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INFLAMMATORY RESPONSES IN ALCOHOLIC LIVER DISEASE

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public discussion in the auditorium PIII, Yliopistonkatu 3, October 21th, at 10 o'clock.

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Hulluuden hämärästä siis etsi järkeä,

sillä viisauspa turhaa lie,

vaan hulluus tärkeä.

P.Mustapää

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LIST OF ORIGINAL PUBLICATIONS

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I Järveläinen HA, Oinonen T, Lindros KO. Alcohol-induced expression of the CD14 endotoxin receptor protein in rat Kupffer cells. Alcohol Clin Exp Res 1997;21:1547-1551.

II Lindros KO, Järveläinen HA. A new oral low-carbohydrate alcohol liquid diet producing liver lesions: A preliminary account. Alcohol Alcoholism 1998;33:1-7.

III Järveläinen HA, Fang C, Ingelman-Sundberg M, Lindros KO. Effect of chronic coadministration of endotoxin and ethanol on rat liver pathology and proinflammatory and anti-inflammatory cytokines. Hepatology 1999;29:1503-1510.

IV Järveläinen HA, Fang C, Ingelman-Sundberg M, Lukkari TA, Sippel H, Lindros KO. Kupffer cell inactivation alleviates ethanol-induced steatosis and CYP2E1 induction but not inflammatory responses in rat liver. J Hepatol 2000;32:900-910.

ABBREVIATIONS

ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
ALDH	aldehyde dehydrogenase
ALT	alanine aminotransferase
BAL	blood alcohol level
BuOOH	tertiary butyl hydroperoxide
CD	cluster of differentiation
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
CYP2E1	cytochrome P450-2E1
ED2	Kupffer cell –specific gene product
$GdCl_3$	gadolinium chloride
GPI	glycosyl-phosphatidyl inositol
GSH	glutathione
HBSS	Hanks balanced salt solution
HPLC	high pressure liquid chromatography
IgA	class A immunoglobulin
IL	interleukin
i.v.	intravenous
KCR	Kupffer cell -specific gene product
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MP	methylpyrazole
mRNA	messenger RNA
NAD	nicotine adenine dinucleotide
NADP	nicotine adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PMN	polymorphonuclear neutrophil
Pu-1	ubiquitous monocyte gene product
RNA	ribonucleic acid
RT	reverse transcriptase
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor

ABSTRACT

The activation of liver macrophages (Kupffer cells) by gut-derived endotoxin and the release of pro-inflammatory mediators have been recognized as crucial events in the development of alcoholic liver disease (ALD). Endotoxin in complex with its carrier protein, lipopolysaccharide binding protein (LBP), is bound mainly to the CD14 receptor on Kupffer cells. This process leads to cell activation and release of potentially cytotoxic pro-inflammatory cytokines.

To study the mechanisms by which ethanol-induced liver injury develops beyond steatosis, male rats for 6 weeks were given a modified ethanol liquid diet. In this diet, the carbohydrate content was lowered from 11% to 5.5% of calories. This low-carbohydrate ethanol diet resulted in consistently elevated diurnal blood ethanol levels and aggravated liver lesions. Liver changes after six weeks consisted of panlobular microvesicular and macrovesicular steatosis, occasional inflammatory foci, and a two- to three-fold elevations of serum liver enzyme activities. In livers from rats on regular 11% carbohydrate ethanol diet, lesions beyond the periportally-located steatosis were rare.

Considering the important role of CD14 endotoxin receptor and LBP in mediating the LPS-induced pro-inflammatory signals and the putative role of endotoxins in ALD, it was studied whether the known sensitivity to endotoxin hepatotoxicity in rats with fatty liver is associated with increased expression of these proteins. After two weeks of ethanol treatment, an increase in immunoreactive CD14 protein in isolated Kupffer cells was observed. The increase was regulated pretranslationally: there was a three-fold elevation in the hepatic level of CD14 mRNA. Hepatic mRNA expression of LBP was also increased by chronic exposure to alcohol.

To gain further understanding about the influence of gut-derived endotoxins on alcohol-induced inflammatory responses and liver injury, ethanol-treated rats were infused with endotoxin via osmotic minipumps for four weeks. Contrary to the acute situation, the ethanol-induced liver damage was not significantly enhanced by the chronic presence of endotoxin. This suggested that there was a development of tolerance to endotoxin in spite of a sustained hepatic expression of pro-inflammatory cytokines (TNF-α and IL-1β). The pro-inflammatory responses are possibly counteracted by the anti-inflammatory cytokines (IL-4 and IL-10) and by a down-regulation of CD14 and LBP.

Gadolinium chloride, which inactivates Kupffer cells, has been found to alleviate experimental ALD. We investigated the mechanism of gadolinium chloride protection after oral feeding of low-carbohydrate ethanol diet. Gadolinium was found to destroy ED2-positive Kupffer cells, that were mainly located periportally, and to significantly alleviate ethanol-induced steatosis. However, focal inflammation and the hepatic CD14 expression, which was mainly perivenous, were unaffected. Gadolinium significantly moderated the ethanol induction of CYP2E1, and this effect correlated to the degree of steatosis.

In conclusion, oral administration of a low-carbohydrate liquid ethanol diet may provide a useful model for studying early pathogenic mechanisms in alcoholic liver disease. The increase in hepatic CD14 and LBP expression suggests a new mechanism by which alcohol increases the LPS-mediated cytokine signaling by the liver macrophages, thus promoting the interaction between alcohol and endotoxins in the development of liver damage. The development of endotoxin tolerance, however, indicates that the chronic presence of endotoxin does not alone seem to be a primary

or a determinant pathogenic factor in ALD. Protection of ALD by gadolinium chloride is through counteraction of steatosis, and perivenously-dominated Kupffer cell populations expressing CD14 and releasing of pro-inflammatory cytokines are not involved in this mechanism. $GdCl_3$ moderated the ethanol-induced CYP2E1 induction, suggesting that protection by $GdCl_3$ could be, at least partly, due to less CYP2E1. The correlation between pathology and CYP2E1 induction suggests a role for this enzyme in the development of ethanol-induced steatosis.

INTRODUCTION

Alcohol abuse remains to be one of the main causes of morbidity and mortality throughout the Western world. Not surprisingly, since the liver is the site for the bulk of alcohol metabolism, most of its toxicological effects are seen in the liver. Liver disease resulting from alcohol abuse caused approximately 350 deaths among men aged 30 to 64 in Finland 1997 (Tilastokeskus, 2000). According to autopsie studies, the number of people having cirrhosis at death may be considerably higher (Savolainen *et al*. 1992). Although the pathogenesis of alcoholic liver disease (ALD) has been increasingly delineated in a number of studies, still relatively little is known about the actual mechanisms for the development and aggravation of ALD. From data obtained from autopsies in numerous epidemiological studies, it appears that between 10 % to 20 % of chronic alcoholics have cirrhosis at the time of death. The relation between the degree of alcohol consumption and risk of developing ALD is complex. There is some evidence for a threshold level of alcohol consumption above which the risk of ALD increases rapidly (Pequignot *et al*., 1978; Sørensen *et al*., 1984).

Since there is a considerable variance in individual susceptibility to the development of liver injury, other factors than alcohol abuse *per se* must clearly be involved in the liver's susceptibility to alcohol. Alcohol-induced fatty liver is thought to progress gradually to hepatitis and cirrhosis. While fatty liver is considered to be a rapid and direct result of alcohol ingestion, the evidence showing that inflammation and liver cell death can develop as a result of alcohol toxicity alone is less convincing. Numerous efforts have been directed at identifying co-existent factors interacting with alcohol, such as immunological, genetic, hormonal, or nutritional conditions, which may be involved in the progression of ALD beyond steatosis.

In recent years, it has become increasingly evident that bacteria in the intestines play a key role in the initiation of ALD. Endotoxin (lipopolysaccharide, LPS) forms an integral part of the cell wall of Gram-negative bacteria. Plasma endotoxin levels are frequently elevated in chronic alcoholics and also in experimental animals chronically receiving alcohol. The mucosal barrier is normally quite impermeable to bacterial products, but supposedly alcohol consumption increases the passage of an endotoxin through the intestinal wall into the portal blood. Upon reaching the liver, endotoxin binds to its carrier protein, LBP (lipopolysaccharide binding protein), and activates liver macrophages (Kupffer cells) through a CD14 protein on the cell membrane. This results in a local synthesis and release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and various other potentially cytotoxic products. The observation that experimental alcoholic liver injury is alleviated by oral treatment with lactobacillus or antibiotics, both of which reduce Gram-negative bacteria, supports the notion that endotoxins are involved in the pathogenesis of the disease. The facts that alcohol consumption increases cytokine levels in the liver and in blood, and that the symptoms cytokines produce in humans are similar to those seen in alcoholic hepatitis, further support the idea that endotoxin and cytokines are involved in the etiology of alcoholic liver disease.

Attempts to reproduce human ALD in animals for the study of the pathogenic mechanisms have been mainly unsuccessful. There is no animal model that is economical and practical, and that creates most of the central features seen in human ALD. In rats ethanol liquid diet administration orally does not produce liver lesions beyond steatosis and blood ethanol levels are low to moderate. High blood ethanol levels, seemingly a prerequisite for more severe lesions, can be obtained by infusing

ethanol via an intragastric cannula. In this way, other histopathological lesions, such as inflammation and necrosis, are achieved. However, this model is characterized by several side effects and complications, and is expensive to use.

The present work was designed to clarify some of the mechanisms that are involved in the progression of ALD beyond steatosis, with particular emphasis on the regulation and induction of the Kupffer cell inflammatory responses. To study these mechanisms, two rat models were developed: a low-carbohydrate ethanol liquid diet model and a model of chronic endotoxemia.

REVIEW OF THE LITERATURE

1 Pathology of ethanol-induced liver injury in man and animals

The histopathological features of alcoholic liver disease (ALD) are very diverse: virtually all forms of liver pathology may be encountered with patients with a history of chronic ethanol intake. The spectrum of human ALD includes fatty liver, hepatitis, fibrosis, and liver cirrhosis. Chronic ethanol administration to rodents has been demonstrated to lead to a number of hepatic changes including steatosis, hepatocellular necrosis, inflammatory cell infiltration, terminal hepatic venular sclerosis, proliferation of the smooth endoplasmic reticulum, and mitochondrial aberrations (Iseri *et al*., 1966; Lieber and DeCarli, 1976; Tsukamoto *et al*., 1985). All these changes also occur in the early phase of human alcoholic liver disease, demonstrating the relevance of the experimental models (MacSween and Burt, 1986; Hall, 1995). On the other hand, several other changes, such as alcoholic hepatitis, Mallory bodies, and advanced liver cirrhosis, are rarely seen in animal models, usually only in the presence of additional toxic factors or in combination with some type of nutritional deficiency.

1.1 Structural and functional organization of the liver

The structural and functional organization of the liver has been described by hepatic lobule and hepatic acinus models, respectively. The hepatic lobule is defined histologically as a hexagonal region of parenchyma which surrounds the central vein at its center. The hepatic lobules are composed of one-cell thick parenchymal cell plates, arranged radically around the central vein, thus forming sinusoidal blood spaces. According to the lobular concept, the blood flows from the periphery of the lobule, i.e., from the portal vein and hepatic artery, through sinusoids, and into the central veins.

Rappaport defined the hepatic acinus as the smallest structural and functional unit in the liver, a term based on the microcirculation in the liver. The simple hepatic acinus is defined as a parenchymal mass organized around the portal triad (Fig. 1), with the portal triad consisting of bile ductules, branches of the portal vein, and branches of the hepatic artery. Within the acinus, blood drains from the portal triad through the sinusoids into the central hepatic vein. The cells of the acinus are arbitrarily divided into three zones: the periportal zone, the intermediate zone, and the perivenular zone. The blood supply is mainly from the portal tract vessels: 80% from branches of the portal vein and 20% of the hepatic artery.

Five intrinsic cell types have been identified in the liver: the parenchymal cells or hepatocytes and four types of non-parenchymal cells. The non-parenchymal cells are the liver resident macrophages; the Kupffer cells; endothelial cells; the Stellate cells (also called Ito- or fat storing cells); and the pit cells or large granular lymphocytes. In the rat, hepatocytes represent for about 60% of the total cell number and 80% of hepatic tissue volume. Non-parenchymal cells in the rat are estimated to constitute about 30% of total cellular population but comprise only 6-7 % of tissue volume due to their small size relative to hepatocytes (Jones, 1996).

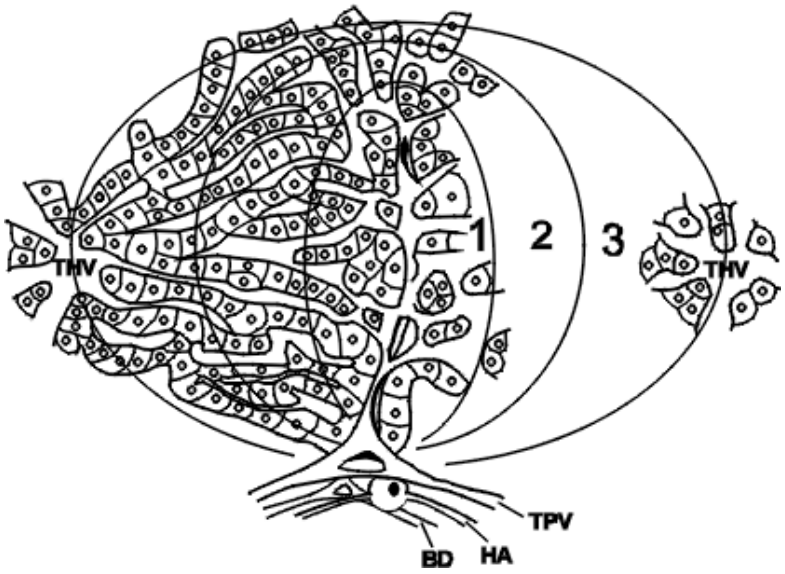

Figure 1. The hepatic acinus is the functional unit of the liver.
Blood drains from the final branches of the terminal portal venule (TPV) and hepatic arteriole (HA) in zone 1, to the terminal hepatic venule (THV) in zone 3.

1.1.1 Zonation of liver injury

Many of the histological abnormalities found in ALD are more prevalent in the perivenular region of the liver lobulus. Several theories have been proposed to identify the mechanisms responsible for this zonal nature of liver damage. The perivenular region is located furthest from the periportal area, which is the entry site for oxygen-rich blood. Accordingly, since the oxygen concentration is often lower in the tissue's downstream region, perivenous cells in the liver are potentially more susceptible to ischemic necrosis. It is suggested that after alcohol consumption, hypoxic conditions sufficient to damage hepatocytes may develop in this area. The oxygen requirement is increased during alcohol metabolism. In addition, this may be combined with its decreased availability (Mezey, 1984; Tsukamoto and Xi, 1989). Indeed, centrilobular necrosis caused by ethanol feeding can be prevented by propylthiouracil, a compound that decreases liver oxygen consumption (Israel *et al*., 1975; Orrego *et al*., 1987).

Another factor suggested to contribute to the zonation of ALD is that expression of CYP2E1, which produces oxy-radicals that may contribute to the injury, is highest in the perivenular region (Kato *et al*., 1990). Furthermore, hepatocytes in the perivenous area contain less antioxidant factors, such as glutathione, and antioxidant enzymes, such as glutathione peroxidase (Kera *et al*., 1987). Thus, while the lipid peroxidation mediated by oxy-radicals is likely to be highest in the perivenous area, the detoxifying capacity of hepatocytes here is reduced; therefore, the production may exceed the

detoxification specifically in the perivenular area. This also might be of importance with respect to liver toxicity induced by a variety of xenobiotics. In addition to ethanol, chemicals that cause liver injury specifically in the perivenous zone in hepatic lobule typically have to be preactivated by cytochrome P450 enzymes in order to cause injury (Lieber, 1997).

Some functional and morphologic heterogeneity has been described in the location of Kupffer cells in the liver acinus. The number of Kupffer cells is highest in the periportal regions. In addition, the ones here are larger, contain more lysosomes, and are more active in phagocytosis than those found in the perivenous region of the liver acinus. The smaller Kupffer cells in the perivenous area are more active in cytokine production and cytotoxicity (Bouwens *et al*., 1986; Fang *et al*., 1998).

1.2 Fatty liver

The most common histological change in alcoholic liver disease is steatosis, a rapid and predictable metabolic response to excessive alcohol consumption. It occurs very commonly in alcoholics: the reported incidence ranges from 10% to 90%. Even in the normal population, the hepatic steatosis has a high prevalence, between 6% to 24%, in most autopsy series (Hilden *et al*., 1977; Underwood Ground, 1984). Fatty liver is thought to be a result of metabolic disturbances, such as decreased fatty acid oxidation, increased triglyceride synthesis, reduced fat export, and mobilization of extrahepatic fat stores (Lieber, 1993). Fatty infiltration is usually macrovacuolar, with one large fat droplet per hepatocyte and lateral displacement of the nucleus (Ishak *et al*., 1991). The term “alcoholic foamy degeneration“, describing microvesicular steatosis, was introduced in 1983 by Uchida et al. The hepatocytes are filled with many small fat droplets (less than 1µm) surrounding centrally placed nuclei (Fromenty and Pessayre, 1995).

