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ROLE OF OXIDATIVE STRESS IN TRIGGERING IMMUNE RESPONSE IN ALCOHOLIC LIVER DISEASE

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Abstract

Immune reactions toward the liver have been implicated in the pathogenesis of Alcoholic Liver Disease (ALD), however the antigens involved, as well the pathogenetic mechanisms, are still poorly characterized. We observed that the titres of IgG recognising epitopes derived from several lipid peroxidation products were significantly higher in alcoholic as compared to non-alcoholic cirrhotics, heavy drinkers with fatty liver only or healthy controls. ALD patients, but not heavy drinkers without liver disease had also peripheral blood CD4+ T-cell response specific for the MDA-HSA adduct that was associated with the presence of anti-MDA-HSA antibodies. This indicated that oxidative stress represents a key step in neoantigen generation in ALD, being able to promote both humoral and cellular immune responses associated with alcoholic liver disease. This latter conclusion was further strengthened by the observation that anti-phospholipid antibodies (aPL) present in a large fraction of ALD patients recognize as antigens oxidized phospholipids.

Oxidative stress could also play a role in promoting auto-immune reactions often associated with ALD. Previous studies have shown that the binding of hydroxyethyl free radicals (HER) to hepatic proteins, including cytochrome P4502E1 (CYP2E1), stimulates the production of specific anti HER antibodies in both alcohol-fed rats and alcoholic patients. Extending these investigations revealed that CYP2E1 modification by HER promoted the development of anti-CYP2E1 auto-antibodies. These auto-antibodies were detectable in about 40% of patients with advanced ALD, but not in heavy drinkers without liver damage. Using molecular modeling and site-directed mutagenesis we have characterized the specificity of these auto-antibodies, showing that they recognized two conformational epitopes on CYP2E1 C-terminal portion corresponding to, respectively, the G-helix and an area formed by juxtaposition of the J' and K" helices. We also observed that the development of anti-CYP2E1 auto-reactivity during ALD was closely associated with a genetic polymorphism of the cytotoxic T-lymphocyte antigen-4 (CTLA-4) suggesting the combined role of ethanol-induced oxidative stress and an impaired control of T-cell proliferation, by CTLA-4 polymorphism, in the breaking of self tolerance during alcoholic liver disease.

All together, these observations indicate the importance of ethanol-induced oxidative stress in stimulating both allo- and auto-immune reactions and suggest a possible role of immunological mechanisms in the progression of hepatic injury by alcohol.

Abbreviations

4-HNE	4-hydroxynonenal
AALD	Advanced Alcoholic Liver Disease
AAOP-HSA	oxidized arachidonic acid - Human serum albumin adduct
ADCC	antibody-dependent cell-mediated cytotoxicity
ADH	alcohol dehydrogenase
ALD	Alcoholic Liver Disease
ALDH2	mitochondrial aldehyde dehydrogenase
ALOP-HSA	linoleic acid - Human serum albumin adduct
AMA	anti-mithocondrial antibodies
ANA	anti-nuclear antibodies
anti-LKM	anti-liver kidney microsomes
anti-SMA	anti-smooth muscle antibodies
aPL	anti-phospholipid antibodies
β_2-GP1	β_2-glicoprotein 1
CL	cardiolipin
CTLA-4	cytotoxic T- lymphocyte antigen-4
CYP2E1	cytochrome P4502E1
ESR	Electron Spin Resonance
ELISA	enzyme-linked immunosorbent assay
GSH	reduced glutathione
H_2O_2	hydrogen peroxide
HD	heavy drinkers
HER	hydroxyethyl free radicals
HER-CYP2E1	CYP2E1- hydroxyethyl radical adducts
HNE-HSA	4-hydroxynonenal - Human serum albumin adduct
HSA	human serum albumin
IL-	interleukin-
LBP	LPS-binding protein
LPS	lipopolysaccarides
LTB4	leukotriene B4
MAA	malonildialdehyde-Acetaldehyde
MDA	malonildialdehyde
MDA-HSA	malonildialdehyde - Human serum albumin adduct
MEOS	microsomal ethanol oxidising system
MIP2	macrophage inflammatory protein-2
$O_2^{\cdot -}$	superoxide anion
$^{\cdot}OH$	hydroxyl radical
pANCA	perinuclear staining anti-neutrophil cytoplasmic antibodies
PBMC	peripheral blood mononuclear cells
PS	phosphatidylserine
ROS	reactive oxygen species
SAME	S-adenosyl-L-methionine
TLR4	Toll-like receptor 4
TXB2	tromboxane B2

List of original papers included in this thesis

1. Mottaran E, Stewart SF, Rolla R, Vay D, Cipriani V, Moretti MG, **Vidali M,** Sartori M, Rigamonti C, Day CP, Albano E. Lipid peroxidation contributes to immune reactions associated with alcoholic liver disease. Free Radic Biol Med 2002; 32:38-45.

2. Rolla R, Vay D, Mottaran E, Parodi M, **Vidali M,** Sartori M, Rigamonti C, Bellomo G, Albano E. Antiphospholipid antibodies associated with alcoholic liver disease specifically recognise oxidised phospholipids. Gut 2001; 49:852-859.

3. Stewart SF, **Vidali M,** Day CP, Albano E, Jones DEJ. Oxidative stress as a trigger for cellular immune responses in patients with alcoholic liver disease. Hepatology 2004; 39:197-203.

4. **Vidali M,** Stewart SF, Rolla R, Daly AK, Chen Y, Mottaran E, Vay D, Cipriani V, Jones DEJ, Leathart JB, Day CP, Albano E. Genetic and epigenetic factors in autoimmune reactions toward cytochrome P4502E1 in Alcoholic Liver Disease. Hepatology 2003; 37:410-419.

5. **Vidali M,** Hidestrand M, Eliasson E, Mottaran E, Reale E, Rolla R, Occhino G, Albano E, Ingelman-Sundberg M. Mapping conformational epitopes recognised by anti-Cyp2E1 auto-antibodies associated with halothane hepatitis and alcoholic liver disease (Submitted).

Introduction

Epidemiology and key morphological features of Alcoholic Liver Disease

Alcoholic Liver Disease (ALD) represents one of the main consequences of a prolonged alcoholic abuse. Epidemiological studies indicate that alcohol-related liver injury is an important cause of morbidity and mortality in either US and Europe. For instance in US alcoholic cirrhosis has a prevalence of 3.6/1,000 and is estimated as the 9th most frequent cause of all deaths in the general population, but the 4th most frequent among people in the active age (25-64 years) in urban areas. Similar data hold true also for Europe where, according to Lelbach (1975), there is rough correlation between the national per capita alcohol consumption and the prevalence of liver cirrhosis. Moreover, changes in the drinking pattern of the population are reflected in corresponding, later occurring, changes in the incidence of liver cirrhosis.

The disease encompasses a wide spectrum of lesions, the most characteristic are: alcoholic steatosis (fatty liver), alcoholic hepatitis, alcoholic fibrosis and cirrhosis.

Alcoholic steatosis is characterised by: a) the presence of giant and distorted mitochondria; b) the accumulation of small lipid droplets (microvescicular), containing mainly triacylglycerols and surrounding the cell nucleus, which, with persistent alcohol ingestion, coalesce into large clear macrovescicular globules, compressing and displacing the nucleus to the periphery of the hepatocytes. Fat accumulation is present virtually in every heavy drinker (men >60 g/day and women >20-40 g/day) and is initially centrilobular but may involve the entire lobule in severe cases. Moreover, with continuous alcohol intake, fibrous tissue may develop around the central veins and extend into the adjacent sinusoids. Up

to the time that fibrosis appears, the steatosis is completely reversible upon abstention from further alcohol consumption.

A minor percentage (17-42%) of heavy drinkers develop hepatitis characterised by the presence of: a) hepatocyte swelling, due to the accumulation of fat, water and proteins that normally are exported; b) hepatocyte necrosis and/or apoptosis; c) the presence of the so called Mallory's bodies, eosinophilic intracytoplasmatic inclusions composed mainly of keratin intermediate filaments; d) infiltration by polymorphonuclear (neutrophil) leukocytes, lymphocytes and macrophages. Alcoholic Hepatitis is almost always accompanied by a rapidly progressing sinusoidal and perivenular fibrosis. Periportal fibrosis may occasionally predominate, particularly following repeated bouts of heavy alcohol intake.

In spite of a continuous and heavy alcohol intake, only a minority of patients (about 10%) with alcohol abuse develop liver cirrhosis, the final and irreversible form of ALD. At the beginning, faint fibrous septa extend through sinusoids from central vein to portal regions, as well as from portal tract to portal tract, while the regenerative activity is limited to a micronodular scale. With time, fibrous septa enlarge and dissect surrounding nodules, progressively leading to a complete distortion of hepatic architecture, with loss of parenchyma and liver function.

