhosis, in two of these as early as two years after start of the alcohol treatment (Lieber et al 1975). In a later report, however, alcoholic hepatitis was not a prominent and consistent finding, and an alternative pathway of progressive pericentral fibrosis to cirrhosis without alcoholic hepatitis was indicated (Popper & Lieber 1980).
This pericentral or perivenular fibrosis (see 2.2.2.3.2.) is composed of deposition of collagen fibres around the central vein and is associated with proliferation of myofibroblasts around the central vein wall (Van Waes & Lieber 1977; Nakano & Lieber 1982). The same perivenular fibrosis with progression to cirrhosis without alcoholic hepatitis has been found to occur in human ALD (Nakano et al 1982), particularly in Japan (Takada et al 1982).
Recently, Ainley et al (1988) have disputed the baboon model as an adequate animal model for human ALD. After 15-60 months of administration of large amounts of alcohol (70% of calories intake) and an adequate diet, only fatty change but no evidence of alcoholic hepatitis or significant fibrosis was found in the livers of their baboons. Daily alcohol intake was 25 g/kg as compared to 4,5-8,3 g/kg body weight (Rubin & Lieber 1974) in Lieber's baboons. Resulting ethanol blood levels were

comparable in both studies, 63-143 mg/dl and 92-184 mg/dl in Ainley's and Lieber's study. Important to note was the failure to gain weight in Lieber's baboons (Lieber et al 1975) whereas Ainley's animals gained weight comparable with non-treated controls. The composition of the diet of both studies differed. This makes comparison and interpretation of the different results of both studies difficult but suggests that composition of the diet may be a key factor in the hepatotoxicity of ethanol (Mezey 1989).

3.7.2.2.2 Rhesus-monkeys

In two long-term studies, using Rhesus-monkeys fed an alcohol containing (40%-50% of calories substituting) liquid diet with choline supplementation, only fatty liver but no inflammation or fibrosis were found.
In the first study, the Macaca mulatta subspecies was studied for 1.5-4.5 years; alcohol-treated monkeys failed to gain weight but the livers showed only fibrosis and cirrhosis when the diet was clearly deficient of lipotropes (methionine, choline and folate) (Rogers et al 1981). In the second study, the Macaca radiata subspecies was studied for 40-48 months; only steatosis but no fibrosis of the livers was found on histopathological and biochemical examination (Mezey et al 1983).

3.7.2.3 Rodents

Rodents, predominantly rats, have been the favoured non-human species to study ALD, mainly because of the lower costs of keeping these animals as compared to monkeys.
Both to overcome the inborn aversion of rats to alcohol and to reach relevant blood levels, Lieber and co-workers have developed in the early sixties a liquid diet, considered to be nutritionally balanced and adequate, in which alcohol was incorporated (Lieber et al 1963). Administration of this diet allowed forced ingestion of large amounts of alcohol that achieved high blood levels and accurate recording of nutrient intake. Moreover, this diet technique facilitates pair-feeding of control animals. Since 1963, this liquid alcohol containing so-called Lieber-DeCarli diet has been adjusted and its composition slightly improved.

The studies describing the effects of alcohol, notably on the liver, using this diet were recently critically reviewed and updated (Lieber & DeCarli 1989).
In 1983, Leo & Lieber reported that administration of the Lieber-DeCarli diet, which was supplemented with a moderate amount of vitamin A, caused liver fibrosis in all alcohol treated rats. Fatty liver and inflammation preceded fibrosis, but the characteristic features of alcoholic hepatitis, as found in the baboon liver, were not shown (Leo & Lieber 1983).
This publication had considerable implications for subsequent experimental alcoholic liver research. Unfortunately, no other research group so far has been able to reproduce these results of Lieber and co-workers in rat liver, and subsequently the validity of the rat model for ALD has been questioned.
Although the liver lesions found by Lieber and co-workers are held by them to be the direct toxic effect of alcohol on the liver, the dispute about the role of nutrition as a major determinant factor of liver fibrosis still remains a central issue (Rao et al 1986; see 4.1).

3.8 The role of nutrition in the generation of alcoholic liver disease and liver fibrosis

3.8.1 General remarks

Studies of the nutritional status of patients with ALD have shown evidence of malnutrition. Deficiencies are of various kind and include proteins, carbohydrates, fats, minerals and vitamins (Leevy et al 1985).
Until about 2 decades ago, it was generally held that the malnutrition found in alcoholics was the cause, not the consequence, of ALD (Rao et al 1986; Lieber 1989). However, evidence grew that several specific nutritional deficiencies of chronic alcoholism were secondary to ALD, and resulted from intestinal and liver dysfunction leading to malabsorption and chronic liver disease, due to the effect of alcohol on the gastrointestinal tract, pancreas and liver.
The increasing awareness of malnutrition of ALD being a secondary phenomenon, was supported by the results of the animal model developed by Lieber and coworkers in baboons and rats, which showed direct hepatotoxicity of ethanol when it was

administered in a nutritionally adequate diet.
On the other hand, malnutrition *per se* adversely effects liver function and structure, and may be presenting as fatty liver histopathologically indistinguishable from ALD (Achord 1987).

3.8.2 Effects of malnutrition and specific nutrients on the liver

3.8.2.1 Proteins

Of the three sources of calories: protein, carbohydrate and fat, protein is the most important to maintain normal structure and function of the liver. Protein deprivation has serious negative effects on the synthesis and content of proteins by the liver. In rats, a choline-deficient diet can produce steatosis, fibrosis and even cirrhosis of the liver (Nakano 1986). However, these effects can not be extrapolated to human liver disease, and choline is not considered to be an essential amino acid in humans (Achord 1987).
Kwashiorkor
Broad protein deficiency in extreme form is known as kwashiorkor, prevailing in many third world countries. It is a syndrome composed of skin and hair alterations, gross hepatomegaly with fatty liver, edema and ascites. This syndrome is now believed to be a manifestation of protein deficiency in the presence of adequate calorie- intake derived from high carbohydrate or fat containing diet (Nayak 1987), and thus contrasts with marasmus due to starvation and overall dietary deficiency.
Although stellate portal fibrosis may be found in some cases of long-standing disease, there is no evidence that kwashiorkor alone leads to liver cirrhosis (Nayak 1987). The syndrome improves dramatically on protein administration and is usually reversible.
Steato-hepatitis
In obese thus not calorically deprived humans, fatty liver with hepatitis ,so-called steato-hepatitis, may be present and may lead to cirrhosis (Galambos & Wills 1978). Steato-hepatitis occurs also in patients with jejuno-ileal bypass surgery for morbid obesity treatment. It is indistinguishable from alcoholic hepatitis (Peters et al 1975, Vyberg et al 1987). Liver injury is progressive (Vyberg et al 1987), and in 10-15% of the patients cirrhosis will develop eventually (Achord 1987). The etiology of this liver

lesion is unknown. The similarity in the sequence of events may suggest a pathogenetic mechanism common to ALD (Vyberg et al 1987). Prevention or correction by protein supplementation and/or antibiotics is not possible (Haines et al 1981).

3.8.2.2 Vitamin A

3.8.2.2.1 General introduction

Vitamin A, or naturally occurring retinoids (synonym retinol), is an essential nutritional component that has an important function in the differentiation and maturation of epithelial tissues. Vitamin A deficiency has been incriminated to be a pathogenetic factor in carcinogenesis (Goodman 1984; Lieber et al 1986).
Vitamin A is mainly generated in intestinal epithelial cells from dietary carotenoids of vegetable origin with provitamin A activity such as beta-carotene. Also, dietary retinylesters mainly of animal origin are hydrolysed in the gut lumen, resulting in vitamin A that subsequently is absorbed by intestinal mucosal cells. Vitamin A is re-esterified with long-chain fatty acids and transported, in association with chylomicrons, via the lymph into the blood circulation for removal by the liver where it is stored primarily in FSC and also in hepatocytes. Vitamin A is re-excreted by the liver into the blood bound to a specific plasma carrier protein, retinol-binding protein.
The liver is the main organ for processing and storage of vitamin A. In intact liver and under normal conditions, vitamin A is processed mainly in the endoplasmatic reticulum of parenchymal cells, while it is stored in lipid droplets of FSC (Hendriks et al 1988).
The amount of vitamin stored in the liver varies markedly depending on nutritional intake. There is very little correlation between plasma and liver vitamin A levels. Plasma levels normally remain fairly constant for a given individual, and abnormal levels reflect extreme vitamin A (nutritional) status when liver content is critically depleted or excessively expanded in the hypervitaminosis range (Goodman 1988).
The role of vitamin A metabolism and storage in the liver has been extensively investigated in our Institute and was recently reviewed by Hendriks et al (1987) and Knook et al (1989).

Both hyper- and hypo-vitaminosis A have been shown to have toxic effects.

3.8.2.2.2 Hypervitaminosis A

Clinical toxicity of hypervitaminosis A may result when the capacity of retinol- binding protein to transport vitamin A is exceeded and excessive retinol is presented to cell membranes in another form than bound to this carrier protein. It occurs when a daily intake of more than 75.000 IU is taken for 6 months. This may happen by (over-) medication in dermatology, notably for psoriasis, and in oncology (cancer prophylaxis) (Bollag 1983).

In the presence of excess of vitamin A, overflow to extra-hepatic organ storage, mainly in lungs and kidneys, will occur.

Although chronic hypervitaminosis A is a well recognised disorder, it is only rarely diagnosed and probably frequently overlooked (Russell et al 1974), and most papers concern one or a few case reports. It is associated with varied rather aspecific clinical signs of toxicity, mainly from extra-hepatic organs, notably upper gastrointestinal tract (nausea, anorexia, weight loss, gingivitis), skin and hair (alopecia, brittle nails), muscles and bones (pain), nervous system (headache, psychiatric disturbances), and, biochemically, hypercalcaemia.

Evidence of toxicity of excess vitamin A in the human liver is less prevalent than may be expected from the liver being by far the most important site of vitamin A storage. Most reports of hepatotoxicity are case studies.

Severe hepatic injury may occur without signs of toxicity in extrahepatic organs (Verneau et al 1984). The usual clinical signs of hepatic involvement are hepato-(spleno-) megaly with biochemical impairment and morphologically pronounced FSC with vitamin A loaded fat vacuoles, occasionally perisinusoidal (pericellular) fibrosis, and even cirrhosis (Guarascio et al 1983; Verneau et al 1984). Portal hypertension may be present in relation to cirrhosis in a late stage of hepatic damage, but it may already be found in an earlier stage being reversible at vitamin A withdrawal (Guarascio et al 1983). As has been evidenced from a study in rats (Ikejiri & Tanikawa 1977), this early portal hypertension may be explained by obstruction to the sinusoidal blood flow due to narrowing of the sinusoids by grossly enlarged bulging FSC (Russell et al 1974).

3.8.2.2.3 Hypovitaminosis A; relation to alcohol

Very low nutritional intake of vitamin A sources may result in a low hepatic level and eventually in low serum levels.
It has been clearly established, that chronic alcohol consumption lowers hepatic vitamin A levels, both in baboons and in rats (Leo et al 1983), as well as in human liver (Leo & Lieber 1982; Bell et al 1989).
The reason why this occurs in the chronic alcoholic, even in the presence of adequate vitamin A intake, is not clear (Lieber et al 1986). Although malabsorption of vitamin A from the gut may be a contributory factor in humans, this was not found to occur in chronically alcohol fed rats with decreased hepatic vitamin A storage (Grummer & Erdman 1983). Lowered hepatic levels in chronic alcohol abuse are more plausibly explained by increased vitamin A metabolism resulting in catabolism (Lieber et al 1986; Weiner et al 1988; Bell et al 1989) and, additionally, by mobilization from the liver to peripheral tissues where vitamin A content then increases (Lieber et al 1986).
Evidence for this explanation has been given by Lieber and coworkers who showed that liver vitamin A metabolism was partially mediated by specific isozymes of the microsomal cytochrome P-450 system (Leo & Lieber 1985) which are induced by chronic alcohol abuse (Leo et al 1985).
This action of ethanol on MEOS can alter hepatic vitamin A requirements which in turn can affect hepatic morphology (Weiner et al 1988). Again, Lieber and coworkers have suggested, mainly based on data gained from rat and human studies (alcoholics with cirrhosis), that vitamin A depletion is probably an important factor contributing to the development of the lesions of ALD, notably to fibrogenesis (Lieber 1987). This hypothesis may also be supported by experimental CCl_4-induced liver injury in rats, showing that developing fibrosis is suppressed by vitamin A supplementation (Senoo & Wake 1985; see also chapter VI).
There is a lot of evidence that vitamin A has an important determinant role in some forms of developing liver fibrosis associated with hepatic injury (inflammation), especially of ALD.
This role of vitamin A in the generation of liver fibrosis or its prevention by vitamin A supplementation is very complex and has been an important issue of our studies. It will be discussed and reviewed, both in relation to CCl_4-induced liver injury and to

ALD later (see 4.2, chapter V, 8.2.2 and 8.2.3).

Other nutritional factors

Deficiency of many nutrients of various kind, such as vitamin B and zinc, is found in chronic alcoholics, partly due to malnourishment and partly to malabsorption from the gut. In contrast with non-drinkers, total calorie intake is generally increased in drinkers, with relative low carbohydrate content due to alcohol calories (Lieber 1987). It is not believed that these factors contribute to the pathogenesis of ALD, but nutritional support is aimed to be an additional factor in the primary treatment by abstinence that in itself dramatically improves the prognosis of both acute and chronic ALD (Lieber 1982).

3.9 Summary of this chapter

Many data have been gained on the composition of the extracellular matrix (EM) of the liver, in health and disease. However, the specific interaction between the cells of origin and the many factors known to be involved in the maintenance or adjustment of the EM to circumstances and needs are only partly understood. This is largely explained by the fact that data derived from studies performed in vitro or in experimental animals in rather artificial conditions, mostly of CCl_4- induced liver damage to rats, cannot be directly extrapolated to human ALD.

All liver cells, parenchymal and sinusoidal (except the pit cell) and even vascular cells, can produce matrix components and therefore, theoretically, be involved in the generation of fibrosis. However, the general conclusion from most studies, both on human and experimental animal livers, is that the fat-storing cell probably is the main precursor cell of LF.

The factors involved in EM-overproduction are still incompletely known but the general trigger is probably parenchymal damage (hepatitis) of some duration. Degradation (turnover) of EM components is probably not decreased and may even be increased, but not sufficiently to prevent accumulation.

Mainly from experimental studies of CCl_4-induced liver damage, vitamin A status of the liver has been established to be important in limiting, or even preventing,

fibrogenesis but also enhancement of liver injury may occur.
There is little doubt that acetaldehyde, being the main metabolite of ethanol, is directly or indirectly the most important trigger to induce liver injury in ALD.
Recently, it has been recognised that release of Ctk following liver damage is a striking phenomenon that could be related to resulting increase of collagen deposition. This may in particular be the case in ALD, in which increased blood levels of several Ctk are consistently found to be associated with active liver disease (alcoholic hepatitis). With respect to ALD, bacterial endotoxins may play an additional important role, since they are a very potent trigger of Ctk release, mainly of TNF. This occurs notably in chronic alcohol abuse in which both endotoxin overload to the liver, by increased absorption from the gut due to alcohol-induced mucosal damage, and alcohol-induced decreased phagocytic capacity of the mononuclear phagocytic system, mainly the Kupffer cell compartment in the liver, occur.
Animal models have been developed to identify and, subsequently, better define the contribution of specific factors involved in the generation of ALD. Therefore, Lieber and coworkers have developed a nutritionally adequate liquid diet, in which at least 40% of total calories were substituted by ethanol. This diet was used in studies with baboons and rats and resulted in high ethanol blood levels.
They reported for the livers of baboons fed with this diet for long periods all features of human ALD, alcoholic hepatitis and cirrhosis included. In rats fed this diet supplemented with vitamin A, they found developing fibrosis in the livers of all animals after 9 months, but no alcoholic hepatitis.
The results of Lieber's group have been an important motive to apply the Lieber-DeCarli diet in our Institute to rats in order to analyze the underlying mechanisms involved in the pathogenesis of developing alcohol related liver disease especially fibrosis, that could be expected (see 4.1.and 4.2). Also other long-term studies were undertaken in rats to analyze the effects on the liver of various alcoholic beverages in combination with malnutrition of various kinds, notably with respect to vitamin A content of the diet (see 8.2.2). Also, short-term rat studies have been performed using the CCl_4-model to analyze the modulating effect of ethanol (chapter V) and of vitamin A (chapter VI).
These results of Lieber's group gained in monkeys and in rats have not been reproduced by other research groups (see also 4.1 and 4.2). The adequacy of this

baboon and rat model for human ALD has been criticized. The conflicting results certainly indicate that the ultimate effects of chronic ethanol overconsumption on the liver depend on nutritional and genetic factors that are still poorly understood and, at present, beyond experimental control.

3.10 References

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CHAPTER IV

CHRONIC ADMINISTRATION OF ETHANOL WITH HIGH VITAMIN A SUPPLEMENTATION IN A LIQUID DIET TO RATS DOES NOT CAUSE LIVER FIBROSIS: 1. MORPHOLOGICAL OBSERVATIONS

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Running title: Alcoholic liver disease and vitamin a: morphology

Acknowledgements

This work was subsidized by Praeventiefonds grant 12-1206, Den Haag, The Netherlands. The authors express their gratitude to Drs. A.E. Becker and J. Bras, Department of Pathology, Academic Medical Center, University of Amsterdam for suggestions and criticism.

Abstract

Rats of 2 strains (BN/BiRij and WAG/Rij) were fed for 16 months with the ethanol-containing Lieber-De Carli liquid diet supplemented with high amounts of vitamin A. In contrast to Lieber and coworkers, who showed liver fibrosis developing within 9

months on the same diet in Sprague Dawley rats, we were unable to demonstrate a histological and biochemical increase in liver collagen in either strain. Steatosis was present to a varying degree in both strains in ethanol-treated rats, but also in control animals. Considerable liver inflammation with focal necrosis accompanied by severe systemic inflammation was observed in 60% of the ethanol-treated WAG rats. This suggests that, at least in rats, the main effects of chronic ethanol consumption on the liver may be secondary to interference with host resistence to infections. The ethanol-high vitamin A Lieber-De Carli liquid diet does not necessarily elicit fibrosis or other characteristic histological abnormalities of human alcoholic liver disease.

Introduction

Alcoholism is a major health problem in modern Western society [1], with a heavy social and economic impact. In spite of this, very little is known about the determinants of the adverse effect of ethanol on the liver [2]. Attempts have been made to develop an animal model as an analogue of human alcoholic liver disease (ALD) for investigations on the pathogenetic factors involved in alcoholic liver fibrosis and, in particular, on the mechanisms involved in the early events of ALD [3,4,5].
In 1983, Lieber and coworkers described a rat model to study ALD, which was based on toxicity of dietary vitamin A in combination with chronic ethanol consumption [5]. Ethanol and high doses of vitamin A were incorporated in a well-defined semi-synthetic liquid diet which results in the ingestion of high amounts of ethanol, at a fixed percentage of total calories. They showed that liver fibrosis could be elicited in 9 months in all rats. However, the highly characteristic histological picture of alcoholic hepatitis in human liver, composed of focal parenchymal cell damage with swelling and polymorphonuclear cell infiltration, was not observed [5].
Using the same experimental approach, but in two other rat strains than that used by Lieber and coworkers, we further investigated the reliability of the model in general to study ALD. We used young adult rats to exclude deleterious effects of high doses of ethanol and vitamin A on maturation, which usually are excluded in human alcoholics. The histopathological findings in the liver were related to pathological alterations of the lung and the kidney. This report focuses on the histopathological

findings. The biochemical results are presented in the accompanying paper [6].

Material and methods

Animals

Male rats of the BN/BiRij and the Wag/Rij strain, aged 3 months, weight 268 ± 19 g (n=20) and 246 ± 13 g (n=20) respectively, were obtained from the Institute's colony (TNO, Rijswijk, The Netherlands). All rats were housed individually. A liquid diet (Bio-Servo Inc, Frenchtown, New Jersey, USA) as the only source of food and water was given. The composition of the diet was the same as used by Leo and Lieber [5] and it is listed in detail in a 1989 review [7,"all purpose diet"]. Briefly, the diet contained 36% of the total caloric amount either as ethanol or as isocaloric carbohydrates (dextrin-maltose). Vitamin A, as retinyl acetate, was added to a final concentration of 28.000 IU/liter, which was the same as administered to rats of the high vitamin A group reported by Lieber and coworkers [5]. In both strains, for each ad libitum ethanol-fed animal there was a paired carbohydrate-fed control. Body weight was measured every week. The rats were sacrificed after 16 months of liquid diet administration, at the age of 19 months. Blood ethanol levels were determined with a commercially available test combination (Boehringer Mannheim, FRG) on heparinized blood collected from the tail vein in the morning (9 a.m.) and in the evening (9 p.m.).

Surgical liver biopsies

Under halothane anaesthesia, surgical liver biopsies were taken at 6, 9 and 12 months of liquid diet administration. Sections of 20 to 30 mg of the median lobe were removed with an iris scissor (Stöpler, Utrecht, The Netherlands) and fixed in 4% buffered formalin for histological examination. The bleeding was staunched with dental foam (Willospon, Will Pharma B.V., Zwanenburg, The Netherlands). The peritoneum and the abdominal wall were sutured with absorbable surgical suture (4-0 Dexon plus, Davis and Geck, Danbury CT, U.S.A.). The skin was closed with wound clips (Clay Adams, Parsippany, NJ, U.S.A.) which were removed one week after surgery.

Organ preparation and histopathology

The animals were fasted overnight before sacrifice. After sacrifice, the right lung was removed and frozen in liquid nitrogen for biochemical assays. Liver tissue was collected after perfusion with phosphate buffered saline (PBS, pH 7.4) through the portal vein for 5 min at 5 ml/min, followed by perfusion fixation. The median lobe was excluded from fixation and frozen in liquid nitrogen for biochemical analysis. The right lateral and caudate lobe of the liver were fixed for electron microscopy using 2.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4, 8 min). The remainder of the liver was fixed for light microscopy in neutral buffered formalin. A routine post mortem examination was carried out and at least kidney, left lung, heart, spleen and brain were fixed in neutral buffered formalin for light microscopy. Tissues were routinely embedded in paraplast and sectioned at four μm thickness. The sections were stained with haematoxylin-phloxin-saffron (HPS). Liver was also stained with PAS, with and without diastase pretreatment, Elastica Van Gieson and Sirius-red F3BA [8] for connective tissue and collagen, respectively.

Light microscopy of the liver

The assessment of morphology was done by a pathologist who had been blinded to the source of the specimens. Sections of liver biopsies and of post mortem liver tissue were analysed for the presence of steatosis, inflammation, fibrosis of the central vein (perivenular fibrosis) and of the adjacent parenchyma (zone 3), the presence of megamitochondria and of portal inflammation. Steatosis and parenchymal inflammation were graded from 0 to '+++'. Gradings '+', '++' and '+++' steatosis reflected an estimated involvement of 1/3, 2/3 and all of the liver parenchymal cells, respectively. Predominance of the steatosis as micro- or macrovesicular change was noted. Inflammation grade '+' reflected the presence of scattered isolated inflammatory cells in the parenchyma, '++' focal collection of inflammatory cells, and '+++' diffuse parenchymal inflammation; predominance of round cell or polymorphonuclear cell infiltration was noted. Intermediate grading of steatosis and parenchymal inflammation was applied when necessary.

Electron microscopy and morphometric analysis

For electron microscopy and morphometric analysis, representative samples were

taken from the right lateral and the caudate lobe. The samples were fixed in glutaraldehyde and postfixed with osmium tetroxyde, dehydrated in graded concentrations of ethanol and embedded in epon LX 112 (Ladd Research Industry Inc., Burlington, VT, USA). Ultrathin fibratome sections were mounted on uncoated copper grids, contrasted with uranyl acetate and lead citrate, and examined under an EM 410 electron microscope (Philips, Eindhoven, The Netherlands). These sections were used to determine the relative numbers of fat storing cells (FSC) and parenchymal cells (PC). 150-200 parenchymal cells were determined per animal and only cells of which nuclei were included in the plane of the section, were counted. The number of FSC was expressed per 1000 parenchymal cells (Ito cell index). For morphometric analysis of the various subcellular compartments in the FSC, photographs were taken of those cells which showed a portion of the nucleus (at 10,750 x final magnification). The total cell body and the areas occupied by lipid droplets, nucleus, mitochondria and rough endoplasmic reticulum (RER) were determined using a digitiser tablet connected with a microcomputer system (MOP-Videoplan) with Kontron software [9]. For each rat, 10 photographs were analysed.