It was long considered that steatosis was a rather benign condition, due to its common occurrence and rapid disappearance upon ethanol withdrawal. Even in the most severe degree of fatty livers, the fat disappeared after 3 to 4 weeks of abstinence (Lieber and Rubin, 1968; Desmet, 1985; MacSween and Burt, 1986). In addition, the metabolic abnormalities in fatty liver were suggested to be insufficient to lead to inflammation (Lieber, 1994). However, later studies have indicated that the cellular changes taking place during the fatty metamorphosis may sensitize the cells to further injury. Thus, it is now suggested that the more fat in the liver, the higher the susceptibility to more severe damage (Day and James, 1998). In fact, both experimental and clinical data suggest an association between the severity of fatty accumulation and the development of damage (Sørensen *et al*., 1984; Nanji *et al*., 1989). For instance, the hepatic triglyceride concentrations are higher in baboons eventually developing cirrhosis than in those which only develop fatty liver (Lieber *et al*., 1975). In alcoholics, the severity of steatosis and the degree of the necroinflammation and fibrosis are positively correlated (Bacon *et al*., 1994; Reeves, 1996; Day and James 1998). However, the harmful effects of lipid excess in the liver are manifested only if additional stressful events occur. The fatty liver is highly vulnerable to oxidative stress or the injury mediated by endotoxin or cytokine action (Bhagwandeen *et al*., 1987; Yang *et al*., 1997; Colell *et al*., 1998). Furthermore, the sensitivity to anoxic injury is increased in alcoholic fatty liver (Caraceni *et al*., 1997).

The presence of microvesicular steatosis is especially indicative of a severe condition. The mechanisms producing microvesicular steatosis have not been

completely elucidated, but severe impairment of fatty acid beta-oxidation in mitochondria is apparent (Fromenty and Pessayre, 1995). Microvesicular steatosis is also seen in a number of other conditions involving mitochondrial aberrations. These include drug toxicity (i.e. high-dose tetracycline and valproate) and Reye's syndrome. Microvesicular steatosis is frequently associated with macrovesicular steatosis, giant mitochondria, and perivenular fibrosis.

In humans, fatty liver is first seen in the perivenous area of the liver lobule (MacSween and Burt, 1986), but this is not consistently seen in animals. In rats either perivenous, periportal, or panlobular fat accumulation is observed, depending upon dietary factors and also upon gender (Thurman *et al*., 1998). Experimental studies have clearly shown that the amount and type of dietary fat affects the development of fatty liver. Thus the degree of steatosis increases along with the long-chain fatty acid content of the diet and the degree of steatosis is decreased by medium-chain triglycerides (Lieber *et al*., 1967). The composition of the dietary fat is reflected in the composition of the lipids accumulating in the liver of ethanol-fed animals (Lieber and DeCarli, 1970). Aggravation of alcohol-induced fatty liver is also seen if the diet is low in protein or carbohydrates (Nanji *et al*., 1989; Sankaran *et al*, 1991).

1.3 Inflammation

About 10-30% of alcoholics develop acute alcoholic hepatitis, the frequency of which has been suggested to be even lower than that of alcoholic cirrhosis (Hislop *et al*., 1983; Savolainen *et al*., 1993). Alcoholic hepatitis, also called sclerosing hyaline necrosis, is a highly characteristic histological condition. The morphological pattern in man consists of the infiltration of polymorphonuclear leukocytes, hepatocyte degeneration, and necrosis. The appearance of Mallory bodies is common, but it is not considered to be an obligatory diagnostic sign (MacSween and Burt, 1986; Hall, 1995). In biopsy steatosis is almost always present with hepatitis, or, if the biopsy is delayed, the mobilization of fat may have taken place (MacSween and Burt, 1986). Alcoholic hepatitis in most cases is a reversible condition, and is not always clinically symptomatic (French and Burbige, 1979).

Hepatitis is considered to be the most important precursor to cirrhosis, the progression of disease appearing to require one or more antecedent episodes of steatohepatitis (Diehl, 1999). The presence of alcoholic hepatitis in the initial biopsy may be of prognostic significance in the progression to cirrhosis; it is estimated that about 50% of patients with hepatitis develop cirrhosis within 10 years (Sørensen *et al*., 1984). However, it is suggested that in some populations, particularly in Japan, fibrosis and cirrhosis may develop without preceding hepatitis (Takada *et al*., 1982; 1993).

The ethanol-induced inflammatory lesions seen in rodents are somewhat different. In rats the corresponding lesion is best described as an inflammatory cell infiltration rather than as hepatitis. In many studies no more than 1-2 mononuclear inflammatory foci per liver lobulus are seen in rats treated chronically with ethanol, and this occasionally is present even in the livers of controls (Nanji *et al*., 1989*b*).

There is no direct evidence that ethanol *per se* causes inflammation. The presence of fat in the liver seems to be a prerequisite to the development of inflammation, possibly because a fatty liver is more vulnerable to various factors that trigger inflammation (Day and James, 1998). For instance, there is evidence for an involvement of bacterial endotoxins and viral hepatitis (Sata *et al*., 1996; Thurman *et*

al., 1998). Furthermore, oxidative stress induced either by dietary polyunsaturated fatty acids or by iron supplementation may aggravate the inflammation (Nanji *et al*., 1994; Tsukamoto *et al*., 1995). A more pronounced inflammatory response is seen in livers from female rats than males, suggesting an immunomodulatory effect of estrogen (Thurman *et al*., 1998; Yin *et al*., 2000). Moreover, studies with rodents suggest that binge-type alcohol treatment enhances the inflammatory response in the liver (Enomoto *et al*., 1998; Bautista and Spitzer, 1999).

1.4 Fibrosis and cirrhosis

Perivenular fibrosis is considered as the first irreversible step in ALD progressing into severe fibrotic changes and eventually to cirrhosis (Worner and Lieber, 1985; MacSween and Burt, 1986). The fibrotic process is characterized by a proliferation of stellate cells (HSCs) and their transformation into myofibroblasts. Hepatic myofibroblasts are the source of both the overproduction of structural proteins that constitute liver fibrosis and of the matrix metalloproteinases which contribute to the remodeling of the hepatic parenchyma (Friedman, 1999). The pathogenesis of alcohol-associated fibrosis remains speculative. Although conclusive evidence is still lacking, Kupffer cell-derived inflammatory cytokines, such as TNF-α and TGF-β, have been implicated as the major cause for liver fibrogenesis (Britton and Bacon, 1999; Friedman, 1999). In addition, oxygen-derived free radicals, ethanol itself, and its metabolite acetaldehyde have been invoked in the stimulation of collagen synthesis (Lieber, 1991; Niemelä *et al*., 1995).

Fibrotic changes seldom appear in rats fed with ethanol and a normal diet, but they can be provoked with dietary manipulations**.** A high-fat ethanol diet marginal in choline, proteins, and vitamins has been reported to cause centrilobular fibrosis within a period of few months (French *et al*., 1988; French *et al*., 1988*b*). This was also achieved by oversupplementation of ethanol liquid diet with vitamin A (Leo and Lieber, 1983) and in studies with baboons and miniature pigs (Porto *et al*., 1989; Lieber *et al*., 1990; Niemelä, 1999)

Cirrhosis, the most severe form of alcoholic liver injury, develops only in 15-25% of alcoholics even after 10-20 years of heavy drinking (Savolainen *et al*., 1993; Becker *et al*., 1996). The most common pattern of human ALD is micronodular cirrhosis, a condition characterized by fibrosis and the conversion of the normal liver architecture into uniform-sized regenerating small nodules (Anthony *et al*., 1978; MacSween and Burt, 1986). Microscopically, the normal liver architecture is distorted by scar tissue which forms bands of connective tissue joining the periportal and perivenous areas. It is generally accepted that eventually micronodular cirrhosis will convert into cirrhosis of the macronodular type. The progression may continue even after cessation of alcohol intake (Gluud *et al*., 1987).

The production of such severe morphological changes by ethanol exposure alone in animal models has proven difficult. In rodents cirrhosis only develops if ethanol is combined with a nutritionally deficient diet, by dietary supplementation with carbonyl-iron, or by inclusion of another hepatotoxin such as carbon tetrachloride (Hall *et al*., 1991; Bosma *et al*., 1994; Tsukamoto *et al*., 1995). In baboon experiments lasting 1-8 years, 20% of animals developed cirrhosis (Lieber *et al*., 1975; Popper and Lieber, 1980; Lieber *et al*., 1990). It is of interest that this resembles the unpredictable precipitation of cirrhosis in humans.

2 Pathogenesis

2.1 Ethanol metabolism

Ethanol is removed from the body mainly by metabolism in the liver. Approximately 90% of the alcohol is metabolized in the liver, while the gastrointestinal tract, lungs, and kidneys play only a minor role (Lieber, 1994). Recent studies have also emphasized the role of bacteria in the colon that are capable of ethanol metabolism (Salaspuro, 1996). In the liver, there are three metabolic pathways involved in the clearance of ethanol. The major pathway, cytosolic alcohol dehydrogenase (ADH), catalyses reversible oxidation of ethanol to acetaldehyde. In normal conditions, it accounts for about 80-90% of ethanol oxidation. The pathway through cytochrome P450-2E1 (CYP2E1) is normally of minor quantitative importance but under situations of sustained exposure to ethanol it can be induced several-fold. The role of peroxisomal ethanol metabolism mediated by catalase is less important under physiologic conditions in humans. Ethanol oxidation may produce pathogenetic effects in several ways as a results of an altered redox state, toxic products made by the induced CYP2E1, and the direct cellular toxicity of acetaldehyde.

2.1.1 Alcohol dehydrogenase

The primary enzymes for ethanol oxidation are the cytosolic alcohol dehydrogenases (ADHs). The most important isoenzyme, class I ADH (ADH_1), has a particularly high affinity for ethanol with a K_m of approximately 0.1 to 1.0 mM. The major consequence of alcohol dehydrogenase-mediated metabolism of ethanol in the liver is the generation of excessive reducing equivalents, primarily as NADH (Forsander, 1970). The increased NADH/NAD+ ratio interfers with the capability of hepatocytes to maintain homeostasis, resulting in a number of metabolic disorders, including hepatic steatosis. This “redox shift” also predisposes the hepatocytes to hypoxic damage (Thurman *et al*., 1984), and favors events leading to oxidative stress and lipid peroxidation (Kukielka *et al*., 1994).

2.1.2 Cytochrome P450-2E1

The second system for ethanol oxidation is NADP-dependent cytochrome P-450 located mostly in the endoplasmic reticulum. The CYP2E1 isoenzyme has been well documented as the ethanol-inducible cytochrome form. However, this enzyme has a very wide substrate specificity. In addition to ethanol, it metabolizes ketones, aldehydes, aromatic compounds, nitroamines, and some carcinogens. Many substrates of CYP2E1 induce their own metabolism. Consequently, due to its inducibility ethanol metabolism by this enzyme becomes more important during long-term use of alcohol. The molecular bases for the induction of CYP2E1 are controversial. Generally, ethanol and other agents acting on CYP2E1 have little effect on the contents of CYP2E1 transcripts in the liver except at extremely high blood ethanol concentrations (above 300 mg/dl) when an additional transcriptional step may be involved (Badger *et al*., 1993). Post-translational regulation is the major mechanism

for CYP2E1 induction. A generally recognized mechanism is the decreased enzyme degradation stemming from substrate stabilization (Eliasson *et al*., 1988; Chien *et al*., 1997). In addition to ethanol and other inducing agents, conditions including diabetes, high-fat diet, and production of ketone bodies have correlated with increased expression of CYP2E1, but it has been suggested that the associated hormones, such as insulin, more likely mediate the regulation of CYP2E1 in these cases (Yun *et al*., 1992; Zangar and Novak, 1997)

Data from several studies indicate a pathogenic role for the induced CYP2E1 enzyme (Ingelman-Sundberg *et al*., 1994). Due to the particularly high NADPH oxidase activity possessed by this enzyme, reactive oxygen species (hydroxyl and hydroxyethyl radicals) are generated during ethanol metabolism. These may initiate membrane lipid peroxidation and cytokine release. Indeed, hydroxyethyl radical production *in vitro* correlates with the degree of lipid peroxidation and liver damage in experimental ALD (Albano *et al*., 1996). Consistently, two CYP2E1 inhibitors, diallylsulfate and phenethyl isothiocyanate, inhibit the progression of experimental ALD. CYP2E1 expression in the liver correlates with the ethanol-induced pathology and lipid peroxidation (Morimoto *et al*., 1995). CYP2E1 is almost exclusively expressed and induced in the area around the central vein, a localization that correlates with the predominant site of tissue damage in ALD. In alcoholics, autoantibodies are formed against CYP2E1, suggesting that this enzyme potentially acts as an autoantigen (Lytton *et al*., 1999). Induced expression of CYP2E1 in alcoholics provides an explanation for the increased susceptibility of these subjects to the hepatotoxic effects of paracetamol, vitamin A, anesthetics, chemical carcinogens, and industrial solvents.

In opposition to this evidence, a recent mouse study argued against the pathogenic significance of CYP2E1 in alcohol-induced liver disease. Similar pathological changes in the liver were observed in ethanol-treated CYP2E1 knock-out and wild-type mice (Kono *et al*., 1999). The power of this finding is, however, weakened first by the fact that hepatic expression of CYP2E1 is relatively low in these mice and second by the rather mild pathological changes observed.

2.1.3 Generation of acetaldehyde

All routes for ethanol oxidation result in the formation of acetaldehyde. Acetaldehyde is further oxidized to acetate, primarily by a low K_m aldehyde dehydrogenase (ALDH2) localized in the mitochondria. Although ALDH2 is very effective in keeping the acetaldehyde levels low, acetaldehyde generated during alcohol metabolism is widely accepted as an agent toxic to hepatocytes (Maddrey, 1995). Many *in vitro* studies have shown that acetaldehyde is potentially a highly reactive substance that has direct cytotoxicity and genotoxicity, and an ability to form protein adducts with several target proteins and macromolecules (Sorrell and Tuma, 1987; Lieber, 1993; Singh and Khan, 1995). The functional consequences of acetaldehyde-modified proteins are suggested to include mitochondrial aberrations, enzyme inactivation, depletion of hepatic antioxidant factors, and the appearance of antibodies against the adducts (Lieber, 1994; Niemelä, 1999). However, the pathogenic relevance of acetaldehyde *per se* was recently questioned in an *in vivo* experimental study. When the effects of acetaldehyde were maximized by giving two different ALDH inhibitors, disulfiram or benzocoprine together with an ethanol liquid

diet, alcoholic liver injury was not aggravated but rather diminished (Jokelainen *et al*., 1998).

2.2 Nutritional aspects

The importance of nutritional factors in the toxicity of alcohol has been a controversial issue for many years (Bunout *et al*., 1983; Lieber, 1984; Rao and Larkin, 1997). Earlier it was though that cirrhosis was primarily a nutritional disorder (Best *et al*., 1949). Although numerous studies have now shown the development of alcoholic liver damage with a nutritionally adequate diet, it is obvious that the alcohol effects in some cases are due to nutritional consequences. It is hard to define whether malnutrition plays a pathogenic role in ALD or whether it is secondary or coincidental to ALD. Malnutrition or mild nutritional deficiency is certainly common among alcoholics, since they obtain close to 50 % of their energy intake as ethanol calories that displace other nutrients; their diet often is poor, especially among lower-income and homeless alcoholics; and their intestinal uptake of vitamins and minerals often is compromised (Nazer and Wright, 1983; Salaspuro, 1993).

A main issue of controversy in the experimental field has been the reduced intake of the liquid diet after the addition of ethanol. To avoid the nutritional differences, which complicate the interpretation of hepatic changes, isocaloric pair-feeding is used. Nevertheless, despite strict isocaloric protocols and regardless of whether ethanol diet was voluntarily consumed (Lieber-DeCarli -model) or force-administered intragastrically (Tsukamoto-French -model), ethanol-fed animals often tend to gain weight less than controls (Morimoto *et al*., 1994; Korourian *et al*., 1999). Interestingly, a similar effect has been demonstrated in a human ward experiment (Pirola and Lieber, 1972). Various explanations in addition to malnutrition have been put forward for this phenomenon, including changed growth regulation and energy wastage (Pirola and Lieber, 1976; Rao *et al*., 1997).

Diets fed to experimental animals are usually based upon the recommendations of the American Institute of Nutrition (AIN, 1977; Reeves, 1997). Since minimal daily requirements for nutrients for rodents consuming alcohol may exceed those for normal animals, oversupply of certain essential nutrients might be indicated. At least the Lieber-DeCarli diet formula seems to be in itself nutritionally adequate, since ethanol-related hepatic changes are not affected by an overall reduction in liquid diet intake (Lieber and DeCarli, 1989), nor by supplementing this diet with extra proteins, essential minerals, and vitamins (Rogers *et al*., 1981; Lieber and DeCarli, 1989). Possible interactions between the diets and alcohol should, however, never be overlooked.

In contrast to malnutrition, an excess of calories leading to obesity of the animals may also be a problem. The incidence of steatosis and steatohepatitis correlates with the degree of obesity (Sheth *et al*., 1997) and genetically obese mice are also sensitive to endotoxin-induced liver injury (Yang *et al*., 1997).