Despite only about 20% of alcohol abusers develop serious liver disease (hepatitis and/or cirrhosis) (Mezey 1982), a recent prospective study has shown that within 48 months of follow-up more than half of the patients with cirrhosis and about two thirds of those suffering of cirrhosis plus alcoholic hepatitis had died (Chedid et al. 1991). Thus, once established, ALD represents a severe disease with an evolution worst than many cancers. Nonetheless, it remains open why the liver of the majority of alcoholics withstands the burden of heavy drinking for decades, while other individuals develop liver disease after a few years of alcohol abuse.

Pathogenesis of Alcoholic Liver Disease

During the past decades many pathogenetic factors, namely alcohol consumption length, dietary factors, sex and associated viral infections, have been suggested to explain alcohol-mediated liver injury. Although it is evident that lifetime alcohol intake, i.e. the product of years of drinking and daily average intake per body weight, is of decisive importance (Lelbach 1975), other data suggest that, beyond a threshold, there is no further influence of alcohol intake on the incidence of advanced ALD (Sorensen 1989).

For long time it was believed that liver disease in the alcoholics was exclusively due both to primary as well as secondary malnutrition, consequent of either maldigestion or malabsorption of nutrients. However, nowadays most of alcohol abusers have adequate diets, while it has been shown that in the absence of dietary deficiencies, and even in the presence of protein-, vitamin-, and mineral-enriched diets, ethanol produces fatty liver with striking ultrastructural lesions both in rats and in human volunteers, and fibrosis with cirrhosis in nonhuman primates (Lieber 1980; Lieber and DeCarli 1991; Lieber 1992).

Although dietary factors may contribute to liver injury (Nanji and French 1986), the great majority of alcohol-related diseases should be attributed to the biochemical changes induced by ethanol or its metabolites in the different tissues. Ethanol, in fact, is readily adsorbed from the gastrointestinal tract and is largely metabolized (90-98%) in the body, mainly in the liver (Lieber and Guadagnini 1990). With the exception of the stomach, extrahepatic metabolism of ethanol is small. This relative organ specificity justifies why ethanol toxicity mostly involves the liver.

The hepatocyte has three main pathways for ethanol metabolism, each located in a different subcellular compartment: a) the alcohol dehydrogenase (ADH) pathway in the cytosol; b) the microsomal ethanol oxidising system (MEOS) in the endoplasmic reticulum and c) the catalase-mediated oxidation in the peroxisomes.

The major pathway for ethanol disposal involves its oxidation to acetaldehyde by ADH, a cytosolic zinc metalloenzyme for which five different classes have been distinguished in human tissues. These classes of the enzyme arise from the association of eight different types of subunits into active dimeric molecules. Ethanol metabolism in the liver largely rests on class I isoenzyme activity, while class IV isoenzyme is mainly responsible for alcohol oxidation in the gastric mucosa. Subsequently, acetaldehyde is transformed into acetate mainly by mitochondrial aldehyde dehydrogenase (ALDH2). Both steps are coupled to the reduction of NAD to NADH.

Ethanol can be also oxidized to acetaldehyde by a cytochrome P450-dependent pathway, known as microsomal ethanol metabolising system (MEOS), that rests on the activity of cytochrome P450 2E1 isoenzyme (CYP2E1), requiring O_2 and NADPH as cofactor. CYP2E1 is mostly present in the centrilobular areas of the liver, but low levels of the enzyme are also detectable in the gastrointestinal tract, in the kidney, in the lung and in the brain. Although the K_m for ethanol of hepatic ADH (0.2-2 mmol/L) is lower than that of CYP2E1 (8-10 mmol/L) (Lieber and De Carli 1970), CYP2E1 activity is increased 2-10 times by alcohol intake as a result of both enzyme stabilisation and increased gene expression (Lieber and De Carli 1970; Lieber and De Carli 1968). Thus, ADH normally accounts for the bulk of ethanol oxidation at low blood ethanol concentrations, while at high ethanol levels, especially during long-term abuse, CYP2E1 contribution becomes also relevant. Catalase oxidises ethanol *in vitro* in the presence of an H_2O_2-generating system (Keilin and Hartree 1945). However, under physiological conditions, catalase does not appear to have a major role in ethanol metabolism in human liver.

The toxic action of acetaldehyde along with the metabolic disorders consequent to the excess production of NADH have been proposed to be responsible for causing the adverse effects of alcohol (Lieber 1982). Acetaldehyde was shown to activate lipocyte collagen production, to affect enzyme inhibition and to impair both mitochondrial oxidative phosphorylation as well as microtubular protein

export, causing this last a decrease in VLDL secretion, thus contributing to steatosis (Lieber 1994; Lindros 1978; Peters and Ward 1988; Lauteberg and Blizer 1988). The excess of reducing equivalents, primarily as NADH, on the other side, overwhelming the hepatocyte ability to maintain redox homeostasis, may determine a number of metabolic disorders (Lieber 1992), including hyperlactacidemia, which contributes to the acidosis and also reduces the capacity of the kidney to excrete uric acid, leading to secondary hyperuricemia. NADH surplus may also impair lipid and glucose synthesis.

Gender and viral infections have also been shown to be important determinants in the pathogenesis of ALD. Indeed, the average alcohol intake associated with an increased risk of cirrhosis and the threshold amount of ethanol are lower in women than in men. Moreover, the disease progression to more severe liver injury is accelerated in women. This increased susceptibility of females has been attributed both to a reduced body mass, hence a more severe damage because of the higher ethanol concentration with equal alcohol intake, as well as to a different hormone-dependent ethanol metabolism (Mezey et al. 1980; Teschke and Wiese 1982; Morgan and Sherlock 1977; Nakamura et al. 1979; Cole-Harding and Wilson 1987; Mishra et al. 1989; Arthur et al. 1984).
In many subjects with alcoholic cirrhosis, there is no evidence of antecedent viral hepatitis, but alcoholism and viral hepatitis B or C are commonly associated (Parés 1990). According to Corrao and Aricò (1998), the interaction between ethanol and HCV in promoting cirrhosis is additive for lifetime daily alcohol intake of about 50g/day, but it becomes synergistic at higher consumption (>125g/day). Nonetheless, a recent report suggests that even moderate alcohol intake can promote the progression of fibrosis in patients with HCV infection. Despite these findings, the exact interplay between hepatitis viruses and ethanol is still largely unknown.

In spite of several decades of researches involving pathogenetic factors of ALD, no definitive conclusion has been reached on the mechanisms of alcohol toxicity. In the recent years a growing interest has concerned the possibility that free radical mediated oxidative damage and inflammation might play a role in the pathogenesis of alcohol-related injury to the liver.

Role of oxidative damage in ALD pathogenesis

The contribution of oxidative injury to ethanol hepatotoxicity was first proposed by Di Luzio in the early 1960 following the observation that the pre-treatment of rats with antioxidants alleviated ethanol-induced liver fat accumulation (Di Luzio 1963). In the subsequent years a number of experimental evidences have confirmed the presence of ethanol-induced oxidative damage in the liver of rats, mini-pigs and baboons chronically fed with alcohol (Dianzani 1985; Albano et al. 1991a; Nordmann et al. 1992; Niemela et al. 1995; Lieber et al. 1997; Pawlosky et al. 1997).