Hydroxyproline determination

300 to 500 mg of the frozen median lobe of the liver was lyophilized overnight, powdered and extracted twice with 20 ml diisopropylether to remove vitamin A (which suppresses the colour development of the assay). Traces of diisopropylether were evaporated in a waterbath at 37°C. Hydrolysis was performed by addition of 5 ml of 6 N HCL per 300 mg "wet" tissue and incubation at 120°C for 16 h in screw-capped plastic tubes (Falcon 2070). After cooling, the hydrolysate was filtered through a 0.22 µm Millipore filter. Each tube and filter were rinsed with the same volume of 6 N HCL, which was added to the first filtrate. The hydrolysates were stored at -20°C.
Hydroxyproline determination was performed using a modification of the method of Woessner [10], as described earlier [9]. Briefly, a 100 µl sample of the hydrolysate was added to a mixture of 100 µl 6 N NaOH and 1.8 ml 0.3 M NaCl. One ml of 0.05 M chloramine T in citrate-acetate buffer (pH 6.0) in the presence of 1.32 M n-propanol was added to this mixture. The solution was mixed and incubated for 20 min at room temperature. The incubation was stopped by adding 1 ml perchloric acid/4-dimethylaminobenzaldehyde solution in n-propanol. The colour was developed by a 15

min incubation at 60°C. After cooling, the absorbance was determined at 550 nm with an Ultraspec 4050 spectrophotometer (LKB, Bromma, Sweden). Hydroxyproline content was calculated on the basis of calibration curves using purified hydroxyproline as the standard.

Statistics

Results are expressed as the mean ± S.E.M. Statistical analysis was performed by the Student t-test for paired observations (significance level 5%, 1% and 0.1%).

Results

Dietary and ethanol intake, and blood ethanol levels

The daily intake of liquid diet per kg body weight showed very little variation within each strain, but was slightly higher for BN rats (Table 1). The growth curves during the treatment period were very similar for ethanol-treated and control rats of both strains (Table 1). A gradual increase in body weight was noted in all rats.

The daily ethanol intake per kg body weight by the BN rats was slightly higher when compared to WAG rats (Table 1). Within the BN strain the ethanol intake per kg body weight gradually increased during the treatment period. A slight gradual decrease was noted in the rats of the WAG strain.

Blood ethanol levels were slightly higher in the WAG rats when compared to BN rats. In both strains, blood ethanol levels were significantly higher at 9 p.m. than at 9 a.m. (Table 1). This may be explained by the fact that the food intake was higher in the hours after fresh diet was given (at about 10 a.m.), i.e. after the morning time point.

Light microscopy

The liver biopsies taken at 6, 9 and 12 months only showed steatosis, which was most pronounced in the ethanol-treated WAG rats. To a lesser degree, steatosis was also present in ethanol-treated BN rats as well as in both control groups. Very occasionally, minor parenchymal inflammation ('+' round cell) was noted in some biopsies of treated and control rats. No parenchymal fibrosis was found. No fibrosis was observed in central vein walls.

TABLE 1

Body weight, dietary and ethanol intake, and blood ethanol levels of BN and WAG rats kept on liquid diet with or without ethanol.

	BN rats		WAG rats	
	control	ethanol	control	ethanol
Body weight (g)				
0 months on diet	271 ± 3	264 ± 7	248 ± 4	243 ± 4
2 months " "	320 ± 8	315 ± 11	317 ± 2	315 ± 6
9 months " "	382 ± 7	386 ± 12	382 ± 5	379 ± 7
16 months " "	434 ± 11	423 ± 19	428 ± 7	426 ± 11
Dietary intake (ml/kg body weight per day)[a]				
2 months	-	180 ± 3	-	175 ± 2
9 months	-	181 ± 6	-	171 ± 6
16 months	-	190 ± 4	-	169 ± 3
Dietary intake (ml)[a]				
2 months	-	4239 ± 126	-	3828 ± 231
9 months	-	18194 ± 474	-	16258 ± 677
16 months	-	35929 ± 1196	-	33507 ± 1262
Ethanol intake (g/kg body weight per day)				
2 months	0	9.0 ± 0.2	0	8.8 ± 0.1
9 months	0	9.1 ± 0.3	0	8.6 ± 0.3
16 months	0	9.5 ± 0.2	0	8.5 ± 0.2
Blood ethanol levels (mg/dl)				
16 months (9 am)	0	33 ± 11	0	36 ± 9
16 months (9 pm)	0	60 ± 8*	0	87 ± 9**

Values are given as mean ± SEM (n =10).
a Given for ethanol-treated rats only: pair fed controls received the same amounts.
* : significantly different from blood ethanol levels at 9 am, $p<0.05$
**: significantly different from blood ethanol levels at 9 am, $p<0.001$

At sacrifice at 19 months, moderate ('+' - '++') to pronounced ('++' - '+++') steatosis of mixed micro/macrovesicular type was present in the ethanol-treated and in control rats of the BN strain with comparable severity (Table 2, Figs. 1a,b,c). The degree of inflammation was the same in both groups of control and ethanol-treated BN rats (Table 2, Figs. 1a,b) and was usually of the round cell infiltrate type.

TABLE 2

Histopathological evaluation of inflammation and steatosis of liver of ethanoltreated and control rats.

Treatment	Strain	Inflammation				Steatosis			
		0	+	++	+++	0	+	++	+++
Control	BN	0	4	6	0	0	4	5	1
Ethanol	BN	0	5	5	0	0	3	6	1
Control	WAG	0	9	1	0	7	3	0	0
Ethanol	WAG	1	3	6	0	1	3	6	0

Values represent the number of rats within each category (n = 10).
Gradings '0' to '+++' are described in "Material and Methods"

In the most severe cases, the inflammation was of the mixed round cell-polymorphonuclear cell type with spotty and occasionally focal necrosis of the liver parenchyma, and increased mixed inflammatory infiltration of portal tracts. Severe inflammation was usually associated with considerable steatosis. However, no fibrosis was present in livers of the ethanol-treated BN rats (Fig. 1c). One ethanol-treated BN rat died after 11 months due to severe ulcerative purulent oesophagitis with microorganisms, accompanied by extensive local necrosis of two liver lobes (not shown).

In the ethanol-treated WAG rats, the degree and pattern of steatosis and inflammation in the liver at sacrifice were comparable with those in the ethanol-treated BN rats (Table 2). In 60 % of the ethanol-treated WAG rats, systemic disease was present in the cases of most pronounced parenchymal and portal liver inflammation with steatosis. However, neither parenchymal fibrosis nor thickening of the central vein walls was present. In particular, the areas of spotty or focal coagulative necrosis of the severely inflamed livers were not fibrotic (Figs. 2c,d). This finding of florid

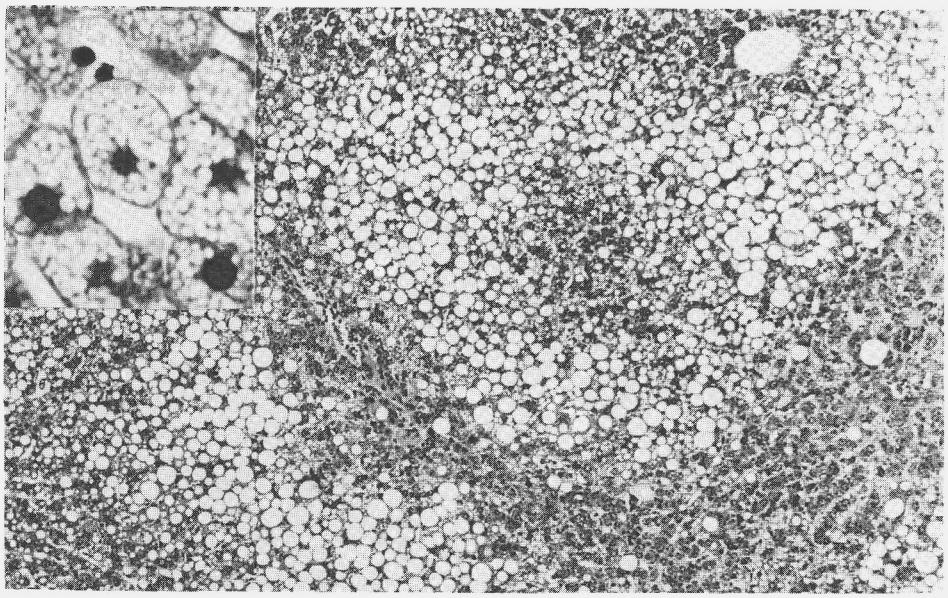

Fig.3.1a Liver of a BN rat, which was administered the ethanol-high vitamin A-containing diet of Lieber-DeCarli for 16 months, shows extensive (++/+++), predominantly microvesicular steatosis and only minor (+/++) inflammation of round cell infiltrate. No fibrosis is seen. HPS 80x; inset shows some parenchymal cells with microvesicular change (500x).

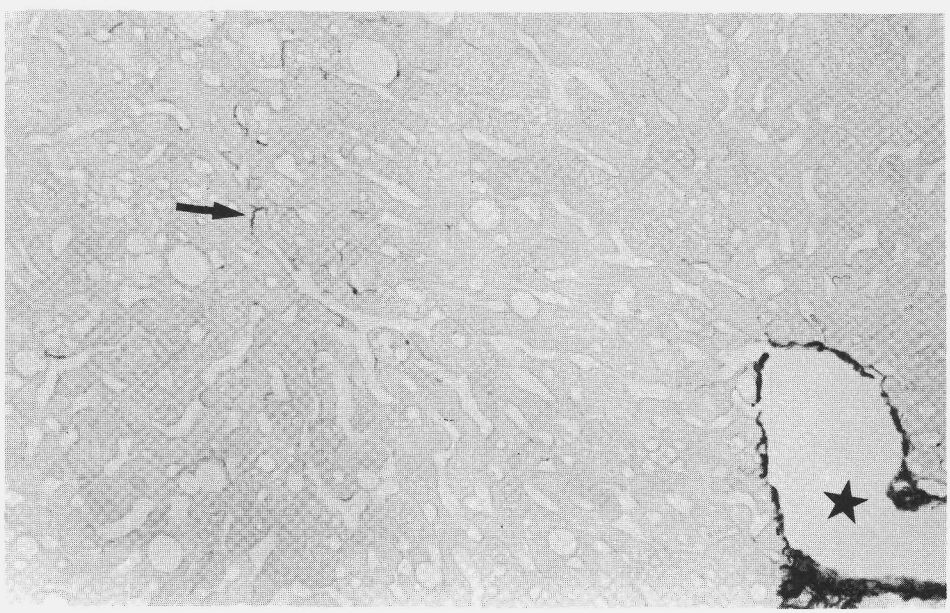

Fig.3.1b Serial section of a. stained for collagen. Only the portal tract (asterisk) shows normal frame work of connective tissue. Occasionally a collagen fiber is seen along the sinusoids (arrow) but no increase in collagen is evident. Sirius Red 210x.

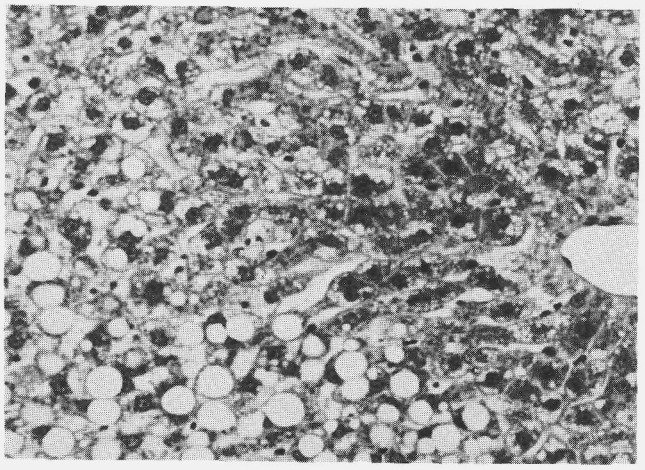

Fig.3.1c Liver of the pair fed control BN rat of fig. 1a-b. There is moderate steatosis (++) of mixed macro-microvesicular type, minor (+/++) inflammation and no fibrosis. HPS 210x.

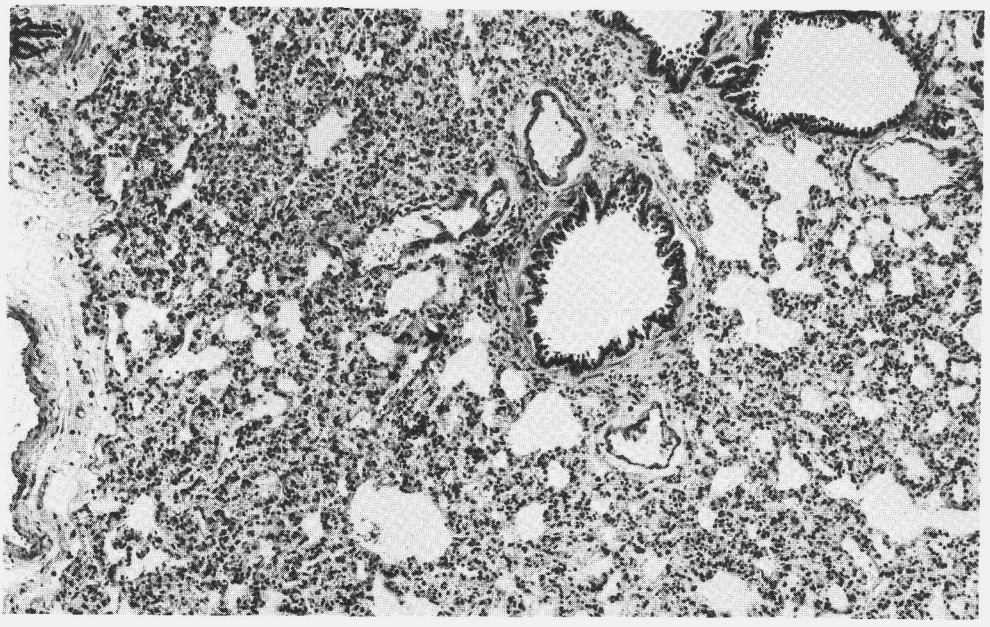

Fig.3.2a Lung of a WAG rat after 16 months of ethanol-containing liquid diet, showing interstitial pneumonia with desquamation of alveolar lining cells and with presence of macrophages in the alveolar spaces. HPS 80x.

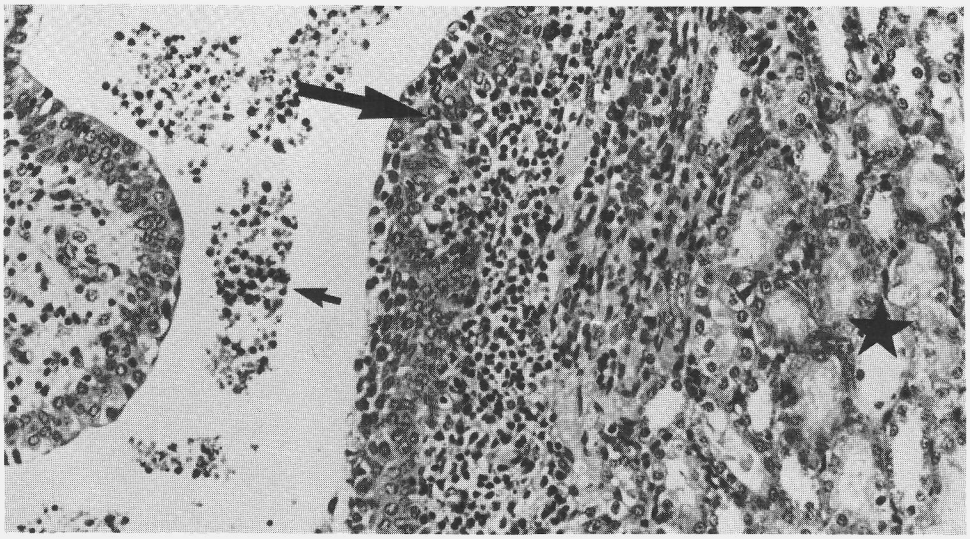

Fig.3.2b Kidney of the same ethanol-treated WAG rat of a., showing medulla (asterisk) and mucosa lining the pyelum with active inflammation (long arrow); in the lumen of the pyelum polymorphonuclear leucocytes are accumulated (short arrow). HPS 210x.

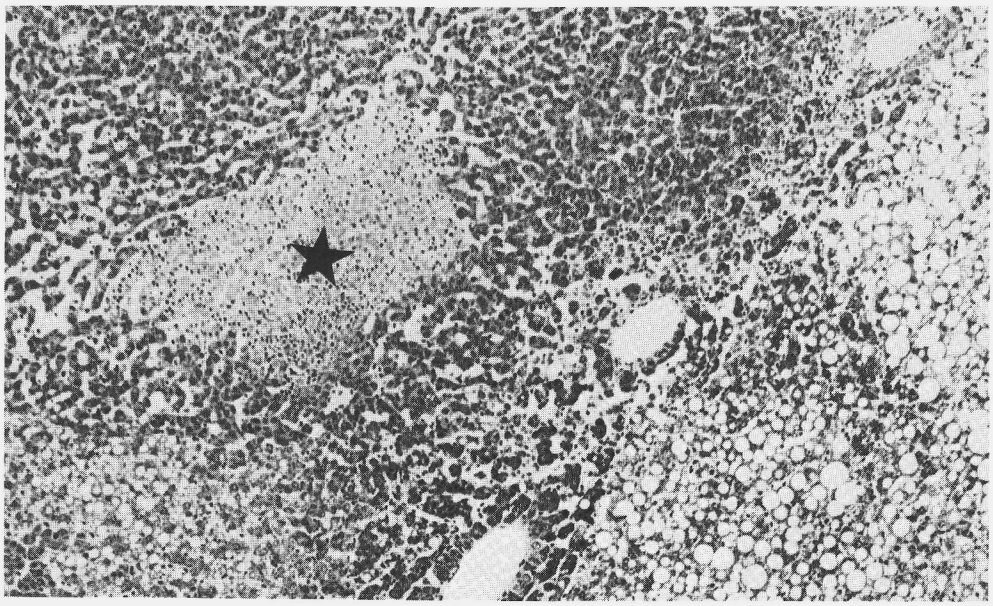

Fig.3.2c Liver of the same ethanol-treated rat with active pneumonia (a) and active pyelitis (b) showing extensive (++/+++) steatosis of mixed macro- microvesicular type with a large focus of coagulative parenchymal necrosis (asterisk). HPS 80x.

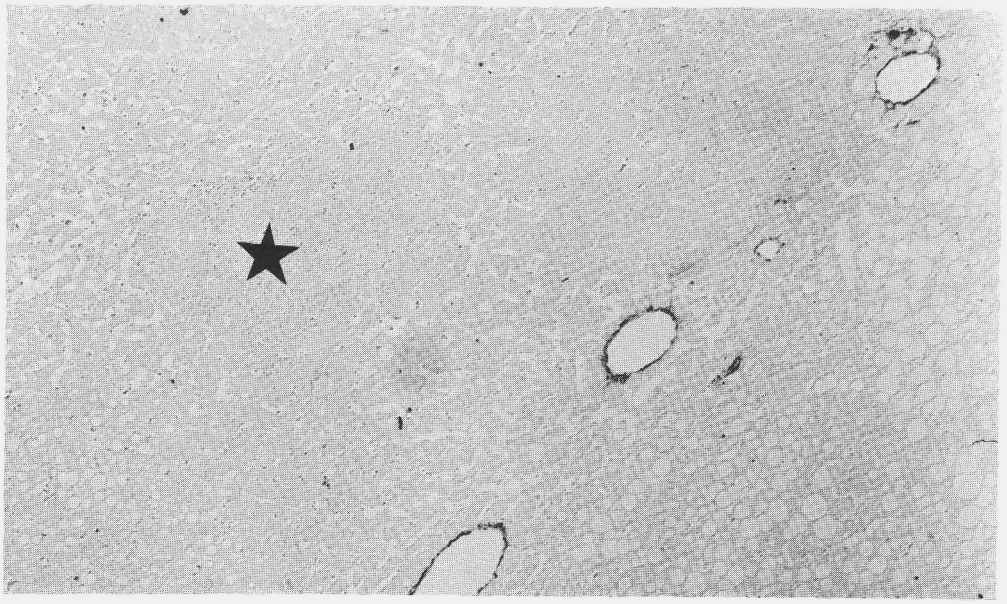

Fig.3.2d A liver section of the same focus of necrosis (asterisk) as shown in 2c, displaying no increase in collagen. Sirius Red 80x.

inflammation, with focal necrosis and without fibrosis, was always associated with systemic disease, usually marked interstitial pneumonia (Fig. 2a) and active chronic pyelitis (Fig. 2b). Steatosis and inflammation, and also associated systemic disease, were considerably less prominent in the control group of WAG rats than in the ethanol-treated group (Table 2). One ethanol-treated WAG rat had hepatocellular carcinoma in 2 liver lobes at autopsy (not shown). Hepatocellular carcinoma is a very rare phenomenon in our rat strains [11].

Fat-storing cells of alcohol-treated and control rats of both strains were roughly equally prominent in lipid droplets content, as judged by light microscopy. No megamitochondria were observed in hepatocytes of either ethanol-treated or control rats of both strains.

Electron microscopy and morphometric analysis

Electronmicroscopical analysis showed that the ultrastructural characteristics of ethanol-treated and control rat livers were qualitatively similar. In the liver lobule, fat-

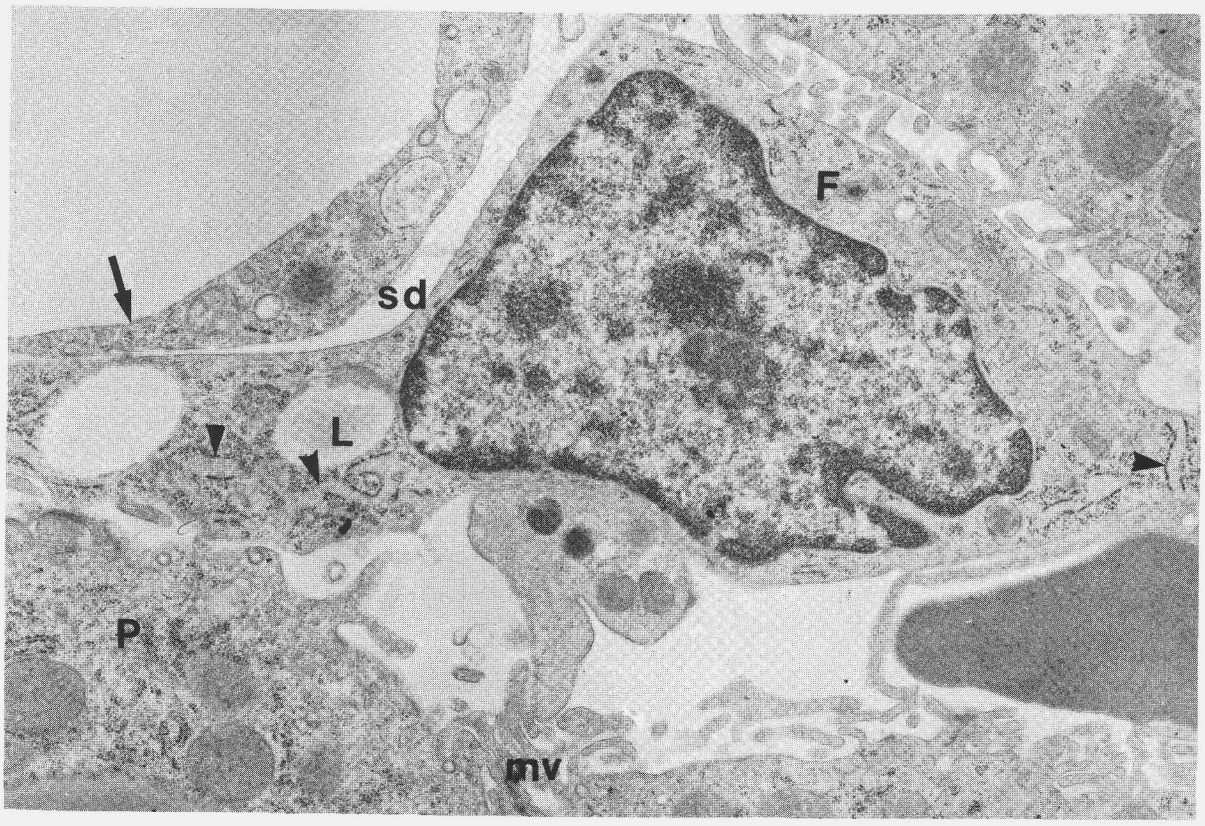

Fig.3.3 Transmission electron microscopy of the liver of a WAG rat treated with ethanol for 16 months. A fat-storing cell (F) is situated beneath the endothelial lining (arrow) in the space of Disse (sd) between the recesses of parenchymal cells (P).
The cytoplasm of the fat-storing cell demonstrates lipid droplets (L), some lysosomes, and vesicular structures. Note the relative abundance of short strands of slightly dilated rough endoplasmic reticulum (RER) (arrowheads). In the space of Disse, collagen fibers were sometimes noted (not present in this illustration). Parenchymal cells display numerous microvilli (mv) at their surface. Note the normal appearance of their cytoplasm containing some short non-dilated strands of RER and lipid droplets. Original enlargement: 6800x.

storing cells, containing many lipid droplets, were seen only in the Disse space. The parenchymal cells also contained lipid droplets (of a higher electron density), but no other major ultrastructural alterations, such as megamitochondria, were evident in these cells (Fig. 3). No increase in the amount of collagen in the space of Disse was evident and no increase in the number of (myo-)fibroblasts was observed in the perivenular area, nor in the adjacent parenchyma (not shown). Quantitative morphometric analysis showed that the relative surface area occupied by fat droplets in fat-storing cells seemed to be decreased in ethanol-treated rats of both strains (Table 3), but this was only significant for the surface area of fat droplets per total cell body in

BN rats. The surface area of the RER in FSC appeared increased by ethanol treatment, especially in WAG rats. No changes were found in the surface area of mitochondria in FSC. The number of FSC per 1000 hepatocytes (Ito-cell index) was significantly lower in the ethanol-fed animals of both strains, particularly in WAG rats (Table 3).