2.2.1 Carbohydrates

In pair-feeding studies, control animals are usually given an isocaloric amount of carbohydrates to replace ethanol. This protocol was first developed by Lieber *et al*. (1963). Thus ethanol-fed animals receive much less carbohydrates. In terms of the deposition of triacylglycerides in the liver, the carbohydrate content rather than the fat

content seems to be critical. Increasing the dietary carbohydrate content prevents steatosis, and also some other damage (Tsukada *et al*., 1998; Yonekura *et al*., 1993). For example, if the carbohydrate content of intragastrically-fed ethanol diet is lowered from 21 to 2.5%, inflammatory changes and elevated transaminase levels are observed (Korourian *et al*., 1999). One mechanism by which carbohydrates have been suggested to prevent steatosis is accelerating the rate of ethanol elimination (Rao *et al*., 1987). It must be pointed out, however, that feeding a carbohydrate restricted control diet as such has no effect on liver morphology or fat content (Lieber *et al*., 1965).

2.2.2 Protein

Casein is the usual source of protein in liquid diets. It is reasonable low in cost and the amino acid composition is adequate except for sulfur amino acids. Therefore, it is necessary to supplement casein-based diets with cystine/cysteine or methionine. A full range of different protein contents have been used in ethanol-containing liquid diets, from a protein deficient diet (4% of calories), to an excess of proteins (25%) (Lieber and DeCarli, 1982). A protein content of 13% is considered to be nutritionally adequate for adult rats, while rapidly growing rats require 17-20% (AIN, 1977; McDonald, 1997; Reeves, 1997).

2.2.3 Fats

A high fat content in the diet and the fatty acid composition influence the development and degree of hepatic injury induced by ethanol. Thus increasing the amount of fat leads to more severe changes, including abnormalities of mitochondrial function and enhanced microsomal ethanol oxidizing capacity (Lieber and DeCarli, 1970; Wahid *et al*., 1980). Consequently, the diet protocols used in experimental ALD models include a high amount of calories as fat. Actually, this high fat diet resembles that consumed by alcoholics in the US (Mitchell and Herlong, 1986).

Several experimental ALD studies have demonstrated that the type of fat is also essential. The proportion of unsaturated fat correlates with the damage. Thus a diet low in corn oil produced less damage than a diet high in corn oil, and virtually no damage was seen if corresponding amounts of saturated fats (tallow oil or lard) were given (Nanji *et al*., 1989*b*). Even more extensive damage, particularly inflammation and necrosis, was seen when giving fish oil that is unusually rich in polyunsaturated fatty acids (Nanji *et al*., 1994). Furthermore, experimental alcoholic liver injury was shown to be reversed by a diet rich in saturated fatty acids (Nanji *et al*., 1995). These data suggests that the combined presence of ethanol and unsaturated fatty acids provokes damage via a high rate of lipid peroxidation.

Dietary fats also modify the ethanol induction of CYP2E1, which is induced by polyunsaturated fatty acids (Yoo *et al*., 1991). The combined exposure of ethanol and unsaturated fatty acids leads to an unusually high induction of the ethanol-metabolizing CYP2E1 enzyme, supporting the notion of a pathogenic role of CYP2E1 in ALD (Takahashi *et al*., 1992; Morimoto *et al*., 1993). Dietary fatty acids have also been shown to modulate macrophage function. Saturated fatty acids suppress phagocytosis (Morrow *et al*., 1985), and eicosapentaenoic acid suppresses inflammatory cytokine production (Billiar *et al*., 1988). Medium-chain triglycerides (saturated fat) also attenuate alcohol-induced inflammatory reactions and liver injury,

possibly by altering CD14-regulated signaling pathway in Kupffer cells (Nanji *et al*., 1997; Kono *et al*., 2000).

2.2.4 Micronutrients

Several changes have made over several years in the composition of the ethanol liquid diet with respect to various vitamins (e.g. vitamin A, Bs, D, E, K), minerals, and trace elements (Lieber and DeCarli, 1989). All commercial diets usually contain adequate amounts of these elements as well as of macronutrients.

The antioxidant capacity of the liver is of particular importance in situations which involve enhanced lipid peroxidation, which is especially marked with a high intake of polyunsaturated fatty acids (Pawlosky *et al*., 1997). Hepatic vitamin E stores are reduced if alcohol is given with a vitamin E deficient diet, leading to accumulation of lipid peroxidation products and increased serum ALAT activity (Sadrzadeh *et al*., 1994). However, high-dose vitamin E supplementation does not reverse alcohol-induced histopathological changes (Sadrzadeh *et al*., 1995).

Vitamin A deficiency orits reduced bioavailability, as well as its excess, can all aggravate alcoholic liver injury (Leo and Lieber, 1983; Lieber and DeCarli, 1989). Thus, in designing diet composition, it is important to keep in mind that the therapeutic window for vitamin A is relatively narrow (Ainley *et al*., 1988).

Choline has received special attention in the pathogenesis of ALD. Choline deficiency in itself causes fatty liver and fibrosis in rodents, and alcohol enhances the choline requirement (Rogers *et al*., 1981). Liver injuries of several etiologies are augmented if the diet is poor in choline (Eastin *et al*., 1997). Severe folate deficiency may secondarily aggravate these changes (Kim *et al*., 1994).

An important goal in experimental studies on ALD is to develop more specific treatment methods. For example, S-Adenosyl-l-methionine (SAM) enhances the synthesis of glutathione, a nonprotein thiol crucial in antioxidant defense (Lu, 1998). Ethanol-induced depletion of glutathione is reversed by administration of SAM in baboons, and recent findings suggest that it may also increase survival in patients with liver disease (Lieber, 1999; Mato *et al*., 1999). Promising results in counteracting the fibrotic process have been achieved with the baboon model. Administration of fatty acid lecithin or its main component phosphatidylcholine may afford protection against alcoholic fibrosis and cirrhosis (Lieber, 1999).

2.3 Inflammatory responses

Inflammation is the body's response to infection or local tissue damage. In most situations it is a protective mechanism that prevents the spread of injury and mobilizes the defense mechanisms of the immune system. However, prolonged and excessive inflammation is a pathological process that may lead to the aggravation of tissue damage. With respect to pathogenesis, many features of alcoholic liver injury are typical of immune-mediated diseases. These include an absence of a clear dose-effect relationship and a remarkable variation in individual susceptibility. Furthermore, in alcoholic hepatitis damage is known to progress even after cessation of alcohol ingestion. As with many other immune-mediated diseases, women are more susceptible to ALD than men. Indeed, a large number of immunological changes have been observed to occur in alcoholic liver disease and also after consumption of alcohol. Recently, the activation of inflammatory cytokine cascades has been

recognized as a central process in the progression of ALD (Thurman *et al*., 1998). It is now becoming evident that gut-derived endotoxins promote inflammatory responses via activation of the resident liver macrophages, Kupffer cells.

2.3.1 Role of Kupffer cells

The mononuclear phagocyte system consists of peripheral tissue macrophages, blood monocytes, and their bone marrow precursors. The most differentiated cells in the system, tissue macrophages, are also functionally the most active ones. Characteristic features for macrophages include their long life span, phagocytic capacity, lysosomes and endocytic vacuoles, and an ability to produce a variety of mediators such as cytokines and prostaglandins. Kupffer cells are the largest population of resident macrophages, constituting more than 80% of the body's total phagocytic activity. They are located in the hepatic sinusoids, where their primary role is the phagocytic clearance of the blood from the portal circulation.

A number of studies have shown that the activation of Kupffer cell is a key event in the initiation and perpetuation of inflammation in liver injury of various etiologies (Rosser and Gores, 1995; Lichtman and Lemasters, 1999). Selective inhibition of Kupffer cells by administration of either gadolinium chloride or methyl palmitate results in abrogation of hepatic injury in many models. Conversely, priming of these cells, for example, by *Propionibacterium acnes*, augments hepatic injury (Laskin *et al*., 1997). Kupffer cells may be activated by gut-derived endotoxins, inflammatory signals, oxidative stress, and hypoxia. In addition to their increased phagocytic capacity, activated Kupffer cells are a source of considerable amounts of biologically active products, including proinflammatory cytokines (TNF-α, IL-1, IL-6), prostaglandins (particularly PGE_2), and reactive oxygen intermediates, such as hydrogen peroxide and superoxide (Smedsrød *et al*., 1994). All these products play important roles in the host's defense against bacteria, but at the same time, they can be detrimental to the tissue locally. Kupffer cells also participate in recruitment and activation of other inflammatory cells, such as monocytes and PMNs, which produce additional injurious effects (Winwood and Arthur, 1993).

Results from several investigations suggest that Kupffer cells are involved in the etiology of ALD. Both the acute and chronic consumption of alcohol affect many Kupffer cell functions, such as cytokine production, phagocytosis, bactericidal activity, and opening of Ca^{2+} channels (Earnest *et al*., 1993). The administration of gadolinium chloride has been reported to prevent the experimental ALD produced by intragastric ethanol administration (Adachi *et al*., 1994). Nimodipine, which prevents Ca^{2+} channel opening essential for Kupffer cell activation, also decreases the extent of experimental ALD (Kawada *et al*., 1992; Iimuro *et al*., 1996).

2.3.1.1 Modification of Kupffer cell function by gadolinium chloride

A trivalent cation of the lanthanide series, gadolinium chloride ($GdCl_3$), is commonly used to deplete Kupffer cells. When rats are pretreated with $GdCl_3$, they are protected from the hepatotoxic effects of endotoxin, paracetamol, carbon tetrachloride, galactosamine, and diethyldithiocarbamate (Towner *et al*., 1994; Ishiyama *et al*., 1995; Laskin *et al*., 1995; Liu *et al*., 1995). In addition, gadolinium chloride has been shown to prevent the progression of ethanol-related liver injury in

rats after chronic intragastric ethanol feeding (Adachi *et al*., 1994). The mechanisms causing the protection, however, are poorly defined. It has been suggested that gadolinium chloride acts by displacing Ca^{2+} ion in the extracellular side of the calcium channel, thus blocking calcium influx and efflux (Hambly and dos Remedios, 1977; Pillai and Bikle, 1992; Roland *et al*., 1999). This is supported by experiments in which chelating agents prevent the blockage of calcium channels (LeGuennec *et al*., 1996). In addition, intrasynaptosomal free Ca^{2+} levels are dose-dependently inhibited by gadolinium (Romano-Silva *et al*., 1994).

Gadolinium chloride is soluble in acidic conditions, but becomes aggregated in the blood stream due to its neutral pH. Microscopic examinations have shown that i.v. administered $GdCl_3$ is largely taken up by Kupffer cells (Husztik *et al*., 1980). Gadolinium chloride inhibits Kupffer cell phagocytosis *in vivo* (Husztik *et al*., 1980), impairs Kupffer cell antigen presentation and generation of reactive oxygen species (Roland *et al*., 1993; Liu *et al*., 1995), and reduces mortality in sepsis (Iimuro *et al*., 1994; Vollmar *et al*., 1996). Moreover, alterations in Kupffer cell phenotypes have been reported. $GdCl_3$ downregulates the expression of ED1, ED2, and the Kupffer cell-specific lectin-binding receptor (KCR), and abolishes peroxidase activity; but the expression of the monocyte-macrophage specific gene product Pu-1 remains unchanged (Hardonk *et al*., 1992; Kohno *et al*., 1997; Koop *et al*., 1997). While the Kupffer cells in the periportal region may be even completely eliminated, gadolinium chloride seem to be ineffective in a subpopulation of Kupffer cells that probably contribute to the proinflammatory cytokine production (Hardonk *et al*., 1992; Rai *et al*., 1996; Ahmad *et al*., 1999). The *in vitro* secretory pattern of isolated Kupffer cells changes after *in vivo* $GdCl_3$, with a decrease in IL-10 and PGE_2 release, and an increase in proinflammatory cytokine TNF-α and IL-6 release (Rai *et al*., 1996). It is intriguing that while gadolinium chloride reduces mortality in sepsis, it does not decrease the level of TNF-α, which is supposed to be the proximal mediator of most of the events in sepsis (Iimuro *et al*., 1994).

2.3.2 Polymorphonuclear neutrophils

Histologically, alcoholic hepatitis in humans is characterized by an infiltration of polymorphonuclear neutrophils (PMNs) into liver tissue. Influx and activation of neutrophils to the focal sites of injury are thought to be pivotal events in the development of hepatocyte damage during inflammation. Neutrophils in a stimulated state are able to release large amounts of proteolytic enzymes and reactive oxygen intermediates (Liu *et al*., 1994). Experimental evidence suggests that polymorphonuclear neutrophils aggravate injury, while their depletion prevents it (Molnar *et al*., 1998). In rat models of alcoholic liver disease, however, the neutrophil infiltration is generally sparse.

2.3.3 Endotoxin

Endotoxin, also known as lipopolysaccharide (LPS), represents the major component of the outer cell wall membrane of all gram-negative bacteria. It forms a hydrophobic barrier that restricts entry of many substances, such as antibiotics, into the cell. This glycolipid is responsible for many of the pathophysiological effects of these bacteria. Endotoxin can be divided into three structural regions: i.e., the O-specific side chain, the core polysaccharide, and the lipid A component. The O-chain,

characterized by repeating polymers of oligosaccharides, is highly variable and antigenic, and is structurally unique for a given bacterial serotype. The core region is structurally less variable and can be divided into the outer and inner core. The major bioactive component of endotoxin resides in the lipid A portion. It is the most conserved part of endotoxin, and includes two glycosamine sugars modified by phosphate and a variable number of fatty acids. Using purified and synthetic preparations of lipid A, it was demonstrated that lipid A is responsible for most of the effects of endotoxin (Shiba *et al*., 1984; Galanos *et al*., 1985).

Endotoxemia, i.e., the excessive presence of endotoxin in blood, is associated with a number of pathophysiological alterations both in experimental animals and in humans. During severe Gram-negative infections, a large amount of endotoxin may cause a condition called endotoxic shock. It is a result of an overwhelming inflammatory response by endotoxin-activated cells, which results in tissue destruction, respiratory distress, profound hypotension, intravascular blood coagulation, and if not reversed, death. The main target organ for endotoxin is the liver. After i.v. administration, 80-90% of endotoxin is taken up rapidly by Kupffer cells, where it is internalized and subsequently modified (Ruiter *et al*., 1981).

The human intestine contains about 10^{14} bacteria, many of them Gram-negative, providing a huge amount of potentially proinflammatory organisms (Goldin, 1990). In normal situations, bacteria continuously translocate through the intestinal wall in low numbers, but these bacteria are readily detoxified by lymphoid organs, such as mesenteric lymph nodes, and eventually by Kupffer cells. When the host immune defense or liver function is impaired, endotoxins may spill over into the peripheral blood stream. Alternatively, the release of endotoxin into circulation is enhanced by proliferation of flora or when bacteria die or lyse, for example, from antibiotics (Evans and Pollack, 1993). In these situations the increased production of endotoxin exceeds the clearance capacity of reticuloendothelial system.

Endotoxin has been shown to be a pivotal cofactor in the development of liver diseases. Experimental cirrhosis in rats due to choline-deficiency can be prevented by sterilizing the gut with antibiotics (Nolan, 1989). The presence of endotoxin is also essential for the acute liver injury associated with exposure to carbon tetrachloride (CCl_4) and D-galactosamine. For example, D-galactosamine toxicity is prevented if the small bowel and colon, the large reservoirs of endotoxin, are resected (Galanos *et al*., 1979). Polymyxin B, which has direct anti-endotoxin properties, protects against CCl_4 toxicity (Nolan and Leibowitz, 1978). However, gentamicin, which does not interact with endotoxin, has no protective effect (Nolan and Leibowitz, 1978). Hepatotoxin sensitivity often correlates with endotoxin sensitivity, as shown by studies with both endotoxin resistant (strain C3H/HeJ) and endotoxin sensitive (strain C3H/HeN) mice (Freudenberg and Galanos, 1991; Essani *et al*., 1995).

Alcoholics, especially those with liver disease, regularly have systemic endotoxemia (Bode *et al*., 1987). The serum IgA anti-lipid A titers from patients with ALD are elevated as an indication of bacterial translocation (Nolan *et al*., 1986). This may be promoted by multiple, synergistic mechanisms. Alcohol consumption may lead to intestinal bacterial overgrowth, or may increase intestinal permeability. Endotoxemia may also occur as a result of the liver's reduced capacity to clear endotoxin from the portal circulation. Experimentally, elevation of portal endotoxin has been detected 2 hrs after an alcohol challenge (Enomoto *et al*., 1999). Systemic endotoxin levels begin to increase 1-2 weeks after the addition of ethanol to the diet in rats, and a correlation between blood endotoxin and pathology is observed (Nanji *et al*., 1993; Adachi *et al*., 1995). The pathogenic importance of gut-derived endotoxin is

supported by experimental studies in which ethanol-induced liver injury is alleviated by administration of antibiotics or *lactobacillus*, both of which reduce endotoxin in the gut (Nanji *et al*., 1994*b*; Adachi *et al*., 1995).

2.3.4 CD14 endotoxin receptor

Endotoxin is not cytotoxic and does not evoke its responses directly. Instead, tissue toxicity results from the synthesis and release by endotoxin-activated cells of various mediators. Studies by Wright *et al*. (1990) demonstrated that CD14 protein functions as a receptor for LPS and for its carrier, the LPS-binding protein (LBP). CD14 is a 55 kDa myeloid membrane glycoprotein, expressed mainly by monocytes and macrophages, and at low levels also on the surface of polymorphonuclear leukocytes (Antal-Szalmas *et al*., 1997; Kitchens, 2000). Upon binding to the CD14 receptor, endotoxin forms a complex with the lipopolysaccharide binding protein (LBP) that promotes features of cell activation, such as cytokine production and the generation of oxygen species (Schumann *et al*., 1990). The association of CD14 to cell activation has been shown in studies in which transgenic mice that overexpressed CD14 protein exhibited hypersensitivity to endotoxin (Ferrero *et al*., 1993), while CD14 knock-out mice were resistant to it (Haziot *et al*., 1996). Furthermore, antibodies against CD14 are able to block binding of LPS to macrophages as well as cytokine production (Wright *et al*., 1990).