Moreover, the possible implication of oxidative damage in human alcoholic liver disease is supported by several clinical studies. In particular, it has been observed that indices of oxidative stress, namely lipid peroxidation products and protein carbonyls, are higher in the liver biopsies or in the serum obtained from alcoholic patients as compared to specimens from non drinker subjects or patients with non-alcoholic liver diseases (Suematzu et al. 1981; Shaw et al. 1983; Situnayake et al. 1990; Baldi et al. 1993; Lecompte et al. 1994; Grattagliano et al. 1996; Aleynik et al. 1998; Hill and Awad 1999). Patients with alcoholic cirrhosis also exhale more pentane, a volatile end products of lipid peroxidation (Letteron et al. 1993). We have observed that among patients with alcoholic liver disease, markers of lipid peroxidation, such as blood levels of lipid hydroperoxides and malonildialdehyde (MDA), are about three times higher in subjects drinking more than 100 g ethanol/day than in those drinking below 100g ethanol/day, irrespectively to the extent of liver injury (Clot et al. 1994). Nonetheless the most relevant contribution in establishing a connection between free radical-mediated

oxidative damage and alcoholic liver disease has been obtained in the recent years by the use of a new experimental model of alcohol toxicity, based on the continuous intragastric administration of high amounts of alcohol, along with a liquid diet rich in fat and poor in carbohydrates (Tsukamoto et al 1985; Tsukamoto et al 1986). Using this experimental model it is possible to reproduce in rats several pathological features of human alcoholic liver disease, including steatosis, inflammatory infiltrates, focal necrosis and, after 16 weeks of treatment, liver fibrosis (Tsukamoto et al 1985; Tsukamoto et al 1986). The studies performed using rats receiving ethanol by intragastric feeding have shown that the development of histological signs of liver damage is associated with an increase in lipid peroxidation and protein carbonyls (Kamimura et al. 1992; Nanji et al. 1994; Rouach et al. 1997; Polavarapu et al. 1998). Furthermore, the replacement of corn oil with ω-3 unsaturated fatty acid rich fish oil stimulates lipid peroxidation and worsens liver pathology in intragastric ethanol fed rats (Nanji et al. 1994). Immunohistochemical analysis performed in the livers of ethanol-fed rats have shown that aldehydes derived from lipid peroxidation are present in the areas of fatty infiltration, focal necrosis and fibrosis. Similar findings have also been obtained in liver biopsies from patients with alcoholic liver disease, further supporting the concept of a causal relationship between oxidative events and the development of alcoholic liver injury (Niemela et al. 1994; Niemela et al. 1995; Tsukamoto et al. 1995; Ohhira et al. 1998; Niemela et al. 1999).

Several free radical species, i.e. oxygen-derived radicals, ethanol and acetaldehyde derived radicals, lipid derived radicals, have been proposed to play a role in causing ethanol-mediated oxidative tissue damage. These radical species can be produced by parenchymal cells as well as by tissue macrophages, endothelial cells and infiltrating phagocytes. On the other side, the impairment of cellular antioxidant defences is also a common feature in tissues exposed to alcohol. Thus the combination of increased free radical production and decreased

cellular antioxidants is probably responsible for the development of oxidative injury associated to alcohol abuse.

The formation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) represents an important cause of oxidative injury in many diseases associated to free radical formation. Several enzymatic systems, including microsomal monoxygenase system, mitochondrial respiratory chain, cytosolic xanthine and aldehyde oxidase have been proposed to produce ROS in cells exposed to ethanol.

The induction of CYP2E1-dependent monoxygenase activity by chronic alcohol exposure can represent an important source of oxygen radicals since, even in the absence of substrates, CYP2E1 has an especially high NADPH oxidase activity, leading to an extensive production of $O_2^{\cdot-}$ and H_2O_2 (Ronis et al. 1996). Indeed, liver microsomes obtained from rats chronically exposed to alcohol are more active than microsomes from untreated animals in producing $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$ and also show an enhanced susceptibility to lipid peroxidation (Cederbaum 1989; Persson et al. 1990). Oxygen radical production and lipid peroxidation can be selectively reduced by antibodies directed against CYP2E1 (Ekström and Ingelman-Sundberg 1989). Interestingly, in either human and rat liver microsomes NADH is equally effective as NADPH in promoting the production of reactive oxygen species (Dicker and Cederbaum 1992). Such a peculiarity can be important during ethanol intoxication because alcohol metabolism leads to an excess formation of NADH (Lieber 1994). Experiments performed using rats chronically treated with alcohol by the Tsukamoto-French model of intragastric feeding support the role of CYP2E1 in promoting ethanol-mediated oxidative stress. In this experimental model CYP2E1 induction by ethanol shows a positive correlation with the stimulation of lipid peroxidation, whereas compounds that interfere with CYP2E1 induction significantly reduce peroxidative damage (French et al. 1993; Morimoto et al. 1995; Albano et al. 1996).

The mitochondrial respiratory chain also represents an important source of superoxide anion in cells (Forman and Boveris 1988). Kukielka and co-workers

(1994) have reported that chronic alcohol consumption increases the production of reactive oxygen species in intact liver mitochondria incubated with NADH or NADPH by stimulating the activity of a rotenone-insensitive NADH-cytochrome c reductase on the outer mitochondrial membrane. The importance of this enzyme in causing ethanol-induced oxidative injury to the mitochondria could be even greater than that of the respiratory chain, since it does not require the transfer of NADH through the mitochondrial membranes. Accumulation of lipid peroxidation products and oxidative modifications of mitochondrial proteins and DNA (mtDNA) can be observed following both acute and chronic exposure of rats to ethanol, confirming that ethanol-mediated oxidative injury of mitochondria can take place *in vivo* (Kamimura et al. 1992; Wieland & Lautemburg 1995; Cahill et al. 1997).

The oxidation by xanthine oxidase of acetaldehyde present in the tissues during ethanol metabolism has been suggested as an alternative pathway for the generation of $O_2^{\cdot -}$ and hydroxyl radicals during ethanol metabolism (Shaw and Jayatilleke 1990; Grattagliano et al. 1996). Indeed, alcohol treatment stimulates the conversion of xanthine dehydrogenase to the $O_2^{\cdot -}$-producing oxidase form (Sultatos 1988; Abbondanza et al. 1989). Although the K_m of xanthine oxidase for acetaldehyde is very high (30 mmol/L) (Fridovich 1989), Puntarulo and Cederbaum (1989) have reported the formation of reactive oxygen species at concentrations of acetaldehyde close to those present in the liver following alcohol intake (about 0.1 mmol/L) (Stowel et al. 1980). Moreover Mira and co-workers (1995) have recently shown that NADH is a better substrate (K_m 28 μmol/L) than acetaldehyde (K_m 1 mmol/L) for $O_2^{\cdot -}$ generation by aldehyde oxidase.

In addition to the intracellular sources outlined above, the activation of phagocytic cells (resident macrophages, infiltrating monocytes and polymorphonuclear granulocytes) during inflammatory reactions might also contribute to the generation of reactive oxygen species in tissues damaged by alcohol (Smith 1994; Bautista and Spitzer 1992; Dorio et al. 1988).

In the presence of trace amounts of transition metals, most frequently iron, $O_2^{\cdot -}$ and H_2O_2, generated from either enzymatic or non-enzymatic sources, undergo to the, so called metal-catalysed Haber-Weiss reaction producing highly reactive hydroxyl radicals ($^{\cdot}OH$) (Aust et al. 1985). Alcohol abuse in humans is often associated with an impaired utilization and with an increased deposition of iron in the liver (Chapman et al. 1983; Irving et al. 1988). Moreover, experiments *in vitro* have shown that the rise in the cytosolic levels of NADH (Tophan et al. 1989) or the generation of $O_2^{\cdot -}$ can release catalytically active iron from ferritin (Shaw and Jayatilleke 1990). Consistently, the addition of ferritin to liver microsomes from ethanol-fed rats greatly stimulates both NADPH and NADH dependent lipid peroxidation. This effect is prevented by the addition of superoxide dismutase, iron chelators and anti-CYP2E1 antibodies (Kulielka and Cederbaum 1996), indicating that CYP2E1-generated $O_2^{\cdot -}$ might also contribute in mobilizing iron from ferritin. However, a role of iron in causing ethanol-induced oxidative stress of intact tissues has still not unequivocally proven.

Beside reactive oxygen species, free radical intermediates can also be originate from both ethanol and acetaldehyde. The metabolic conversion of ethanol to carbon-centred 1-hydroxyethyl free radical was first demonstrated in 1987 by two independent studies applying Electron Spin Resonance (ESR) spectroscopy and spin trapping technique to the analysis of NADPH-dependent pathway of ethanol metabolism in rat liver microsomes (Albano et al. 1987; Reinke et al. 1987). These observations have been confirmed by several other reports (Albano et al. 1988; Reinke et al. 1990; Rao et al. 1996) and by the demonstration that hydroxyethyl radicals can be generated *in vivo* in the liver of ADH-deficient deer-mice (Knecht et al. 1990) or of alcohol-fed rats (Moore et al. 1995; Knecht et al. 1995; Reinke et al. 1997a) receiving an acute dose of ethanol. CYP2E1 is mostly responsible for the formation of hydroxyethyl radicals in rat and human liver microsomes and anti-CYP2E1 antibodies or CYP2E1 inhibitors greatly reduce the spin trapping of these radicals (Albano et al. 1991; Albano et al. 1994a, Albano et al. 1996). So far, the mechanisms responsible for hydroxyethyl

radical production by CYP2E1 have not yet been completely elucidated. The results of *in vitro* experiments indicate that the interaction of reactive oxygen species ($O_2^{\cdot -}$ and H_2O_2) originating as a result of NADPH-oxidase activity of CYP2E1 with iron might be responsible for hydroxyethyl radical formation (Albano et al. 1988; Reinke et al. 1990; Albano et al. 1991; Knecht et al. 1993; Rao et al. 1996; Persson et al. 1990; Albano et al. 1988). Alternatively, a direct one-electron oxidation of ethanol by $O_2^{\cdot -}$ might also account for hydroxyethyl free radical formation (Knecht et al. 1993; Rao et al. 1996; Reinke et al. 1997b). As mentioned above, xanthine oxidase and aldehyde oxidase metabolize acetaldehyde with the formation of reactive oxygen species. In the presence of iron, $O_2^{\cdot -}$ and H_2O_2 can lead to the formation of $^{\cdot}OH$ radicals that are then responsible for attacking another molecule of acetaldehyde, giving rise to a carbon centered free radical, identified as methyl carbonyl species ($CH_3C^{\cdot}O$) (Albano et al. 1994b). Thus, acetaldehyde might act at the same time as source of reactive oxygen species, being substrate for xanthine oxidase, as well as target for $^{\cdot}OH$ radicals.