TABLE 3

Ito cell index and morphometric analysis of the relative surface areas of RER, lipid droplets and mitochondria in FSC.

	BN		WAG	
	control	ethanol	control	ethanol
Ito cell index [a]	214 ± 16	179 ± 9*	209 ± 21	140 ± 16*
Nucleus included [b]:				
Nucleus	16.9 ± 0.5	2.5 ± 0.8*	14.8 ± 1.1	19.3 ± 3.4
Lipid	61.2 ± 1.2	53.2 ± 1.7*	67.2 ± 2.4	55.6 ± 7.1
RER	0.4 ± 0.1	0.8 ± 0.3	0.2 ± 0.1	0.8 ± 0.2*
Mitochondria	1.5 ± 0.3	1.7 ± 0.3	1.7 ± 0.3	2.1 ± 0.5
Nucleus excluded [c]:				
Lipid	73.5 ± 1.6	67.6 ± 2.2	78.9 ± 2.1	67.5 ± 6.2
RER	0.4 ± 0.1	1.1 ± 0.4	0.3 ± 0.1	1.0 ± 0.3*
Mitochondria	1.8 ± 0.3	2.3 ± 0.4	2.0 ± 0.3	2.6 ± 0.6

Values are given as means ± SEM (n=5).
*) Significantly different from control ($p < 0.05$).
a) Expressed as number of FSC per 1000 parenchymal cells.
b) Expressed as percentage of the total cell body (nucleus included).
c) Expressed as percentage of the cytoplasm (nucleus excluded).
Abbreviations:
RER: Rough endoplasmic reticulu

Biochemical observations

Biochemical results revealed no significant increase in the hydroxyproline content of liver tissue from ethanol-treated rats (Table 4). This is in agreement with the histopathological observations of the absence of liver fibrosis. Results of an extensive biochemical analysis, presented in the accompanying paper [6], show that the effects of ethanol treatment on vitamin A metabolism and storage and on the appearance of liver enzymes in serum are less severe than reported [5].

TABLE 4

Hydroxyproline content of the liver of ethanol fed and pair fed control rats.

Hydroxyproline content	BN		WAG	
	control	ethanol	control	ethanol
µg/ g liver	220 ± 20	247 ± 35	196 ± 23	193 ± 17
µg/ total liver	2518 ± 400	3783 ± 679	1775 ± 209	2140 ± 154

Values are given as mean ± SEM (n=8).

Discussion

The present study with a liquid diet containing ethanol and a high amount of vitamin A was initiated to further analyse the mechanisms and critical early events of ethanol-induced liver fibrosis in a rat model. This ethanol-containing diet was reported to be nutritionally adequate [5,7]. In 1983, Leo and Lieber [5] showed that perivenular fibrosis of the liver, with thickening of the central vein, developed within 9 months in all Spraque Dawley rats treated with this diet supplemented with a high amount of additional vitamin A.

We have applied the same treatment protocol in two rat strains, BN and WAG, which are bred and commonly used in our institute, and differ from the strain (Sprague-Dawley) used by Lieber and coworkers. In both strains, we have not succeeded in eliciting fibrosis with the ethanol-high vitamin A-containing Lieber-De Carli diet, even

after the duration of the experiment was extended to 16 months. It is unlikely that the absence of fibrosis is due to a strain specific response, since earlier studies performed in our institute have shown that both BN and WAG rats are at least as sensitive to the induction of liver fibrosis by CCl_4 [12] and heterologous serum (unpublished results) as reported for other strains, including Sprague-Dawley [13]. In BN rats, it was also established that ethanol could potentiate liver fibrosis previously induced with CCl_4 [9]. In a preliminary experiment with the ethanol-containing Lieber-De Carli liquid diet, which contained twice the normal amount of vitamin A and was carried out in the same two rat strains and in Sprague-Dawley rats, we found no fibrosis after 12 months and saw only minor to moderate steatosis of the liver (unpublished results). The intake of the ethanol-high vitamin A diet per kg of body weight of both strains was very similar to the intake of the Sprague-Dawley rats fed the same diet in Leo and Lieber's experiments [5]. They reported that the total dietary intake of the Sprague-Dawley rats kept on the ethanol-high vitamin A diet for 9 months was only 2/3 of that consumed by the rats on the ethanol-normal vitamin A diet for the same period. Although the data for the ethanol-high vitamin A group were not calculated by Leo and Lieber [5], it can be inferred from Lieber's 1989 update [7] that rats fed the ethanol-high vitamin A diet consumed about 8-10 g/kg body weight and reached blood levels of 66-100 mg ethanol/dl blood. This shows that the absence of hepatic fibrosis in the present study is not attributable to a lower ethanol intake or lower blood ethanol levels.

The induction of liver fibrosis by the ethanol treatment also might be dependent on the age of the rats at the start of treatment. In our experience, administration of ethanol to weanlings affected their maturation and growth, and resulted in bad general health (data not shown). These alterations were not observed when the diet was administered to 3-month-old rats. Developing animals may be more sensitive to ethanol and vitamin A toxicity in general and this could contribute to the development of liver fibrosis.

In the present study, the only histological findings in the liver common with those reported by Lieber's group [5] were steatosis and round cell inflammatory infiltration. In the most pronounced cases, steatosis and inflammation were located in the same areas of the liver as reported by Lieber and coworkers (zone 2 and 3 according to Rappaport's classification; Figs. 1a,b). In the BN rats, however, steatosis and

inflammation were roughly equally prevalent in the pair fed controls and in the ethanol-treated animals. In the WAG rats, steatosis was clearly more pronounced in the ethanol-treated group. Steatosis was not present in the control rats used by Leo and Lieber [5].

Steatosis has been interpreted as a direct effect of ethanol on the liver [14]. However, a high fat content of the diet and the fatty acid composition may strongly influence the development and the degree of steatosis [15,16]. Our finding of steatosis in livers of control rats supports this.

Striking features in the present study were severe focal necrosis in the steatotic liver of ethanol-treated rats. These lesions were not, however, reminiscent of human alcoholic hepatitis because of their sparsity, large size, and coagulative nature and because they lacked the highly characteristic [17] fibrosis of the lesions of the human disease. Focal necrosis was only seen in the livers of ethanol-treated rats with severe extrahepatic inflammation. Therefore, chronic ethanol administration in rats might, as in humans, have an indirect effect on the liver by making the organism more sensitive to noxious agents (infections, toxins). In this way it could have an effect on the rat liver similar to (non-specific) reactive hepatitis observed in human liver in the course of infectious diseases such as pneumonia or septicaemia.

In the reports of the experiments of Lieber and coworkers, the histopathological findings of the extrahepatic organs at sacrifice were not mentioned [5]. Therefore, it is possible that the liver fibrosis in their experiments is the result of a combination of malnutrition [as argued by Rao et al.,18], of genetic factor(s) specific for the rat strain used by Lieber's group (Sprague-Dawley), and of superimposed systemic or infectious disease related to chronic ethanol consumption. This conclusion is in line with results of recent epidemiological studies [19]. In humans, overconsumption of ethanol is often associated with malnutrition and with increased susceptibility to infectious disease due to interaction of ethanol intake with the immune system [14,19].

Quantitative morphometric analysis of the livers of ethanol-fed rats of both strains revealed dilatated RER and reduced lipid droplet content of fat-storing cells, which can be considered as very early signs of transition of fat-storing cells to fibroblast-like cells [12,20,21]. In our study, however no increase in such precursor cells of fibrosis was found, and there was no increase in collagen deposition. The observed decrease in Ito-cell index might be explained by the increased liver volume due to steatosis,

rather than to an absolute decrease in the number of FSC. In ALD, another important precursor cell of liver fibrosis has been identified, called myofibroblast [4, 5, 21, 22]. These cells are a normal part of the adventitia of the central vein and are held reponsible, by proliferation and migration, for the fibrosis of the wall of the central vein (perivenular fibrosis or sclerosis) and for the pericellular parenchymal fibrosis of zone 3 [22, 23]. In our rats, however, no such alterations of myofibroblasts were found.

Our results show that long-term treatment of rats with ethanol in the vitamin A supplemented Lieber-De Carli diet does not necessarily result in liver fibrosis. Therefore, the conclusions of Leo and Lieber [5] cannot be generalized and the role of vitamin A as a major factor in the pathogenesis of alcoholic liver fibrosis should be reconsidered. Other factors, including age, the occurrence of extrahepatic lesions and immunological, nutritional and metabolic characteristics probably play a more important role.

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CHRONIC ADMINISTRATION OF ETHANOL WITH HIGH VITAMIN A SUPPLEMENTATION IN A LIQUID DIET TO RATS DOES NOT CAUSE LIVER FIBROSIS: 2. BIOCHEMICAL OBSERVATIONS

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Running title:
ALCOHOLIC LIVER DISEASE AND VITAMIN A: 2. BIOCHEMISTRY

Acknowledgements

This work was subsidized by Praeventiefonds grant 12-1206, Den Haag, The Netherlands. The authors wish to thank the excellent technical assistance of Ms. I. Bock.

Abstract

The inability of the "ethanol/high vitamin A Lieber-DeCarli diet" to induce liver

fibrosis in two different rat strains was further evaluated by determining changes in parameters of liver cell damage and of retinoid and lipid metabolism. In the ethanol/-vitamin A treated group, slight but constant hepatic cell damage, as indicated by elevated alanine aminotransferase, aspartate aminotransferase and glutamate dehydrogenase activities in blood, was already observed at 6 months and maintained until the time of sacrifice at 16 months. Serum gamma glutamyl transaminase activities were not raised. The moderate parenchymal liver cell damage was not accompanied by fibrosis. Hypertriglyceridemia or hypercholesterolemia were observed at 6-16 months of chronic alcohol administration. This response was strain dependent. In ethanol-treated rats of both strains, total liver retinoids and serum retinol concentrations were not altered. Therefore, the hypothesis that interaction between alcohol and retinoids is a major factor in the pathogenesis of alcoholic liver disease, needs to be reconsidered.

Introduction

In the accompanying paper, we have reported that long-term treatment of rats with the ethanol/high vitamin A diet does not necessarily produce fibrosis or other severe morphological alterations of the liver [1]. These results were in apparent contrast with those obtained by Leo and Lieber using the same diet in another experimental set-up [2]. In this study we report on our biochemical studies in the same rat model, which were focussed on parameters of cell necrosis and of vitamin A and lipid metabolism. These studies were conducted to further delineate the conditions and mechanisms that determine the degree of liver damage and disease that is inflicted by chronic alcohol consumption, with emphasis on the contributory role of vitamin A toxicity.

Material and methods

Experimental set-up

Animals, housing conditions, treatment schedules and methods for organ preparation at sacrifice are described in detail in the accompanying paper [1].

Retinoid analysis

The content of retinol and the three main retinyl esters, i.e. retinyl palmitate, retinyl stearate and retinyl oleate, were determined in liver, lung and serum using high performance liquid chromatography (HPLC) [3]. Serum concentration of retinol-binding protein (RBP) and liver concentrations of RBP and cellular retinol binding protein (CRBP) were analysed by specific radioimmunoassays [4,5].

Biochemical analysis of blood and serum

Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyl transaminase (GGT), triglyceride (TG) and cholesterol (CHOL) were determined in heparinized blood from the tail vein using the Reflotron system (Boehringer Mannheim, F.R.G.) after 6, 9, 12 and 16 months of liquid diet administration.

At the time of sacrifice, blood was collected from the dorsal vein. Serum glutamate dehyrogenase (GDH) activity was assayed by the method of Ellis and Goldberg [6]. Lactate dehydrogenase (LDH), triglyceride (TG), cholesterol (CHOL) and phospho-lipids (PL) were determined with commercial test combinations (Boehringer Mannheim, FRG). Serum lipoproteins were fractionated by density gradient centrifugation ($1.006<d<1.210$) according to the method of Redgrave [7]. Briefly, 0.65 g KBr was added to 2 ml serum resulting in a density of 1.21 and an additional 1.8 ml KBr/NaCl solution with the same density was added. Three 2.7 ml layers of KBr/NaCl solution with densities of 1.050, 1.019 and 1.006 respectively, were stacked on the serum fraction. After centrifugation (200,000 x g, 22 h, 18°C), fractions of 0.6 ml were collected. Of each fraction, protein [8], cholesterol, triglyceride and phospholipid (PL) content were determined. The apolipoproteins of each fraction were analysed by polyacrylamide gel electrophoresis according to the method of Laemmli [9].

Statistics

Results are expressed as the mean ± S.E.M. Statistical analysis was performed by the Student t-test for paired observations (significance level 5%, 1% and 0.1%).

Results

Morphological alterations in liver and other tissues

A detailed description of the morphology is given in a separate paper [1]. None of the rats fed the ethanol-containing diet showed liver fibrosis and the total hydroxyproline content of the livers of both strains were not significantly elevated. Fatty liver and reactive changes in liver parenchyma, with foci of congulative parenchymal necrosis and mixed cellular inflammatory infiltration, were observed and were explained as an effect of extrahepatic diseases, i.e. interstitial pneumonia and pyelitis.

Retinoid content of tissue and serum

Retinoid contents (retinol and retinyl esters) of liver, lung and serum are shown in Table 1. In the WAG rats, retinol and retinyl ester concentrations per gram liver were significantly lower in the ethanol-treated animals as compared to controls; however, due to a higher liver weight, the total retinoid liver content did not change. In the BN rats, neither the concentration nor the content of retinoids did change. The total amount of retinoids stored in the liver at the time of sacrifice represented approximately 10-16% of the total intake of retinoids administered to the diet. In both strains, the retinyl ester composition of the liver was altered by ethanol administration. An increased oleate and stearate ester content and a decreased palmitate ester content were noted. No changes were observed in the retinol and CRBP concentrations.

The retinoid concentration of the lungs of BN rats fed the ethanol-containing diet was significantly higher than of the controls (Table 1), but the retinyl ester composition was identical. In contrast, the retinoid concentration of the lungs of ethanol-fed WAG rats was not significantly different from their pair-fed controls, but the retinyl ester composition was changed. The percentage palmitate esters were higher and the percentage stearate esters were lower. Retinol was not detectable in lungs of WAG rats, but in BN rats of both groups 6-8% of the retinoids consisted of retinol.

Serum retinoid levels were not affected by the ethanol consumption (Table 1) and no change in retinoid composition was found; retinol was the main retinoid present in serum (95-100%). The ratio between the concentration of retinol and retinol-binding protein in serum was also not affected by ethanol treatment, which indicates that no alterations in the degree of protein binding of retinol had occurred.

TABLE 1

Effect of ethanol administration on body weight, liver weight and on retinoid levels and retinyl-ester composition in liver, lung and serum of the BN and WAG strain at the time of sacrifice.

	BN		WAG	
	control	ethanol	control	ethanol
Body weight (g)	434 ± 11	423 ± 19	428 ± 7	426 ± 11
Liver weight (g)	11.6 ± 0.7	14.0 ± 0.7*	8.1 ± 0.3	11.5 ± 1.5*
% (liver weight/body weight)	2.67 ± 0.09	3.51 ± 0.14**	1.90 ± 0.08	2.61 ± 0.29*
Retinoid and cellular retinol binding protein (CRBP) levels and retinyl ester composition (% of total retinoids) in LIVER				
retinol equivalent (μg/g liver)	2687 ± 430	2988 ± 250	5423 ± 290	3134 ± 312**
retinol equivalent (μg/liver)	30620 ± 6940	41902 ± 4440	44254 ± 3014	37753 ± 3731
% (liver ret. eq./consumed ret. eq.)	10.1 ± 2.3	13.8 ± 1.5	15.6 ± 1.1	13.3 ± 1.3
CRBP (g/g liver)	24.1 ± 0.5	33.2 ± 5.4	47.8 ± 7.6	33.1 ± 3.2
% retinol	1.1 ± 0.8	0.8 ± 0.3	1.1 ± 0.8	1.2 ± 0.9
% r. oleate	3.9 ± 0.6	7.9 ± 0.3***	3.6 ± 0.7	6.9 ± 0.9***
% r. palmitate	81.4 ± 10.8	74.6 ± 0.6***	81.5 ± 10.8	75.8 ± 10.6**
% r. stearate	13.6 ± 1.4	16.8 ± 0.6***	13.7 ± 1.7	16.1 ± 1.9*
Retinoid levels and retinoid ester composition (% of total retinoids) in LUNG				
retinol equivalent (μg/g lung)	13.6 ± 3.0	22.2 ± 2.2**	20.4 ± 3.8	24.2 ± 2.8
% retinol	8.9 ± 4.0	6.4 ± 3.8	0.0	0.0
% r. oleate	5.9 ± 2.4	5.4 ± 1.3	3.3 ± 1.3	5.0 ± 0.7
% r. palmitate	60.2 ± 3.3	65.6 ± 3.0	63.7 ± 1.3	66.8 ± 1.4*
% r. stearate	25.0 ± 2.6	22.6 ± 1.0	33.0 ± 0.6	26.7 ± 0.8***
Retinoid and retinol binding protein levels (RBP) in SERUM				
retinol equivalent (μg/dl serum)	24.2 ± 3.0	22.9 ± 5.3	9.9 ± 2.7	11.1 ± 1.7
RBP (mg/dl serum)	1.84 ± 0.1	1.86 ± 0.2	1.12 ± 0.1	1.25 ± 0.2
sRBP serum/retinol (mmol/mmol)	1.15 ± 0.2	1.51 ± 0.3	2.33 ± 0.6	1.59 ± 0.2

Values are given as mean ± SEM.

*) significantly different from control, $p<0.05$

**) significantly different from control, $p<0.01$

***) significantly different from control, $p<0.00$

Biochemical analysis of blood and serum

ALAT, ASAT, GGT and GDH activities were determined in serum as indicators of parenchymal liver cell damage (Table 2). ALAT activity was slightly increased in both the BN and WAG rats which were fed the ethanol-containing diet for 6 months. The increased activity was maintained until the animals were sacrificed. The ASAT activity was also slightly increased in the rats fed the ethanol-containing diet for 9 and 12 months, but this activity was not longer significantly different from that in controls at the time of sacrifice. The activity of GGT, a liver enzyme that frequently is elevated in sera of alcoholics, was at all time points below detection limit (< 5.00 IU/l, 37 °C) in all rats. The liver mitochondrial enzyme GDH was slightly elevated in serum of ethanol-fed rats of both strains. These biochemical data indicate that a small but consistent parenchymal liver cell damage was induced in the ethanol-fed rats of the two strains studied.

The influence of ethanol intake on lipid metabolism, as reflected by serum triglyceride, cholesterol and phospholipid concentrations, was strain dependent (Table 2). TG, but not CHOL and PL were increased in the BN rats fed the ethanol-containing diet for 16 months. In contrast, the serum TG concentrations did not change in the ethanol-fed WAG rats, but CHOL and PL levels were significantly increased at the time of sacrifice. Analysis at intermediate time points showed that the ethanol-induced elevations of lipids described above were already evident after 9 and 12 months of treatment (data not shown). Fractionation of the serum lipoproteins of the ethanol-fed WAG rats revealed increased cholesterol levels in fractions with a density corresponding to that of HDL (fig. 1a-c). The phospholipid and triglyceride content were not significantly altered in either the HDL, LDL or VLDL rich fractions. No alterations were observed in the fractions isolated from the ethanol-fed BN rats (data not shown). Analysis by SDS-polyacrylamide gel electrophoresis of the apolipoprotein profiles revealed no qualitative differences due to ethanol intake (data not shown) in either strain.

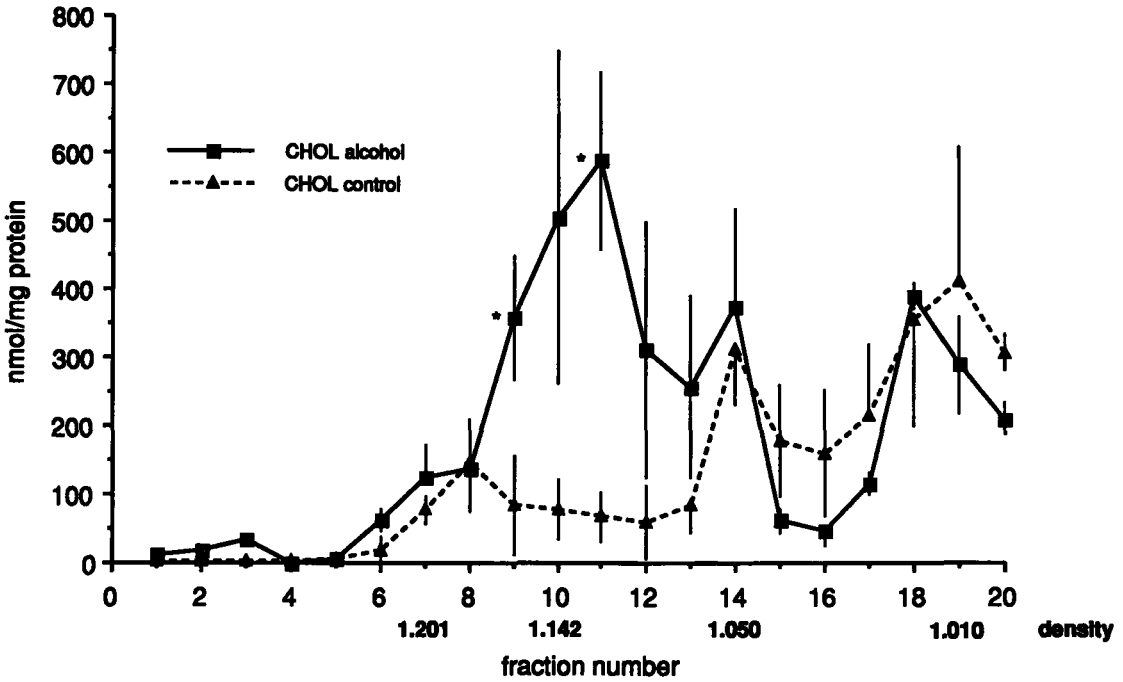
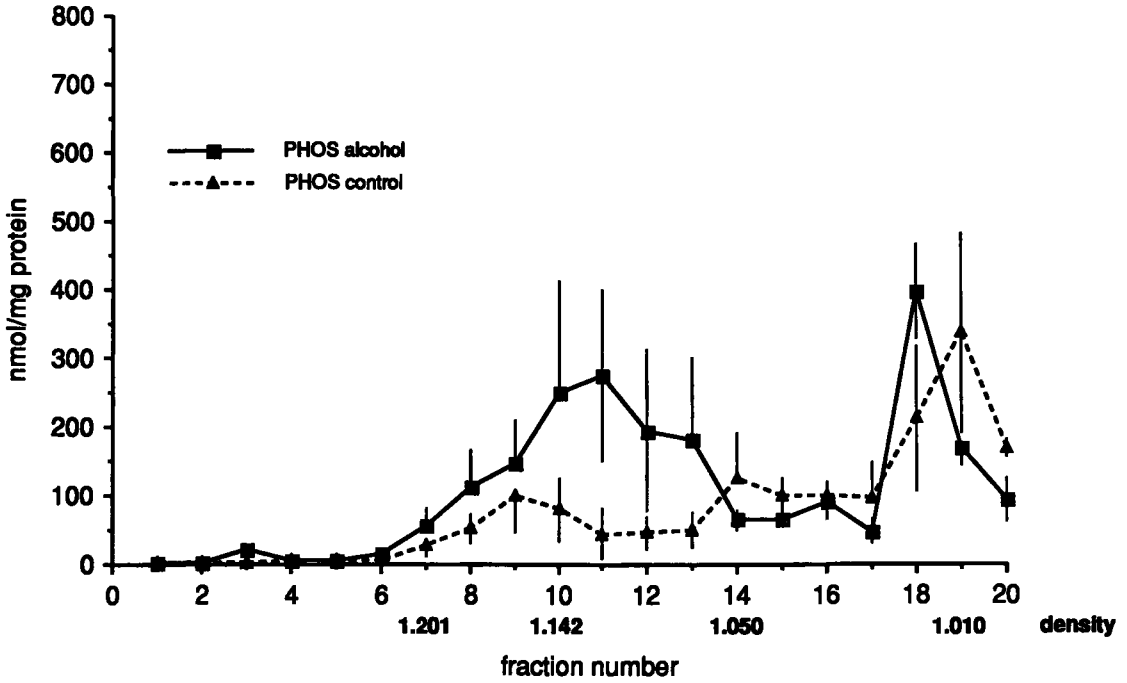
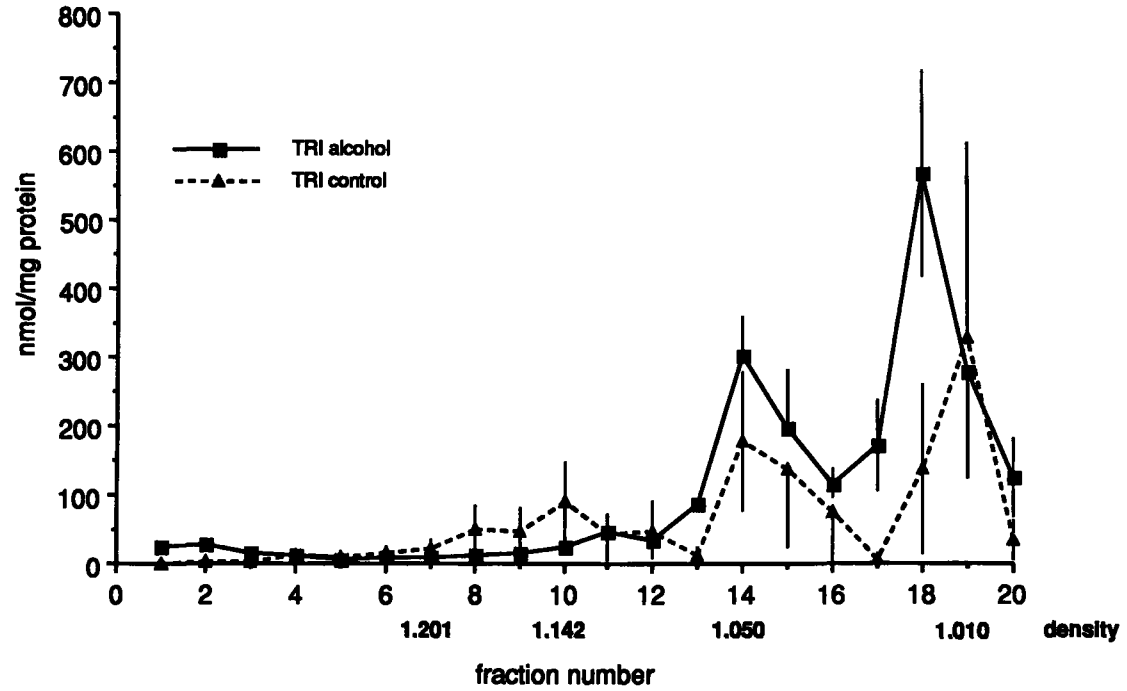


Fig. 4.1 Density fractionation of the serum lipoproteins of the control- and ethanol-fed WAG/Rij rats. Cholesterol content (CHOL) of the ethanol-fed rats as significantly increased in the fractions with a denstity of approximately 1.142, corresponding to the HDL-rich fraction of the WAG rats. The phospholipid (PHOS) and triglyceride (TRI) contents were not significantly altered. Values are given as mean ± SEM. *) significantly different from control, $p<0.05$.
a. cholesterol content



b. phospholipid content



c. triglyceride content

TABLE 2

Values of some blood[1] and serum parameters of liver damage (ALAT, ASAT, GDH and LDH) and lipid metabolism (TG, CHOL and PL) for rats of both strains fed the control or ethanol diet for 6, 9, 12 or 16 months.