The inhibition of LPS-induced cytokine release by an antibody to CD14 is most pronounced at low LPS concentrations, and is only partial at higher LPS concentrations (Kitchens, 2000). A possible explanation for this observation is that cell stimulation occurs at high, possibly supraphysiological, LPS concentrations via both CD14-dependent and independent pathways (Su *et al*., 1995). LPS is also bound to some extent by other membrane proteins, such as members of the CD11/CD18 family and scavenger receptors, but a clear association to cell activation has been demonstrated only with CD14.

Comparatively little is known about intracellular signaling pathways after binding of LPS to CD14. CD14 is anchored to the cell surface by linkage to glycosyl-phosphatidyl inositol, and lacks the intracellular protein sequence required for signal transfer (Ulevitch and Tobias, 1995). It seems that many endotoxin-induced CD14-dependent effects are mediated via an early tyrosine kinase-dependent protein phosphorylation step (Dong *et al*., 1993; Weinstein *et al*., 1993). The next step is the activation of the transcription factor, NF-κB. This results from phosphorylation and degradation of an inhibitor protein, I-κB. The release of NF-κB from I-κB permits translocation of NF-κB into the nucleus, where it binds to DNA enhancer motifs and regulates the transcription of a variety of genes, for example, those of proinflammatory cytokines (Chen *et al*., 1999).

Although the tyrosine kinase step is dependent upon CD14, the mechanisms by which binding of LPS to CD14 leads to activation of tyrosine kinases remains unknown, since CD14 does not have intrinsic tyrosine kinase activity (Liu *et al*., 1994*b*). This has led to the hypothesis that another membrane protein(s) is (are) also involved as a co-receptor. New evidence indicates that TLR4, a recently cloned member of the human transmembrane Toll-like receptors, may be the endotoxin co-receptor (Chow *et al*., 1999; Hoshino *et al*., 1999). In vitro transfection with the TLR4 gene conferred endotoxin responsiveness in cells, and this was augmented by addition of CD14.

A soluble form of receptor, sCD14, has also been found in humans at a plasma concentration of about 2 to 6 μg/ml (Öesterreicher *et al*., 1995; Burgmann *et al*.,

1996). The soluble form is produced by shedding the cell surface form, which results in an approximately 48 kDa molecule (Haziot *et al*. 1988; Labeta et al., 1993). The biological functions of sCD14 are not yet fully elucidated. *In vitro* studies show that sCD14 can bind to LPS, and these complexes are able to stimulate non-myeloid cell types, such as endothelial cells and some epithelial cells, that do not express membrane CD14. This stimulation is thought to occur through an unidentified receptor, leading to the cell activation (Pugin *et al*. 1995; Tapping and Tobias, 2000). Alternatively, it was proposed that sCD14 may function by neutralizing endotoxins, by inhibiting endotoxin binding to monocytes or by delivering endotoxin to high-density lipoprotein particles and phospholipid vesicles (Maliszewski, 1991; Wurfel *et al*., 1995).

In addition to endotoxin, CD14 recognizes a set of microbial ligands with similar structural features. Indirect studies have suggested that CD14 acts as a receptor not only to the Lipid A of endotoxin but also to certain bacterial envelope components found in Gram-positive bacteria. These include peptidoglycan, lipoteichoic acid and phosphatidylcholine (Yu *et al*., 1997; Dziarski *et al*., 2000). In this respect, CD14 functions as a "pattern receptor" of the innate immune system. However, the *in vivo* significance of these structures is doubtable, since up to 1000-fold higher concentrations of peptidoglycan, for example, than LPS may be required (Hamann *et al*., 1998).

Kupffer cell CD14 protein expression is normally low, but increases in various conditions, such as in cholestasis, and in acute and chronic active hepatitis (Tomita *et al*., 1994; Tracy and Fox, 1995). The level of CD14 expression, originally defined as a myeloid differentiation marker, also depends upon the stage of cell maturation (Trinchieri *et al*., 1987; Pan *et al*., 1999). Mouse Kupffer cell and human monocyte CD14 have been shown to be upregulated by LPS, suggesting that the proinflammatory response could be enhanced by this mechanism. The importance of the CD14-dependent pathways in the development of liver damage has been confirmed in studies, in which the endotoxin-induced hepatocellular necrosis and hepatitis are alleviated in CD14 knock-out mice (Woltmann *et al*., 1999).

2.3.5 Lipopolysaccharide binding protein (LBP)

Endotoxin in circulation forms complexes with the lipopolysaccharide binding protein (LBP), which binds to the lipid A portion of LPS with a high affinity (Tobias *et al*., 1989). Transfer of endotoxin to CD14 on the membranes of macrophages is mediated by this protein, cell activation being 100 to 1000 more efficient in the presence of LBP (Heumann *et al*., 1992; Hailman *et al*., 1994). Especially at low endotoxin concentrations, LBP has a pivotal role in the target cell endotoxin recognition. LBP is synthesized by hepatocytes as a 50 kDa polypeptide, and is released after glycosylation into the plasma in the form of a 60 kDa protein (Ramadori *et al*., 1990; Schumann *et al*., 1990). The amino-terminal half of LBP is responsible for the specific binding to LPS, while the carboxyl-terminal half of the protein interacts with CD14 (Kirkland *et al*., 1993; Han *et al*., 1998). LBP has many properties of a type I acute phase protein, since the serum levels rise substantially during a stress response (Grube *et al*., 1994). The synthesis of LBP by hepatocytes is upregulated during endotoxemia and inflammation, for example, by cytokines IL-6 and TNF-α (Grube *et al*., 1994; Geller *et al*., 1993). The importance of LBP in the pathophysiological events of sepsis is indicated in many studies. LBP knock-out mice

are unable to combat intraperitoneal Gram-negative infections (Jack *et al*., 1997). Depletion of serum LBP by anti-LBP monoclonal antibodies suppressed the binding of LPS to monocytes (Heumann *et al*., 1992). Furthermore, the administration of anti-LBP antibodies *in vivo* prevents lethality in endotoxemia (Gallay *et al*., 1993).

2.3.6 Cytokines

One of the major responses to endotoxin *in vivo* is the rapid production and secretion of soluble mediators of inflammation, cytokines. Cytokines are low-molecular (less than 50 kDa) polypeptides, produced mainly by the monocyte-macrophage lineage, the major population of which are Kupffer cells. Especially during endotoxemia, Kupffer cells are thought to be the most important contributor to the overall cytokine production. In addition to macrophages, several cytokines are also produced to some extent by lymphocytes, endothelial cells, and Ito cells.

The clarification of the specific roles and functions of each cytokine is difficult because of their large number and their overlapping or synergistic activities. Cytokines are needed in the immune defense against microorganisms, and to combat localized infections. They also have, however, been increasingly implicated in the pathogenesis of various forms of liver injury. Cytokines can be divided into those that mainly induce inflammatory and immune mechanisms (proinflammatory cytokines, such as TNF-α, IL-1, IL-8) and those that have the capacity to inhibit them (anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and TGF-ß).

2.3.6.1 Proinflammatory cytokines

Tumor necrosis factor alpha (TNF-α) is recognized as the initial and the most important mediator of many inflammatory processes. Administration of recombinant TNF-α to experimental animals or humans mimics several of the responses attributed to endotoxin itself, such as symptoms of the sepsis syndrome, including fever, anorexia, muscle wasting, and neutrophilia (Remick *et al*., 1987). Antibodies against TNF-α are able to protect against the lethality of either endotoxin or gram-negative bacteria (Beutler *et al*., 1985; Tracey *et al*., 1987).

TNF-α was originally identified as a macrophage-derived factor that caused hemorrhagic necrosis in murine tumors (Carswell *et al*., 1975). Later a 17 kDa protein was isolated that caused severe wasting in infected animals; it was termed cachectin (Beutler and Cerami, 1986; Tracey *et al*., 1988). TNF-α is unique among proinflammatory cytokines due to its capacity to induce cell necrosis and apoptosis directly (Leist *et al*., 1994; Wang *et al*., 1995). Secreted monomers of TNF-α are thought to associate to form a biologically active trimer. It can bind to either of the two TNF-α receptors expressed on the target cells, TNF-R1 (55 kDa) or TNF-R2 (75 kDa). There is no homology between the intracellular parts of the two receptors, suggesting that the signal transduction pathways are different (Fiers, 1991). TNF-R1 has been recognized to be responsible for most of the cellular effects of TNF-α, such as cytotoxicity, stimulation of cytokine synthesis and upregulation of various cell surface molecules (Tartaglia *et al*., 1993). The TNF-R1–deficient mice are resistant to lethal endotoxin shock (Pfeffer *et al*., 1993). TNF-R2 signaling seems to be restricted mostly to T-cell proliferation (Grell *et al*., 1998).

Many studies have suggested a role for proinflammatory cytokines, especially TNF-α, in the development and progression of alcoholic liver disease. Several of the

clinical manifestations of ALD resemble the biological effects following the *in vivo* administration of proinflammatory cytokines, suggesting that cytokines contribute to the clinical complications and to the liver injury (McClain *et al*., 1999). Indeed, elevated serum levels of cytokines, such as TNF-α, are observed in acute hepatitis patients, or even in alcoholics without liver disease (Bird *et al*., 1990; Khoruts *et al*., 1991). The serum concentrations of TNF-α correlate with the severity of liver injury in patients with alcoholic hepatitis and are also a significant predictor of its clinical outcome (Felver *et al*., 1990; Rodriguez-Rodriguez *et al*., 1995).

A number of animal experiments studying the involvement of TNF-α in alcoholic liver disease have been performed. Induction of hepatic mRNA expression of TNF-α is evident in experimental alcoholic liver disease (Kamimura and Tsukamoto, 1995). Moreover, the incidence of an increase in hepatic mRNA is associated with the development of ethanol-induced liver injury (Nanji *et al*., 1994*c*). The importance of TNF-α as a proximal mediator of ethanol-induced liver damage has been demonstrated in rats in which administration of TNF-α antibodies prevented liver necrosis and inflammation, but interestingly not steatosis (Iimuro *et al*., 1997). Knockout mice lacking TNF-R1 receptors are protected from inflammatory responses associated with chronic ethanol administration (Yin *et al*., 1999). However, ethanol does not necessarily promote TNF-α production directly. The addition of ethanol to monocyte cultures inhibits TNF-α production (Basista *et al*., 1993).

In addition to TNF-α, proinflammatory cytokines IL-1 and IL-6, and the chemokine IL-8 are considered to be important mediators in alcoholic liver disease (McClain *et al*., 1999). Each one may enhance the effects of the others and act synergistically. Although interleukin-1 (IL-1) appears to participate in the pathogenesis of ALD, it does not seem to cause liver injury itself but rather acts in a synergistic fashion with TNF-α (McClain *et al*., 1999). As with TNF-α, alcoholic hepatitis patients show increased plasma levels of IL-1 and an enhanced endotoxin-induced release of IL-1 by monocytes (McClain *et al*., 1986; Bird *et al*., 1989; Khoruts *et al*., 1991). IL-1 exists as two distinct and separate gene products, IL-1α and IL-1β, both of which are produced by activated monocytes and macrophages (Roux-Lombard, 1998).

2.3.6.2 Anti-inflammatory cytokines

To counterbalance the overshooting inflammatory processes, the effects of proinflammatory cytokines are endogenously modulated by anti-inflammatory mediators. Thus the presence of proinflammatory cytokines does not necessarily imply that inflammatory processes are occurring in body. It seems to be important to characterize the balance between both proinflammatory and anti-inflammatory cytokines in order to assess the nature of inflammatory responses. A number of cytokines have inhibitory actions on a wide range of monocyte-macrophage functions. These include interleukins 4, 10, and 13 and transforming growth factor beta (TGF-β) (Opal and DePalo, 2000).

IL-10 is considered to be the most potent anti-inflammatory molecule: it inhibits the production of TNF-α and IL-1 and suppresses the activation of NF-kB (Moore *et al*., 1993; Burger and Dayer, 1995; Chernoff *et al*. 1995). In addition, it reduces macrophage production of nitric oxide and reactive oxygen intermediates (Bogdan *et al*., 1991; Cenci *et al*., 1993), eicosanoid synthesis (Niiro *et al*., 1994), expression of

adhesion molecules (Willems *et al*., 1994) and chemokine synthesis (Kasama *et al*., 1994; Wang *et al*., 1994).

Interleukin-10 is not normally expressed in the liver, but its synthesis by Kupffer cells is upregulated early in the course of liver injury and by endotoxin and proinflammatory cytokines (Knolle *et al*., 1995; Nanji *et al*., 1999). The ability of endogenous IL-10 to protect against hepatotoxicity has been shown in several models of experimental liver injury, including those induced by endotoxin and galactosamine, and also in hepatic ischemia/reperfusion injury (Louis *et al*., 1997; Yoshidome *et al*., 1999). Furthermore, IL-10 knockout mice developed enterocolitis and colon cancer spontaneously with aberrant cytokine production (Berg *et al*., 1996).

In addition to its pivotal role in fibrogenesis, TGF-β antagonizes specific effects of proinflammatory cytokines and has multiple other effects as a negative immunoregulatory agent (McCarthy, 1994; McCartney-Francis *et al*., 1998). It is expressed mainly by Kupffer cells and activated stellate cells (De Bleser *et al*., 1997). Hepatic TGF-β expression is increased in liver biopsies from patients with liver disease, and in isolated Kupffer cells after 10 weeks of ethanol treatment with the intragastric ethanol feeding model (Kamimura *et al*. 1995).

2.3.7 Priming, sensitization, and tolerance to endotoxin

For endotoxin-induced liver damage to develop, it is considered that priming and/or sensitization to the effects of endotoxin is required (Tracey and Cerami, 1993; McClain *et al*., 1999). Primed inflammatory cells release massive amounts of TNF-α and other cytokines in response to stimuli. Not only Kupferr cells but also the target cells – the hepatocytes – must be sensitized to injury. Hepatocytes are normally resistant to the cytotoxic effects of TNF-α, but they become susceptible to normally innocuous amounts of TNF-α injury by mechanisms termed “sensitization” (Tiegs *et al*., 1989; Shedlofsky *et al*., 1991). TNF-α has been shown to be cytotoxic in a dose-dependent fashion to sensitized Hep G2 cells (Hill *et al*., 1995). However, repeated endotoxin confrontation leads to an attenuation of almost all pathophysiological effects that are mediated by endotoxin and proinflammatory cytokines, producing a state called endotoxin tolerance (Ziegler-Heitbrock, 1995).

2.3.7.1 Priming

One way to prime Kupffer cells is by pretreatment with *Corynebacterium parvum* (Smith *et al*., 1993). As a result, the production of TNF-α and IL-1 is strongly enhanced in response to endotoxin challenge. Chronic ethanol may have a priming effect on monocytes and macrophages. Monocytes isolated from patients with ALD have been observed to have an enhanced release of TNF-α compared to healthy controls (McClain and Cohen, 1989). Kupffer cells isolated from ethanol-treated rats exhibited enhanced TNF-α release in response to endotoxin (Enomoto *et al*., 1998). In livers of chronically ethanol-treated rats, enhanced transcriptional activity and serum levels of cytokines are seen in response to an acute endotoxin challenge (Honchel *et al*., 1992; Hansen *et al*., 1994; Pennington *et al*., 1997).

2.3.7.2 Sensitization

Sensitization occurs via several discrete mechanisms that culminate to the defective adaptation to oxidative stress and inflammatory factors, such as TNF- α (Hansen *et al*., 1994; Colell *et al*., 1998). Classic examples of hepatocyte sensitization to TNF-α involve protein or mRNA synthesis inhibitors cycloheximide or D-galactosamine (Hill *et al*., 1995). Mice treated with D-galactosamine become sensitive to the lethal effects of submicrograms of endotoxin or TNF-α (Galanos *et al*., 1979; Lehmann *et al*., 1987). Prior administration of anti-TNF-α antibodies attenuated histological changes in the liver and serum transaminase activity caused by galactosamine/endotoxin administration (Hishinuma *et al*., 1990; Fiedler *et al*., 1992). It has been postulated that sensitized hepatocytes failed to make the protective antioxidant enzymes that are necessary to withstand cytotoxicity to TNF-α (Wong *et al*., 1989; Leist *et al*., 1994). In addition, TNF-R1 upregulation has also been suggested as a mechanims for hepatocyte sensitization (Nagaki *et al*., 1999).

A phenomenon resembling hepatocyte sensitization is also seen during chronic alcohol exposure. Fatty livers from chronically ethanol-fed rats are extremely susceptible to endotoxin: a single minimal dose of endotoxin induced inflammation and fulminant necrosis that were not seen in rats fed control diet (Bhagwandeen *et al*., 1987). Furthermore, fatty hepatocytes isolated from chronically alcohol-treated rats have been demonstrated to become sensitive to TNF-α -induced cell death *in vitro*, in a similar way as to LPS *in vivo* (Pennington *et al*., 1997; Colell at al.,1998). The mechanisms by which ethanol increases cell death caused by TNF-α may be partly explained in terms of selective reduction of hepatic mitochondrial GSH (Colell *et al*., 1998).