The lowering of liver antioxidant defences might significantly contribute to the development of ethanol-induced oxidative damage. A decrease in the hepatic content of reduced glutathione (GSH) is a common feature in ethanol-fed baboons (Shaw et al. 1981) as well as in alcoholic patients, in which GSH loss is independent from the nutritional status or the degree of liver disease (Shaw et al. 1983; Jewell et al. 1986; Situnayake et al. 1990). Recent studies have demonstrated that rats receiving alcohol chronically either by traditional pair feeding (Fernandez-Checa et al 1987) or by intragastric nutrition (Tacheshi et al. 1992) undergo a progressive decrease in the GSH pool of liver mitochondria. Such a selective depletion of mitochondrial GSH appears to depend upon a defect in the transfer of the tripeptide from cytosol to the mitochondrial matrix (Fernandez-Checa et al. 1991) due to a decreased efficiency of an ATP dependent GSH transporter in the inner mitochondrial membrane (Colell et al.

1997). Mitochondrial GSH depletion is more evident in centrilobular hepatocytes (Garcia-Ruitz et al. 1994) and precedes the development of lipid peroxidation and of functional alterations in ATP production (Tacheshi et al. 1992). This suggests that the effects of ethanol on mitochondrial GSH homeostasis might significantly contribute to the development of oxidative damage in these organelles (Fernandez-Checa et al. 1997). The importance of GSH homeostasis in preventing alcohol toxicity and oxidative injury is further supported by the observation that the liver GSH depletion favours lipid peroxidation and acute alcohol toxicity (Kera et al. 1989; Strubelt et al. 1987), while stimulation of GSH re-synthesis by treatment with S-adenosyl-L-methionine (SAME) reduces alcohol hepatotoxicity (Vendemiale et al. 1989; Lieber et al. 1990).

The lowering of liver and plasma levels of the liposoluble antioxidant vitamin E is often detectable during chronic alcohol administration to rats (Bjørneboe et al. 1987; Kawase et al. 1989; Sadrzadeh et al. 1994; Rouach et al. 1997) and has been also documented in patients with alcohol abuse with or without overt signs of liver disease (Tanner et al 1986; Bell et al. 1992; Lecompte et al. 1994; Clot et al. 1994). The mechanisms responsible for vitamin E decrease during alcohol intake have not yet been completely elucidated. However, Kawase and co-workers (1989) have proposed that an increased oxidation of α-tocopherol to the corresponding quinone might account for the loss of this antioxidant during alcohol exposure. Such an interpretation is consistent with the presence of an inverse correlation between the levels of α-tocopherol and lipid peroxidation products in either the liver of intragastric ethanol-fed rats or the plasma of patients with alcoholic cirrhosis (Sadrzadeh et al. 1994; Rouach et al. 1997; Clot et al. 1994). Nonetheless, the actual importance of vitamin E loss in the development of alcohol toxicity is still uncertain.

A number of studies have also investigated the effect of ethanol on the enzymes devoid to the detoxification of reactive oxygen species. The results of these studies are rather inconclusive. Acute alcohol intoxication lowers catalase, superoxide dismutase and glutathione S-transferase activities in several tissues,

but these effects are not constantly observed following chronic alcohol treatment (Nordmann 1994).

Although, in recent years, several studies using intragastric ethanol-fed rats have clearly demonstrated that markers of oxidative stress are positively correlated with the extent of histological liver lesions (Kamimura et al. 1992; Nanji et al. 1994a; Albano et al. 1996; Rouach et al. 1997; Polavarapu et al. 1998), the mechanisms by which free radical reactions contribute to the pathogenesis of alcoholic liver disease are still largely unknown.

Morphological and functional abnormalities of mitochondria represent one of the earliest manifestations of hepatocyte injury following chronic ethanol intoxication (Ishak et al. 1991). As discussed above, in the presence of ethanol, mitochondria can represent an important source of reactive oxygen species. Mitochondrial GSH loss also precedes the development of functional alterations on these organelles during chronic ethanol feeding, which can be partially prevented by restoring mitochondrial GSH by rat supplementation with SAME (Fernandex-Checa et al. 1997). Oxidative mitochondrial damage might affect the activity of the enzymes involved in mitochondrial fatty acid β-oxidation (Fromenty and Pessayre 1995). Moreover, by causing mutations in mtDNA, reactive oxygen species might reduce the efficiency of NADH oxidation by the respiratory chain enzymes, thus contributing to the lowering in ATP production, and the permeability transition of inner mitochondrial membranes (Rosser and Gores 1995). All these alterations can be regarded as possible causes for hepatocyte killing during ALD. It has also been proposed that oxidative mitochondrial damage, by releasing cytochrome c and other intramitochondrial proteins, can trigger liver cell apoptosis (Green and Kroemer 1998). On the other hand, oxidative modifications of mitochondrial proteins and mutations of mtDNA, resulting from oxidative attack, are likely responsible for the decrease in the activity of several mitochondrial enzymes and for the depression in the levels of mitochondrially-encoded sub-units of the electron transport chain observed in

animals exposed to ethanol (Coleman et al. 1994). These events can affect ATP production and lead to a premature ageing of mitochondria that respond less efficiently to ethanol-induced centrilobular hypoxia (Ji et al. 1982).

Recent studies in cultured human and rat hepatic stellate cells have shown that malonildialdehyde (MDA) and 4-hydroxynonenal (4-HNE) derived from lipid peroxidation are able to stimulate collagen type 1 production by activating gene transcription (Poli and Parola 1996). The mechanisms responsible for 4-HNE stimulation of procollagen gene expression involve the binding of 4-HNE to the 46 kD and 54 kD isoforms of c-Jun terminal kinase and the subsequent activation and translocation of these proteins into the nucleus where they induce AP-1 activation (Parola et al. 1998). Despite the difficulties in reproducing alcoholic fibrosis in experimental models, several studies have shown that the biochemical and immunohistochemical detection of MDA and 4-HNE in the liver of intragastric alcohol-fed rats or alcohol-fed mini-pigs precedes the appearance of the initial signs of hepatic fibrosis (Kamimura et al. 1992; Niemela et al. 1995). Although the very attractive hypothesis suggested by these and other reports about a relationship between oxidative stress and alcohol-induced liver fibrosis data are still far from conclusive.

Inflammation and ALD

One of the mechanisms of alcohol hepatotoxicity that has received increasing attention in the recent years concerns the role played by non-parenchymal liver cells, particularly by Kupffer cells, and the possibility that alcohol can lead to the activation of these cells by the action of lipopolysaccarides (LPS) derived from the external wall of Gram-negative bacteria (Nolan et al. 1980; Nolan an Camara 1988; Tsukamoto et al. 1984). Large amounts of LPS are commonly present in the gut as a result of the turn-over of the bacterial flora and the small portion physiologically adsorbed in the intestine is catabolised in the liver by Kupffer cells (Nolan an Camara 1988). Clinical studies have shown that increased plasma levels of LPS (endotoxemia) are frequently detectable both in patients with ALD,