	BN		WAG	
	control	ethanol	control	ethanol
ALAT (U/l)[1]				
6	29.5 ± 0.6	50.2 ± 3.7**	28.2 ± 3.1	62.4 ± 20.7**
9	39.0 ± 3.0	54.2 ± 3.7**	47.2 ± 2.8	65.4 ± 2.8**
12	33.7 ± 1.5	53.0 ± 3.9*	46.0 ± 2.9	58.3 ± 1.7*
16	34.3 ± 2.1	59.9 ± 9.1*	33.4 ± 2.1	51.7 ± 2.2*
ASAT (U/l)[1]				
6	70.4 ± 20.5	108.7 ± 35.7	61.0 ± 20.7	86.5 ± 6.4
9	88.7 ± 6.1	112.5 ± 5.7*	72.5 ± 5.6	98.2 ± 3.6**
12	70.4 ± 2.3	113.4 ± 9.0*	77.0 ± 7.0	82.3 ± 3.2
16	88.8 ± 5.4	124.8 ± 17.2	72.4 ± 2.8	81.7 ± 3.2
GDH (U/l)				
16	2.2 ± 0.4	19.5 ± 3.2***	2.3 ± 1.0	16.8 ± 6.5***
LDH (U/l)				
16	1625 ± 461	1446 ± 444	1435 ± 278	1409 ± 388
TG (mmol/l)				
16	1.55 ± 0.21	2.51 ± 0.45*	1.55 ± 0.24	1.72 ± 0.20
CHOL (mmol/l)				
16	2.24 ± 0.1	2.33 ± 0.1	2.03 ± 0.10	2.98 ± 0.23***
PL (mmol/l)				
16	1.72 ± 0.10	1.97 ± 0.10	1.54 ± 0.07	2.07 ± 0.15*

Values are given as mean ± SEM.
* : significantly different from control, $p<0.05$
** : significantly different from control, $p<0.01$
***: significantly different from control, $p<0.001$
ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; GDH = glutamate dehydrogenase; LDH = lactate dehydrogenase; TG = triglyceride; CHOL = cholesterol; PL = phospholipid.

Discussion

The morphological data reported in the accompanying paper [1], showed that the ethanol induced abnormalities in liver were much less severe in our rats as compared with rats kept on the same diet by Lieber and coworkers [2]. This biochemical study was undertaken to investigate alterations in key parameters that reflect effects of alcohol on the liver function. The results are discussed in relation to the apparent discrepancy between the two studies, to further clarify the mechanisms responsible for the pathogenesis of alcoholic liver fibrosis.

Liver cell damage

The slightly elevated ALAT, ASAT and GDH activities and the low GGT activities in blood of the ethanol-high vitamin A fed rats of both strains indicated that slight but constant parenchymal liver cell damage was present during the major part of the experimental period. This corresponds well with the histology of the liver biopsies, which showed minimal non-specific reactive inflammation and local parenchymal cell loss [1]. Leo and Lieber found that ALAT, ASAT and GDH activities of Sprague-Dawley rats were markedly elevated after 9 months treatment with the ethanol/high vitamin A diet [2]. Since parenchymal cell damage is a recognized factor in liver fibrogenesis (10,11), the relatively low degree of liver damage could partly explain the absence of fibrosis in the livers of the rat strains used in our experiments.

Retinoids and their metabolism

The liver retinoid contents of the two rat strains fed high vitamin A diet for 16 months were 3 times higher than those in rats of the same age fed ad libitum with normal lab chow [12]. The retinoid content was comparable to that of livers of Sprague Dawley rats which were administered the diet for 9 months [2]. This indicated that there are no strain related differences in the normal intestinal absorption and hepatic storage of dietary vitamin A that should be taken into account. Both short and long-term administration of ethanol can seriously affect retinoid metabolism [13-16]. The ethanol induced reduction in hepatic stores of up to 30%, which has been described by several groups [13-16], was absent or insignificant in our study. In accord with others, there was less palmitate and more oleate and stearate

among retinyl esters in liver, but the shift was relatively small.
Plasma levels of retinol and retinol binding protein RBP were not altered by chronic ethanol intake in rats of either strain. This indicates that, a) the regulation of the secretion of retinol-RBP complex by the liver was not affected, b) there is no risk of toxicity due to free retinol in plasma, and, c) availability of retinol for peripheral tissues was not affected in the ethanol-fed rats. Retinoid content in lungs was somewhat elevated in BN rats treated with ethanol and the retinyl ester composition of lung was altered in WAG rats. These changes appear to be due to a local effect of ethanol on retinoid metabolism in the lung, rather than to an increased or altered transport of retinol from liver to lung, as suggested by Lieber and coworkers [2].
From these results it is obvious that the effects of long-term ethanol feeding on retinoid metabolism in rats are dependent on the genetic background. Some ethanol induced alterations of retinoid metabolism were observed, but these were of minor significance. Fatty liver, which is the result of increased retention of dietary fatty acids in the liver [17], was mildly induced by chronic ethanol consumption in our rats. Since oleate constituted about 55% of the dietary fat, the change in the acyl pattern of hepatic retinyl esters may be due to a relative increase in oleate over palmitate as substrate for lecithine:retinol acyltransferase [18].
Our results show that high vitamin A intake per se does not necessarily form a toxic co-factor for the hepatotoxicity of ethanol. In as far as vitamin A can act as co-factor, as described by Leo and Lieber, this is probably restricted to conditions in which there is a clear disturbance of retinoid metabolism, notably a decrease in retinyl ester content of the liver. The key event may be the catabolic oxidation of retinoids into toxic metabolites by ethanol induceable microsomal oxidases, such as the microsomal ethanol-oxidising system (MEOS) [19]. In our rats, catabolism of retinoids was probably not increased by ethanol, since liver stores were not affected.
The results confirm and extend earlier observations on the dual role of vitamin A in chemically induced liver damage and fibrogenesis [20,21]. In BN rats vitamin A combined with CCl_4 displayed acute toxicity, but under the right conditions vitamin A could significantly reduce liver fibrosis [20]. Vitamin A appears to interact with fibrogenesis in various, antagonistic ways; by metabolic interaction with hepatotoxins resulting in cell damage, and by modulating inflammatory processes and tissue repair. Alterations in function, differentiation and proliferation of both Kupffer and fat-

storing cells are likely to be involved.

The influence of ethanol on serum lipids and lipoproteins

Depending on the rat strain, our study showed that long-term ethanol consumption induced hypertriglyceridemia or hypercholesterolemia. These results reflect those obtained from clinical and epidemiologic studies in alcoholic patients, in which hypertriglyceridemia and/or hypercholesterolemia were commonly observed [22-24]. The mechanism behind these effects of ethanol is not clear. Hypertriglyceridemia has been suggested to be the result of changed lipoprotein metabolism in the alcoholic liver [25,26]. Ethanol administration in baboons enhanced hepatic production of VLDL triglicerides [27]. In the ethanol-fed rats of the BN strain, hypertriglyceridemia was associated with slight liver cell injury, but there were no indications of enhanced, specific production of VLDL triglycerides in these rats as indicated by the lipoprotein fractions. No significant elevations were observed in either the VLDL, LDL or HDL fractions.

This study also shows that ethanol-induced liver cell damage in the rat does not necessarily lead to hypertriglyceridemia. Hypercholesterolemia was observed in the ethanol-fed rats of the WAG strain without a rise of triglyceride levels. Fractionation of the serum lipoproteins revealed increased cholesterol levels of the HDL fractions. In vivo [28] and in vitro [29] studies indicate that HDL promotes cholesterol efflux from cells, which has been associated with a lower incidence of coronary heart disease [30], suggesting that ethanol consumption may exert protective cardiovascular effects.

The rat shows different aspects of the interaction between ethanol and lipid metabolism, and the ultimate effect was dependent on the genetic background of the strain in use. In view of the close resemblance to a variety of effects observed in humans, the use of different rat strains may contribute to the explanation of the interaction of ethanol, lipid metabolism and genetic background.

Concluding remarks

These experiments show that long-term treatment of BN and WAG rats with ethanol in the retinoid supplemented Lieber-DeCarli diet does not result in liver abnormalities that are characteristic for human ALD. The slight inflammatory response of the liver

parenchyma was of a non-specific type and had no characteristics of alcoholic hepatitis as observed in humans [1]. The results also cast doubt on the hepatotoxic potential of retinoids in alcoholic liver disease [2]. The role of retinoids in the pathogenesis of liver fibrosis should be reconsidered. As has been observed in experiments with other fibrotic agents like CCl_4 and heterologeous serum [20,21], it is possible that high levels of retinoids in the livers of the ethanol-treated rats may suppress the induction of liver fibrosis. This may be due to influences of retinoids on the metabolism of fat-storing cells [20]. Orally administered retinoids are normally transported to and stored in fat-storing cells [12,31,32]. Several studies have shown that fat-storing cells are involved in collagen synthesis during fibrogenesis. Furthermore, the fibroblast-like cells associated with the fibrotic septa may be derived from fat-storing cells by migration, transformation and cell division [33-35]. High retinoid concentrations, stored in the fat-storing cells, may suppress the collagen synthesizing capacity of these cells and prevent their transformation to fibroblast-like cells as has been seen in cultured fat-storing cells which were loaded with retinoids before isolation [36]. In ethanol-fed rats of both strains, neither proliferation of fat-storing cells nor the appearance of fibroblast-like cells were observed, which may be due to the maintenance of high retinoid content in the fat-storing cells.

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JOURNAL OF PATHOLOGY, VOL. 156: 15–21 (1988)

CHAPTER V

SYNERGISM BETWEEN ETHANOL AND CARBON TETRACHLORIDE IN THE GENERATION OF LIVER FIBROSIS

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Received 10 November 1987
Accepted 15 February 1988

SUMMARY

In this study, alcohol-induced histological lesions in a short-term experimental rat model were compared with those characteristic of human alcoholic liver disease. In the rat model used, pretreatment with carbon tetrachloride (CCl_4) for 6 weeks was employed possibly to sensitize the liver for the effects of alcohol and shorten the time of induction of alcoholic liver disease. After 6 weeks of CCl_4 treatment, subsequent maintenance on drinking water containing up to 10 per cent alcohol for 7 weeks potentiated liver fibroplasia as compared with non-alcohol-treated rats. However, steatosis and alcoholic hepatitis, as histological evidence for alcoholic liver disease as seen in humans, were not observed. In non-CCl_4-pretreated control animals, alcohol administration had no effect on liver histology.

It can be concluded that in the model used, CCl_4 pretreatment sensitizes the liver to increase collagen deposition following alcohol administration, but not to steatosis or alcoholic hepatitis as seen in human alcoholic liver disease. In this experimental set-up, direct metabolic interaction of CCl_4 with alcohol as a cause of the increased fibroplasia can be excluded.

KEY WORDS—Ethanol, liver fibrosis, carbon tetrachloride, collagen, alcohol, liver disease, experimental rats.

INTRODUCTION

Alcoholic liver disease (ALD) is one of the most prevalent diseases of the Western world, with serious socio-economic impact on society. Much research on ALD in humans, and also in experimental animal models, has been done especially concerning hepatic fibrosis in later stages. However, important questions still remain to be resolved regarding the pathophysiological mechanisms which lead to early alcoholic fibrosis.

In animals, many studies on the effects of ethanol have been done in rats. Although in some histological studies in rats, steatosis, necrosis, and later fibrosis were produced by chronic alcohol administration,[1,2] these changes were not dependent on alcohol administration alone. Alcohol administration per se appeared not to be hepatotoxic without additional modulation of the diet. Furthermore, in none of these studies was the typical histological picture of alcoholic hepatitis, as observed in man, seen.

Whether this makes the rat model an inappropriate tool to study human ALD is, however, doubtful, since the aetiology of human ALD is also multifactorial[3] and in humans heavy chronic alcohol consumption may only lead to (end-stage) ALD in a minority of the cases.

In this paper, we report attempts to study the effect of alcohol on developing fibrosis in a short-term experimental model. The results show that, after sensitization of the liver by pretreatment with CCl_4, subsequent alcohol administration enhances liver fibrosis in all animals of the group but that no other histological evidence of human ALD, viz. steatosis and alcoholic hepatitis, is induced.

MATERIALS AND METHODS

Animals

Four groups of eight male BN/BiRij rats, 11 weeks old, weighing 260 ± 27 g (mean $\pm$ SD), were

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0022–3417/88/090015–07 $05.00

Table I—Consumption of alcohol-containing drinking water by CCl_4-treated and control rats

Period (days)	Alcohol concentration (%)	Treatment group	
		CCl_4	Controls
1–3	2·5	177±28*	115±9
4–7	5	139±23*	103±9
8–10	5	119±27*	90±5
11–14	7·5	113±19*	88±7
15–17	10	95±18	91±8
18–21	10	104±17	97±11
22–24	10	95±17	98±8
25–28	10	103±18	99±9
29–31	10	101±13	94±8
32–35	10	90±13	91±16
36–38	10	105±29	96±12
39–42	10	97±11	96±16
43–45	10	97±14	99±10

Animals were treated with CCl_4 or paraffin oil (controls) for 6 weeks prior to alcohol administration.

Values are given as ml of fluid per kg body weight per day ±SD ($n=8$).

*Value differs significantly from control value ($P<0·01$).

kept in individual cages during the entire experiment. An almost constant temperature of about 22°C, and an almost constant air humidity of about 60 per cent were maintained. The animals were kept on standard laboratory chow which was given *ad libitum*. Fluid intake was recorded daily and body weight weekly.

Treatment of animals

Induction of liver fibrosis by CCl_4 was accomplished essentially as described by Geerts *et al.*[4] Two groups of rats were injected subcutaneously twice a week with 200 µl of CCl_4 diluted in paraffin oil (50 per cent v/v) per 100 g body weight for 6 weeks. The two control groups were injected with paraffin oil only. After this treatment schedule was completed, one group of the CCl_4-treated rats and one of the control rats were given ethanol in drinking water, the other groups normal drinking water. The initial concentration of ethanol was 2·5 per cent (v/v) which was increased stepwise to 10 per cent in 2 weeks and maintained at that concentration for another 5 weeks (Table I).

Liver tissue histology

Under ether anaesthesia, the rats were subjected to total body perfusion with saline and Karnovsky fixative through the aorta. The entire medial liver lobe was excluded from fixation by ligation during preperfusion with saline. This lobe was immediately frozen in liquid nitrogen for biochemical analysis. The perfusion-fixed remainder of the liver was post-fixed in Karnovsky for light microscopy and routinely processed and embedded in paraplast. Four µm thick sections were stained with haematoxylin–phloxin–saphrane (HPS), Gordon and Sweet (for reticulin) to evaluate the liver architecture. Sirius red–F_3BA[5] and Van Gieson Elastica were used for staining of collagen. For Sirius red staining, the mounted sections were first rinsed with SUSA fixative solution[5] (20 min, 20°C) to suppress non-specific (nuclear) staining.

Hydroxyproline determination

Part of the frozen medial lobe of the liver was freeze-dried overnight. Hydrolysis was performed by adding 5 ml of 6 M HCl to 60 mg of dried tissue followed by incubation at 100°C for 16 h in screw-capped glass tubes. After cooling, the hydrolysate was filtered through a 22 µm Millipore filter. Each tube and filter were rinsed with 5 ml of 6 M HCl which was added to the first filtrate. The hydrolysates were stored at −20°C.

Hydroxyproline determination was performed using the method of Woessner[6] modificd according

to Stegemann and Stalder[7] and Meistovich *et al.*[8] Briefly, a 100 μl sample of the hydrolysate was added to a mixture of 100 μl of 6 M NaOH and 1·8 ml of 0·3 M NaCl. One ml of 0·05 M chloramine T in citrate–acetate buffer (pH 6·0) in the presence of n-propanol (1·32 M) was added to this mixture. The solution was mixed and incubated for 20 min at room temperature. The incubation was stopped by adding 1 ml of perchloric acid/4-dimethylaminobenzoaldehyde solution in n-propanol. The colour was developed by incubation for 15 min at 60°C. After cooling, the absorption was determined at 550 nm with an Ultraspec 4050 spectrophotometer (LKB, Cambridge).

Hydroxyproline content was calculated from calibration curves, using purified hydroxyproline as the standard.

Morphometry

The degree of dissection in fibrotic livers was analysed by computer-aided morphometry using a MOP-Videoplan, a microcomputer system with standard Kontron software, a digitizer tablet, and a microscope with a drawing tube. Surface areas of liver parenchyma within fibrous septa were measured in Sirius red–F_3BA-stained sections. Measurements were taken directly from microscopic images using a red light-emitting diode fitted to the digitizer cursor. The image of the diode was superimposed on the field of view through the drawing tube and measurements were made by tracing the diode spot along fibrous septa, encircling each area of parenchymatous tissue. For each specimen, 30–40 areas were measured.

RESULTS

Alcohol and water intake

The water intake of untreated rats was 100–130 ml per kg body weight per day. During the 2-week period of adaptation to alcohol, the CCl_4-treated rats consumed somewhat more alcohol than the untreated rats. For both groups, the alcohol consumption was relatively stable at 7·5–8·5 g of pure alcohol per kg body weight per day from day 15 onwards, when the animals were given 10 per cent alcohol (Table I).

Liver histology

The liver sections of all control rats treated with paraffin oil showed normal liver histology; occasionally, a small focus of predominantly round cell inflammatory infiltrate was found in the parenchyma, as can be found in untreated animals of this strain (not shown). Normal liver morphology was also seen in all control rats post-treated with alcohol only (Figs 1a and 1b).

Six weeks after the start of the CCl_4 treatment, the liver showed diffuse swelling necrosis of the parenchyma, with ballooning of liver cells and occasional acidophilic bodies, and slight inflammatory infiltrate mainly composed of mononuclear cells. There was dissecting fibrosis of the lobules of the slender septa staining positively with Sirius red (Figs 2a and 2b).

The livers of all rats treated with CCl_4 followed by administration of normal drinking water showed extensive dissecting fibrosis (Fig. 3), but less than immediately after 6 weeks CCl_4 treatment. Collagen bridges connected central and portal areas of the lobule but there was no evidence of cirrhosis. Pericellular fibrosis and necrosis of the parenchyma outside fibrous septa were not observed. Steatosis was absent.

In the rats treated with CCl_4 followed by ethanol administration (Fig. 4), dissecting fibrosis was more pronounced than that in the rats treated with CCl_4 only and was also comparable with rat liver directly after 6 weeks CCl_4 treatment; the liver parenchyma was subdivided into smaller fields, but nodule formation by regeneration or cirrhosis was not observed. Steatosis and necrosis were absent, as were inflammation or foci of alcoholic hepatitis and pericellular fibrosis outside the fibrous septa.

Morphometrical and biochemical evaluation of liver fibrosis

The degree of liver fibrosis was evaluated by determination of the hydroxyproline content and the degree of dissection by morphometrical analysis. Treatment of control rats with ethanol did not alter the liver hydroxyproline content (Table II). Rats treated with CCl_4 had a significantly higher hydroxyproline content than the control rats treated with paraffin oil only. In CCl_4-treated rats, the post-treatment with ethanol caused a substantial additional increase in liver hydroxyproline content.

The morphometric data in Table III show that the surface area of liver parenchyma within fibrous septa is much smaller in CCl_4 rats post-treated with ethanol, compared with rats post-treated with water. The liver parenchyma was subdivided into significantly smaller fields by the fibrous septa.