2.3.7.3 Tolerance

In endotoxin tolerance, various responses of Kupffer cells to endotoxin are downregulated and lethality is reduced (Ziegler-Heitbrock, 1995). Tolerance may be induced by a single or repeated injections of endotoxin or recombinant TNF-α (Patton *et al*., 1987; Schade *et al*., 1995). Two distinct phases of tolerance are recognized. Early endotoxin tolerance, in which monocytes/macrophages play a primary role, develops within 24h of the first endotoxin challenge. It is dependent upon lipid A and is characterized by an attenuation of increase in proinflammatory cytokine levels upon a second endotoxin challenge (LaRue and McCall, 1994; Yoza *et al*., 2000). In addition, secretion of anti-inflammatory cytokine IL-10 and production of PGE_2 are enhanced (Hafenrichter *et al*. 1994). Early endotoxin tolerance disappears within 12 days after the initial LPS exposure (Sanchez-Cantu *et al*., 1989; Ziegler-Heitbrock, 1995). The late phase of the tolerance is mediated by formation of antibodies specific for the O-antigen polysaccharide side chain, and lasts from weeks to months. In endotoxin tolerance, target cells also become less vulnerable, since TNF-α -susceptible cell lines can be made TNF-α -resistant *in vitro* by pre-exposures to TNF-α (Zimmerman *et al*., 1989; Imanishi *et al*., 1997).

Suppression of the immune system is characteristic for alcohol under certain circumstances (Spitzer and Bautista, 1993; Szabo, 1999). The conditions by which alcohol inhibits inflammatory responses and how this is related to immune activation are yet not clear. Especially in acute alcohol intoxication, an attenuation of endotoxin-induced cytokine and superoxide production has been observed (Spitzer and Bautista,

1998). The plasma ethanol concentrations show an inverse correlation to the endotoxin-induced peak TNF-α activities (D'Souza *et al*., 1989). In fact, acute alcohol may even protect the liver from the hepatotoxicity of LPS (Bautista and Spitzer, 1996). These effects have a highly time-dependent nature. Kupffer cells were shown to be in a hyporeactive state during the first hours after ethanol administration, as the LPS-induced TNF-α -release is diminished (Enomoto *et al*., 1998). However, after 24 hours, Kupffer cell were in a stimulated state as shown by enhanced TNF- α and Ca^{2+} release, and induced CD14 expression. All these effects were blunted by antibiotics to sterilize the gut, demonstrating that endotoxin, but not ethanol *per se*, stimulates the cytokine production.

2.4 Oxidative stress and hepatic antioxidant defense

2.4.1 Generation of reactive oxygen species

Oxidative stress occurs when there is an imbalance between pro-oxidants and antioxidants, either due to an overwhelming generation of oxidizing species or due to a relative lack of antioxidant defense capacity. There is considerable clinical and experimental evidence for increased oxidative stress in alcoholic liver disease. It is a common consequence of both acute and chronic ethanol administration to rats and also to humans (Nakajima *et al*., 1992; French *et al*., 1993; Meagher *et al*., 1999). Ethanol-induced production of free radicals in the liver has been detected by a sensitive assay based upon spin-trapping agents (Knecht *et al*., 1990). Although increased oxidative stress is well documented, the precise pathogenic significance of it remains a subject of controversy (Ishii *et al*., 1997). Oxidative stress may cause tissue injury directly by initiating a chain of peroxidation processes in the cells, or potentially it may activate inflammatory responses by stimulation of the transcription of pro-inflammatory cytokines.

Following chronic ethanol administration, induction of CYP2E1 leads to an increased microsomal free radical generation. Although the quantitative significance of CYP2E1 in ethanol oxidation may be small, its potential to generate oxygen-derived free radicals through NADPH oxidase activity may be high, and it is suggested to be responsible for the hepatotoxicity after chronic ethanol administration (Morimoto *et al*., 1995; Lieber, 1997). This notion is supported by the finding that the sensitivity of isolated hepatic microsomes to iron-catalyzed lipid peroxidation is increased in conjunction with CYP2E1 induction (Castillo *et al*., 1992). Furthermore, CYP2E1-mediated generation of α-hydroxyethyl radical adducts *in vitro* and *in vivo* correlates with lipid peroxidation (Albano *et al*., 1996). Another major intracellular source of oxidative stress during alcohol metabolism may be the mitochondrial respiratory chain, which generates superoxide anions in response to a decrease in the NAD/NADH ratio (Nordmann *et al*., 1992). NADH is also capable of inhibiting the activity of xanthine dehydrogenase, which results in a shift of purine oxidation to xanthine oxidase. Recent study with allopurinol pointed to xanthine oxidase as the major intracellular source of reactive oxygen species in experimental ALD (Kono *et al*., 2000*b*).

A number of *in vivo* and *in vitro* studies have presented a role for activated Kupffer cells and polymorphonuclear neutrophils as a significant source of extracellular reactive oxygen species production in the liver. Inflammatory cells generate reactive

oxygen species by NADPH oxidase in response to stimuli, such as endotoxin and pro-inflammatory cytokines. Indeed, the generation of oxygen-derived free radicals by the liver is evident during endotoxemia (Jaeschke *et al*., 1996). Enhanced formation of reactive oxygen species by Kupffer cells and neutrophils is seen in ALD (Bautista and Spitzer, 1999). Consistently, inhibition of Kupffer cell function with GdCl diminishes free radical formation during alcohol intoxication by over 50% (Knecht *et al*., 1995). It is suggested that the activation of free radical release is mediated directly by alcohol in the acute situation, but after chronic alcohol through endotoxin and cytokines (Bautista and Spitzer, 1999).

2.4.2 Hepatic antioxidant defense

Oxidative stress may be harmful to the liver only if the defensive capacity is not able to cope with the increased free radical generation. Under normal conditions, hepatic oxidative stress is efficiently counteracted by a variety of protective antioxidant factors. Antioxidants may be insufficient, however, either if there is an increased demand, or if antioxidant enzymes are inactivated due to the reactions with free radicals.

Glutathione (GSH) peroxidase, which detoxifies hydrogen peroxide, is an important enzyme in the cell antioxidant defense system. Reduced GSH peroxidase capacity may thus result in a diminished capacity to remove hydrogen peroxide. Glutathione peroxidase is very susceptible to inactivation by oxidation from peroxides and oxygen-derived free radicals generated during ethanol metabolism, presumably by binding to the active site of the enzyme (Blum *et al*., 1985; Pigeolet *et al*., 1990; Tabatabaie and Floyd, 1994; Kinter and Roberts, 1996). Indeed, a decrease in glutathione peroxidase activity has been observed in rats after ethanol administration (Ribiere *et al*., 1985; Ishii *et al*., 1997).

Glutathione is the major cellular nucleophile, and provides an efficient detoxification pathway for reactive substances (Deneke and Fanburg, 1989; Uhlig and Wendel, 1992). Depletion of mitochondrial glutathione, which is observed after chronic ethanol feeding, has been suggested to play a significant role in the pathogenesis of ALD (Fernandez and Videla, 1981; Fernández-Checa *et al*., 1997; Rouach *et al*., 1997). The hepatic concentration of glutathione is an important factor in the protection against oxidative stress, since a lowering of GSH results in enhanced cytotoxicity to ethanol in CYP2E1-overexpressing cells (Chen and Cederbaum, 1998). Accordingly, the glutathione precursor, L-2-oxothiazolidine-4-carboxylic acid, was found to protect rats from alcohol-induced liver injury (Iimuro *et al*., 2000).

3 Animal models of ethanol-induced liver damage

Animals have been administered ethanol chronically by various methods in attempts to try to develop liver lesions resembling those seen in human ALD. Simple inclusion of ethanol in the drinking fluid seldom causes high and sustained elevation of blood ethanol levels and only a moderate rise in liver triglycerides is observed (Lieber *et al*., 1989). If the concentration of ethanol in the drinking fluid is increased above a certain level, intake decreases sharply, leading systemic dehydration, reduced food intake, and ceased or reduced growth rate. One apparent exception to this rule is with rats weaned to an ethanol solution as their sole fluid (Landrigan *et al*. 1989; Batey and Patterson 1991; Sinclair and Suomela 1994). The animals continued to gain

weight with ethanol concentrations up to 50%, showed increased ethanol metabolism, and sustained elevated blood ethanol levels, but liver damage occurred at most in only a minority of the animals.

To overcome the failure of most animals otherwise to consume higher amounts of ethanol voluntarily, it has been administered in a nutritionally adequate liquid diet that provided a maximum of 35-40 % of total calories from alcohol. This situation resembles that of many alcoholics, who often receive more than 50 % of their total energy as ethanol (Patek *et al*., 1975; Salaspuro and Lieber, 1980). In rats this has been achieved by administration of ethanol as a component of the liquid diet, either orally or by forced intragastric infusion. Some of the currently used animal models of ALD are summarized in Table 1.

3.1 Oral liquid diets

The method forces rats to consume high amounts of ethanol by its inclusion in a balanced liquid diet that contains sufficient water and all necessary nutrients. It was developed almost four decades ago (Lieber *et al*., 1963) and proved to be very useful in studies of the pathogenesis of early ethanol-induced changes. Controls were pair-fed an equicaloric amount of diet with ethanol replaced by carbohydrates (Lieber *et al*., 1989). The improved formula consisted of casein (providing 18% of calories) supplemented with methionine and cysteine, a mixture of dextrin and maltose (providing 11% and 47% of calories for ethanol and control diets, respectively), and fat (35% of calories, mainly olive oil, corn oil, and safflower oil). All essential vitamins (A, D, E, K, Bs), minerals, and fiber were present (Lieber and DeCarli, 1986; Lieber *et al*., 1989). The amount of ethanol in the diet was gradually increased during the first week to provide 36% of total calories.

This so called “Lieber-DeCarli” formula has been extensively used in rodent studies. The average daily ethanol intake, 12-15 g/kg, resulted in fatty liver and in metabolic tolerance, i.e. their ethanol elimination rate was increased (Lieber and DeCarli, 1970*b*). A 6-fold increase in hepatic triglycerides was observed after 1 month of feeding, an effect that persisted for 22 weeks (Lieber and DeCarli, 1970). For proper fatty liver to develop, at least 21% of the calories had to be derived from fat (Lieber and DeCarli, 1970), although even a low-fat (13%) ethanol diet causes some steatosis (Di Luzio and Hartman, 1969). The incorporation pattern of dietary fatty acids in liver triglycerides indicated that most fat comes from the diet (Lieber *et al*., 1966), and much less from hepatic lipogenesis (Tsukamoto *et al*., 1984). Lesions beyond steatosis are rare in this model. For example, rats fed for up to 9 months had no fibrotic changes (Leo and Lieber, 1983). This probably is a consequence of the rather modest blood ethanol levels achieved with this regimen (Lieber *et al*., 1989). The levels fluctuate with the circadian rhythm between 0 – 1.5 ‰.

Sustained high blood alcohol concentrations indeed seems to be a prerequisite for the progression of alcoholic liver disease process beyond steatosis (Lieber and DeCarli, 1976; French *et al*., 1995). Evidence for this was obtained by adding a low dose of 4-methyl pyrazole (4-MP) to the diet to inhibit alcohol metabolism. Rats on this diet had elevated ethanol levels and developed steatosis, inflammation, and mild necrosis in 12 weeks (Lindros *et al*., 1983). A later study demonstrated exacerbated damage with an increased fat content (Takada *et al*., 1986). The possibility cannot be excluded, however, that 4-MP, even at low doses, may have had side effects (Lieber

and DeCarli, 1970*c*), including the potential induction of CYP2E1, which may be pathogenic (Koop *et al*., 1985; Dicker *et al*., 1991).

3.2 Intragastric ethanol feeding model

In this model ethanol liquid diet is fed through a permanent indwelling intragastric catheter. By regular monitoring of blood ethanol levels, the ethanol infusion rate can be titrated (to an average of 12-13 g/kg/d) and high blood ethanol levels achieved. As in the oral feeding model, controls are infused with isocaloric amounts of ethanol-free diet, with carbohydrates replacing ethanol.

The gastrostomy tube is usually implanted on adult rats weighing 300-400g (Tsukamoto *et al*., 1985), but rats weighing 200-250g have also been used (French, 1993). In younger animals the liver injury was found to be more severe and the fibrotic activity stronger (Takahashi *et al*., 1990). Either single or double gastrostomy cannulas have been inserted via the neck into the stomach. The tube is connected to spring coils and swivels to protect the cannulas and permit free movement of the infused animal (Tsukamoto *et al*., 1985). Daily monitoring of alcohol intoxication is necessary, since the rate of ethanol infusion needs to be adjusted to achieve consistently high, yet tolerable ethanol levels. Monitoring of ethanol inebriation is by jugular blood or urine sampling and also by visual inspection of the animals (Badger *et al*., 1993*b*, Yin *et al*., 1999).

Approximately 30-50 % of the rats on ethanol diet developed macrovesicular and microvesicular steatosis, focal necrosis, and mononuclear inflammation (Tsukamoto *et al*., 1985; Tsukamoto *et al*., 1986; French *et al*., 1988*b*). Early perivenous fibrogenesis starts to develop in 3-6 months, provided that a high-fat diet with 42% to 49% of total energy as ethanol, is infused (French *et al*., 1986; Tsukamoto *et al*., 1986; Kamimura *et al*., 1992). Addition of carbonyl iron to the diet further aggravates injurious changes (Tsukamoto *et al*., 1995).

A peculiar feature of the intragastric feeding procedure is that the blood alcohol levels (BAL) go up and down over a 5-day cycle despite a constant ethanol dose. The extent of ethanol fluctuation has been found to correlate to the degree of hepatic damage (Tsukamoto *et al*., 1985*b*).

The intragastric feeding technique was recently applied to mice (Zhang-Gouillon *et al*., 1998). One problem is that their metabolism of alcohol is so rapid (i.e. 25 g/kg/d) that it is difficult to feed them enough ethanol without compromising their nutritional balance. This may explain the frequent loss of animals reported during intragastric ethanol feeding to mice (Zhang-Gouillon *et al*., 1998).

3.3 Primate models

The baboon model, developed in the early seventies (Lieber and DeCarli, 1974), should be superior to other animal models, since extrapolation to human ALD can be expected to be more straight forward. The baboons received up to 50 % of their calories as ethanol and could be kept on ethanol liquid diet for years. Peak blood alcohol levels in the baboons kept on a regimen with 11% carbohydrates approached 4 ‰. The baboons developed more damage than seen in rodent studies. After 3-8 years on a diet with approximately 50% of the calories as ethanol, fatty liver, increased mononuclear inflammatory activity, and perivenular fibrosis was seen in most of the animals. In one or two out of five animals fibrosis eventually progressed

to cirrhosis (Lieber and DeCarli 1974; Lieber *et al*., 1975; Popper and Lieber, 1980; Lieber *et al*., 1990). This prevalence is approximately of the same magnitude as among human alcoholics (Morgan, 1994). However, some of the histological features typically seen in human alcoholic hepatitis, such as heavy infiltration of neutrophil polymorphs and formation Mallory bodies, were not observed (Popper and Lieber, 1980). This is in contradiction with the opinion that alcoholic hepatitis is an essential step in the transition to cirrhosis.

The data were not replicated in another baboon study. Ainley *et al*. (1988) treated baboons for up to 60 months, but no cirrhosis or even fibrosis developed. These authors used a nutritionally different diet, the Mazuri primate diet supplemented with lipotropes, vitamins, and minerals. They suggested that the diet used by Lieber *et al*. was nutritionally inadequate and that this could explain the differences between the studies. However, a follow-up baboon study demonstrated that supplementation with massive amounts of choline and methionine failed to protect against ethanol hepatotoxicity (Lieber and DeCarli, 1994).

Contradictory results have also been obtained with rhesus monkeys. Ethanol given with a high-fat, low-choline diet produced cirrhosis in 8 months, but damage was avoided by choline supplementation (Cueto *et al*., 1967). Several negative results have been reported, in spite of high alcohol consumption (up to 50% of calories) and extended study periods of 1-4.5 years (Rogers *et al*., 1981; Mezey *et al*., 1983). In a recent rhesus monkey study, a diet marginal in antioxidants and containing 24% of calories as ethanol resulted in macrovesicular and microvesicular steatosis and mild fibrotic changes after 18-36 months (Pawlosky *et al*., 1997; Pawlosky and Salem, 1999).

3.4 Minipigs

Since pigs seem to tolerate ethanol better than most animals and their metabolism is considered to resemble humans closer than rats, they should be very useful for ALD studies. Minipigs given alcohol in a slurry-type low-fat diet consumed 6.0 g ethanol/kg per day, exhibited somewhat elevated hepatic triglyceride levels but only minimal histological changes after 8 weeks (Kusewitt *et al*., 1977). However, when the fat content was increased (to 35-40% of calories) and the treatment time prolonged to 12 months, steatonecrosis developed and in some animals interstitial and perivenous fibrosis was observed (Halsted *et al*., 1993; Niemelä *et al*., 1995; Niemelä, 1999). Thus the minipig may provide a manageable model to study ALD.