as well as in heavy drinkers without signs of liver damage (Bode et al. 1987). Although it is still not clear how ethanol consumption can cause endotoxemia, experiments in alcohol fed rats have shown that the reduction of gut bacterial flora by the administration of antibiotics or supplementation of the diet with lactobacilli significantly reduced endotoxemia and effectively prevented the development of liver injury (Adachi 1995; Nanji et al. 1994; Thurman 1998). The mechanisms by which LPS might participate to ethanol hepatotoxicity is believed to involve the activation of Kupffer cells that represent about 80% of resident macrophages in the liver. In the plasma, in fact, LPS interact with a 60 kD protein, known as LPS-binding protein (LBP), and the complex is specifically recognized by the CD14 receptor and the Toll-like receptor 4 (TLR4) on the plasma membrane of Kupffer cells that trigger their activation (Jarvelainen et al. 1997; Su et al. 1998). Consistently, alcohol-induced liver injury is attenuated in homozygous (-/-) TLR4 knockout mice (Yin et al. 2000; Uesugi et al. 2000), while in humans a polymorphism in the promoter of CD14 gene, that increases the gene expression, is associated with an enhanced susceptibility to ALD in the Finnish population (Jarvelainen et al. 2001). Kupffer cells, as most other macrophages, respond to LPS by synthesizing and releasing cytokines (IL-1, IL-6, TNF-α, TGF-β1), eicosanoids, reactive oxygen species and nitrogen oxide (NO) (Tilg and Diehl 2000). In addition, the recruitment of inflammatory cells by the action of interleukin 8 (IL-8) and macrophage inflammatory protein-2 (MIP2) along with an up-regulation in the expression of leucocyte adhesion molecules E-selectin, and ICAM-1 can contribute to phagocyte accumulation in livers exposed to alcohol (Bautista 2002). Consistently increased production of TNF-α, leukotriene B4 (LTB4) and tromboxane B2 (TXB2) can be observed in ethanol-treated rats as well as in the blood of patients with ALD. Experimentally, the role of cytokines released by Kupffer cells in the development of alcoholic liver damage is supported by the attenuation of hepatic injury in chronically alcohol fed animals by the inactivation of Kupffer cells with gadolinium chloride (Adachi et al. 1994; Thurman 1998). Furthermore, liver injury induced by

chronic intragastric alcohol feeding can be reduced by the administration of antibodies against TNF-α or in mice knockout for TNF-R1 receptor.

In spite of the evidences discussed above, the role of LPS in promoting inflammation during ALD is still questionable on the light of recent evidence showing that in two experimental models of ALD, liver injury and inflammation develops even in the absence of endotoxemia (Ronis et al. 2003). Furthermore, Kupffer cell activation by endotoxins might unlikely be the only factor responsible for maintaining inflammatory response in the liver, since the continuous exposure to endotoxins causes the development of tolerance (Ziegler-Heitbrock 1995) and the chronic administration of ethanol in combination with endotoxins fails to increase alcohol hepatotoxicity (Jarvelainen et al. 1999).

Humoral and cellular immune responses in ALD

It is broadly recognized that chronic alcoholics often display increased serum immunoglobulin levels (Paronetto 1993; McFarlane 2000). The major classes of immunoglobulin IgA, IgG and IgM can all be elevated. Typically, IgA are increased both in alcoholics without liver disease and in ALD, while IgG are elevated in ALD and IgM only in ALD with active disease, such as alcoholic hepatitis. Moreover, the presence of auto-antibodies recognizing both liver and non-organ-specific antigens have been widely reported in ALD patients. Among these auto-antibodies, IgG against alcohol dehydrogenase and hepatic asialoglycoprotein receptor are present in 25-50% of ALD patients, while non-organ specific auto-antibodies such as ANA, anti-SMA, anti-LKM, AMA and pANCA are much less frequent (1-10%) (McFarlane 2000). A separate mention should be deserved to anti-phospholipid antibodies (aPL), an heterogeneous group of auto-antibodies with apparent specificity for negatively charged phospholipids (Harris 1990; Mc Neil 1991). Recent studies have shown that high aPL titres are frequent in patients with alcoholic hepatitis or cirrhosis and the presence of aPL in these patients seems to reflect the disease progression, showing significant correlation with the disease severity (Chedid et al. 1994).

However, aPL can also be detected in alcoholics without liver damage (Chedid et al. 1994; Biron et al. 1995; Zima et al. 1998). Lymphocyte infiltration is also frequently observed in advanced ALD, with activated CD8+T cells being a significant proportion of the infiltrate. T cells isolated from the peripheral blood of patients with ALD become activated on exposure to liver homogenates, and lymphocytes isolated from peripheral blood of ALD patients can kill autologous hepatocytes in *in vitro* systems (Cochrane et al. 1977, Izumi et al. 1983). These findings raise the possibility that cellular immune responses may, at least in a subgroup of ALD patients, directly contribute to hepatocyte damage (Chedid et al. 1993). Furthermore, in a recent study involving 200 ALD patients without serological markers of B and C viral hepatitis, a predominantly lymphocyte portal infiltrate was observed in 40% of cases, being significantly correlated with the extent of portal and septal fibrosis even after adjustment for age, gender, steatosis and alcohol consumption (Colombat et al. 2002).

A possible link between alcohol and the immune reactions described above has emerged from the observation that acetaldehyde is able to react with amino groups of lysine giving rise to stable protein adducts (Gaines et al. 1977; Nomura and Lieber 1981; Stevens et al. 1981; Kenny 1982; Donohue et al. 1983; Tuma and Sorrel 1985; Tuma et al. 1987a; Tuma et al. 1987b). Pioneering studies by Israel and colleagues (1986) have shown that the adducts originating from acetaldehyde binding to proteins cause the production of specific antibodies when injected into experimental animals. The presence of anti-acetaldehyde antibodies has subsequently been confirmed in rat chronically exposed to alcohol (Israel et al. 1986) and in alcoholic patients (Niemela et al. 1987; Koskinas et al. 1992), particularly in those with severe liver damage (Viitala et al. 1997). Furthermore, Yokoyama and co-workers (1993) have reported that the immunization of ethanol-fed guinea pigs with acetaldehyde-modified haemoglobin reproduces several features of alcoholic hepatitis. However, the interest in the immune response generated by acetaldehyde adducts is dampened

by the uncertainty regarding the identity of the antigens involved and by the low specificity for ALD, being indeed these antibodies detectable, although at lower titres, in heavy drinkers with no liver disease and patients with non-alcoholic liver disease (Klassen et al. 1995). More recently studies in our laboratory have shown that protein adducted by hydroxyethyl radicals (HER) are also immunogenic and lead to the formation of specific antibodies. These antibodies have been detected in rats chronically fed with ethanol (Albano et al. 1996) as well as in the sera of patients with alcoholic cirrhosis, but not in patients with non-alcoholic liver diseases (Clot et al. 1995). In alcoholic patients the formation of anti-hydroxyethyl radical antibodies shows a good correlation with CYP2E1 activity, as measured by chlorzoxazone hydroxylation (Albano et al. 1996; Clot et al. 1996). Experiments using immunofluorescence and laser confocal microscopy have demonstrated that anti-hydroxyethyl radical IgG from alcoholic patients react with epitopes present in the outer side of the plasma membrane of intact hepatocytes incubated *in vitro* with ethanol (Clot et al. 1997). Western blot analysis of plasma membrane proteins from these cells has allowed the recognition of three main plasma membrane protein bands, one of which corresponds to CYP2E1-hydroxyethyl radical adducts (Clot et al. 1997). Hydroxyethyl radical-CYP2E1 adducts on hepatocyte plasma membranes can also be detected by the co-localization of the immunofluorescence following combined cell immunostaining with anti-hydroxyethyl radical and anti-CYP2E1 antibodies (Clot et al. 1997). The presence of these plasma membrane adducts might have a role in the development of immune-mediated cytotoxicity, since isolated rat hepatocytes exposed *in vitro* to ethanol can be killed by antibody-dependent cell-mediated cytotoxicity (ADCC) reactions upon the addition of sera from alcoholic patients and normal human blood mononuclear cells (Clot et al. 1997). This suggests that, during alcohol abuse, the development of immune-toxic reaction towards hydroxyethyl radical-derived antigens might contribute to liver damage. On this respect, a clinical survey among alcoholic patients has

associated the presence of antibodies reacting with alcohol-modified hepatocytes with an increased risk of developing liver cirrhosis (Takase et al. 1993).

The observation that HER adducts with proteins are able to induce an antibody responses suggest the possibility that alcohol-induced oxidative damage might have a role in promoting immune reactions associated with ALD. It is well established that protein adducts with lipid peroxidation-derived products such as MDA, 4-HNE, acrolein and oxidized lipids have strong immunogenic properties (Palinski and Witztum 2000; Viitala et al. 2000). Immunohistochemistry studies have shown that these lipid peroxidation adducts are present in the liver during ALD and are localized in the areas of fatty liver infiltration, focal necrosis and fibrosis (Niemela et al. 1994). Moreover, the accumulation of MDA in the liver of intragastric alcohol-fed rats is associated with the development of antibodies recognizing protein-MDA adducts (Albano et al. 1996). Recent observation that chronic ethanol exposure in both experimental animals (Tuma et al. 1996; Xu et al. 1998) and humans (Rolla et al. 2000) is associated with the development of specific IgG, recognizing epitopes derived from MDA and acetaldehyde interaction (MAA), support the hypothesis that lipid peroxidation-derived adducts might contribute to the immune response in chronic alcoholics.