DISCUSSION

The aim of this study was to determine whether histological liver lesions that are considered to be

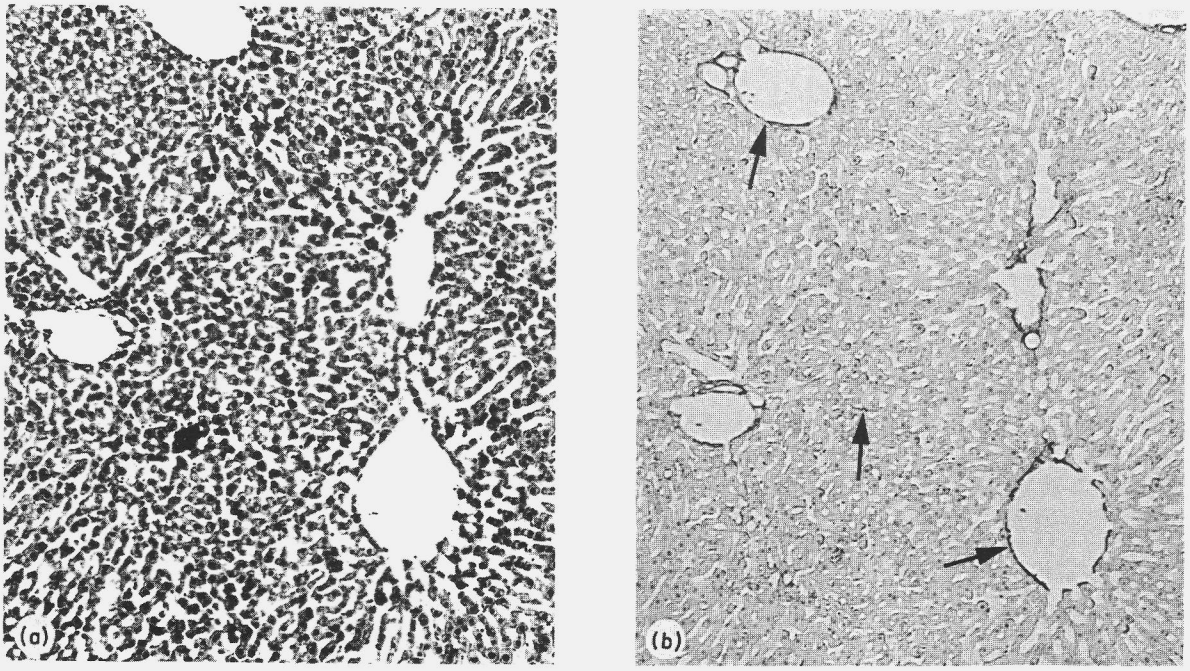

Fig. 1—Rat liver after 6 weeks treatment with paraffin oil followed by 7 weeks alcohol-containing drinking water shows normal histology. No necrosis, steatosis, or increased collagen deposition is seen, with a normal pattern of collagen distribution. Staining with Sirius red (b, arrows) is present in the portal tracts, in the wall of central veins, and, very sparsely, as tiny fibres along the sinusoids of the lobules. (a) HPS; (b) Sirius red

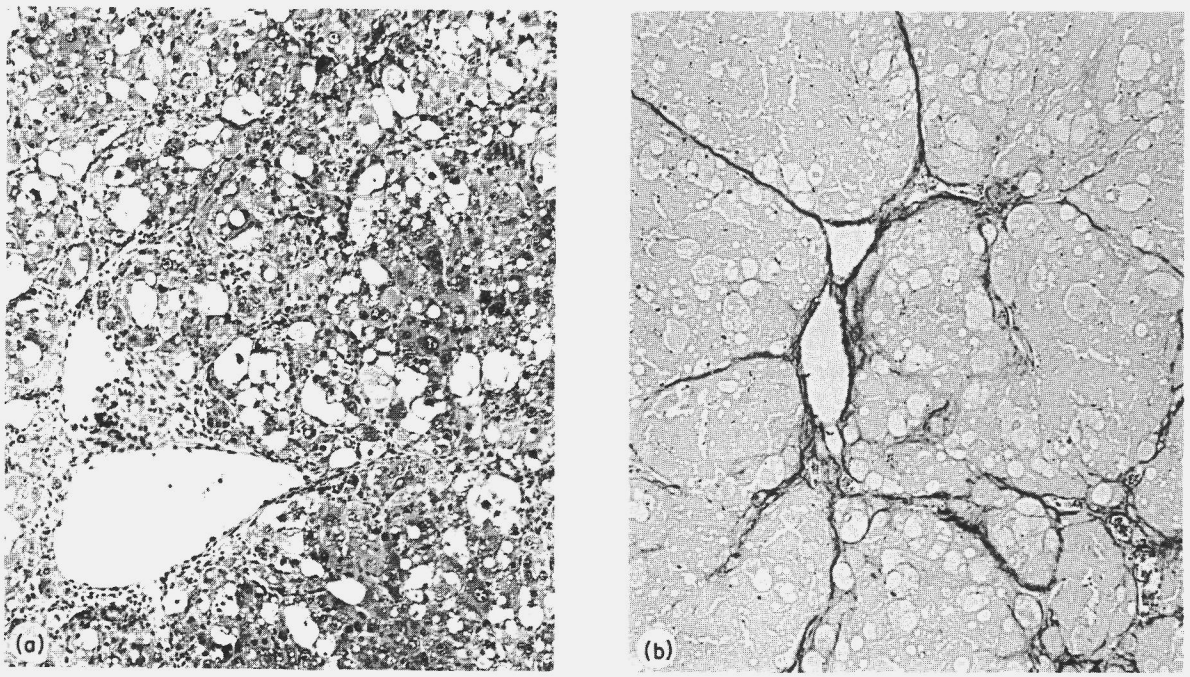

Fig. 2—Rat liver after 6 weeks treatment with CCl_4. Severe parenchymal damage with cell swelling, necrosis with occasional acidophilic bodies, and (slight) inflammatory infiltrate, mainly mononuclear cells. Dissecting fibrosis is extensive (b). (a) HPS; (b) Sirius red

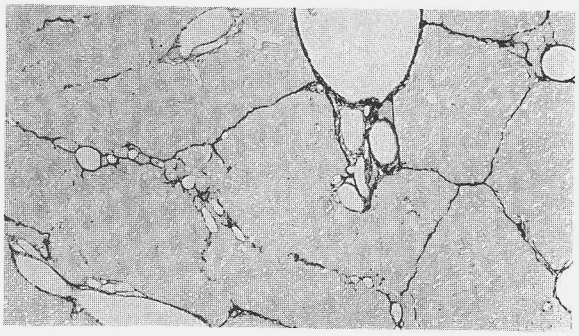

Fig. 3—Rat liver after 6 weeks treatment with CCl_4 followed by 7 weeks normal drinking water without alcohol. Diffuse dissecting fibrosis with slender septa is present but less extensive than that in Fig. 2. Sirius red

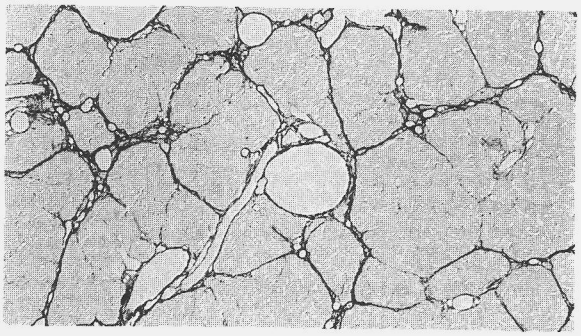

Fig. 4—Rat liver after 6 weeks treatment with CCl_4 followed by 7 weeks alcohol-containing drinking water. Fibrosis is more extensive than that in Fig. 3 but also more extensive than that in Fig. 2, with subdivision of the parenchyma by thicker fibrous septa into smaller fields. However, no regeneration nodules are seen; steatosis and inflammation, as seen in human alcoholic liver disease, are absent. Sirius red

Table II—Hydroxyproline content of rat liver after sequential treatment with CCl_4 and ethanol

Treatment	Hydroxyproline content (μg/g wet weight)
Paraffin oil; water	315 ± 64 (7)
Paraffin oil; ethanol	294 ± 64 (7)
CCl_4; water	535 ± 204 (8)*
CCl_4; ethanol	721 ± 164 (8)*,†

Values given are the mean ± SD (n).

Rats were treated with paraffin oil or CCl_4 twice weekly for 6 weeks, followed by maintenance on normal drinking water or water containing ethanol for 7 weeks.

*Differs significantly ($P < 0{\cdot}005$, Mann–Whitney U-test) from both groups receiving paraffin oil.

†Differs significantly ($P < 0{\cdot}05$, Mann–Whitney U-test) from the CCl_4–water group.

Table III—Morphometric analysis of the degree of dissection in fibrotic rat liver after CCl_4 treatment with or without sequential treatment with ethanol

Treatment	Surface area of liver parenchyma within fibrous septa (arbitrary units)
CCl_4; water	21·0 ± 8·5 (5)
CCl_4; ethanol	7·7 ± 3·8 (6)*

Values given are mean ± SD (n).

*Differs significantly ($P < 0{\cdot}001$, Mann–Whitney U-test) from the CCl_4–water group.

characteristic of human alcoholic liver disease could be elicited in a rat model by alcohol administration.

In rats, several histological effects of ethanol on the liver have been described. The nature and severity of these effects vary with the route of alcohol administration and the composition of the diet, especially regarding fat content[2] and vitamin A supplementation to the diet.[1] The histopathological effects of alcohol in rats are comparable to some extent with the effects observed in human alcoholic liver disease. In rats, these effects may include steatosis with or without pericellular fibrosis, swelling and necrosis of hepatocytes with predominantly mononuclear cell infiltrate, and perivenular and dissecting fibrosis. However, the histological picture of human alcoholic hepatitis[9,10] composed of damage of parenchymal cells with Mallory body formation and inflammatory response with mainly polymorphonuclear cells, and pericellular fibrosis, has not been described in a rat model. This highly characteristic histological picture of alcoholic hepatitis in human liver is still generally considered to be the hallmark of progression to cirrhosis.[11] However, from experimental studies in primates[12,13] and more recently also from studies in man,[14–16] it has been suggested that this characteristic histological picture of alcoholic hepatitis is not the obligatory precursor lesion of progressive alcoholic liver disease leading to cirrhosis. The possibility remains that alcoholic hepatitis with polymorphonuclear cellular infiltrate in man is only a secondary

phenomenon, possibly mediated by leucocytotactic factor(s), which would make it invaluable as a histological prognosticator of progressive ALD.

CCl_4 is known as a potent hepatotoxic agent and as an inducer of hepatic fibrosis.[17] As early as 2 weeks after starting CCl_4 treatment, collagen deposition can be observed on light microscopy. In order possibly to sensitize the liver for alcohol and shorten the time span of induction of ALD, we pretreated rats with CCl_4 which resulted in dissecting liver fibrosis but without transition to cirrhosis. When after CCl_4 treatment rats were given ethanol-containing drinking water, the dissecting liver fibrosis was more severe in all animals as compared with the fibrosis in those that received CCl_4 treatment only; this was also reflected by the difference in hydroxyproline content of the liver in both groups. In rats post-treated with alcohol, liver fibrosis was more extensive than directly after CCl_4 treatment. In contrast, post-treatment with normal drinking water resulted in partial recovery. These results indicate that administration of ethanol to CCl_4-treated rats results not only in inhibition of the recovery of the liver but also in a further progression of collagen deposition in fibrous septa. As only histological evidence of ALD (increased) fibrosis was seen; cirrhosis was not observed within the 7-week period of alcohol administration.

Other studies on the combined effects of ethanol and CCl_4 have shown that during simultaneous treatment of rats with these agents ethanol augments the hepatotoxic action of CCl_4 as reflected by serum transaminases[18,19] and liver hydroxyproline content.[18] Since, in these studies, ethanol was given from the start of CCl_4 treatment, a direct effect of ethanol on the metabolism of CCl_4 was considered to be the most likely cause for the enhanced toxicity.[20,21] In our study, ethanol was shown also to be effective when given after CCl_4, which excludes metabolic interaction as a major determinant.

It can be concluded that CCl_4 pretreatment sensitizes the liver of rats to increased collagen deposition by alcohol administration but not to steatosis or alcoholic hepatitis as seen in human ALD.

This study shows that in a rat model alcohol consumption can be an important determinant of the course of liver fibrosis even in a short-term experiment. This experimental approach appears to be particularly useful for studying the effects of alcohol consumption on liver fibrosis induced by other agents, such as methotrexate and other hepatotoxins.

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Cells of the Hepatic Sinusoid, Vol. 2, Editors: E. Wisse, D.L. Knook, K. Decker

CHAPTER VI

DUAL ROLE OF VITAMIN A IN EXPERIMENTALLY INDUCED LIVER FIBROSIS

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ABSTRACT

The effects of vitamin A on carbon tetrachloride (CCl_4) induced liver damage and fibrosis was examined. Vitamin A administered prior to, or during CCl_4 treatment aggrevated the hepatotoxicity of CCl_4, and 6/6 and 4/9 animals respectively died during the CCl_4 treatment. None of the rats died when treated with CCl_4 only. Steatosis and necrosis with inflammation of the liver parenchyma were increased as compared to rats treated with CCl_4 alone. In the rats which were pretreated with vitamin A, liver fibrosis was not observed at the time of death, whereas liver fibrosis was induced in rats which were simultaneously treated with vitamin A and CCl_4. The degree of fibrosis in the livers of the last group was at least as high as in rats treated with CCl_4 only. On the other hand, vitamin A posttreatment (6 weeks) was not hepatotoxic in rats with CCl_4 induced liver fibrosis. Neither steatosis nor necrosis of the liver parenchyma was observed. Furthermore, vitamin A posttreatment strongly reduced hydroxyproline content of the fibrotic livers compared to treatment with CCl_4 only. Light microscopy showed that the septa of mature collagen became thin and the fibroblast-like cells in contact with the reduced septa, were extremely filled with fat droplets containing vitamin A. Electron microscopically, these fibroblast-like cells strongly resembled fat-storing cells. These results further stress the hepatotoxic potential of vitamin A in experimental liver disease and the dual role of vitamin A in liver fibrosis particularly; depending on the time of administration, vitamin A potentiates parenchymal cell damage and reduce liver fibrosis.

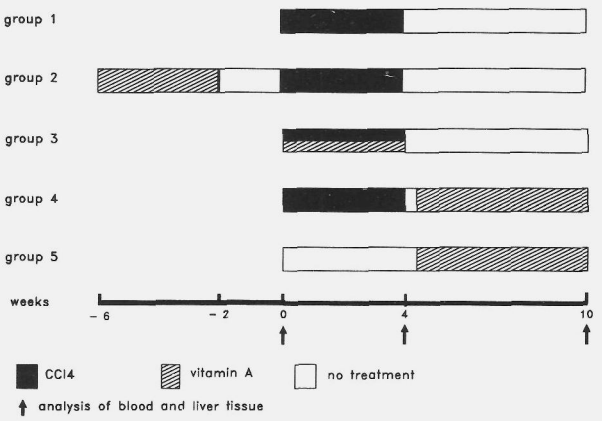
INTRODUCTION

Conflicting results have been reported on the effects of vitamin A administration on liver fibrogenesis and the effects of hepatotoxins in rats. Studies showed that vitamin A may act synergistically with ethanol in the generation of liver injury and fibrosis (10), while it was also reported to suppress induction of fibrosis by CCl_4 or heterologous serum (13). In this study, vitamin A was administered prior to, during or after CCl_4 treatment. Effects of vitamin A on the induction of liver fibrosis and on established fibrosis were examined.

MATERIAL AND METHODS

Experimental set up. Female rats of the BN/BiRij strain, aged 3 months, weighing 160 ± 11 g (mean ± SEM, n=33), were obtained from the institutes colony (Rijswijk, The Netherlands). Rats were maintained under "clean conventional" conditions and were fed a standard laboratory chow ad libitum. Animals were divided into five groups (fig. 1). Rats of group 1 to 4 received s.c. injections of CCl_4 twice weekly for 4 weeks

EXPERIMENTAL SET UP



(200 µl diluted in vegetable oil (1:1 v/v) per 100 g body weight). Rats of group 2 were pretreated with 12.500 IU retinyl palmitate per 100 g body weight in vegetable oil orally twice a week for 4 weeks. The third group was given 12.500 IU retinyl palmitate in vegetable oil per 100 g body weight twice weekly for 4 weeks at the same time the CCl_4 injections were given. The fourth group was posttreated with 50.000 IU of retinyl palmitate in vegetable oil 5 times a week for 6 weeks, started a half week after the last injection of CCl_4. The fifth group was not treated with CCl_4 but received 50.000 IU retinyl palmitate in vegetable oil 5 times a week for 6 weeks.

Surgical liver biopsies. Surgical liver biopsies were taken under halothane aneasthesia at 1 week before CCl_4 treatment and at the end of the CCl_4 treatment (n=4; five groups). Wedge shaped sections of 40 to 50 mg of the median lobe were removed with an iris scissor (Stöpler, The Netherlands). A part of each liver biopsy was fixed in 4% buffered formalin for light microscopy. The remainder was stored at -70°C until analysis for vitamin A and hydroxyproline. The bleeding was staunched with dental foam (Willospon, Will Pharma B.V., Zwanenburg, The Netherlands) and the peritoneum and the abdominal wall were stitched with absorbable surgical suture (4-0 Dexon plus, Davis + Geck, U.S.A.). The skin was cramped with wound clips (Clay Adams, U.S.A.) which were removed one week after surgery.

Liver preparation. Before sacrifice, the animals were fasted overnight. The liver was perfused with PBS through the portal vein for 5 min at 5 ml/min. The median lobe was then ligated, excised, weighed and stored at -70°C until biochemical analysis. The left lateral lobe was temporarely excluded from perfusion by a lose ligation. The right lateral and the caudate liver lobe were then fixed by perfusion with 2.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4, 5ml/min for 8 min) for electron microscopy. The ligation of the left lateral

lobe was removed and this lobe was then perfusion fixed with 4% buffered formalin for light microscopy.

Hydroxyproline determination. Liver collagen content was measured by hydroxyproline determination. 30 to 40 mg of liver biopsy or 300 to 500 mg of the frozen median lobe of the liver were lyophilized overnight. The lyophilized liver was powdered and subsequently extracted 5 times with 2 ml or 20 ml diisopropylether, respectively, to remove vitamin A, which suppresses the colour development in the assay. Traces of diisopropylether were evaporated at 37°C. Subsequently, the lyophilized tissue was hydrolyzed by 5 ml 6 N HCL per 300 mg wet weight for 16 h at 120°C. The hydrolysate was filtered through a 0.22 µm Millipore filter. The hydrolysates were stored at -20°C. Hydroxyproline determination of the hydrolysates was performed as described earlier (1). Results were expressed as µg hydroxyproline/ g liver tissue or µg hydroxyproline/ total liver.

Light and electron microscopy. For light microscopy, 4µm sections were cut from paraplast embedded tissue. Sections were stained with haematoxylin-phloxin-saphrane (HPS) and Sirius-red F3BA for collagen (8). For electron microscopy, representative samples were taken from the right lateral and the caudate lobe which were postfixed in 1% osmium tetraoxide, dehydrated in graded concentrations of ethanol and embedded in epon LX 112 (Ladd Research Industry Inc., Burlington, VT). Ultrathin fibratome sections were mounted on uncoated copper grids, contrasted with uranyl acetate and lead citrate, and examined under an EM 410 electron microscope (Philips, Eindhoven, The Netherlands).

Biochemical analysis of blood. Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyl transaminase (GGT) were measured in heparin-blood by the Reflotron system (Boeringer Mannheim, F.R.G.) both at the time a liver biopsy was taken and at the time of sacrifice.

RESULTS

No animals died after CCl_4 treatment alone (group 1). However, when CCl_4 was preceeded by or given simultaneously with vitamin A, 6/6 and 4/9 rats respectively died in the third week of CCl_4 treatment(groups 2 and 3). None of the rats died when vitamin A was administered after CCl_4 treatment (group 4).

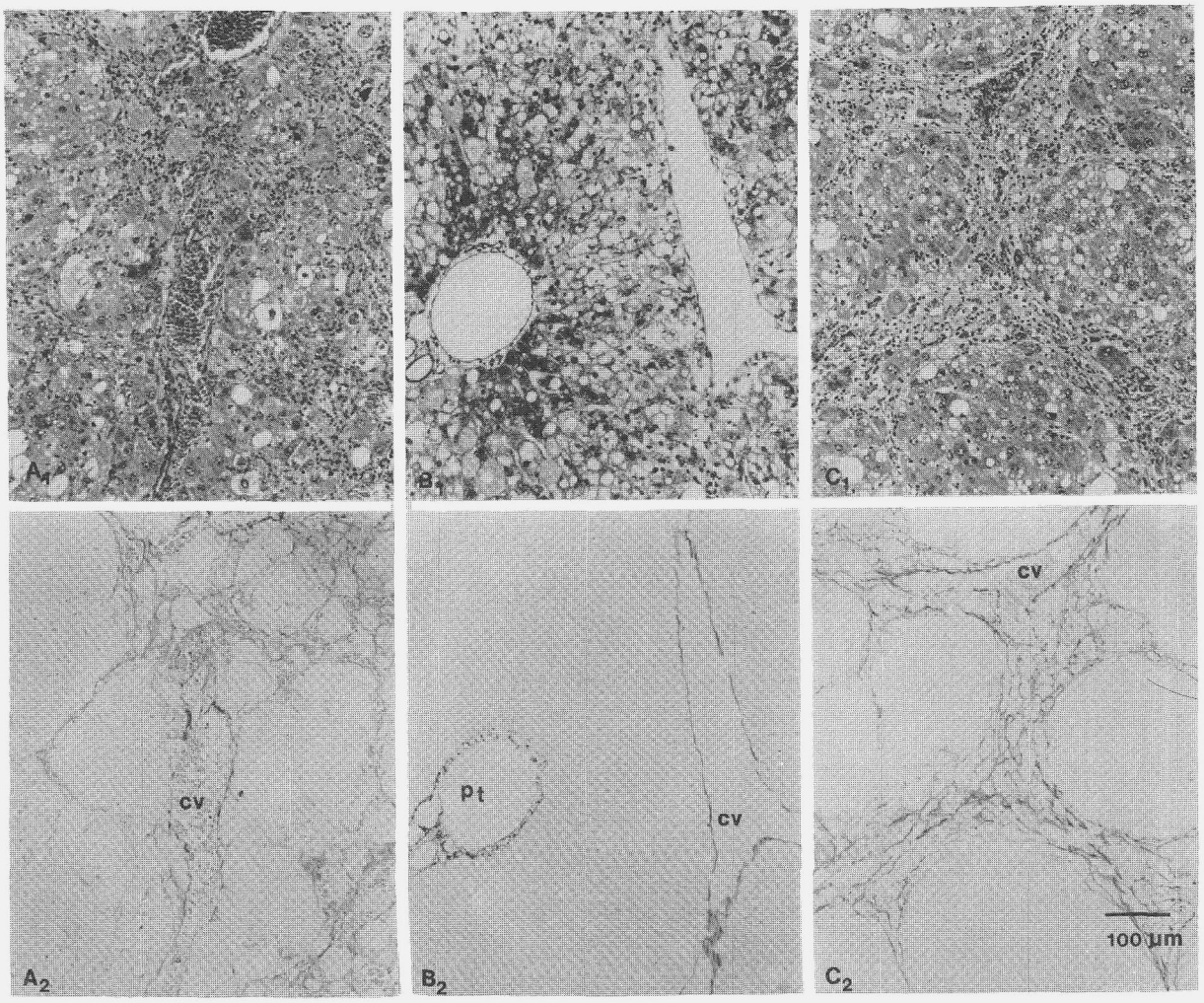


Figure 2: Rat liver after 3 weeks of CCl_4 treatment. (A1,2) Parenchymal cell swelling, necrosis and inflammatory infiltrate is seen. Diffuse dissecting fibrosis is present. (B1,2) After vitamin A pretreatment, lesions in the liver parenchyma are more severe than after CCl_4 treatment only, but no fibrosis is seen. (C1,2) Vitamin A simultaneously administered with CCl_4 treatment also enhance CCl_4 toxicity in the liver parenchyma. In contrast to vitamin A pretreatment, extensive fibrosis with septa is present. (1) HPS; (2) Sirius red; (pt) portal tract; (cv) central vein.

Histological evaluation of the rat livers of groups 2 and 3 showed severe steatosis and parenchymal cell necrosis in both portal and central areas and also a severe infiltration of polymorphonuclear and round nuclear blood cells (figs. 2B and C). The lesions were more severe than those observed in livers of rats treated with CCl_4 only (fig 2A), in which parenchymal cell necrosis mainly occurred close to the central veins. ALAT, ASAT and GGT activities in blood of rats of groups 2 and 3 were more elevated than in those of group 1 (table 1). Acute liver failure is most probably the cause of death of the animals of groups 2 and 3.

Liver fibrosis was not observed in animals of group 2 (fig 2B). Also, liver hydroxyproline content remained at the level of untreated control rats (table 2). In group 3, both the animals which died spontaneously and those which survived had developed dissecting fibrosis with connective tissue septa staining for collagen (fig. 2C). At the time of sacrifice, the hydroxyproline content of these livers was comparable to the livers of the CCl_4 treated animals (group 1; table 2). In livers of animals of both group 1 and 3, connective tissue septa connected central with portal areas of the liver lobule (figs 2A and C). Only septa formation and no pericellular parenchymal fibrosis was observed.

The rats which were posttreated with vitamin A (group 4), received a much higher dose than animals of groups 2 and 3 (total dose of 1.5×10^6 and 1.6×10^5 IU, respectively). In spite of the high dosis, no signs of toxicity were observed. Neither steatosis nor parenchymal cell necrosis was observed at the time of sacrifice (fig 3B). Blood ALAT, ASAT and GGT activities were normal. Histological examination showed only thin connective tissue septa (fig 3B). The hydroxyproline content was significantly lower than in the livers of rats treated with CCl_4 only (table 2). The FSC in the liver parenchyma as well as the fibroblast-like cells present in the septa were filled with lipid droplets. Cisternae of the rough endoplasmic reticulum were not swollen in either cell type, suggesting that the synthesis of secretory proteins, such as collagens, was relatively low. The morphology of the fibroblast-like cells resembled that of FSC (fig 4).