Table 1. Characteristics of animal models of alcoholic liver disease

Alcohol administration model	Duration , species and gender	Daily ethanol consumption (g/kg body wt.)	Blood ethanol levels (mg/dl)	Fold-elevation of ALT	Fatty liver	Focal inflam-mation	Focal necrosis	Fibrosis	Cirrhosis	Comments
Oral Lieber-DeCarli liquid diet (Lieber *et al.*, 1989)	4-6 weeks male rats	12-18	~0-150	-	**X**	-	-	-	-	Modest BALs.
Oral 4 –MP – liquid diet model (Lindros *et al.*, 1983)	3 months male rats	12	100-350	not determined	**X**	**X**	**X**	-	-	Sustained elevation of BALs. Effect of 4-methylpyrazole (4-MP) on CYP2E1 ?
Oral low-carbohydrate liquid diet (this study)	6 weeks male rats	12-18	150-250	2-3 x	**X**	**X**		-	-	Sustained high BALs.
Oral LOC model (Järveläinen et al., 2000)	6 weeks female rats	12-18	150-350	7 x	**X**	**X**	**X**	-	-	Sustained high BAL, advanced liver injury
Intragastric alcohol feeding model, (Tsukamoto *et al.*, 1985)	6 weeks male rats	11-13	~0-500	2-3 x	**X**	**X**	**X**	-	-	High, but cycling BAL. Strict pair-feeding possible. Technical difficulties may hamper use.

Intragastric alcohol feeding, prolonged treatment (Tsukamoto *et al*., 1986)	3-6 months male rats	11-13	~0-500	2-10 x	**X**	**X**	**X**	**X**	-	In addition to above: fibrotic changes in rat
Intragastric intubation daily (Enomoto et al., 1999)	6 weeks female rats	5	~0-350	2-3 x	**X**	**X**	**X**	-	-	
Ethanol vapour inhalation (Goldin *et al*., 1987)	3 weeks female mice	not determined	40-160	(2.5 x)	**X**	**X**	**X**	-	-	Requires individual BAL monitoring
Knock-out mice, intragastric alcohol feeding model (Kono et al, 1999)	4 weeks male mice	28	0-400	2-3	**X**	**X**	**X**	-	-	Risk for high mortality.
Minipig model (Halsted *et al*., 1993)	1 year male minipigs	5-6	~180	2 x	**X**	**X**	**X**	**X**		Fibrosis with a high-fat diet
Baboon model (Lieber *et al*., 1975)	2-4 years male baboons	25	- 380	2-3 x	**X**	**X**	**X**	**X**	**X**	Fibrosis in most animals, cirrhosis in some.

AIMS OF THE PRESENT STUDY

Although the adverse effects of ethanol on the liver have been well known, the precise mechanisms of ethanol toxicity are not well understood. The specific aims of the present work were as follows:

1. The mechanism of enhanced sensitivity to endotoxin after chronic ethanol was investigated by studying the hepatic expression of the CD14 endotoxin receptor and of lipopolysaccharide binding protein (LBP).
2. To investigate whether modifying the contents of carbohydrates and polyunsaturated fatty acids in the oral ethanol liquid diet lead to higher blood alcohol levels and aggravation of alcohol-induced liver lesions, and to find out whether this model is also applicable to the study of alcohol-induced inflammatory mechanisms in rats.
3. In order to study how chronic endoxemia acts on alcoholic fatty liver, a low-dose of endotoxin was chronically infused to alcohol-receiving rats, and the Kupffer cell-derived pro-inflammatory and anti-inflammatory responses were investigated.
4. The evidence for an involvement of Kupffer cells in ALD is at present based mainly on the intragastric alcohol feeding model, in which destruction of Kupffer cells by gadolinium chloride attenuates liver damage. The mechanisms of gadolinium chloride protection were studied here after oral ethanol liquid diet feeding.
5. Considering the zonal and cellular heterogeneity of the pathogenetic events of alcohol-induced damage, the distribution of CD14 and LBP in the liver acinus was studied. In addition, the level of oxy-radical producing CYP2E1 induction and its distribution in the liver acinus were compared with the key enzymes involved in protection against peroxidation.

MATERIALS AND METHODS

Animals and ethics

Male Wistar rats were obtained from the breeding colony of the University of Helsinki. The room temperature in the animal room was 21 ± 1°C and relative humidity 50 ± 10%. The lighting rhythm was 12 hours on, 12 hours off with lights on at 6:00 AM. During the experiments, the rats were individually housed in stainless steel wire-bottom cages. When sacrificed, the rats were anesthetized with pentobarbital (50-60 mg/kg intraperitoneally).

The procedures were approved by the Animal Experimentation Committee of the National Public Health Institute, Helsinki, Finland, and the studies were conducted in accordance with the principles of the Declaration of Helsinki.

Liquid diet administration

For chronic administration of ethanol, rats were pair-fed a nutritionally adequate liquid diet. In study I, the "Lieber-DeCarli" diet (NC diet) was used, containing 18 % (Joules) protein, 35 % fat, and 47 % carbohydrate (controls); or 11 % carbohydrates and 36 % ethanol (ethanol-fed). For studies II-IV, this diet was modified by reducing the content of carbohydrate to 5.5% and increasing the fat content correspondingly to 44%, by addition of an equicaloric amount of corn oil (LC diet). For technical reasons the protein content of the latter diet was slightly lower (16%). A diet containing 16 % protein is nutritionally adequate according to feeding regimes for the laboratory rat (National Research Council, 1978). Casein, vitamins, and minerals were added to match the composition of the NC diet. In addition, 0.4 % carboxymethylcellulose (CMC) was added as a stabilizer to increase viscosity.

Table 2. Basic composition of the normal-carbohydrate (NC = Lieber-DeCarli) and low-carbohydrate (LC) diets. Caloric contributions (%) of protein, fat, carbohydrate and ethanol.

Diet	Protein	Fat	Carbo-hydrate	Ethanol
NC control	18	35	47	0
NC ethanol	18	35	11	36
LC control	16	44*	40	0
LC ethanol	16	44*	5.5	34.5

*Additional fat (corn oil) was added to the NC diet (consisting of olive, safflower, and corn oil).

Chronic low-dose endotoxin treatment

In study III, two weeks after the beginning of 5% ethanol liquid-diet treatment, some rats also were infused with endotoxin via osmotic minipumps for an four

additional weeks by a modification of the protocol of Fish and Spitzer (1984). Shortly, the surgery to implant minipump was performed under halothane anesthesia. An osmotic pump (Alzet 2ML4, Alza Corp., Palo Alto, CA) dosed with endotoxin to deliver 0.1 mg/kg/day (based on the pre-surgery body weight) was implanted subcutaneously on the back of the rats and connected to the right jugular vein. A 3 cm long PE-10 tube (Clay Adams, Parsippany, NJ) was connected to a 22 cm PE-60 tube, which was filled with pyrogen-free saline to provide a 36h post-surgical endotoxin-free recovery. Control rats had a minipump with saline.

Acute endotoxin treatments

In study I, the acute endotoxin treatment rats received an intraperitoneal injection (3mg/kg) 29 and 5 hours before Kupffer cell isolation.

In study III, the control rats (ATx) were treated with an intraperitoneal injection of a small dose of endotoxin (0.5 mg/kg) 4 hours before sacrifice.

Gadolinium chloride treatment

To cause inactivation of Kupffer cells, gadolinium chloride ($GdCl_3$) was injected (10 mg/kg in acidic saline) into the tail vein every third day.

Compounds used in the studies

Compounds from the following sources were used: Percoll was obtained from Pharmacia Biotech (Uppsala, Sweden); Collagenase type V, endotoxin (Salmonella abortus equi), gadolinium chloride hexahydrate and casein (technical grade) were from Sigma Chemical Co (Saint Louis, MO, USA); mouse anti-rat ED2 and ED9 monoclonal antibodies were from Serotec (Oxford, U.K.); streptavidin-coupled fluorescein was from Boehringer (Mannheim, Germany); liquid diets from Purina Mills (Richmond, IN, USA); CMC was from Metsa Specialty Chemicals Oy, Äänekoski, Finland; pentobarbital (Mebunat®) from Orion-Farmos (Turku, Finland); and digitonin from ICN Chemicals (Cleveland, OH, USA).

Histopathology

After sacrificing the animals, a piece of the liver was fixed in 10% neutral buffered Formalin. Following fixation, the samples were embedded in paraffin and processed routinely for histopathological examination. Light microscopy sections stained with hematoxylin/eosin were graded blindly for the degree of fatty change and focal inflammation, following the protocol by Nanji *et al.* (1989). Ten low-power fields were examined per liver. The severity of steatosis was assessed as follows: 1+ = <25% of cells containing fat, 2+ = 26-50%, 3+ = 51-75%, 4+ = >75%. Steatosis was graded from 0-4, with 0 depicting no fat present and 4 depicting that >75% of cells contain fat. Focal inflammation was graded as 1+ if there was one focus per low power field or 2+ if there were two or more foci per field. For study III, the number of neutrophil polymorph infiltrates was determined by counting infiltrating cells in 10 high power fields (×400) and is expressed as neutrophils per 100 hepatocytes. The liver sections were also stained with van Gieson for the evaluation of fibrosis.

Isolation of Kupffer cells

The liver was perfused *in situ* through the portal vein with calcium-free and magnesium-free Hanks balanced salt solution (HBSS) followed by normal HBSS supplemented with collagenase (50 mg/100 ml, Sigma) for 8-10 min (I). The papilliform lobe was ligated before collagenase, and samples were frozen in liquid nitrogen for protein analysis and RNA isolation or transferred into buffered formalin for histological examination. After redispensing the liver in cold PBS, removing the capsule and gentle shaking, the cell slurry was filtered to remove debris and then centrifuged twice for 3 min. at 50 x g. Nonparenchymal cells were prepared from the first two supernatants by isopycnic centrifugation in Percoll as described in detail by Smedsrød and Pertoft (1985). Kupffer cells were isolated by selective adherence to glass cover slips and either frozen for immunofluorescense or removed, centrifuged and collected in aliquots for Western blot and protein determinations. Kupffer cells were identified by phase-contrast microscopy and their purity (> 90 %) controlled by endogeneous peroxidase staining using a Zymed (San Francisco, USA) peroxidase staining kit.

Collection of liver samples and preparation of periportal and perivenous cell lysates

The liver was perfused *in situ* with saline through the portal vein for 1-2 min, the papilliform lobe ligated and removed, and liver samples collected in buffered formalin solution or frozen in liquid nitrogen and stored at -70°C (II, III).

Periportal and perivenous cell lysates (IV) were obtained by a modified (Saarinen *et al.*, 1993) dual digitonin pulsing method (Quistorff and Grunnet, 1987). Briefly, periportal cells were lysed by infusion of 6.7 ml/kg b.wt. of 3.5 mM digitonin via the portal vein and the lysate collected by immediate retrograde flushing. Perivenous cell lysates were obtained by infusing 10 ml/kg b.wt. digitonin solution via the upper vena cava followed by antegrade flushing. The length (penetration depth) of the digitonin pulse was determined empirically to lyse approximately one-fourth to one-third of the cells along the plate in either the proximal or distal part of the sinusoid. The zonal origin of the cell lysates was controlled by assaying the periportal marker enzyme, alanine aminotransferase (ALT, EC 2.6.1.2.) as in (Lindros and Penttilä, 1985).

Immunoblotting

For analysis of CD14 protein in isolated Kupffer cells (I), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run in 8.7 % gels. Proteins were electroblotted on nitrocellulose filters and blocked using the TROPIX (Bedford, USA) Western Light® protocol and probed with 1:100 dilution of mouse anti-rat ED9 monoclonal antibody (Serotec, Oxford, U.K) specific for CD14 (Tracy and Fox, 1995). The filters were incubated with chemiluminescence substrate (TROPIX) and exposed to Kodak (New York, USA) X-ray OMAT film.

For analysis of CYP2E1 protein (IV), 2.5-5 µg protein from each sample supernatant was subjected to 10% SDS-PAGE on a Bio-Rad Mini Protein II apparatus using published methods (Laemmli, 1970). Separated proteins were blotted onto nitro-cellulose membranes (Towbin *et al.*, 1979) and probed for CYP2E1 as previously reported (Badger *et al.*, 1993*b*). Immunoreactive bands were visualized by enhanced

chemiluminescence according to manufacturer's specifications (Amersham, Little Chalfont, Buckinghamshire, England). The amount of CYP2E1 apoprotein was quantified by a personal densitometer (Molecular Dynamics, USA) and expressed as relative amount of arbitrary units.

Immunochemistry

In study I, isolated Kupffer cells adhering to glass cover slips were treated with 7 % sucrose for 30 min and frozen at - 80º C. For staining, cell preparations were air-dried for 30 min at room temperature, washed for 10 min with absolute acetone and then washed with PBS . The cell preparations were then incubated with the ED9 antibody, diluted 1:100 in 0.5 % bovine serum albumin, at + 4 ºC for 20 h and washed with PBS. For detection, an avidin-biotin complex immunohistochemistry kit (Zymed, San Francisco, USA) followed by streptavidin-coupled fluorescein was used according to manufacturer's instructions. After mounting, the sections were coverslipped and examined with a fluorescence microscope Olympus BH-2, equipped with a BH2-RFL-T3 burner. For control staining, PBS was used instead of ED9 antibody.

For study IV, frozen liver sections (5 μm) were air-dried at room temperature for 30 minutes and then fixed in cold acetone for 10 minutes. Endogenous peroxidase activity was blocked by treating the sections for 10 min in methanol containing 0.3% hydrogen peroxide. Rehydrated sections were incubated overnight at +4˚C with the Kupffer cell specific ED2 (Dijkstra *et al*., 1985) monoclonal antibody. Visualization was carried out using a Histostain™ SP immunohistochemistry kit (Zymed, San Francisco, CA, USA).

Enzyme assays

The catalytic activity of p-nitrophenol hydroxylase (IV), which reflects CYP2E1 enzyme activity, was estimated from liver microsomes by measuring the hydroxylation of p-nitrophenol using 0.2 mg microsomal protein (Koop, 1986).

Glutathione transferase (GST) activity (IV) was assayed as conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 25°C in phosphate buffer. Selenium-dependent glutathione peroxidase (Se-GPx) activity (IV) was measured with tertiary butyl hydroperoxide (BuOOH) by the glutathione reductase-coupled system at 37°C in phosphate buffer, pH 7.0). The final glutathione concentration was 2.5 mM. Serum alanine aminotransferase (ALT, EC 2.6.1.2.) activities (II, III) were determined using a commercial kit (Boehringer, GMBH, Mannheim, Germany).

RT-PCR

A semi-quantitative RT-PCR was used to estimate the levels of CD14, LBP, CYP2E1, and β-Actin mRNA in liver samples (I, III, IV). For these studies, total RNA was isolated from whole liver or cell lysate samples using commercially available RNA isolation kits according to the instructions given by the manufacturers. The integrity of isolated RNA and the determination of the RNA concentration (A_{260}) were validated by RNA electrophoresis in formaldehyde denatured agarose gel. cDNA was produced from identical amounts of total RNA with Promega's (Madison,

WI, USA) Reverse Transcription system according to the manufacturer's instructions. Control experiments were performed to assure that the PCR amplification reaction was linear with respect to the amount of cDNA and to the number of cycles used and that the PCR conditions (annealing temperature and time, Mg2+, primers) were optimal. The primer sequences were chosen from the coding region of a published rat mRNA sequences. Conditions, reagents, and oligonucleotide primer sequences are given in each study (I, III, IV). The size of the PCR product was analyzed by agarose gel electrophoresis in 4% NuSieve GTG (FMC BioProducts, Rockland, ME, USA) followed by ethidium bromide staining. Relative quantification of the products was done by anion exchange HPLC (Katz and Dong, 1990). All compared samples were run in the same series of cDNA synthesis and PCR. The interseries variation was reduced by normalizing the relative CD14 mRNA amplification of each sample to that obtained with ß-actin primers (I). Normalization to ß-actin mRNA was not used in studies III and IV because low-carbohydrate alcohol liquid diet treatment was found to increase ß-actin mRNA expression. Instead, the interseries variation in quantification of CD14 and LBP -PCR products was reduced by normalizing them mathematically as described before (Lindros *et al*., 1997). The four independent PCR runs (CD14, LBP, CYP2E1 and ß-actin) were used to normalize the data.

A modified competitive PCR was used to quantify mRNA expression of cytokines TNF-α, IL-1β, IL-4, IL-10, and TGF-β (III, IV). In the technique, PCR mimics were constructed with composite primers (45-62 bp) containing 2 target gene primer sequences instead of one, so that the same PCR product (mimic) can be applied for 2 different competitive PCRs when the appropriate PCR primers were used. The mimics are nonhomologous DNA fragments more than 100 bp longer or shorter than the target DNA and served as internal standards in the competitive PCR. The optimal conditions for the competitive PCR were selected from the pilot tests. To monitor the efficiency of competitive PCR and to produce standard curves for calculation of the relative amount of mRNA, threefold serial dilutions of mimic were coamplified with a constant amount of cDNA mixtures from control and treated samples, and the appropriate amounts of different mimics that gave equal intensities of target and mimic bands were determined respectively when the ratio of target to mimic was 1:1. The PCR products were electrophoresed through an agarose gel with ethidium bromide. Band intensity data were converted to ratios of target to mimic, then normalized by the ratio of house keeping gene cyclophilin, which served as a control for the quantity of RNA sample variations and the efficiency of reverse transcription.