Project aims

The aim of my Ph.D. project has been the characterization of the role played by oxidative stress in promoting immunological reactions associated with alcoholic liver disease. In particular main goals of the project were:

1) to investigate the possibility that lipid peroxidation-derived adducts might contribute to both humoral and cellular immune responses associated with alcoholic liver disease;

2) to determine the possible role of oxidative stress in the development of auto-immune responses often detected in human alcoholics.

Results

Effect of lipid peroxidation in the development of immune reactions in ALD (Paper I)

The potential contribution of oxidative stress to the humoral immune responses observed in patients with alcoholic liver disease was initially investigated by measuring the titres of antibody towards protein adducted with several lipid peroxidation products in two groups of 50 patients with either alcoholic or non-alcoholic liver cirrhosis matched for sex, age and disease severity together with the same number of healthy controls. For these experiments a microplate enzyme-linked immunosorbent assay (ELISA) was developed using as antigen human serum albumin (HSA) modified *in vitro* by the reaction with 50 mmol/L of malonildialdehyde (MDA-HSA), 3 mol/L of 4-hydroxynonenal (HNE-HSA) and 3 mg of oxidized arachidonic acid (AAOP-HSA) or linoleic acid (ALOP-HSA). The results obtained demonstrated that patients with alcoholic cirrhosis had IgG, but not IgA or IgM, against MDA and HNE adducts significantly ($p<0.001$) higher ($abs._{490\ nm}$ 0.73 ± 0.25 for anti-MDA-HSA IgG; 0.10 ± 0.09 for anti-HNE-HSA IgG) than patients with non-alcoholic cirrhosis ($abs._{490\ nm}$ 0.61 ± 0.28 for anti-MDA-HSA IgG; 0.06 ± 0.02 for anti-HNE-HSA IgG) or control sera ($abs._{490\ nm}$ 0.52 ± 0.18 for anti-MDA-HSA IgG; 0.04 ± 0.02 for anti-HNE-HSA IgG). No statistically significant difference was instead observed between non-alcoholic cirrhotic and healthy control antibody titres. Cirrhotics with alcohol abuse, but not patients with non-alcoholic cirrhosis also displayed a significant increase in IgG recognising HSA complexed with oxidation products derived from arachidonic acid (AAOP-HSA) ($p<0.001$) or linoleic acid (LAOP-HSA) ($p<0.01$), whereas antibody titres against HSA complexed with other lipid peroxidation products, such as acrolein, 2-hexenal and methylglyoxal, were not different among groups. The frequency of sera with antibody titres against the different lipid peroxidation-derived epitopes above the 95^{th} percentile of the

control population was also significantly higher in alcoholic cirrhosis patients (55-72%) than non-alcoholic cirrhotics (8-13%).

In order to better characterise the relationship between the presence of antibodies against lipid peroxidation products and the severity of alcohol-related liver damage, we then investigated a group of long-term heavy drinkers with biopsy-proven fatty liver (n=23) or advanced liver disease (cirrhosis or extensive fibrosis with or without hepatitis; n=28). The titres of IgG against MDA, HNE and AAOP adducts were lower in patients with fatty liver compared to those in patients with advanced liver injury. Furthermore, the titres of antibodies towards HNE- and AAOP-derived epitopes in the fatty liver group were not different from those in the control group. From these results we concluded that antigens derived from lipid peroxidation actually contribute to the development of immune responses associated with alcoholic liver disease. In this study we also attempted a preliminary characterization of the epitopes involved, showing that, despite alcoholics with high IgG titres to one adduct tended to have high titres to all the others (correlation ranging from $r=0.54$ to $r=0.78$ $p<0.001$), the antigens recognised by the different antibodies were structurally unrelated. We also demonstrated that the anti-MDA IgG present in ALD patients did not recognise MDA-cross linked lysine epitopes frequently present in humans, but involved instead newly developed antigens.

Effect of oxidative stress in anti-phospholipid antibody development (Paper II)

High titres of antibodies targeting cardiolipin, phosphatidylserine, and phosphatidyletanolamine are frequent in patients with alcoholic hepatitis or cirrhosis, but can also be detected in a fraction of heavy drinkers with milder liver damage (Bird et al. 1994; Chedid et al. 1994; Biron et al. 1995; Zima et al. 1998). The observation that patients with alcoholic liver disease have antibodies

against oxidized fatty acids prompted us to investigate whether the presence of anti-phospholipid antibodies (aPL) might be associated with oxidative stress. The results obtained revealed that ALD patients with aPL had markers of oxidative stress higher than aPL-negative subjects and that the individual levels of aPL were correlated with anti-HSA-MDA and anti-HSA-AAOP titres ($r=0.67$ and 0.60, respectively; $p<0.0001$). ALD-associated aPL recognize as antigen cardiolipin modified by the peroxidation of the unsaturated fatty acid moiety, but not oxidation-protected cardiolipin ($abs._{490\ nm}$ 0.49 ± 0.34 vs 0.27 ± 0.18; $p<0.0001$). Statistically significant difference in the antibody titres between control and ALD sera was only observed when oxidised cardiolipin was used as antigen. The titres of IgG against oxidised cardiolipin were significantly ($p<0.0002$) higher in ALD patients with severe liver injury (Maddrey's DF index>90) as compared to patients with moderate or mild liver damage (Maddrey's DF index<90). A statistically significant difference ($p<0.01$) was also observed when the same patients were sub-grouped according to the Child-Turcotte classification. Conversely, no reactivity towards oxidized cardiolipin was instead evident in either heavy drinkers with steatosis only or in non-alcoholic cirrhotics.

It is known that the recognition of phospholipids by aPL is largely mediated by β_2-glycoprotein 1 (β_2-GP1), a plasmatic 50 kD glycoprotein which readily binds to anionic phospholipids (Hughes et al. 1993; Greaves 1999). The preadsorption of ALD sera with β_2-GP1 reduced by 80% the reactivity to oxidized cardiolipin, without affecting that antibody binding to other lipid peroxidation-derived antigens. On the other hand, the preadsorption of ALD sera on plates coated with oxidized cardiolipin did not affect the recognition of antigens formed by the reaction of HSA with oxidation products originating from the autoxidation of linoleic acid (HSA-LPP), the main unsaturated fatty acid of cardiolipin, while it decreased by 82% the binding to the same antigen. Thus, aPL detectable in ALD patients are specifically directed against complexes formed by oxidized phospholipids and β_2-GP1.

Altogether these data indicated that aPL recognize oxidized phospholipids complexed with β_2-GPI and suggested the involvement of oxidative mechanisms in the development of aPL associated with alcohol liver injury.

Oxidative stress and cellular immune response in ALD (Paper III)

Since lymphocyte infiltrates represent a common histological feature in advanced ALD, we have investigated whether oxidative stress might also contribute to the development of a cellular immune response. To this aim, we characterized the proliferative response of peripheral blood T-cell (PBMC T-cell) isolated from patients with advanced ALD (AALD), heavy drinkers without liver disease (HD) and mild/moderate drinking healthy controls to human serum albumin adducted with acetaldehyde (Aca-HSA) or with malonildialdehyde (MDA-HSA). PBMC T-cells from patients with AALD showed a significantly higher ($p=0.01$) mean proliferative response to MDA-HSA than those from HD or controls. Moreover, 10/28 (36%) of AALD patients had significant T-cell proliferative responses to MDA-HSA compared to 0/14 (0%, $p=0.02$) of the NALD group and 2/22 (9%, $p<0.05$) of controls. Conversely, no significant differences in proliferative response to Aca-HSA were seen between the three subject groups. Furthermore, the patients demonstrating positive lymphocyte proliferative response to MDA-HSA also had significantly higher antibody titres to the same antigen as compared to T-cell non-responders ($p<0.005$).

These results indicated that, beside humoral immune response, oxidative stress may represent an important stimulus for the development of cell-mediated reactivity associated with advanced ALD.

Anti-CYP2E1 auto-antibodies in ALD: prevalence and immuno tolerance breaking mechanisms(Paper IV)

Auto-immune responses are often associated with alcoholic liver disease (Paronetto 1993; McFarlane 2000). However little is known about the mechanisms that promote the loss of self tolerance. Experiments performed in chronic intragastric-fed rats previously showed that these animals developed circulating antibodies recognizing cytochrome P450 (CYP) isoenzymes CYP2E1 and CYP3A and the titres of anti CYP2E1 IgG correlated with the extent of hepatic injury (Lytton et al. 1999). Since anti-CYP auto-antibodies have been observed in patients with drug-induced hepatitis (Bourdi et al. 1990; Beaune et al. 1987; Leeder et al. 1992; Lytton et al. 2002; Beaune et al. 1994; Leeder et al. 1996; Choudhuri et al. 1997; Manns et al. 1997; Robin et al. 1997; Eliasson et al. 1996; Lecoeur et al. 1996), we investigated whether anti-CYP2E1 auto-reactivity might be associated to alcohol liver damage in humans and the mechanisms possibly involved in causing the loss of immune tolerance.