ASAT, ALAT AND GGT (IU/L) ACTIVITIES IN BLOOD

GROUP	1	2	3	4	5
BEFORE CCl4					
ASAT	33 ± 1	37 ± 1	43 ± 4	44 ± 2	30 ± 3
ALAT	78 ± 5	97 ± 4	86 ± 5	94 ± 6	76 ± 4
GGT	< 5	< 5	< 5	< 5	< 5
	n=6	n=6	n=6	n=6	n=6
AFTER 3 WEEKS CCl4					
ASAT	409 ± 55	1565 ± 177*	440 ± 58	389 ± 45	-
ALAT	1785 ± 254	4600 ± 165*	2035 ± 184	1623 ± 174	-
GGT	23 ± 1	29 ± 7	34 ± 2	21 ± 1	-
	n=6	n=3	n=5	n=6	
SACRIFICE					
ASAT	49 ± 3	x	48 ± 3	37 ± 2	42 ± 3
ALAT	94 ± 3	x	98 ± 5	74 ± 3	93 ± 4
GGT	< 5	x	< 5	< 5	< 5
	n=6		n=5	n=6	n=6

*: significantly higher than after CCl4 treatment only ($p < 0.05$)
-: no CCl4 treatment
x: rats died spontaneously

HYDROXYPROLINE CONTENT IN LIVER

GROUP	1	2	3	4	5
BEFORE CCl4					
HP µg/g liver	117 ± 20	89 ± 24	149 ± 40	127 ± 24	98 ± 14
	n=4	n=4	n=4	n=4	n=4
AFTER 3 WEEKS CCl4					
HP µg/g liver	413 ± 122	116 ± 60*	485 ± 98	418 ± 157	-
	n=4	n=3	n=4	n=4	
SACRIFICE					
HP µg/g liver	414 ± 138	x	339 ± 72	243 ± 73&	132 ± 37
HP µg/ liver	2943 ± 1128	x	2343 ± 578	1968 ± 610&	1051 ± 243
	n=6		n=5	n=6	n=6

*: significantly lower than three weeks CCl4 only ($p < 0.05$)
-: no CCl4 treatment
x: rats died spontaneously
&: significantly lower than after CCl4 treatment only ($p < 0.05$)

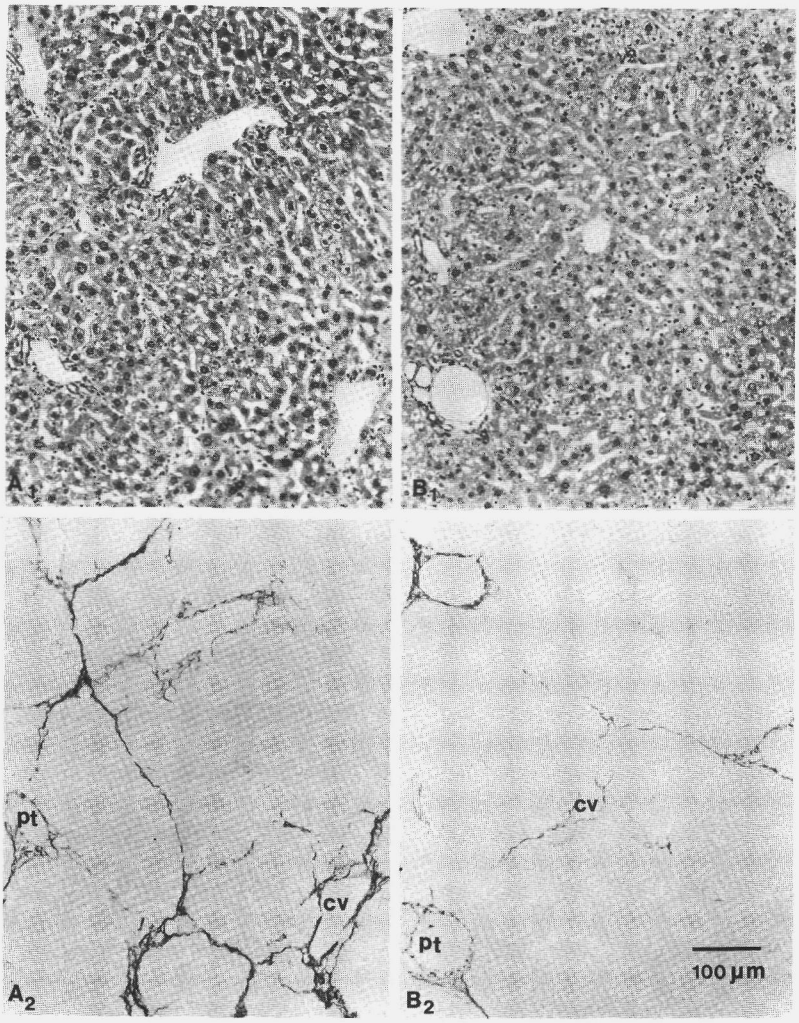


Figure 3: Rat liver after 4 weeks of CCl_4 treatment followed by 6 weeks of no treatment (A1,2) or followed by 6 weeks of vitamin A administration (B1,2). In both livers, neither steatosis nor necrosis of the parenchyma is present. Regression of the fibrotic septa is seen after vitamin A posttreatment (B2). (1) HPS; (2) Sirius red; (pt) portal tract; (cv) central vein.

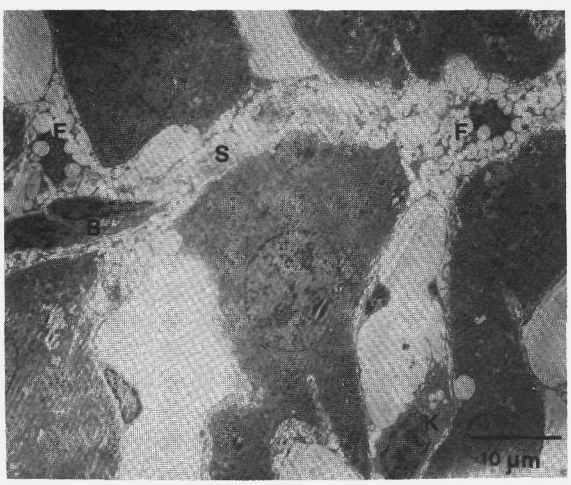


Figure 4: Electron micrograph of a liver after 4 weeks CCl_4 treatment followed by 6 weeks of vitamin A administration, showing a connective tissue septum (S). Fibroblast-like cells (F), which resemble fat-storing cells, are in contact with the septum. In the CCl_4 treated groups bile duct cell (B) proliferation is sometimes observed. (K) Kupffer cell

DISCUSSION

In these experiments, we observed a dual effect of vitamin A on the hepatoxicity of CCl_4 which was related to the time of administration: 1) Vitamin A enhanced the hepatoxicity of CCl_4 when administered prior to or during CCl_4 treatment. Significant mortality was found in contrast to CCl_4 treatment alone. 2) vitamin A was not hepatotoxic when administered after the CCl_4 treatment. Furthermore, administration of high doses resulted in an enhanced removal of CCl_4 induced fibrotic septa. These results are partly in contrast with those reported by Senoo (13). Although suppressional effects of vitamin A on the induction of liver fibrosis were observed, however, toxicity due to vitamin A administration in combination with CCl_4 was not mentioned. No mortality was reported. The apparent discrepancy between our results and those of Senoo may be the result of minor differences in treatment schedules or strain differences.

The mechanism of enhanced toxicity of vitamin A in combination with CCl_4 is not known. CCl_4 is hepatotoxic through the formation of free radicals by cytochrome P-450 in the liver endoplasmic reticulum (5,15). It has been shown that chronic excess of vitamin A also can be hepatotoxic (4,9,12). Vitamin A administration may produce moderate changes in parenchymal cells, such as endoplasmic reticulum proliferation and enlargement of mitochondria (10). It was proposed that vitamin A is metabolized by liver microsomes, especially when cytochrome p-450 activity is stimulated by hepatotoxic agents such as ethanol (11).The produced vitamin A metabolites might play a role in the increased hepatotoxicity of CCl_4.

Other studies showed that high doses of vitamin A stimulate Kupffer cell function in vivo (2,7). Hypervitaminosis A enhanced the plasma clearance of colloids and led to changes in Kupffer cell morphology suggestive for Kupffer cell activation (2). Plasma clearance of endotoxin was more rapid than in normal rats, but small doses of endotoxin which caused only minimal parenchymal cell injury in normal rats, produced significantly more parenchymal cell necrosis in hypervitaminosis A rats. It was demonstrated that activated Kupffer cells produce mediators which induce cell damage (7). The mechanism of both enhanced toxicity as well as a decreased liver fibrosis might be related to Kupffer cell activation. Enhanced toxicity of vitamin A in the liver during CCl_4 treatment may activate Kupffer cells resulting in an enhanced mediator production leading to an increased cell damage. An enhanced production of acute phase reactants by Kupffer cells as a consequence of activation both by vitamin A and by the uptake of necrotic material in the damaged liver, may lead to a more severe damage of the liver parenchyma. However, in the absence of CCl_4, Kupffer cell activation by excess vitamin A may have a benificial effect by stimulating collagen breakdown in fibrotic septa.

The suppressional effect of vitamin A on the induction of liver fibrosis by CCl_4 (group 2) and the effect on the reduction of liver fibrosis (group 4), may be due to influences of vitamin A on the metabolism of FSC in normal liver and of both FSC and fibroblast-like cells in the fibrotic liver. In normal liver, orally administered vitamin A is transported to and stored in the FSC (6). Our study shows that, in fibrotic livers, newly administered vitamin A can also be stored in fibroblast-like cells which provides new evidence for the idea that these cells are derived from FSC. Stored in these cells, vitamin A may reduce their collagen synthesis, as was seen in cultured FSC which were loaded with vitamin A before isolation (14). Suppression of the induction of liver fibrosis did not occur when vitamin A was administered simultaneously with CCl_4 (group 3). This suggests that CCl_4 may prevent the simultaneously added vitamin A to accumulate in the FSC and fibroblast-like cells.

Several studies have shown that the fibroblast-like cells associated with fibrotic septa may be derived from FSC by migration, transformation and cell division (3,16). Our study shows that these fibroblastic cells have, indeed, the capacity to store large amounts of vitamin A. Furthermore, the storage of vitamin A alters their ultrastructural morphology to make it very similar to that of FSC. These findings further support the involvement of FSC in the formation of fibrotic septa.

In conclusion, a high level of vitamin A in FSC and fibroblastic cells in fibrotic septa probably suppresses the potential of these cells to contribute to fibrogenesis of the liver. However, vitamin A may potentiate the hepatotoxicity of agents such as CCl_4 through metabolic interactions in parenchymal cells or by affecting the reactions of Kupffer cells.

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Histopathology 1989, **14**, 81–90

CHAPTER VII

Massive pericellular collagen deposition in the liver of a young female with severe Crohn's disease

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Accepted for publication 13 May 1988

BOSMA A., MEUWISSEN S.G.M., STRICKER B.H.CH. & BROUWER A. (1989) *Histopathology* **14**, 81–90

Massive pericellular collagen deposition in the liver of a young female with severe Crohn's disease

We report a patient with Crohn's disease in whom repeated liver biopsies taken at surgery for the bowel disease showed progressive hepatic fibrosis of an unusual type. Dense collagen was deposited in the perisinusoidal space often surrounding individual hepatocytes. The aetiology and pathogenesis of this abnormality remain speculative.

Keywords: Crohn's disease, liver fibrosis, alcohol, medication

Introduction

Chronic idiopathic inflammatory bowel disease—ulcerative colitis and Crohn's disease—has been associated with several histological types of liver disease. We present a case of Crohn's disease in whom diffuse pericellular fibrosis without accompanying necrosis and inflammation was present in an otherwise normal liver.

Case report

In a female negro patient, born in 1954 in Surinam and living in The Netherlands since 1971, the diagnosis of Crohn's disease of the colon was established in 1976. She

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was treated with salazosulphapyridine 3 g daily. In 1979 symptoms of diarrhoea, weight loss and fever increased and she was treated for 5 months with prednisone (20 mg/day) and azathioprine (50 mg/day). In 1982 she was again treated with this regimen for 6 months because of a relapse. In December 1982 she was re-admitted with cachexia and severe diarrhoea, anaemia and hypoalbuminaemia. The liver function tests were normal. A barium enema showed a gastrocolic fistula and extensive involvement of the colon. A proctocolectomy with formation of an ileostomy was performed on 15 December, 1982. Because of the frequent association of liver pathology with chronic inflammatory bowel disease a (first) biopsy was taken from the liver which had a normal appearance. In May 1984, despite prednisone, clinical deterioration occurred. Because of this, cyclosporin-A (600 mg daily) was added from August 1984 and later increased to 700 mg. Four weeks later she suffered episodes of biliary colic with mild fever (38°C). On ERCP gallstones were found, without choledocholithiasis; there was no evidence of sclerosing cholangitis. Laboratory findings were as follows: haemoglobin, 7.4 mmol/l; ESR, 51 mm; bilirubin, normal; alkaline phosphatase, 161 U/l ($n < 100$ U/l); γ-GT, 94 U/l; AST, 6 U/l; ALT, 3 U/l. Serum immunoelectrophoresis did not show an abnormal band. Cholecystectomy was performed in September 1984. The liver surface appeared smooth with a normal colour; a second (needle) biopsy was taken. The cyclosporin therapy was discontinued at the end of October 1984. In July 1985, a fistula appeared at the edge of the ileostomy, requiring surgical excision in March 1986. However, ileus developed and she was re-operated. A third per-operative liver biopsy was taken. Throughout her illness alcohol intake was estimated to be one unit (10 mg) per day; in the period between November 1985 and February 1986 she admitted to drinking 2–4 units of alcohol (whisky) daily; there were no signs of developing portal hypertension and, notably, splenomegaly was absent.

HISTOPATHOLOGY

The first liver biopsy (Figure 1) showed a normal liver architecture with no liver cell necrosis and no inflammation. Collagen stains, however, showed slight pericellular collagen deposition predominantly in the perivenular areas. The portal triads showed a mild mononuclear cell infiltrate; bile ducts were normal. In the second liver biopsy, more marked perisinusoidal deposition of collagen was observed (Figure 2). Stains for amyloid were negative. The collagen appeared to be in the space of Disse and gave an appearance of fixed patency to the mildly dilated sinusoids. Hepatic veins appeared to be slightly thickened by fibrosis of the wall without evidence of thickening of the intima.

The third liver biopsy showed still more marked, diffuse and virtually panacinar perisinusoidal fibrosis (Figure 3). There was focal fixed patency of sinusoids with some dilation. Perivenular fibrosis was present but no occluded hepatic veins were seen. Mild fatty change was present in perivenular hepatocytes; no Mallory bodies were seen. Some portal triads showed mild mononuclear inflammation but were otherwise unremarkable. In all three biopsies, there was no evidence of nodular regenerative hyperplasia.

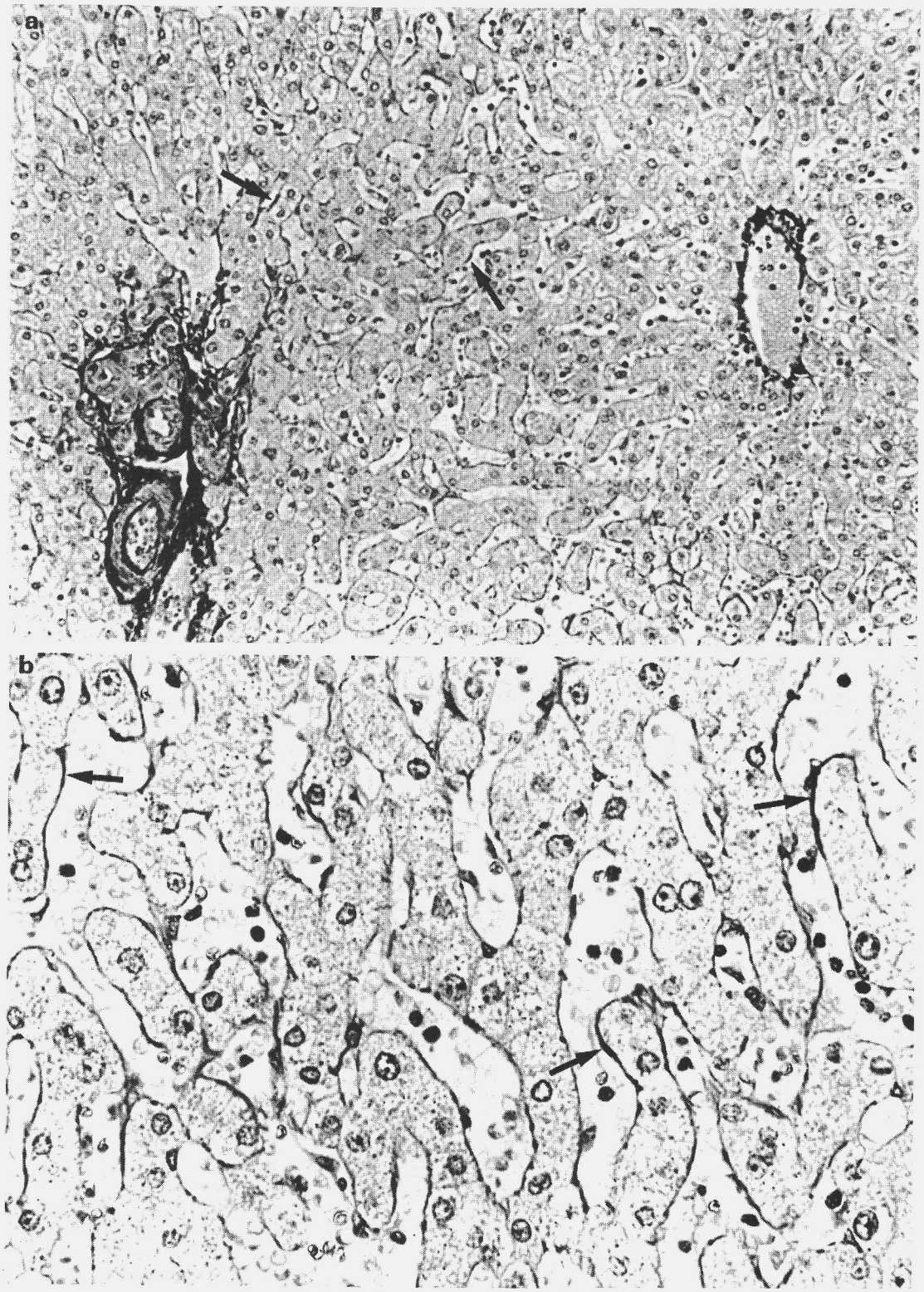

Figure 1. First liver biopsy (December 1982). **a** Focal deposition of collagen fibres (arrows) around liver cell plates is present; there is no parenchymal necrosis or inflammation. Portal tracts appear normal. **b** Higher magnification of area with pericellular fibrosis. Elastica van Gieson. **a** × 200. **b** × 450.

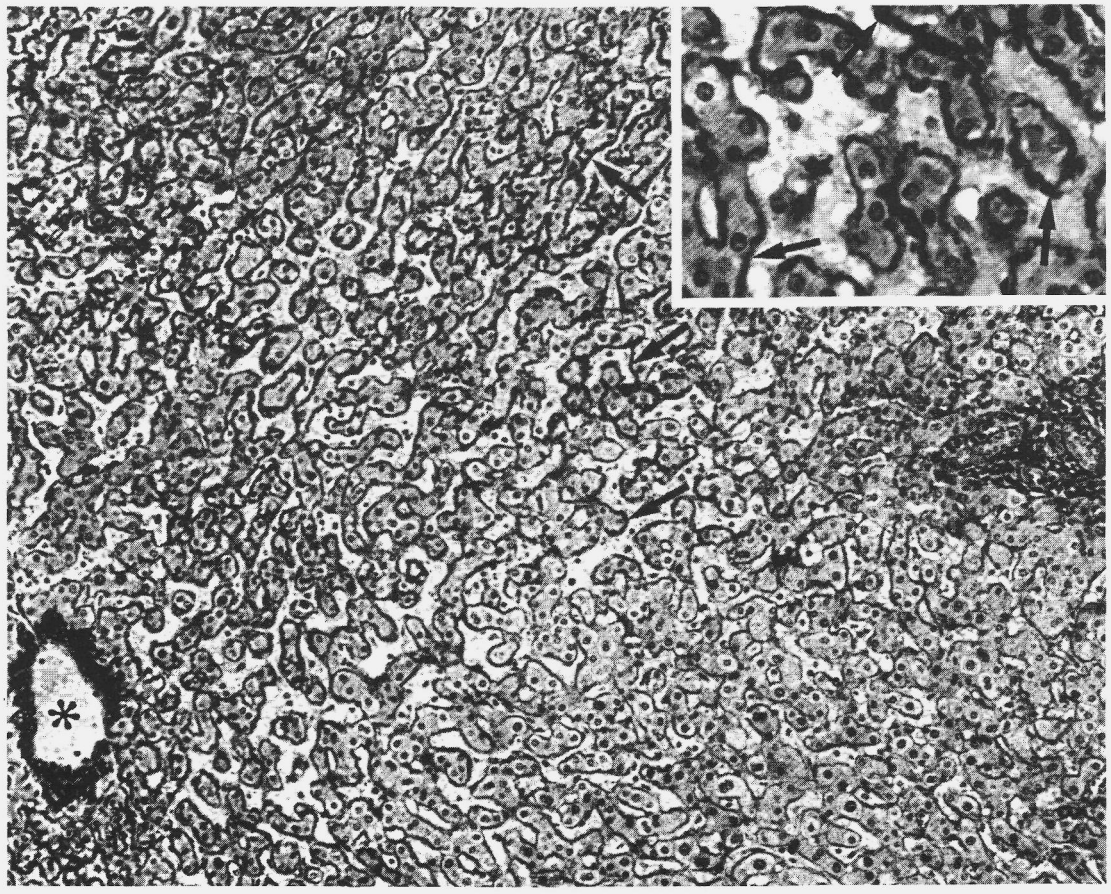

Figure 2. Second liver biopsy (September 1984). Diffuse and marked deposition of collagen (arrows) around liver cell plates and hepatic venule (asterisk) with thickening of the vessel wall. There is mild focal portal tract inflammation. Elastica van Gieson. × 145. *Inset.* × 360.

Electron microscopy of the third liver biopsy showed parenchymal cells with focal lipid granules. A variation in mitochondrial sizes was noted, but there were no megamitochondria; many of the larger ones contained non-specific crystalline inclusions. The parenchymal cell surfaces were active but biliary canaliculi were unremarkable. The most striking feature was the presence of a dense band of well-formed collagen fibres surrounding many of the parenchymal cells (Figure 4). No amyloid fibrils were detected. Unfortunately, immunohistochemical characterization of the collagen could not be carried out.

Discussion

This case is remarkable because of the unusual type of liver fibrosis encountered and the fact that virtually all known causes for hepatic fibrosis could be excluded. Liver

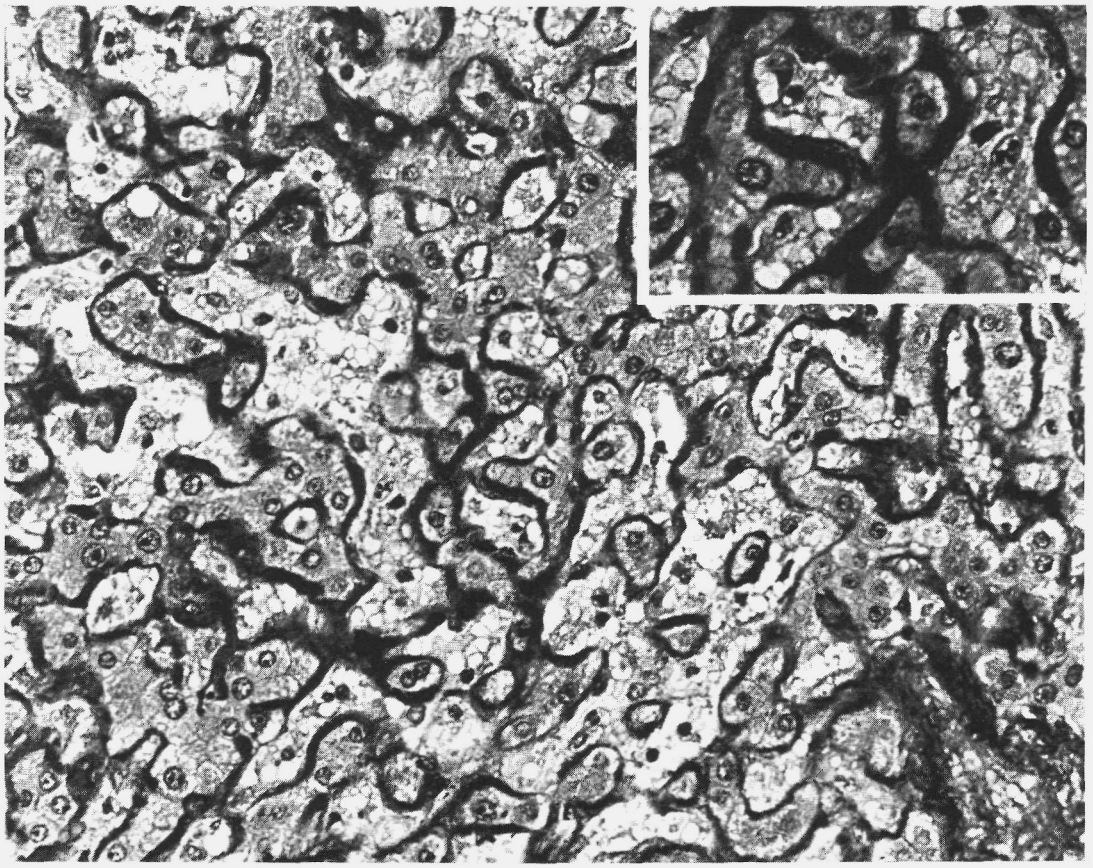

Figure 3. Third liver biopsy (June 1986). Fibrosis in the same pattern but increased as compared with Figure 2. There is focal mild steatosis. Elastica van Gieson. × 320. *Inset.* × 510.

fibrosis with perivenular or pericellular fibrosis is a common finding, but the extent with progression of pericellular fibrosis to more than one zone combined with virtual absence of other morphological alterations has not been described, to our knowledge, in human liver. Three consecutive liver biopsies over a 4 year period showed progressive fibrosis with a diffuse pericellular distribution of collagen alongside the liver cell plates and was not limited to a particular acinar zone. Except for mild focal steatosis in the third biopsy, liver histology was otherwise unremarkable; in particular parenchymal necrosis, inflammation and bile duct pathology were not found.