Other methods

For determination of endotoxin, blood was aseptically collected by heart puncture into pyrogen-free blood collection tubes containing heparin (Endo Tube ET, Chromogenix, Mölndal, Sweden). These were immediately placed on ice in order to minimize the rapid inactivation of LPS which occurs in whole blood at 37 ºC (Steverink *et al*., 1994; Rivera and Thurman, 1998). Platelet-rich plasma (PRP) was obtained by centrifugation at 180 g for 10 min in a refrigerated centrifuge. The PRP was then removed into pyrogen-free storage tubes (N201 Test Tubes, Biowittaker, Walkersville, Maryland), and stored at -80 °C until assay. To inactivate inhibitors, plasma was diluted 1:10 in pyrogen-free water (Biowittaker) and heated to approximately 75 °C in a water bath before assay. Endotoxin levels in the serum

samples were measured by the Limulus Amebocyte Lysate (LAL) sensitive chromogenic assay with a detection limit of 0.1 pg/ml (Biowhittaker QCL 1000) and the microplate (Costar) method using a Multiscan RC microplate reader (Labsystems, Helsinki, Finland).

Liver microsomes were prepared by homogenizing liver tissue in 10mM sodium/potassium phosphate buffer, pH 7.4, containing 1.14% KCl. The microsomal pellet obtained after ultracentrifugation at 105.000 g was washed once and resuspended in 50 mM potassium phosphate buffer, pH 7.4.

Blood ethanol levels (I-IV) were determined by head space gas chromatography as described before (Hu *et al*., 1995). Serum GSTα activity (IV) was measured with a commercial ELISA kit (HEPKIT™, Biotrin, Dublin, Ireland) according to the instructions. Protein was determined fluorometrically (Böhlen *et al*., 1973).

Statistics

The groups were statistically compared by one-way or two-way analyses of variance (ANOVA; with repeated measures where appropriate) followed by the Student's *t*-test or the Mann-Whitney test. Student's *t*-test was used when only two groups were compared. Correlations were made using Pearson's or Spearman's correlation coefficients where appropriate.

RESULTS

Ethanol liquid diet model

In this work, ethanol was administered together with a nutritionally adequate liquid diet. Consumption of the ethanol liquid diet intake had no apparent effect on the health status of the animals. For example, a steady increase in the body weight was observed in all groups during all studies. The mean daily ethanol intakes of the ethanol-treated rats were mostly between 10 - 14 g/kg b.w., and the rats had consistently elevated blood ethanol levels. The mean blood ethanol levels were generally 150 - 200 mg/dl.

Histopathological evaluation of liver specimens from rats fed the regular (NC) ethanol diet, containing 11% of calories as carbohydrates, confirmed earlier observations (Lieber *at al.*, 1965; Rao and Larkin, 1984; Lieber and DeCarli, 1989) that rats receiving ethanol on this regimen developed only steatosis, which was mainly periportal (Fig. 3a in II).

However, the histopathological picture observed in livers of rats given ethanol in the low-carbohydrate diet was markedly different. Panlobular microvesicular/macrovesicular steatosis of pronounced severity was present in most of the ethanol-treated rats. Occasionally, foci of inflammation were observed (Fig. 3b in II). The predominant inflammatory cell type was a mononuclear cell. Neutrophils were also occasionally found. Ethanol treatment increased serum liver enzyme alanine aminotransferase (ALT) –two- to three-fold in male rats. No parenchymal fibrosis was found in any of the samples. The overall pathological score was significantly higher ($p < 0.01$) in livers of rats on the LC diet than in rats on the NC diet. Furthermore, animals on ethanol-LC diet were found to exhibit a significantly higher liver/body weight ratio than the corresponding controls.

CD14 and LBP expression (II, III)

The CD14 protein is normally expressed at a very low level in the liver, on the membrane of Kupffer cells and infiltrating monocytes/macrophages. Consequently, isolation of Kupffer cells was necessary to allow immunological detection of CD14. In Kupffer cell preparations from untreated animals, weak staining of immunoreactive protein with the expected molecular weight (55 kD) was detected. In contrast, Kupffer cell preparations from rats treated 2 weeks with ethanol liquid diet were more intensively stained. The preparation from rats treated acutely with endotoxin showed the strongest staining. The difference in the immunocytofluorescense staining pattern between Kupffer cells from untreated and ethanol-treated animals was also observed. The controls showed only weak fluorescence. Cell preparations from ethanol-treated animals, however, regularly exhibited marked plasma membrane fluorescence. RT-PCR analysis revealed that ethanol feeding significantly ($p< 0.01$) increased the hepatic mRNA expression of CD14 to three-fold, suggesting transcriptional activation. Chronic ethanol administration also significantly increased (approx. 2–fold) the expression of lipopolysaccharide binding protein (LBP) mRNA (III, IV).

Chronic endotoxemia (III)

Chronic endotoxin infusion had no apparent effect on the health status of the animals. Preliminary experiments, using endotoxin doses between 0.05 - 3.0 mg/kg per day, were performed to find a dose that caused no undesirable health effects and only minimal liver injury. The endotoxin dose chosen (0.1 mg/kg/d) resulted in a 50- to 100–fold elevation of blood endotoxin over control levels in the end of the study. The blood endotoxin levels in control-endotoxin rats were 388.4 ± 97.5 pg/ml and in ethanol-endotoxin rats 512.8 ± 80.4 pg/ml. This range of blood endotoxin can be considered as a moderately severe endotoxemia. Ethanol alone also caused a small, but significant ($p < .01$) elevation in blood endotoxin when compared to control animals (9.3 ± 1.5 pg/ml and 3.1 ± 1.0 pg/ml, respectively).

Histopathological analysis of the livers revealed that endotoxin treatment alone had no effect on steatosis, mononuclear inflammation, or necrosis. However, occasionally damage to sinusoidal cell lines was observed, resulting in the accumulation of blood cells, especially in the midzonal region. A significant increase in the frequency of polymorphonuclear cells after the chronic endotoxin treatment was also observed. This was not, however, associated with hepatocyte necrosis. Ethanol treatment with or without chronic endotoxin resulted in a 2- to 3-fold increase in serum ALT. Therefore, in contrast to studies in which endotoxin is given as a bolus to chronically alcohol-fed animals, liver damage was not clearly potentiated in endotoxin-ethanol treated animals. These results suggest a development of tolerance to endotoxin.

Since macrophages are known to be involved in the generation of endotoxin tolerance (Freudenberg and Galanos, 1988), an examination was made as to whether CD14-regulated signaling pathways are down-regulated more during chronic endotoxemia than following an acute challenge. The effect of chronic endotoxin on the expression of the mRNAs for CD14 endotoxin receptor and lipopolysaccharide binding protein (LBP) was investigated by HPLC from the amplified products obtained by RT-PCR. There was no significant increase of CD14 or LBP mRNA after chronic endotoxin treatment (Fig. 4 in III). This result contrasted to that seen after acute challenge. Four hours after a low acute dose of endotoxin (0.5 mg/kg), the amount of CD14 mRNA was five-fold higher ($p < 0.001$) and that of LBP mRNA nine-fold higher ($p < 0.001$) than in controls.

Changes in the hepatic expression of cytokine mRNAs were quantified using competitive RT-PCR. Four weeks of endotoxin treatment resulted in a markedly elevated expression of all the cytokines investigated (Fig. 3 in III). Both pro-inflammatory cytokines were increased about 7-fold ($p< .01$ for both IL-1β, and TNF-α) after endotoxin alone and 9- and 11-fold, respectively, after combined ethanol-endotoxin treatment. Ethanol alone did not significantly increase these cytokines or the expression of the anti-inflammatory cytokines IL-4 and IL-10. However, after chronic endotoxin, a 28-fold increase of IL-10 mRNA was found ($p < .05$) and IL-4 increased –3-fold ($p < .05$); additional ethanol treatment did not potentiate these changes. Endotoxin treatment also increased the expression of TGF-β_1 much more (about 8-fold; $p < .01$) than ethanol did (about 2-fold; $p < .05$). Additional ethanol treatment attenuated the endotoxin-induced increase to about 3-fold ($p= .05$).

Effect of gadolinium chloride (IV)

Administration of $GdCl_3$ every third day to rats has previously been shown to efficiently inactivate the liver macrophage population (Hardonk *et al.*,1992). This was

confirmed in the present study by staining liver cryostat sections with a monoclonal antibody against resident hepatic macrophages ED2 (Heuff *et al*., 1993). In rats treated with gadolinium, very few cells stained positively for ED2. In contrast, in livers from untreated rats, strong positive staining was seen. A lobular pattern of ED2-staining was apparent. The cells were larger and more frequent around the portal triad, while around the perivenous regions cells appeared fewer and smaller.

Gadolinium treatment significantly reduced the level of ethanol-induced steatosis. However, additional treatment with gadolinium did not alleviate the ethanol-induced increase in the frequency of inflammatory foci. Neither was the GST-α activity, indicating liver cell damage, different in rats treated with gadolinium chloride.

As shown in numerous earlier studies, ethanol treatment caused marked induction of CYP2E1. The induction of CYP2E1 protein, as estimated by Western blot analysis, was 8.4-fold and that of catalytic activity, as determined by p-nitrophenol (pNP) hydroxylase activity from microsomes, was 5.1-fold (Fig.4 in IV). Additional treatment with $GdCl_3$ significantly decreased the CYP2E1 induction, an effect seen both at the protein level (to 6.3-fold, $p < 0.05$) and as catalytic activity (to 3.3-fold, $p < 0.05$). Gadolinium treatment alone had no effect on CYP2E1, neither at the protein nor the activity level. Protein and activity data correlated strongly ($r = 0.85$, $p < 0.001$). Of importance, within the two ethanol-treated groups of animals, the CYP2E1 activity correlated to steatosis (Fig. 5 in IV). The correlation coefficient by Spearman's analysis was 0.7 ($p < 0.05$). The correlation was significant also within the ethanol-gadolinium group ($r = 0.6$, $p < 0.05$). Consistent with the effects on activity, the protein expression of CYP2E1 was significantly reduced by $GdCl_3$.

Considering the zonal and cellular heterogeneity of the liver macrophage population (Laskin, 1997), the possibility that $GdCl_3$ could act zone-specifically was evaluated by comparing the mRNA expression of CD14 and LBP in perivenous and periportal cell lysates. The efficiency of the digitonin-pulse technique to obtain zone selective lysates was verified by measuring the activity of the periportal marker alanine aminotransferase (ALT) in the eluates. The activity of ALT in periportal eluates was 7-14 times higher than in the perivenous samples, and this distribution was seen in all four groups (results not shown). RT-PCR analysis revealed that the expression of CD14 mRNA was significantly ($p < 0.05$) higher in the perivenous eluates than the periportal samples, regardless of treatment (Fig. 6 in IV). The perivenous/periportal ratio varied between 1.9 to 2.9. The ethanol-induced increase in hepatic CD14 mRNA was observed in eluates, both in periportal and perivenous samples, indicating that the ethanol effect was panlobular. Interestingly, although the ED2-staining demonstrated efficient inactivation of Kupffer cells by $GdCl_3$, the expression of CD14 or its zonation was unaffected.

In contrast to CD14 mRNA, the expression of LBP mRNA exhibited no zonation (Fig. 6 in IV). Ethanol treatment significantly increased LBP mRNA ($p < 0.05$), as seen both by analysis of liver samples and eluates. Additional gadolinium treatment led to a further increase in LBP expression ($p < 0.05$ vs. ethanol). Analysis of the relative effect of ethanol in the periportal and perivenous eluates suggested that ethanol affected the perivenous area more. Thus while ethanol increased LBP mRNA in the periportal area by 3.9-fold (E vs. C) and 3.3-fold (EG vs. CG), the increase in the perivenous area was 6.8-fold (E vs. C) and 9.4-fold (EG vs. CG ($p < 0.05$).

The hepatic mRNA expressions of the pro-inflammatory cytokines TNF-α and IL-1β were quantified by competitive RT-PCR. Although there was a tendency for ethanol to increase the expression of both TNF-α (2.5-fold) and IL-1β (1.5-fold), and

a tendency for $GdCl_3$ to reduce this effect (to 1.9-fold for TNF-α and to 0.9-fold for IL-1β), these effects were not statistically significant (Table 1 in IV).

To study the possible effect of $GdCl_3$ on the defense capacity of the liver against ethanol/CYP2E1-induced oxidative stress and its zonation, the catalytic activities of the two major GSH-associated enzymes, GSH transferase (GST) and the selenium-dependent GSH peroxidase (Se-GPx), were determined. In agreement with previous observations (Ishii *et al.*, 1997; Rouach *et al.*, 1997), chronic ethanol treatment increased the GST activity (which is higher in the perivenous region) while the activity of Se-GPx (which is higher in the periportal region) was reduced (Table 2 in IV). However, ethanol affected these activities in both acinar areas to approximately the same extent. Furthermore, $GdCl_3$ treatment had no significant effect on either the total activity or on the zonated distribution of these enzymes.

DISCUSSION

Low-carbohydrate ethanol liquid diet model

The chronic administration to rats of an ethanol-containing liquid diet low in carbohydrates (5.5% of calories) but comparably high in unsaturated fatty acids (mainly corn oil) results in distinctly different liver changes than those observed in numerous earlier studies using an alcohol diet with a normal content of carbohydrates. After six weeks, male rats on this liquid diet regimen developed panlobular microvesicular/macrovesicular steatosis, focal inflammation, and a several-fold elevation of serum liver enzymes (II-IV). This suggests that either the carbohydrate content by itself or the carbohydrate/fat ratio is a crucial factor in the development of damage. In the modified formula, the carbohydrate/fat ratio was 1:8, as compared to 1:3 in the Lieber-DeCarli diet (Lieber and DeCarli, 1989). It is well documented that ethanol-induced fatty infiltration is reduced when the dietary carbohydrate content is increased (Stanko *et al*., 1978; Yonekura *et al*., 1993). Thus inhibition of the development of steatosis might be a crucial mechanism in the protection from more advanced injury (Day and James, 1998). A carbohydrate free diet does not by itself produce any lesions (Lieber *et al*., 1965), indicating that a relative lack of carbohydrates only becomes important in combination with chronic ethanol feeding. The dose-response relationship for ethanol in the development of liver damage, however, remains to be determined.

These results also support the pathogenic role of persistently elevated blood ethanol levels. The rats on the low carbohydrate ethanol diet had significantly higher blood ethanol levels than rats on the normal carbohydrate ethanol diet. The blood ethanol levels approached those reported in the intragastric ethanol feeding model (up to 2.5 – 3 ‰). In addition, these levels were continuously elevated, while in the Lieber-DeCarli liquid diet model the blood ethanol levels are known to fluctuate with the circadian rhythm between 0 – 1.5 ‰ (Lieber and DeCarli, 1989). Ethanol levels also fluctuate in the intragastric feeding model, but not diurnally. Instead, they cycle with a characteristic 4-5 day frequency from almost zero to 3-4 ‰ (Tsukamoto *et al*., 1985*c*; Badger *et al.*, 1993). Elevated blood ethanol levels may be explained by the finding suggesting that a low carbohydrate content in the diet may reduce the rate of ethanol metabolism (Rao *et al*., 1987). In fact, when ethanol is administered together with carbohydrates, lowered blood ethanol levels have been observed, possibly as a consequence of accelerated hepatic metabolism (Sankaran *et al*., 1991; Yonekura *et al*., 1993).

This protocol of oral administration of a low-carbohydrate liquid ethanol diet may provide an affordable alternative to the technically demanding intragastric ethanol feeding model for experimental studies of alcoholic liver disease. In addition to the high blood ethanol levels, the daily intake of the ethanol di*et al*so was high: on the average 11-13 g/kg, similar to the ethanol exposure achieved in intragastric feeding model.

Another factor influencing the ethanol effects appears to be gender. Later studies with this oral ethanol feeding model have showed that the pathology is even more aggravated in female rats. The incidence of spotty/focal necrosis was frequent, and serum liver enzymes (ALT) were increased 7 –fold by ethanol in a study with female rats (Järveläinen *et al*., 2000).

Extrapolation of rodent studies to the human disease process should, of course, be done cautiously. However, an adequate daily supply of total carbohydrates may be an important factor when treating ALD patients. This might be of importance also during alcohol consumption. Indeed, individuals drinking alcohol with meals had much lower risk of developing alcoholic liver disease than individuals consuming alcohol without food (Bellentani *et al*., 1997).

CD14 endotoxin receptor and LBP

Immunocytochemistry and immunoblot analysis of isolated Kupffer cells from ethanol-treated rats showed an enhanced expression of CD14 protein. This was associated with a significant increase in the amount of hepatic CD14 mRNA (I, III). This is in line with earlier observations that the upregulation of CD14 is transcriptionally regulated (Matsuura *et al*., 1994; Su *et al*., 1998). Ethanol treatment also increased the amount of lipopolysaccharide binding protein (LBP) mRNA expression in liver samples (III, IV). Since it is known that the increase in CD14 and LBP in the liver is accompanied by an enhanced response to endotoxin (Martin *et al*., 1994; Treon *et al*., 1994; Louis *et al*., 1998), these two complementary responses might work together in the increased sensitivity to the hepatotoxic effects of endotoxin and the induction of inflammatory responses in the liver. Considering the important role of endotoxin in the etiology of ALD, priming of Kupffer cells with endotoxin by a CD14-dependent mechanisms might be a novel mechanism by which alcohol and endotoxins interact in the initiation of alcoholic liver disease. A recent genetic study brings further support to the significance of CD14 in pathogenesis of ALD. Alcohol consumers with a polymorphic C→T (-159) form in the promoter region of the CD14 gene that confers to the increased CD14 expression had a much higher prevalence of alcoholic cirrhosis (Järveläinen *et al*., 2000*b*). Observation of a high expression of CD14 mRNA in the perivenous region (IV) suggests that the higher CD14-regulated inflammatory activity in the perivenous area may contribute to the induction of liver injury specifically in that zone.