ELISA tests, performed using as antigen the human recombinant cytochrome P4502E1 (CYP2E1), have shown that IgG reactivity was not different between heavy drinkers without liver damage (HD) ($o.d._{490nm}$ 0.519 ± 0.119) and control groups ($o.d._{490nm}$ 0.474 ± 0.176). Conversely a statistically significant increase ($p<0.001$) in the titres of anti-CYP2E1 auto-antibodies was appreciable in alcoholic patients with advanced liver disease (ALD) ($o.d._{490nm}$ 0.773 ± 0.353). Anti-CYP2E1 IgG titres above the 95^{th} percentile of the controls were seen in 40% of ALD, but in only 11% of HD subjects. Furthermore, in ALD patients anti-CYP2E1 IgG titres were not significantly different between patients with cirrhosis and those with fibrosis only ($o.d._{490nm}$ 0.782 ± 0.364 vs 0.654 ±).

Subsequent experiments showed that the titres of anti-hydroxyethyl radical (anti-HER) IgG were also significantly higher in ALD ($o.d._{490nm}$ 0.147 ± 0.126) and HD ($o.d._{490nm}$ 0.132 ± 0.065) groups than in controls ($o.d._{490nm}$ 0.080 ± 0.035; $p<0.0005$). Interestingly, ALD patients positive for the anti-HER reactivity

displayed IgG titres significantly higher ($p<0.001$) and had a 4 times increased risk (OR 4.4; CI 1.8-10.9; $p=0.002$) of developing anti-CYP2E1 auto-antibodies than subjects without anti-HER immunity. This indicated that the immune response towards epitopes originating from CYP2E1 modification by hydroxyethyl free radicals is one of the factors contributing to the development of anti-CYP2E1 auto-reactivity in patients with severe alcohol liver injury.

In these same patients we also studied, in collaboration with Prof. Day's group at the University of Newcastle, the possible influence of genetic factors in the breaking of immune tolerance by genotyping, the patients for IL-10 (-627 C→A; -1117 G→A) and CTLA-4 (A→G exon 1) polymorphisms. Our analysis revealed that anti-CYP2E1 auto-reactivity was significantly ($p<0.05$) increased in ALD patients possessing at least one copy of the CTLA-4 G allele compared to A/A homozygotes. These patients were also more likely than A/A homozygotes to have anti-CYP2E1 IgG titres above the 95th percentile of the control group (OR 3.8; [1.4-10.3]; $p=0.011$). Neither association with IL-10 polymorphisms was found. Moreover, none of the genetic polymorphisms investigated had any effect on the development of IgG towards HER-derived epitopes.

However, in ALD patients with the CTLA-4 G allele the concomitant presence of IgG against HER epitopes was associated with the development of anti-CYP2E1 auto-antibodies, being increased by 23 times (OR 22.9; [4.2-125.6]; $p= 0.0001$) the risk of developing anti-CYP2E1 IgG as compared with subjects negative for both of these factors.

These results indicated that autoimmune reactions involving CYP2E1 are present in a significant fractions of subjects with advanced alcoholic liver disease. Moreover we demonstrated the antigenic stimulation by HER-modified CYP2E1 combined with an impaired control of T-cell proliferation by CTLA-4 mutation could promote the breaking of the immune-tolerance during alcohol liver exposure.

Characterization of the epitopes targeted by anti-CYP2E1 auto-antibodies (Paper V)

Beside patients with advanced alcoholic liver disease, anti-CYP2E1 auto-reactivity has also been detected in subjects with halothane hepatitis (Bourdi et al. 1996; Eliasson et al. 1996. The observation that CYP2E1 auto-antibodies target functionally active CYP2E1 present on the outer layer of hepatocyte plasma membrane (Eliasson et al. 1996) prompted us to investigate the epitope specificity of CYP2E1 auto-antibodies in order to understand more about their formation and eventually to get information of value for the development of more specific diagnostic tests.

We initially selected sera from 5 patients with alcoholic liver disease (ALD) and 5 patients with halothane hepatitis having high titres of IgG against recombinant human CYP2E1 in ELISA assays. The antibody specificity for CYP2E1 was confirmed by immunoprecipitation experiments using [^{35}S]-methionine-labelled CYP2E1 translated *in vitro* using the rabbit reticulocyte system. However, subsequent experiments showed that these sera recognize mainly conformational epitopes since anti-CYP2E1 reactivity was lost using CYP2E1 constructs with the N-terminal (222 as) or C-terminal (271 as) moieties or in Western blot under denaturating and reducing conditions.

To characterize these conformational epitopes, we studied the effects of single amino acid substitutions on the antigenic capacity of the whole molecule. A computer simulated structure of CYP2E1 was generated using the Swiss-Model automated comparative protein modeling server and the crystal structure of rabbit CYP2C5, that shares 59% amino acid sequence homology with CYP2E1. Potential residues for mutagenesis were selected by combining theoretical, bioinformatics and experimental approaches. In order to produce major changes in the configuration of the possible epitopes without disrupting the tertiary structure of the molecule, we replaced with alanine charged residues of lysine, arginine and glutamic acid.

Immunoprecipitation experiments performed using CYP2E1 variants correctly folded, as assessed by spectrophotometric reading at 450nm, showed that Ala substitutions of Lys272, Lys342 and Lys420 affected the antibody binding to the highest extent. The effects of Ala substitutions for Lys324, Arg374, Phe421 and Lys440 were less consistent, being evident with few sera. Although the mutations able to reduce CYP2E1 antigenicity involved amino acids located far away from each other in the J'-helix and the β-sheet between K and L helices, respectively, computer simulations revealed that the positions of Lys342, and Lys420 on the tertiary structure were rather close (about 25-30 nm) and identified an area in CYP2E1 surface compatible with the presence of a distinct conformational epitope. A further epitope was identified by the combined substitution of Lys243, Glu244, Glu248 and Lys251.

Taken together these results indicated that anti-CYP2E1 auto-antibodies recognize at least two conformational epitopes located in the G helix and between J' and K" helices in the C-terminal portion of the molecule, respectively. Furthermore, the orientation of CYP2E1 in relation to the membrane shows that these epitopes were both on the outer portion of the molecule and well accessible to antibody recognition.

Discussion

Oxidative stress and immune response

Oxidative stress is recognised to play an important role in the pathogenesis of hepatic injury by alcohol. In humans alcohol-induced oxidative damage is documented by an increase in serum markers of liver lipid peroxidation, such as conjugated dienes, malonildialdehyde (MDA), 4-hydroxynonenal (4-HNE) and F_2-isoprostanes as well as by the immunohistochemical detection of proteins adducted by lipid peroxidation products in the areas of liver fatty infiltration, focal necrosis and fibrosis (Niemela et al. 1994; Tsukamoto et al. 1995; Lettèron et al. 1993; Clot et al. 1994; Aleynik et al. 1998; Meager et al. 1999; Niemela et al. 1999). According to the notion that lipid peroxidation-derived protein adducts have strong immunogenic properties (Palinski et al. 1990; Palinski et al. 2000), we have observed the presence of elevated titres of circulating IgG towards MDA, 4-HNE and oxidized arachidonic acid adducts in a large fraction (55-70%) of patients with biopsy proven advanced alcoholic liver disease (alcoholic hepatitis and/or cirrhosis), but only in few (8-13%) subjects with fatty liver only, irrespective of the magnitude and the duration of alcohol intake. This observation is consistent with experiments using intragastric alcohol-fed rats revealing an association between the accumulation of malonildialdehyde (MDA) within the liver and the development of antibodies recognizing protein-MDA adducts (Albano et al. 1996). A significant proportion (36%) of ALD patients also have peripheral blood CD4+ T-cell responses specific for the MDA-HSA adduct. Such response is absent from all heavy drinkers without liver disease, and from the vast majority of normal drinking controls. The absence of MDA-HSA-specific PBMC T-cell responses in the NALD group does not reflect a global lack of T-cell proliferative capacity since the responses to the pan-T-cell stimulating agent OKT3 is similar in all subject groups. Interestingly anti-MDA-HSA antibody levels are significantly higher in patients demonstrating a positive T-cell

response to MDA adducts than in apparent T-cell non-responders, indicating that oxidative stress represents a key step in neoantigen generation in ALD, being able to promote both humoral and cellular immune responses.