Liver fibrosis is usually caused by primary liver disease (e.g. viral, toxic or autoimmune hepatitis) or secondary liver disease (e.g. congestive heart failure or haematological disorders) but may theoretically also be the result of collagenase deficiency. Although not excluded biochemically the latter can be presumptively discarded because of normal wound healing in this patient. Viral and autoimmune hepatitis were excluded serologically and by the liver histology. Perisinusoidal fibrosis has been found in haematological disorders, notably thrombocytopenic

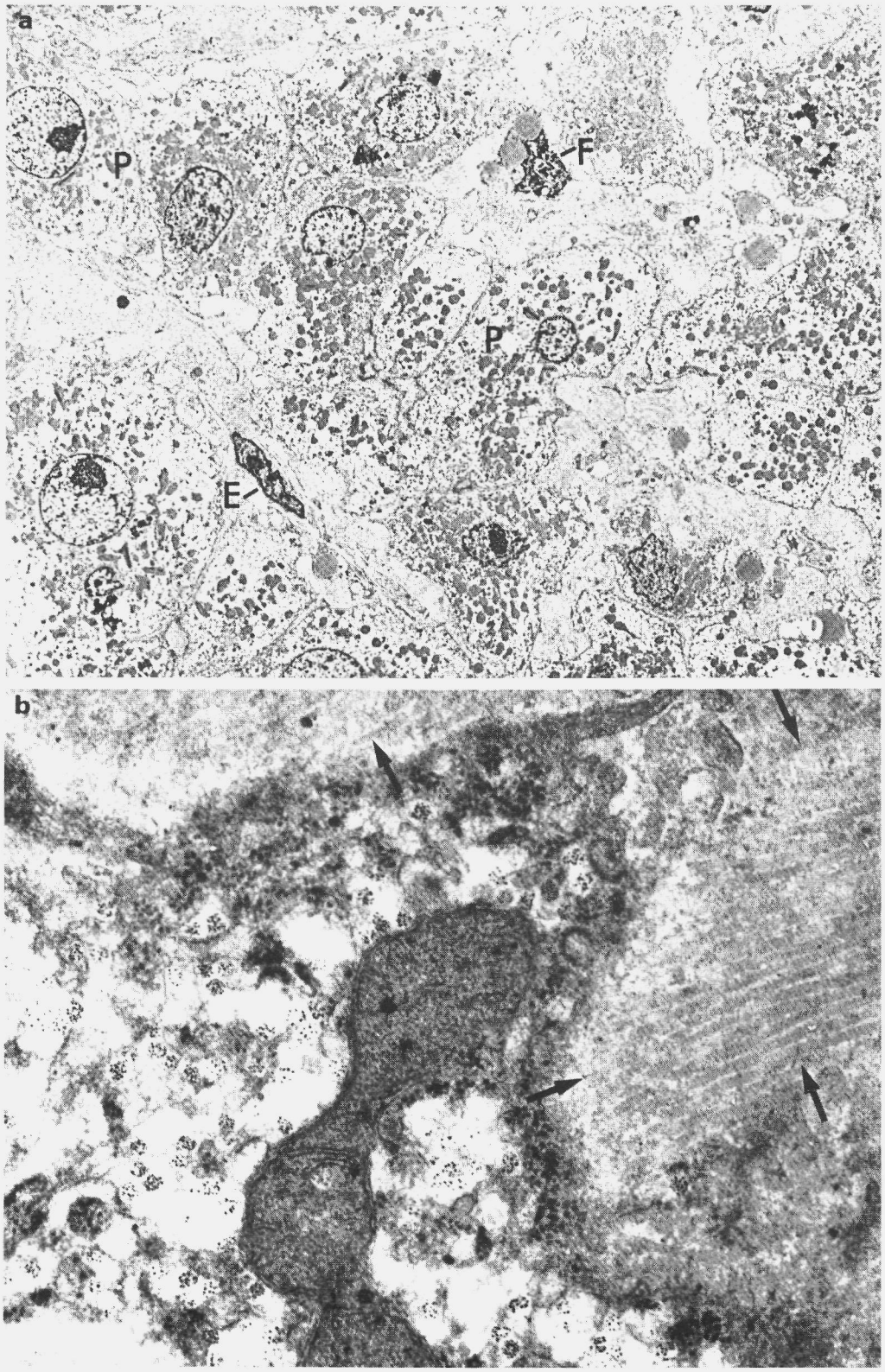

Figure 4. Transmission electron micrographs of the third biopsy. **a** Overview with parenchymal (P) and non-parenchymal (F = fat-storing cell; E = endothelial cell) cells. **b** Strand of mature collagen with collagen bundles (arrows) in close proximity to parenchymal cells. **a.** × 18 000. **b** × 45 000.

purpura, characterized by massive platelet destruction with release of platelet factors and activated Kupffer cells (Lafon *et al.* 1987). Our patient, however, did not show a disorder of this kind. Medical treatment was initially with salazosulphapyridine only. Hepatotoxicity of this drug, widely used in inflammatory bowel disease, has been reported as a rare complication occurring as part of a systemic hypersensitivity reaction comprising skin rash, arthralgia, lymphadenopathy and disturbed liver function tests (Fich *et al.* 1984, Stricker & Spoelstra 1985). Such a hypersensitivity response did not occur in our patient. Although she had used several immunosuppressive drugs, these are unlikely *per se* to be the cause of the liver fibrosis since they were either used for a limited period before, or were started after the appearance of fibrosis. Moreover, except for prednisone, their intake by the patient was temporally unrelated to the progressive panacinar fibrosis. Although azathioprine has been associated with veno-occlusive disease, mainly in patients with a renal transplant (Stricker 1987), in our case the drug was used for a relatively short period and fibrosis progressed despite its discontinuation. Prednisone may cause steatosis but the latter was only present in a mild form in the third liver biopsy. Moreover, these drugs inhibit immune-mediated inflammatory response and resulting fibrosis.

The patient occasionally drank alcohol with (moderate) over-consumption during one period of 3–4 months, but overall the intake was limited because it was found to aggravate the patient's intestinal symptoms. Furthermore, liver histology at the onset of the Crohn's disease was virtually normal without features of alcoholic liver disease. The pattern of the liver fibrosis, especially in the second biopsy, is reminiscent of the early perivenular sclerosis of alcoholic liver disease as described by Van Waes & Lieber (1977) and Popper & Lieber (1980) in baboons and by Nakano, Worner & Lieber (1982) in humans. In alcoholic liver disease progressive perivenular fibrosis without alcoholic hepatitis is probably an 'alternative' pathway to advanced dissecting fibrosis and cirrhosis (Nakano *et al.* 1982, Worner & Lieber 1985); alcoholic hepatitis is still generally regarded as the predominant pathway leading to advanced liver disease with fibrosis and cirrhosis (Lieber 1986). In our patient, fibrous septum formation, which is characteristic of late stage alcoholic liver disease, was lacking, while a diffuse pericellular disposition of collagen was progressively seen.

As far as other potential hepatotoxic agents are concerned, a careful dietary history was unremarkable. There was no history of ingestion of arsenicals and 24 h urinary excretion of arsene in 1987 was normal. Excessive vitamin A ingestion was denied and serum levels in 1987 were normal; hypervitaminosis A, especially in combination with alcohol abuse, is known to be hepatotoxic resulting in fibrosis (Leo & Lieber 1983). There was no occupational exposure to vinyl chloride monomer.

The association of hepatobiliary disorders and chronic inflammatory bowel disease is well established and affects about 10% of patients, although the exact prevalence and incidence are unknown (Desmet & Geboes 1987). Active chronic inflammatory bowel disease is accompanied by chronic bacterial and toxin overload of the liver from the 'leaky' gut. This may result in recurrent toxaemia and sepsis,

reflected by hypergammaglobulinaemia; these features were not shown in our patient. Malnutrition may also result from long-standing and active bowel disease but malnutrition of appreciable severity and duration had not been present in our case. Moreover, while the aetiological relationship of fatty liver and malnutrition is well established, it is presently regarded as very doubtful whether malnutrition *per se* may lead to fibrosis and cirrhosis of the liver (Nayak 1987).

In a recent review, Desmet & Geboes (1987) discuss the various liver lesions associated with bowel disease. In chronic inflammatory bowel disease fatty liver, pericholangitis, sclerosing cholangitis, chronic active hepatitis, fibrosis and cirrhosis, granulomas, amyloidosis and nodular regenerative hyperplasia are mentioned. Of these features only fibrosis and minor steatosis were present in our patient, but the pattern of diffuse pericellular fibrosis was not mentioned in this review. In addition fibrosis and cirrhosis in chronic inflammatory bowel disease are thought to be sequelae of chronic active hepatitis and primary sclerosing cholangitis, features of which were not seen in our case. The patient, at the start of her illness, had a gastro-colic fistula functionally comparable to a jejuno-ileal bypass; this may be associated with liver abnormalities including steatosis and fibrosis (Galambos & Wills 1978, Hocking *et al.* 1983). Although intestinal shunting by an internal fistula cannot be fully excluded as having played a role in the pathogenesis of the liver pathology in our case, this possibility was clinically unlikely.

Thus, no satisfactory explanation can be given for the pattern of diffuse and progressive pericellular hepatic fibrosis we have reported. Alcohol could be the cause but seems unlikely as a single cause in view of the short episode of moderate alcohol over-consumption. It is possible that the fibrosis is multifactorial with an interaction of factors related to inflammatory bowel disease, medication, but also the alcohol consumption. Parenchymal fibrosis of the liver is commonly thought to originate from Ito cells (fat-storing cells) which have a strong capacity to synthesize collagen, at least *in vitro* (De Leeuw *et al.* 1984, Friedman *et al.* 1985, Shiratori *et al.* 1986). However, there is also experimental *in vitro* evidence that not only Ito cells may give rise to fibroplasia in the liver but also that parenchymal liver cells, under defined conditions, are capable of the production and the extracellular deposition of collagen (Tseng *et al.* 1982, Tseng, Smuckler & Stern 1983, Diegelmann *et al.* 1983, Clément *et al.* 1984, Chojkier 1986). In the liver biopsies in the present case, a strikingly close relationship appeared to be present between the collagen deposition and the hepatocytes. Non-parenchymal cell alterations were not prominent and, in particular, Ito cells were inconspicuous and fibroblasts were not seen in the parenchyma. Therefore, the histopathological findings are compatible with the speculation that human parenchymal liver cells, under particular conditions, are capable of collagen synthesis.

Acknowledgements

The authors wish to thank Prof. R.N.M.MacSween, Department of Pathology,

University of Glasgow, for his interest and participation in the investigations of this patient and for his help in the preparation of the light micrographs. We are indebted to Dr I.A.R.More, of the same department, for undertaking the electron microscopy and preparing the photographs. Prof. A.E.Becker, Department of Pathology, Academic Medical Center, University of Amsterdam, is thanked for critical reading of the manuscript and for valuable suggestions. We are grateful to Mrs G.A.Hofland for typing the manuscript.

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CHAPTER VIII

SUMMARY AND DISCUSSION

8.1 Alcoholic liver disease. General introduction

8.1.1 Clinical and epidemiological aspects

Although alcoholic liver disease (ALD) and liver fibrosis (LF) - the hallmark of ALD - are very common disorders, which rank highly as causes of morbidity and mortality, very little is understood of the pathogenesis at present.
From long clinical experience and countless studies, it has become clear that alcohol abuse is damaging the liver in only a fraction of the alcohol abusers, probably 20-25%. However, why the livers of the majority is spared from - or perhaps even protected against - adverse effects of alcohol, is unresolved.
Scarcety of real knowledge may be partly explained by the lack of prospective studies of the general alcohol consuming population. Most data originate from studies of alcohol abusers who attended out - patients clinics or were hospitalised for evidence of liver disease, that was directly related to alcohol or concomitantly present.
From these studies it has been established that ALD is positively correlated with the total amount of consumed alcohol and the duration of alcohol abuse. Moreover, it appeared that the female sex is predisposed to a more severe course of ALD, as compared with the male 'controls'.
Thus, whereas alcohol abuse and contingent ALD are notoriously 'subclinical' or aspecific in presentation, virtually all knowledge of the natural history and factors involved in ALD and LF is selectedly derived from clinically manifested cases. This evidently limits the value of available studies and makes an adequate experimental animal model for human ALD highly desirable.

8.1.2 Morphological aspects

The liver biopsy is the best diagnostic tool to trace ALD and to stage the severity of the liver damage incurred by alcohol ingestion. Although ALD may be expressed in many forms, three main histopathological stages are recognised. The first and early stage is steatosis, producible in each liver following heavy alcohol consumption and reversible after stopping drinking.
The second stage is alcoholic hepatitis with fibrosis. It is usually detected many years later and is supposed to develop at a given moment after years of sustained alcohol abuse.
Alcoholic hepatitis contains characteristic hepatocyte damage and pericellular fibrosis.

It is considered to be the predominant road to progressive liver injury, more specifically to extensive fibrosis and cirrhosis, but a second road without apparent inflammation has been identified in human and baboon livers (Van Waes & Lieber 1977). In non-alcoholic liver disease, inflammation (hepatitis) is the phenomenon that in general mediates to end-stage fibrosis and cirrhosis. That inflammation is not always found in the course of progressive LF is also demonstrated in chapter VII.

8.1.3 Cells and factors involved in alcoholic liver disease and liver fibrosis

The parenchymal cell and most sinusoidal cells of the liver have fibrogenetic potential. This has been established mostly in experimental or *in vitro* conditions but may also occur *in vivo* in human liver (see chapter VII).
Of the parenchymal and sinusoidal cell types, the FSC is still maintained by most experts to be the prevailing collagen producing cell. This also holds true for most cases of ALD which are accompanied by inflammation (alcoholic hepatitis), but in the second 'alternative' pathway of progressive ALD without inflammation, myofibroblasts (Nakano et al 1982) around the central hepatic vein are likely the cells which are responsible for LF (Desmet 1985), at least initially.
Several humoral factors have been identified as being involved in fibrogenesis, but their interaction in inducing and effecting LF is unsatisfactorily revealed as yet. Chronic inflammation clearly is a major trigger, and the concomitant release of cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF), has recently been established to be important in eliciting fibrosis in liver disease of various etiology, including ALD.
In addition to inflammation, gut-derived endotoxin is probably a very important factor in enhancing the release of cytokines. This has been shown to occur in ALD, both experimental and human, in particular with respect to release of TNF.
In human liver disease, it is often difficult and sometimes impossible to point out the etiology and the cells and pathogenetic factors which have caused increased hepatic collagen deposition. Such a case is presented in chapter VII.
It deals with a young female patient with severe Crohn's disease. Crohn's disease is a chronic inflammatory bowel disease of unknown etiology which predominantly affects the small and large intestine, but may involve the whole digestive tract and may have complications, both gastrointestinal and of other organ systems, notably the liver. In the liver of this patient, progressive fibrosis was found in the successive biopsies which were taken over a 4 years period. Liver fibrosis is often associated with parenchymal cell necrosis and inflammation, but these features were not found.
Active inflammatory bowel disease is associated with a 'leaky gut'. Intestinal endotoxins play an important role as mediators of acute and chronic hepatic injury by release of cytokines by activated macrophages (Nolan 1989). Portal and systemic endotoxaemia are important features in patients with diseases of the gut, especially of the large bowel (Van Deventer et al 1988). Continuous triggering of the mononuclear

phagocytic system (MPS), predominantly of its largest body compartment formed by the Kupffer cells, by endotoxin released from, or passed through, the severely damaged colonic wall into the portal blood may be expected to occur. In our patient, however, clinical evidence of chronic endotoxaemia, notably reflected by hypergammaglobulinaemia and associated sepsis, were not shown and histopathologically evidence of activation of the MPS was absent since Kupffer cells were inconspicuous in the liver biopsies. No clue with respect to other etiological factors could be found. A short period of alcohol abuse was very unlikely to be the cause. The same held true for the drugs used for the medical treatment. These were mainly immunosuppressive (prednisone and azothioprine), and as such inhibitors of immune- mediated inflammation and fibrosis. Moreover, in this case of unexplained LF, not only the lack of parenchymal cell damage and inflammatory response was remarkable, also the particular type of fibrosis was unusual. It showed progressively pericellular collagen deposition in Disse's space around individual hepatocytes. A close association of collagen deposition with parenchymal liver cells was found in the absence of alterations of non- parenchymal cells, notably Ito-cells, and of fibroblast-like cells. These findings are compatible with the hypothesis that, in this particular case, hepatocytes had been responsible for the fibrosis instead of non-parenchymal liver cells which are considered to be the usual precursor cells of the fibroblasts that effect parenchymal LF.

8.1.4 Pathogenetic factors specific for alcoholic liver disease

Much evidence has been gathered that acetaldehyde, the metabolite of alcohol, has to be incriminated in ALD as the main trigger to liver.
Overload of alcohol to the liver results in profound metabolic alterations of the parenchymal liver cells, histologically reflected by steatosis. However, the mechanisms responsible for liver damage beyond the early stage of fatty liver to inflammation and fibrosis are unresolved, notably an immune-mediated one is unlikely (see 2.2.5.2).
Also, nutritional deficiency has been abandoned as a decisive or important (co-) factor that is, in combination with alcohol, capable to cause liver injury with fibrosis. This view on the minor role of nutrition in the pathogenesis of ALD has mainly been established from the results of studies in baboons and rats by Lieber's group in New York. Their experimental model of chronic alcohol administration in a liquid diet, that is supposed to be nutritionally adequate, resulted in liver injury including fibrosis.
These results, accomplished with this model in rat and baboon livers, were the main motive for the liver research group of our Institute to apply use of the liquid diet in our rats, in order to study the events of developing LF and to confirm the relevance of this model for human ALD as it was presented by Lieber and coworkers.

8.2 Experiments and results

8.2.1 Induction of liver fibrosis in different rat strains by various treatments

The possibility to elicit liver fibrosis in the rat strains BN/BiRij and WAG/Rij, which are commonly used in our Institute, has been established in several short- and long-term experiments.

8.2.1.1 The carbon tetrachloride model

In chapter V, a detailed report is given of a short-term study of 13 weeks duration on developing LF in BN/BiRij rats. LF was induced by repeated subcutaneous administration of the xenobiotic carbon tetrachloride (CCl_4) for 6 weeks. CCl_4 is known as a potent hepatotoxic agent and inducer of LF (Perez- Tamayo 1983). Extensive dissecting fibrosis, composed of centro-central and centro-portal collagen bridges, developed from the fourth week on. It was preceded by parenchymal inflammation, which started in the central perivenular area and was accompanied by mixed polymorphonuclear and mononuclear inflammatory cell response, and by pericellular fibrosis as early as two weeks after the start of the experiment.

8.2.1.2 Administration of carbon tetrachloride followed by alcohol

The fibrogenetic potential of alcohol on the liver of this rat strain was demonstrated in the CCl_4-model. The initial CCl_4-pretreatment of the liver for six weeks was followed for seven weeks with administration of alcohol, which was added to the drinking water to a concentration up to 10% (vol/vol). This procedure resulted in a significant increase in number and volume of the fibrous septa which were previously induced by CCl_4.

8.2.1.3 Heterologous serum-induced liver fibrosis

Our rat strains were also shown to be normally sensitive to induction of LF by repeated injections of heterologous (pig) serum. Within about 12 weeks, LF of at least comparable severity as described by Ballardini et al (1983) was found (not shown).

8.2.2 Longterm experiments with alcohol

8.2.2.1 The Lieber-DeCarli model

8.2.2.1.1 Introduction

Chapter IV (4.1 and 4.2) gives a detailed report of the morphological and biochemical results gained from a longterm experiment with the alcohol-containing Lieber-DeCarli diet.
Rats were fed for 16 months with a diet as developed by Lieber and coworkers, as the only source of food and water. This so-called Lieber-DeCarli diet was introduced in 1963 (Lieber et al 1963) and has since been steadily improved (Lieber & DeCarli 1989). It is a liquid diet composed to be nutritionally adequate for the animal species in question. In this diet, alcohol is incorporated substituting carbohydrates at a concentration of 5 g/dl or 36% of total dietary calorie content. A sustained high daily alcohol intake of 12-18 g/kg body weight can be reached, with blood levels of 100-150 mg/dl. This diet allows pair-feeding with control animals.
We decided to apply this Lieber-DeCarli model to rats in the assumption that it would provide us with a good model to study the liver lesions induced by alcohol, notably fibrosis and more specifically, the kinetics of the cells and the factors involved in the development of LF.
Lieber and coworkers had reported that in this rat model LF did develop within 9 months and in the livers of all alcohol-treated animals (Leo & Lieber 1983). However, this rat model of alcohol administration is a rather artificial one as compared with human alcohol consumption. One reason is the involuntary, imposed pattern of alcohol-diet feeding of the rats, the other is the high vitamin A content of the diet required for the development of fibrosis in rat liver. The large extra quantum of five times the vitamin A content that is considered normal for rats, was added to the diet.
Vitamin A status is known to be lowered in human and in experimental ALD, and is likely to be an important factor, perhaps even a determinant, (co-) factor in the development of LF that is induced by hepatotoxins, such as CCl_4 (see chapter VI).
We have undertaken experiments with the Lieber-DeCarli diet in several rat strains with the same treatment protocol as applied by Lieber and co-workers. In addition to the experiment with the high vitamin A supplementation, we have carried out an experiment with a low vitamin A supplementation to the diet.

8.2.2.1.2 Low vitamin A supplementation

Thirty rats, with an equal number of paired controls, of three strains were fed the Lieber-DeCarli diet. Vitamin A supplementation to this diet was two times the normal amount and contained 11.200 IU per litre liquid diet as retinyl acetate.
The three rat strains used were BN, WAG and Sprague-Dawley, the latter being the

strain used by Lieber's group. Liver biopsies, taken at regular intervals, showed only steatosis, but neither fibrosis nor inflammation. The experiment was terminated after 12 months.

8.2.2.1.3 High vitamin A supplementation

This experiment was carried out according to the standard procedure of Lieber's group in which vitamin A supplementation to the diet amounts to 28.000 IU per litre. For practical reasons (availability of strains at that moment), the experiment was done in 2 rat strains, BN and WAG, and not in the Sprague-Dawley rats used by Lieber's group.
The course and results of this long-term experiment are reported and discussed in chapter IV.
Only minor to moderate steatosis was found in the liver biopsies of both alcohol-treated and control rats, even after the critical time span of nine months. We decided to extend the duration of the experiment and to terminate it at 16 months. Even at that time, no fibrosis or other liver lesions, that could be interpreted as direct effect of alcohol on the liver, were recognised.
Thus, we were not able to reproduce the findings of Lieber and co-workers, who demonstrated that LF developed as the result of the direct effect of alcohol on the liver of their rats.
The contrasting results gained by our and Lieber's group might be explained by factors related to: a different genetic predisposition (that influences susceptibility and metabolic responses to alcohol) of the rat strain (Sprague-Dawley) used by Lieber's group; to the younger age of the rats (weanlings) at the start of the experiment in Lieber's study, at which age (in our experience) alcohol administration may interfere with maturation and growth and result in bad general health, but also in malnutrition with liver pathology, including steatosis and fibrosis (see 8.2.2.2 preliminary results); additionally, hypervitaminosis A may, in combination with alcohol, result in enhanced toxicity in developing animals, including liver fibrosis. From our results in this experiment we concluded that this rat model not necessarily leads to alcohol-induced LF. The lack of reproducibility of the fibrosis, as found by Lieber's group, and of other characteristic features of human ALD, notably alcoholic hepatitis, strongly limits the value of this animal model to study ALD.

8.2.2.2 Nutrition, alcohol and ageing

Effects of malnutrition on the liver
Malnutrition, as such, is not generally believed to result in LF (see chapters II and III). However, the role of nutrition, especially of malnourishment, is often not well defined in the human situation, in which alcohol abuse is frequently found to be associated with an abnormal nutritional state. This is particularly so in chronic

alcoholics, in which evidence of malnourishment is prevalent but is considered to be a secondary effect of alcohol abuse. In chronic alcohol abuse, malnutrition is due to abnormal food and poly-deficient nutrient intake, with calories greatly derived from ingested alcohol, and to alcohol-induced gastro-intestinal disease with malabsorption, rather than to alcohol-induced liver disease.

Ageing and alcohol

Since the effects of alcohol on the liver and other organs generally occur after longterm exposure and in late middle age or later, the interaction of chronic alcohol consumption and malnutrition with ageing processes should be considered.

Both alcohol abuse and ageing are striking characteristics of our society.

Although alcohol abuse is less prevalent in elderly than in middle-aged people (Scott & Mitchell 1988), it is more difficult to detect and more harmful in elderly people due to pharmacokinetic factors and to increased tissue sensitivity to alcohol (West et al 1984).

Despite the effects of ageing per se on the liver functions are limited, an age-related decrease in biochemical and metabolic reserve capacity, by diminished blood flow, infections, drugs or toxins cannot be excluded, by lack of sufficient studies (Scott & Mitchell 1988).