The factors that regulate the induction and expression of CD14 in the liver are not well understood. Endotoxin is known to upregulate Kupffer cell CD14 mRNA expression (Matsuura *et al*., 1994). Recent study showed that upregulation of the CD14 by alcohol is also mediated by endotoxin, since intestinal sterilization with antibiotics (polymyxin B and neomycin) prevented the effect (Enomoto *et al*., 1998). The evidence for a cytokine-mediated increase in CD14 expression is equivocal. Some cytokines, i.e. IL-4 and IFN-γ, have even been found to downregulate CD14 (Landmann *et al*., 1990). It remains to be clearly delineated if the increase in CD14 expression is a result of the invasion of cells into the liver or the increase in CD14 expression by the Kupffer cells that were already present in the liver. Chronic ethanol is reported to increase the number of Kupffer cells, an effect that by itself would increase liver CD14 (Shiratori *et al*., 1989).

The hepatic CD14 mRNA expression is most likely derived from Kupffer cells, since several studies indicate that its expression in the liver, both at the protein and mRNA level, is restricted to Kupffer cells (Matsuura *et al*., 1994; Tomita *et al*., 1994; Su *et al*., 1998; Kitchens, 2000). Staining of multiple tissues, including liver, tonsil, lymph node, spleen, thymus, skin, pancreas, lung, kidney, colon, and cerebellum, showed that in addition to tissue macrophages, only interstitial cells of skin and lung stained for CD14 (Bordessoule *et al*., 1993). In a study based upon rats acutely treated

with a massive (10 mg/kg) endotoxin dose, hepatocytes were found to express CD14 mRNA (Liu *et al*., 1998), and this was suggested to be a source for soluble CD14 during endotoxemia. However, hepatocytes do not seem to express CD14 under more physiological conditions. Further investigations are needed in order to exclude the possibility that the hepatocyte CD14 expression in that study was due to passive absorption of CD14 or due to contaminating Kupffer cells.

Chronic infusion of low-dose endotoxin

Previous studies, based upon both animal experiments and clinical observations, have suggested aggravation of ALD by chronically elevated levels of circulating endotoxins, which act as a continued stimulus for pro-inflammatory cytokine production. For example, if gut is sterilized by oral antibiotic treatment, experimental alcoholic liver injury is alleviated (Adachi *et al*., 1995). Several studies have demonstrated that acute endotoxin in combination with chronic alcohol administration augments the development of ALD (Bhagwandeen *et al*., 1987; Tanaka *et al*., 1992; Pennington *et al*., 1997). However, the effects of combined long-term exposure to endotoxin and ethanol on liver pathology and gene expression has not been studied before.

In this study endotoxin was given at a rate that caused moderate endotoxemia, in an attempt to mimic the continuous endotoxemia in alcoholics. The dose by itself caused only minimal, yet observable, damage. The changes, consisting of damage to the endothelial cell lining and accumulation of blood cells, are in agreement with earlier observations (Nayaar *et al*., 1989). The levels of circulating endotoxin that were needed (200-500 pg/ml), were 20-50 times higher than those found in ethanol-treated animals (mean 9.3 pg/ml) and were also much higher than in those studies using the intragastric ethanol feeding model (30-70 pg /ml) (Rivera and Thurman, 1998). However, this dose of endotoxin did not significantly enhance steatosis or focal inflammation/necrosis in ethanol-fed animals.

The failure of this model to potentiate the damage suggests that marked tolerance may develop to the continuous presence of relatively high levels of circulating endotoxin. Attenuation of the inflammatory response and of immunological functions has indeed been observed after prolonged endotoxin administration (Friedman *et al*., 1992). The continuous presence of LPS may lead to adaptation at the level of LPS signal transduction in Kupffer cells. Indeed, in the present study, while acute endotoxin markedly enhanced the expression of both LBP and CD14 mRNA, no such increase was seen after chronic endotoxin administration, suggesting that Kupffer cells from chronically endotoxin-treated rats were apparently in the low-responsiveness state. Similarly, in LPS tolerant monocytes, CD14 expression is unchanged (Ziegler-Heitbrock, 1995). Changes in Kupffer cell CD14 expression seem to be reflected in the pro-inflammatory cytokine release (Enomoto *et al*., 1998).

Endotoxin by itself caused occasional sinusoidal polymorphic cell infiltration. This is interesting from the clinical point of view, since neutrophil infiltrates are prominent in alcoholic hepatitis. However, while in the acute endotoxin/chronic ethanol model polymorphonuclear neutrophil infiltration is associated with coagulative necrosis, in this study, after chronic endotoxin challenge, there was no additional injury in the sites of neutrophils. Therefore, as Kupffer cells, also neutrophils are presumably in a hyporeactive, non-cytotoxic state. The observations that only activated neutrophils are

cytotoxic against hepatocytes suggest that events *in vivo* are preceded by Kupffer cell activation (Mavier *et al*., 1988; Schlayer *et al*., 1988; Liu *et al*., 1995).

A major goal of this study was to evaluate how the chronic co-administration of ethanol and endotoxin and the early pathological signs of ALD are reflected in the cytokine profiles. Hepatic mRNA expressions of proinflammatory cytokines TNF-α and IL-1β, both of which are considered to be important mediators in liver injury, were elevated after chronic endotoxin exposure. However, there was no significant increase in the enhanced expression of TNF-α and IL-1β in animals also receiving ethanol, in contrast to the large increase after acute endotoxin administration to animals chronically fed alcohol (Pennington *et al*., 1997). Thus the hepatic cytokine activation does not necessarily associate with liver damage, as has been suggested by several authors (Thurman *et al*., 1998; 1999; McClain *et al*., 1999).

Enhanced expression of anti-inflammatory cytokines has been observed previously in endotoxin tolerance. The absence of damage could be related to the high expression of potent anti-inflammatory cytokine IL-10, suggesting that it is important to consider the relative expression of both pro-inflammatory and anti-inflammatory cytokines. However, additional chronic ethanol treatment may disturb the balance between endotoxin-induced expression of proinflammatory and anti-inflammatory cytokines, thus promoting the development of damage. In our study this effect was seen with the anti-inflammatory cytokine TGF-β, which was significantly lower after ethanol-endotoxin than endotoxin alone.

It has been suggested that continuous endotoxemia leading to an uninterrupted stimulus for inflammatory cytokine production is more likely than an acute presence of endotoxin to produce liver damage in conjunction with alcohol (Thurman *et al*., 1998). The results of the present studies are not consistent with this suggestion: continuous administration of endotoxin did not produce more damage than acute administration. Actually, there has been no clinical study showing a correlation between endotoxin and TNF levels in patients with liver disease, suggesting that TNF release may be modified by various mechanisms. Furthermore, alcoholics with high plasma endotoxin levels do not necessarily show the severe clinical findings typical of endotoxemia. The different drinking pattern of the alcoholics may, however, cause temporal variations in their endotoxin levels, which could modify the development of tolerance. Endotoxin derived from acute bacterial infections may also potentially play a important role in the induction of inflammation in fatty livers. The further characterization of anti-inflammatory cytokines will be important to define the mechanisms governing the autoregulation of hepatic inflammation and might hopefully lead to new therapeutic approaches for alcoholic liver disease.

A complete understanding of the mechanism involved in cytokine release and its action in chronic endotoxemia will be critically important in the development of better therapeutic options for patients with ALD in the future. This experimental model of endotoxin tolerance offers a tool for exploring these mechanisms.

Mechanisms of gadolinium chloride protection

Since gadolinium chloride significantly reduced the severity of ethanol-induced fatty liver, but did not alleviate the inflammatory responses, it seems that the mechanisms by which $GdCl_3$ protects from the injury is primarily antisteatotic. The counteracting effect of $GdCl_3$ on steatosis may nevertheless be a crucial protective mechanism, since evidence is accumulating that fat accumulation is a less benign

condition than previously thought (Day and James, 1998). Particularly the microvesicular form of steatosis, also seen in this study, may propagate the development of a more severe damage (Day and James, 1998).

The persistence of CD14 expression in spite of ED2 depletion indicates that $GdCl_3$ destroys selected ED2-positive macrophage populations rather than all macrophages uniformly. In fact, studies on phenotypic alterations on Kupffer cells have shown that although $GdCl_3$ downregulates the expression of ED1, ED2, and the Kupffer cell-specific lectin-binding receptor KCR, the expression of the monocyte-macrophage specific gene product Pu-1 is unchanged (Hardonk *et al*., 1992; Rai *et al*., 1996; Roland *et al*., 1999).

Observation of a high expression of CD14 mRNA in the perivenous region fits with the recent observation that chronic ethanol treatment increases the expression of cytokines more in the perivenous region (Fang *et al*., 1998). Large ED2 positive Kupffer cells, probably those that are involved in phagocytosis and thus are capable of destroying the gadolinium aggregates, are located mostly in the periportal region (Hardonk *et al*., 1992; Laskin, 1997; Ahmad *et al*., 1999). These were also efficiently eliminated by gadolinium treatment as shown with the ED2 immunohistochemistry. The fact that $GdCl_3$ treatment did not affect CD14 expression suggests that gadolinium has little effect on the smaller, round-shaped, CD14-positive, cytokine-producing Kupffer cells, that are more numerous in the perivenous region (Tomita *et al*., 1994; Rai *et al*., 1996; Rüttinger *et al*., 1996). This could also explain why in this study $GdCl_3$ had little effect on inflammation. However, the large ED2-expressing periportal Kupffer cells that are most efficiently eliminated by $GdCl_3$ probably secrete a different pattern of mediators, such as eicosanoids, which affect hepatic carbohydrate and fat metabolism (Enomoto *et al*., 2000). It has been recently shown that while gadolinium increases the proportion of macrophages secreting TNF-α *in vitro*, it decreases the Kupffer cells secreting PGE_2 (Roland *et al*., 1999). It is conceivable that the effect of $GdCl_3$ on steatosis is mediated by this pathway.

A previous study by Koop *et al*. (1997), based upon the intragastric ethanol feeding model for ALD, reported that $GdCl_3$ alleviated ALD without affecting CYP2E1. In contrast, this study showed that both ethanol-induced steatosis and CYP2E1 induction were attenuated by $GdCl_3$. This suggests that protection by $GdCl_3$ could be, at least partly, due to less CYP2E1. However, $GdCl_3$ has multiple actions, and the mechanisms of CYP2E1 induction are complex. It is still possible that the association between CYP2E1 activity and fatty changes is circumstantial.

Although moderate CYP2E1 expression is found in Kupffer cells (Koop *et al*., 1991; Koivisto *et al*., 1996), the contribution of these cells to total liver CYP2E1 expression is most probably too low to explain the reduced CYP2E1 activity produced by $GdCl_3$. On the other hand, $GdCl_3$ treatment has been shown to increase Kupffer cell expression of TNF-α (Rai *et al*., 1996), and cytokines are known to reduce most hepatic P450 forms *in vivo* (Simpson *et al*., 1997; Morgan, 1997). In this study, however, no significant effect on the mRNA expression of the pro-inflammatory cytokines TNF-α and IL-1β by gadolinium was discerned. Alternatively, since $GdCl_3$ has been shown to reduce total P450 in the liver (Badger *et al*., 1997), $GdCl_3$ could act by reducing CYP2E1 directly in hepatocytes. It is intriguing that $GdCl_3$ seems to protect against compounds which have P450-dependent hepatotoxicity, i.e. CCl_4, but not against many other hepatotoxic compounds, i.e. $CdCl_2$ (Badger *et al*., 1997).

Data based upon the intragastric ethanol feeding model have indicated that the Kupffer cells play a key role in the inflammation produced and even in spotty necrosis

(Adachi *et al*., 1994; Koop *et al*., 1997). It is a characteristic feature of this model that the blood ethanol levels cycle with an amplitude of up to 5 ‰. This may cause an artificially recurring activation of Kupffer cells (Abril *et al*., 1997; Enomoto *et al*., 1998). In fact, daily administration of one large dose (5 g/kg) of ethanol for two months was recently reported to result in liver damage resembling that seen after intragastric ethanol feeding (Enomoto *et al*., 1999). In the oral low-carbohydrate liquid diet model used in the present study, the blood ethanol levels are continuously elevated but fluctuate much less (1.5 – 2.5 ‰), and Kupffer cell activation and their contribution to the pathogenesis may be less prominent, as suggested from the present data.

CYP2E1 and hepatic antioxidant defense

Much evidence suggests that alcohol-induced liver injury is associated with oxidative stress and free radical-mediated tissue damage. The ethanol-metabolizing P450 enzyme, CYP2E1, is suspected to play a role, since it is strongly induced by ethanol and possesses a high NADPH-oxidase activity. Indeed, much experimental evidence support the notion of an involvement of CYP2E1 in the pathogenesis of ALD (Lieber, 1997). Increased production of reactive oxygen species by microsomes from ethanol-fed rats correlates with the amount of CYP2E1 (Ekström and Ingelman-Sundberg, 1989). Studies obtained using intragastric ethanol feeding model also indicate a pathogenic role for CYP2E1 in ALD. For example, the extent of CYP2E1 induction correlates between lipid peroxidation or pathology score (Ingelman-Sundberg *et al*., 1988; Morimoto *et al*., 1995; Albano *et al*., 1996). In addition, two CYP2E1 inhibitors, diallyl sulfide and phenethyl isothiocyanate, alleviated steatosis, and also a colocalization of CYP2E1 expression and steatosis within the liver lobulus has been reported (Morimoto *et al*., 1995).

Orally administered alcohol liquid diet protocols have often been criticized because the histological changes do not correlate with the biochemical changes (Thurman *et al*., 1998). However, in the present study, CYP2E1 activity correlated with the extent of steatosis observed (IV). Although these findings do not prove causality, they fit the previous *in vivo* and *in vitro* studies suggesting the notion of an oxy-radical mediated pathogenic role for CYP2E1.

Among the antioxidant enzymes involved in the protection of hepatocytes against oxidative stress, the selenium-dependent glutathione peroxidase (Se-GPx) plays a key role, especially in the detoxification of lipid peroxides (Reinke *et al*., 1990). The activities of GSH peroxidase and of the other key antioxidant enzyme, GSH transferase (GST), were measured from the whole liver samples and from the periportal and perivenous zone lysates (IV). In agreement with previous findings, the activity of GST exhibited a perivenous dominance and was increased by ethanol, while that of GPx was lowered by ethanol and exhibited a periportal dominance (Kera *et al*., 1987). The reduction of glutathione peroxidase activity specifically in the perivenous zone might exacerbate the lesion, since CYP2E1 is expressed and induced almost exclusively in the perivenous region. A direct interaction between CYP2E1 and GPx is also possible. Ethanol oxidation by CYP2E1 produces 1-hydroxyethyl radicals, which have been shown to inactivate GPx (Puntarulo *et al*., 1999). GPx may also be inactivated by peroxides and oxygen-derived free radicals, presumably by binding to the active site of the enzyme (Blum and Fridovich, 1985; Pigeolet *et al*., 1990; Tabatabaie and Floyd, 1994; Kinter and Roberts, 1996). It is thus tempting to

speculate that oxygen species derived from ethanol-induced oxidative stress by CYP2E1 are involved in the reduced GPx activity, and that they contribute to cell damage.

SUMMARY

1. Oral administration of an ethanol liquid ethanol diet with a low carbohydrate content leads to persistently high blood ethanol levels, elevation of serum liver enzymes, and pathological changes beyond fatty liver. This procedure may offer a new, convenient model for studying the pathogenic mechanisms of alcohol-induced liver damage.
2. The increase in hepatic expression of CD14 and LBP should sensitize Kupffer cells to the hepatotoxic effect of endotoxin. We propose that this is a novel mechanism by which alcohol and endotoxins interact in the initiation of alcoholic liver disease.
3. Chronic exposure to endotoxin and ethanol lead to a marked attenuation of the inflammatory responses. Little hepatic damage was observed in spite of enhanced expression of pro-inflammatory cytokines. It is conceivable that a high expression counterbalancing anti-inflammatory cytokines and low-expression of CD14 endotoxin receptor and LBP may help to protect against injury along with the development of tolerance to endotoxin. This study suggests that chronically elevated endotoxin alone may not be a primary or determinant factor in alcohol hepatotoxicity.
4. Gadolinium chloride alleviates ALD primarily by decreasing ethanol-induced steatosis. Furthermore, the correlation of this effect to CYP2E1 induction lends support to the proposed pathogenic role for CYP2E1. In the present oral feeding model, attenuation of the Kupffer cell mediated pro-inflammatory effect does not seem to be directly involved, since $GdCl_3$ had no effect on ethanol-induced early inflammatory responses. The finding that the endotoxin receptor CD14 exhibited a marked perivenous expression and that $GdCl_3$ treatment did not affect its expression suggests that $GdCl_3$ inactivates selectively the periportally-located phagocytosis-mediating ED-2 expressing Kupffer cells while the perivenous cytokine-mediating CD14 expressing macrophages are less affected.

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