It is well established that both chronic alcohol abuse and chronic nutritional deficiencies often contribute to extra-hepatic tissue damage. Poorly nourished people are more sensitive to infectious disease and they recover more slowly from various diseases and traumata than well nourished people. A poor nutritional state is interfering with normal cell replacement and energy requiring enzymatic and other metabolic processes, tissue integrity and immunity (Achord 1988). Thus, malnourishment, as accompanying effect of alcohol abuse, may be expected to influence the process of normal ageing, and to increase morbidity and associated mortality at an earlier lifetime.

To study the effects of nutrition and alcohol on the liver and on ageing, we have carried out in young adult rats several other longterm experiments with real alcoholic beverages in combination with various diets, including the introduction of severe malnourishment. Of all alcoholic drinks, we have chosen (cheap) whisky alone or in combination with beer because in Scotland severe chronic alcoholism, with ALD is frequently found to be associated with the abuse of these particular alcoholic drinks (personal communication professor MacSween, Glasgow). These studies are currently being worked out and will be described in detail later on. Preliminary results demonstrate that all rats which were treated with whisky and beer and normal lab chow showed phenomena of premature ageing and died before the age of 22 months, whereas all rats which were treated with these alcoholic drinks and food restriction (60% of normal lab chow) were still alive after 30 months. Food restriction increased survival of both alcohol and non-alcohol treated rats. Liver morphology of all rats,

whether they died spontaneously during the course of the experiment or by sacrifice at termination of the experiment after 30 months, was normal. From this longterm experiment might be concluded that alcohol has no adverse effects on the liver morphology but has a strong adverse effect on ageing and survival. Another experiment with whisky was combined with severe malnutrition of the rats fed a diet which was composed of mashed potatoes and mayonnaise and contained 25% of calorie content as alcohol. The effects of this treatment were impressive. Growth and further maturation were retarded and evidence of bad general health and malnourishment were soon obvious and progressive, particularly in the alcohol-treated rats, for which reasons the experiment had to be terminated after 12 months. At autopsy, the livers of all rats showed severe steatosis and dissecting fibrosis with transition to cirrhosis. Fibrosis and cirrhosis were more pronounced in the alcohol-treated rats. From these experiments may be concluded that alcohol has a definite fibrogenetic effect on rat liver only if another fibrogenetic factor: malnutrition is also present.

8.2.3 Vitamin A and liver fibrosis

The relation of vitamin A with liver fibrosis is complex. Extremes in vitamin A status, both hypo- and hyper-vitaminosis A, are hepatotoxic and often associated with liver abnormalities, both experimentally and in humans.
In chapter VI, the results are reported of experiments with the CCl_4-model, which was used to investigate the particular relation between LF and vitamin A.
Three months old female BN-rats were treated with CCl_4 for four weeks with subcutaneous injections twice weekly. Some rats received supplements of vitamin A as retinyl palmitate in vegetable oil in addition to standard lab chow.
Vitamin A supplementation was given prior to, during or after CCl_4-treatment. The different effects of this variant vitamin A treatment regarding toxicity and effect on the CCl_4- treated liver were striking.
Vitamin A status, as reflected by the vitamin A content of the liver, proved to be decisive for the severity of the CCl_4-induced liver injury and fibrosis. Low vitamin A status was associated with severe liver disease. However, high vitamin A status, by supplementation prior to or during CCl_4-administration, resulted in lesser hepatic damage and fibrosis but was associated with a considerable mortality by evidence of vitamin A toxicity, as compared with rats that were only treated with CCl_4 or high vitamin A.
On the contrary, rats that were given high vitamin A after CCl_4-treatment showed considerable reduction of liver fibrosis and no evidence of vitamin A toxicity.
Additional experiments in the same set-up have been done with beta-carotene (provitamin A), in a dose comparable with retinyl palmitate. These experiments showed that beta-carotene also reduced liver damage and fibrosis, that are induced by

CCl_4, but is not toxic in contrast with retinyl palmitate. Proof of lack of toxicity of beta-carotene was the absence of mortality during the experiment.
Two conclusions can be drawn from these experiments with the CCl_4-model and vitamin A in rats: a good vitamin A status, as reflected by storage of vitamin A in the liver in fat-storing cells, either blocks the potential of fat-storing cells to differentiate into fibroblasts, or stimulates the collagen breakdown.

8.2.4 Treatment of alcoholic liver disease and liver fibrosis

Total abstinence from alcohol consumption is the only effective treatment of ALD to prevent further liver damage. It has been established by several studies that it improves dramatically the prognosis for both acute and chronic ALD (Lieber 1985).

Medical treatment
In spite of the awareness that the mechanisms responsible for increased hepatic collagen deposition remain unknown and that thus there is no established therapy for hepatic fibrosis (Brenner & Chojkier 1988), several drugs have been used in order to prevent the progression of fibrosis or to cure existing fibrosis in liver diseases of various etiology, including ALD.
Drugs of such various kinds as corticosteroids, testosterone, anabolic-androgenic steroids, propylthiouracil, and colchicine have been applied in trials of medical treatment of ALD. This was done mainly in patients admitted with (severe, life-threatening) alcoholic hepatitis or cirrhosis.
Most results have been negative or conflicting, and the side-effects of these drugs have been numerous. Corticosteroids (methylprednisolone) did not improve the mortality rate of patients with severe acute alcoholic hepatitis (Theodossi et al 1982). Oral testosterone treatment to men with alcoholic cirrhosis had no beneficial effect on survival and liver biochemistry (Gluud et al 1986). Longterm treatment with propylthiouracil - an antithyroid drug which has been reported to protect against hypoxia- related hepatocellular necrosis that is induced by alcohol - has shown to reduce ALD-related mortality (Orrego et al 1987).
Colchicine is an alkaloid herbaceous drug that has been used for centuries in the treatment of gout. Although its modes of action are not fully understood, it has shown to have anti-inflammatory and anti-fibrogenetic activities, both in experimental, CCl_4-induced, fibrosis and in human liver cirrhosis (Rojkind at al 1984). The results of a longterm study (up to 14 years) of colchicine treatment of patients with cirrhosis of various etiology, mainly post-hepatitic and alcoholic, was promising. It showed a significant increase in survival, with more than doubled 5- years survival rate (75 versus 34%), and histopathological liver improvement in 18% (and even return to normal histology) (Kershenobich et al 1987).

Nutritional support
Alcohol may increase the daily requirement of nutrients, notably proteins, and amino acids such as choline, and vitamins such as folate. Nutritional deficiency may promote the hepatotoxic effect of alcohol on the liver by depletion of hepatic amino acids and enzymes (Sherlock 1984). In this way, increased daily requirement of several nutrients may prevail in active ALD and in the recovery phase. Good nutrition does not protect against the hepatotoxic effect of alcohol but malnourishment, which very often complicates ALD, interferes with rapid repair in a negative way and nutritional support in a positive way (Achord 1987).
'Stable' alcoholics with liver damage, who fail to abstain, should be encouraged to take a well-balanced diet containing at least 2000 calories and 1 g protein/kg body weight with modest multivitamin supplementation, containing vitamin A, B-complex, C, D, folate and K1 (Sherlock 1984).

8.3 General conclusions and outlook for the future

The pathogenesis of ALD and, more specifically, of LF is far from resolved, and this thesis is an attempt to clarify some of the mechanisms that could be involved. The inclusion in this thesis, among experimental studies of fibrosis in rat liver, of an extensively analyzed, but unexplained, case of severe progressive fibrosis of human liver, is not misplaced in that respect.
It must be apparent, however, from the reviews of the literature and the studies included in this thesis, that simplicity with regard to alcoholic LF cannot be expected. LF induced by alcohol is the resultant from the interference in a very complex and delicate network of processes which regulate the microenvironment, called extracellular matrix of the liver. The outcome of this interference is difficult to predict for any individual, and even so for a given experimental condition.
The exact nature of the processes that lead to excess collagen deposition into the matrix are as yet not adequately defined, neither are the triggering factors of these processes. In alcoholic liver disease, the initial factor for induction of parenchymal damage and/or fibrosis is most probably the ethanol-metabolite acetaldehyde.
The rat model of Lieber and co-workers, which makes use of a liquid, nutritionally balanced, alcohol-containing diet, has to be discarded as an generally adequate experimental model for studying the effect of alcohol (per se) on the human liver. Moreover, nutrition, c.q. malnutrition, does appear from the results of our studies on malnutrition and alcohol in rats to be a determinant (co-) factor for enhanced liver fibrosis. This finding contrasts with current opinion which considers prevailing malnutrition to be a secondary phenomenon of alcoholic liver disease.
Our experimental finding about the role of the nutritional state could have implication for the pathogenesis and also for the treatment of human alcoholic liver disease.

In our longterm experiments in rats, the damaging effect of alcohol per se on the liver was absent or minor (steatosis). Notably fibrosis was never found. This is not unlike the human situation in which only a limited number of chronic alcohol abusers will develop liver disease (in contrast with the impairment of other organ systems which occurs at much higher frequency). In these experiments, premature ageing and ageing-related morbidity and mortality, characteristic for the rat strains which were used, were striking findings. This premature ageing effect of alcohol use could also be relevant for our increasingly ageing and alcohol abusing society. The effect of alcohol in accelerating the ageing process will probably be enhanced if malnutrition exists.
The role of vitamin A in relation to alcoholic liver disease and liver fibrosis remains enigmatic. Both hyper- and hypo-vitaminosis A as such are hepatotoxic. Chronic alcohol abuse evidently is associated with an increased risk of a low vitamin A status. This is likely to be an unfavourable factor in the course of alcohol-related liver disease. Vitamin A supplementation appears to increase the rate of recovery of CCl_4-induced fibrosis in rat liver. In the human situation, vitamin A could be considered for treatment support of ALD provided that the inducer (alcohol) is removed; otherwise toxic interactions may occur.

Outlook for the future
Well defined animal models certainly will be needed to further study the interaction of specific cells and factors involved in the generation of alcoholic liver disease and liver fibrosis. Also the process of collagen breakdown, which normally is in balance with collagen formation, has to be more clarified in order to possibly influence it to reverse developing, or even established, fibrosis.
With respect to ALD: prospective epidemiological studies of alcohol-consumption in the general population in relation with the occurrence of this disease are a high priority. These studies are requested to detect which individuals are sensitive to develop ALD in relation to characteristics of alcohol consumption, nutrition, composition of the population, and other as yet unknown factors. It is very likely that the sensitivity to develop ALD has a genetic basis and that, therefore, genetic studies will be needed to map the molecular-biological basis of this sensitivity.

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BRIEF SUMMARY

This thesis deals with clinical and experimental alcoholic liver disease and liver fibrosis and is focussed on the pathogenetic aspects of these diseases. For many clinically relevant diseases of the liver, fibrosis is the main pathway to progressive disease and thus to end-stage cirrhosis. This is particularly so for liver damage incurred by chronic alcohol abuse. Extensive liver fibrosis and cirrhosis of the liver, notably due to alcohol abuse, are very prevalent in our society and carry a high morbidity and mortality. General health is affected, bleeding from oesophageal varices due to portal hypertension and liver failure may occur, both associated with a high mortality rate. Unfortunately, little is known about the pathogenesis of liver fibrosis in general and of alcoholic liver disease in particular. The experimental studies described in this thesis were designed to identify some of the factors which are important for the pathogenesis of liver fibrosis notably in relation to alcohol consumption.

Chapter I gives an general account of the aim of the thesis and the specific experiments and studies described and discussed in the following chapters.

Chapter II presents a review of alcoholic liver disease as known from the copious literature on this subject. Briefly summarised, it concludes that scarcely more is established than that alcohol, or its metabolite acetaldehyde, is the main inducer of liver damage in chronic abuse, but only so in a rather small percentage of those unknown individuals who are "susceptible" to incur liver damage. However, the determining factors for this damage are unknown and nutritional factors are not considered at present to play such a role.

Chapter III reviews the literature on experimental animal models for alcoholic liver disease and liver fibrosis and the mechanisms and cell types, which may be involved in these diseases. It is concluded that real knowledge of the extracellular matrix of the liver in health is still scarce and that the interaction between cells and factors which results in matrix overproduction, or fibrosis, is even less understood. Moreover, extrapolation of findings in experimental animals to the human situation appears hazardous. However, vitamin A is reported to be an established factor in fibrogenesis which can influence liver fibrosis both positively and negatively, depending on experimental conditions. With regard to alcoholic liver disease, Lieber and coworkers

from New York reported on an experimental model for studying the effects of alcohol *per se* on the liver. They applied a nutritionally adequate alcohol containing liquid diet to baboons and rats and found several histological features of human alcoholic liver disease, including alcoholic hepatitis in the baboon livers. These results of Lieber and coworkers were an important motive for us to apply this model to rats for studying the damaging effects of alcohol and, possibly, the mechanisms involved.

In Chapter IV, our negative experience with Lieber's rat model is reported and discussed. In this long-term experiment with the same experimental approach but in different rat strains than that used by Lieber's group, no direct hepatotoxic effect of alcohol on the liver was found and the validity of this rat model for studying human alcoholic liver disease is doubted.

Chapter V reports on a short-term experiment in which alcohol administration was found to enhance liver fibrosis in rats pretreated with carbon tetrachloride.

Chapter VI reports on the dual effect of vitamin A administration on the extent of liver fibrosis in carbon tetrachloride-induced liver injury in rats. In this experimental model, a good vitamin A status of the liver proved to protect this organ against the induced inflammation and fibrosis.

Chapter VII presents an unusual case history of a patient with progressive liver fibrosis which remained unexplained.

Chapter VIII gives the general summary and discussion. The pathogenesis of alcoholic liver disease and of liver fibrosis is complex and far from resolved at present. In the near future much basic, molecular-biological research and clinical, epidemiological and genetic studies are needed to unravel the pathogenetic factors which are involved in the generation of liver fibrosis and alcoholic liver disease.

The results of our experimental studies are in direct contrast to the general belief in the literature, which is mainly based on the reports of Lieber's group. With regard to alcoholic liver disease, it appears that in addition to the direct toxic effect of alcohol on the liver, other factors must be taken into account, notably nutritional factors.

KORTE SAMENVATTING

Dit proefschrift heeft klinisch en experimenteel alcoholisch leverlijden en leverfibrose als onderwerpen en is vooral gericht op de pathogenetische aspecten van deze ziekten. Voor vele klinisch relevante leverziekten geldt dat fibrose de belangrijkste "pathway" is tot progressie van de ziekte en op deze wijze tot het ontstaan van cirrhose als eindstadium. Dit is speciaal het geval bij leverschade opgelopen door chronisch alcoholmisbruik. Uitgebreide fibrose en cirrhose van de lever, in het bijzonder die welke zijn ontstaan als gevolg van alcohol-misbruik, komen veel voor in onze maatschappij en brengen veel ziekte en sterfte met zich mee. De gezondheid wordt er door aangetast; bloeding uit slokdarmvarices op basis van portale hypertensie en leverfalen kunnen optreden, beiden samengaand met een hoog sterftecijfer.

Helaas is slechts weinig bekend over de pathogenese van leverfibrose in het algemeen en van alcoholisch leverlijden in het bizonder. De experimentele studies van dit proefschrift waren bedoeld om enkele van de factoren op te sporen die van belang zijn voor het ontstaan van leverfibrose, vooral met betrekking tot alcoholgebruik.

Hoofdstuk I is een algemene weergave van het doel van het proefschrift en van de verschillende experimenten en studies die in de volgende hoofdstukken worden beschreven en besproken.

Hoofdstuk II is een beschouwing van de zeer uitgebreide literatuur betreffende alcoholisch leverlijden. Kort samengevat wordt de conclusie getrokken dat nauwelijks meer zeker is dan dat alcohol, of het stofwisselingsproduct acetaldehyde, de belangrijkste "inducer" van leverschade is bij chronisch alcoholmisbruik, maar dat dit slechts het geval is in een vrij klein percentage van die onbekende individuen die "gevoelig" zijn voor het oplopen van leverbeschadiging. Echter, de factoren welke bepalen of deze schade zal optreden zijn onbekend, wel worden voedingsfactoren tot heden niet alszodanig aangemerkt.

Hoofdstuk III geeft een overzicht van de literatuur betreffende experimentele diermodellen voor alcoholisch leverlijden en leverfibrose, en van de mechanismen en celtypen die bij deze ziekten betrokken zouden kunnen zijn. Er wordt vastgesteld dat feitelijke kennis van de extracellulaire matrix van de lever onder normale

omstandigheden nog schaars is en dat begrip van de interactie tussen cellen en factoren die resulteert in overproductie van matrix, oftewel fibrose, zelfs nog minder is. Bovendien lijkt het riskant om gegevens verkregen bij proefdieren te extrapoleren naar de mens. Echter, vitamine A wordt in de literatuur een zekere factor genoemd voor het ontstaan van leverfibrose die de fibrose, afhankelijk van de experimentele omstandigheden, zowel positief als negatief kan beinvloeden.

Met betrekking tot alcoholisch leverlijden hebben Lieber en medewerkers uit New York bericht over een experimenteel model om het effect van alcohol *per se* op de lever te bestuderen. Zij gaven een volwaardig alcohol bevattend vloeibaar dieet aan bavianen en ratten en vonden verscheidene histologische kenmerken van alcoholisch leverlijden, inclusief alcoholische hepatitis in de levers van de bavianen. Deze resultaten van Lieber en medewerkers waren voor ons een belangrijk motief om dit model toe te passen bij ratten teneinde de schadelijke effecten van alcohol en, zo mogelijk, de betrokken mechanismen te bestuderen.

In hoofdstuk IV wordt onze negatieve ervaring met het rattemodel van Lieber beschreven en besproken. In dit langdurende experiment, met dezelfde experimentele benadering maar met gebruik van andere rattestammen dan door Liebers groep, werd geen direct schadelijk effect van alcohol op de lever gevonden en werd de geldigheid van dit rattemodel voor de bestudering van alcoholisch leverlijden van de mens in twijfel getrokken.

Hoofdstuk V is het verslag van een kortdurend experiment waarin gevonden werd dat alcoholtoediening leverfibrose van ratten die voorbehandeld waren met tetrachloorkoolstof versterkte.

Hoofdstuk VI beschrijft het tweeledige effect van vitamine A toediening op de mate van leverfibrose van leverschade die door tetrachloorkoolstof was geinduceerd bij ratten. In dit experimentele model werd aangetoond dat een goede vitamine A status van de lever dit orgaan beschermde tegen de opgewekte ontsteking en fibrose.

Hoofdstuk VI is de weergave van een uitzonderlijke ziektegeschiedenis van een patiente met toenemende leverfibrose waarvoor geen verklaring kon worden gegeven.

Hoofdstuk VIII presenteert de algemene samenvatting en discussie.

De pathogenese van alcoholisch leverlijden en leverfibrose is complex en is heden allesbehalve opgehelderd. In de nabije toekomst zullen veel basaal, moleculair-biologisch, onderzoek en klinische, epidemiologische en genetische studies nodig zijn

om de pathogenetische factoren die betrokken zijn bij ontwikkeling van leverfibrose en alcoholisch leverlijden te ontwarren.

De resultaten van onze experimentele studies vormen een directe tegenstelling met de algemene opvatting in de literatuur die hoofdzakelijk is gebaseerd op de publicaties van Liebers groep.
Ten aanzien van alcoholisch leverlijden blijkt dat, behalve het direct schadelijke effect van alcohol op de lever, ook andere factoren in aanmerking moeten worden genomen, in het bizonder voedingsfactoren.

ABBREVIATIONS

ACTH	adrenocorticotrope hormone (or corticotropin)
ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
APP	acute phase protein(s)
APR	acute phase response
ALD	alcoholic liver disease
CCl_4	carbon tetrachloride
Ctk	cytokine(s)
EM	extracellular matrix
FSC	fat- storing (or Ito) cell(s)
GP	glycoprotein(s)
HBV	hepatitis B virus
HGG	hypergammaglobulinaemia
HLA	human leucocyte antigen
IC	immune complexe(s)
Ig	immunoglobulin(s)

Kc	Kupffer cell(s)
LF	liver fibrosis
LPS	lipopolysaccharide
Mb	Mallory body (ies)
MPS	mononuclear phagocytic system
NAD	nicotinamide adenine dinucleotide
NADH	reduced NAD
PIIIP	aminoterminal procollagen type III peptide
PGly	proteoglycan(s)
PVS	perivenular sclerosis (or fibrosis)
IL- 1	interleukin 1
IL- 6	interleukin 6
PAF	platelet- activating factor
RER	rough endoplasmatic reticulum
TNF	tumor necrosis factor
TSH	thyroid stimulating hormone (or thyrotropin)
T4	thyroxine

ACKNOWLEDGEMENTS

This thesis is largely the product of joint efforts of members of the Livergroup at the Institute for Experimental Gerontology (IVEG) of IVVO-TNO where the experiments were carried out. I would like to thank them all for the cooperation and enthusiasm which I experienced during the weekly day of the last six years at which I happily switched from clinical (human) pathology to the animal surroundings of the IVEG. Of all, I am most indebted to Adriaan Brouwer. His analytic power and flexibility in the approach and the solution of not merely scientific problems of very different kinds still astonish me, but I have appreciated most of all his patience and friendship. Wilfried Seifert was a very pleasant companion in working out the experiments and their results for publication, I hope that he will soon finish the road to his thesis.

Of my two promotors, I want to thank Prof.Dr. D.L. Knook for introducing me at the IVEG and giving me the opportunity to apply my clinical and pathological knowledge to experimental animal models. Dick, I admire it that you, the Institute's captain, are able to guide the IVEG boat and its crew safely and usually in good spirits to uncertain harbours, which are often imposed upon you nowadays by political dictators who distribute the money that is needed to keep the boat afloat and that you nevertheless can maintain to warrant the scientific quality of the load. During the past years at TNO, I have considered one day of every week as being on a holiday cruise despite often pretty firm exertions.

I want to thank my second promotor, Prof. J.H.P. Wilson, an expert in liver disease with an international reputation, for being willing to police the extrapolation of the results of experimental animal models to the human situation. Paul, it is always a pleasure to be in your friendly company and to learn from your broad and deep experience of liver disease.

I feel very honoured and much indebted to Prof.Dr. V.J. Desmet, of Louvain, former president of the International Association for the Study of the Liver (IASL), that he was prepared to be the referent of this thesis.

Of all other IVEG workers who have contributed to this thesis, I would like to mention Roel Barelds and Christa van Thiel-de Ruiter for their contribution to the experimental work and for all other help. Mr. Tom Glaudemans is thanked for the

excellent photography. I am very grateful to Mrs. Ted Hofland for her help in the final editing and finishing touch of the thesis.

CURRICULUM VITAE

Anne Bosma werd geboren in Friesland op 8 juni 1943 te Grouw, gemeente Idaarderadeel, en is ook anderszins een Fries. Na het eindexamen gymnasium- beta in 1961 te Groningen werd met de studie geneeskunde begonnen in Amsterdam aan de Vrije Universiteit en werd het kandidaatsexamen afgelegd in mei 1985. De studie werd vervolgd aan de Universiteit van Amsterdam alwaar het doctoraal examen werd afgelegd in januari 1968. Als semi-arts werd een klinisch assistentschap van 8 maanden doorgebracht in het Nederlands Kanker Instituut (Anthoni van Leeuwenhoek Ziekenhuis). Het artsdiploma werd verkregen in juli 1970.
Tot mei 1972 volgden voorbereidende cursussen en klinische assistentschappen voor de te werkstelling als arts in de medical departments van een groot regionaal opleidingsziekenhuis in Ilesha, West Nigeria; dit verblijf in de tropen werd voortijdig beëindigd door virale hepatitis (B).
Tot juli 1978 werd opleiding gevolgd in de interne geneeskunde met als subspecialisatie gastroenterologie en hepatologie (Academisch Ziekenhuis Leiden), direct gecontinueerd door de opleiding tot patholoog in het Leids Laboratorium onder leiding van Prof.Dr. Th.G. van Rijssel met inschrijving in het specialisten register op 1 januari 1982.
Na een korte periode van werkzaamheid als algemeen patholoog in de periferie werd in april 1983 de functie aanvaard als hoofd van de diagnostiek aan het Pathologisch Laboratorium van de Vrije Universiteit (hoofd: Prof.Dr. C.J.L.M. Meijer). Vanaf 1985 vond gedurende 1 dag per week detachering plaats bij de Levergroep van het Instituut voor Experimentele Gerontologie (IVEG) van IVVO-TNO te Rijswijk (directeur: Prof.Dr. D.L. Knook); hier werd het experimentele werk van dit proefschrift verricht. In december 1987 werd de overstap van Vrije Universiteit naar Universiteit van Amsterdam gemaakt vanwege het zwaartepunt maag-darm en leverziekten in het Academisch Medisch Centrum (AMC); hier is de auteur van dit proefschrift sindsdien hoofdzakelijk verantwoordelijk geweest voor de patientenzorg van de afdelingen Gastroenterologie en Hepatologie (hoofd: Prof.Dr. G.N.J. Tytgat) en Hepato-biliaire Chirurgie (hoofd: Prof.Dr. M.N. van der Heyde).