The exact nature of the antigens responsible for stimulating the immune response in humans is still uncertain. For instance, the reaction of MDA with lysine groups leads to the formation of several products, including N^{ε}-β-lysyl-amino-acrolein, N^{ε}-propenal-lysine, N-lysyl-4-methyl-1,4-dihydro-pyridine-3,5-dicarbaldehyde, and amino-3-imino-propene-lysine cross links (Esterbauer et al. 1991; Uchida et al. 2000; Slatter et al. 2000). Moreover, the combination with arginine gives rise to N^{δ} -(2-pyrimidyl)-L-ornithine adducts (Slatter et al. 2000). Human sera from healthy controls show extensive immune reactivity toward MDA-modified proteins. This reactivity is attributable largely to the presence of IgG and IgM recognising amino-3-imino-propene bridges between lysine residues (Vay et al. 2001). However, our data demonstrate that the increase in anti-MDA IgG observed in patients with alcoholic liver disease does not involve these antibodies and is evident also using MDA adducted HSA prepared under reducing conditions that does not contain amino-3-imino-propene cross-links. Among the possible epitopes, N-lysyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde is likely to play an important role. Indeed, preliminary experiments indicate that the preadsorption of ALD sera with this compound reduces by about 50% the reactivity toward MDA-HSA. It is also possible that the T-cell response against MDA-HSA observed in ALD patients might actually involve as antigen N-lysyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde. On this latter respect it is noteworthy that the methyl-pyridine adduct is also generated by the combined reaction of MDA and acetaldehyde with lysine and is responsible for antibody production in both alcohol-fed animals and human alcoholics (Xu et al. 1998; Rolla et al. 2000). The structure of the epitopes responsible for the development of anti-HNE reactivity in patients with alcoholic liver disease remains even more unclear. HNE is known to generate immunogenic adducts with both lysine and

histidine (Uchida et al. 2000). However, experiments using a monoclonal IgG against HNE-histidine hemiacetal largely exclude the recognition of this epitope by the human anti-HNE antibodies. Thus, a role for 2-pentyl-pyrrole generated by the reaction between HNE and lysine can not be excluded. Patients with alcoholic liver disease have appreciable titres of IgG against protein complexed with oxidised arachidonic acid and linoleic acid. Our pre-absorption experiments demonstrate that these antibodies are unrelated to those directed against MDA and HNE adducts, despite the fact that both aldehydes originate during unsaturated fatty acid peroxidation. According to a recent study in patients with macular degeneration is possible that the reactivity against lipid hydroperoxide-modified albumin might involve epitopes with the structure of 2-carboxyethyl- and 2-carboxyheptyl-pyrroles (Gu et al. 2003).

The reasons why alcohol-induced oxidative damage promotes the formation of such a variety of antibodies are still unclear. The onset of an antibody response requires that the peptide antigens might be presented to $CD4^+$ T lymphocytes in conjunction with molecules of the class II mayor histocompatibility complex (MHC) and accessory signals. Therefore, only professional antigen presenting cells, that in the liver are represented by hepatic dendritic cells, Kupffer cells and sinusoidal endothelial cells, are capable to this task (Ting and Trowsdale 2002). On this latter respect, Schneiderhan and co-workers (2001) have recently reported that human hepatic stellate cells (HSC) have the capacity of specifically recognize MDA-modified proteins through the interaction with CD36 scavenger receptor. Such an interaction might be important in promoting the immune response, since human HSC have recently been shown to act as antigen presenting cells and are capable of stimulating lymphocyte proliferation (Vinas et al. 2003). Despite hepatocytes normally do not express MHC class II molecules, an aberrant expression is often seen in conjunction with chronic liver diseases, including ALD (Chedid et al. 1993). The possible significance of this phenomenon in relation to the development of both allo- and auto-immune

response has emerged from a recent study showing that hepatocytes expressing MHC class II also have co-stimulatory B7.1 (CD80) molecules and can function as antigen presenting cells capable to activate $CD4^+$ T lymphocytes (Herkel et al. 2003). Thus, in the course of ALD protein modification by HER or lipid peroxidation products inside the hepatocytes might directly trigger the activation of $CD4^+$ T cells infiltrating the liver and promotes antibody production by the Th2 immune response.

Little is known about the mechanisms leading to production of anti-phospholipid antibodies (aPL) associated with ALD. A growing body of evidence indicates that apoptotic cells can represent an important source of auto-antigens (Savill et al. 2002). In particular, the generation of anti-DNA and anti-phospholipid antibodies has been associated with an immune response towards apoptotic cells (Dicker et al. 2002; Rauch et al 2000). Oxidative modifications of membrane phospholipids have also been implicated in the mechanisms responsible for the recognition of apoptotic cells by phagocytes (Fadok and Chimini 2001). We have observed an association between the presence of anti-phospholipid antibodies and the extent of oxidative stress in ALD patients (Rolla et al. 2001). ALD-associated anti-phospholipid antibodies specifically recognise as antigen phospholipids modified by the peroxidation of the unsaturated fatty acid moiety alone or in combination with β_2-glycoprotein 1 (β_2-GP1), a plasmatic 50 kD glycoprotein which readily binds to anionic phospholipids (Rolla et al. 2001). According to Manfredi and co-workers (2002) the failure to clear apoptotic cells might result in secondary necrosis and in the stimulation of inflammatory reactions that favour the presentation of self-antigens to the immune system.
Such mechanisms might contribute to the promotion of auto-immunity in ALD, since hepatocyte apoptosis is greatly increased in alcoholic hepatitis and correlates with the severity of liver injury (Natori et al. 2001; Ziol et al. 2001). Moreover, McVicker and colleagues (2002) have recently reported that chronic ethanol feeding reduces by about 50% the capacity of hepatocytes to recognise

and remove adjacent apoptotic cells through the asialoglycoprotein receptors. Thus, it is possible that phospholipid oxidation in apoptotic hepatocytes might be responsible for the generation of anti-phospholipid antibodies in ALD patients.

Besides developing antibodies directed towards a variety of allo-antigens, patients with ALD not rarely have signs of auto-immune reactions (Paronetto 1993). Both lymphocyte-mediated response to autologous human hepatocytes (Izumi et al. 1983) and circulating antibodies directed against self-antigens, such as alcohol dehydrogenase, hepatic asialoglycoprotein receptor are detectable in a significant fraction of patients with alcoholic hepatitis or cirrhosis (see McFarlane 2000 for review). Moreover IgG directed against cytochrome P450 (CYP) isoenzymes CYP2E1 and CYP3A have been detected in chronic intragastric ethanol-fed rats (Lytton et al. 1999). In this experimental system, the titres of anti-CYP2E1, but not those of anti-CYP3A IgG, were associated with the severity of alcohol liver damage and the inhibition of CYP2E1-mediated ethanol metabolism by chlormethiazole prevented both liver injury and anti-CYP2E1 auto-reactivity. These observations have been confirmed in humans showing that the titres of IgG against human recombinant CYP2E1 are increased in patients with advanced ALD but not in healthy controls or heavy drinkers without clinical evidence of liver damage (Vidali et al. 2003). Anti-CYP auto-reactivity is not uncommon in liver diseases and one of the mechanisms proposed to explain the formation of anti-CYP auto-antibodies postulates that CYP alkylation by reactive drug metabolites promotes not only a humoral immune response against the modified protein, but also favours the activation of normally quiescent auto-reactive lymphocytes, leading to the production of antibodies to the native CYP molecules (Van Pelt et al. 1995; Griem et al. 1998). Indeed, auto-antibodies against conformational epitopes in CYP2E1 and CYP2C9 are often present in the sera of subjects with halothane or tienilic acid hepatitis along with antibodies recognizing trifluoroacetyl-CYP2E1 and tienilic acid-CYP2C9 adducts (Eliasson and Kenna 1996; Lecoeur et al. 1996). Accordingly, we have

observed that ALD patients with antibodies against hydroxyethyl radical-derived (HER) antigens have higher titres and a 4 times increased risk of developing anti-CYP2E1 auto-reactivity as compared to patients that do not develop allo-immunity against HER-derived epitopes (Vidali et al. 2003). This indicates that CYP2E1 alkylation by HER is critical for the development of anti-CYP2E1 auto-reactivity in ALD patients. Nonetheless, besides oxidative damage, our studies also indicate the contribution of genetic factors in causing the loss of immune tolerance. In particular we have observed that the presence of a single nucleotide mutation (+49 A→G transition) in exon 1 of the immunoregulatory cytotoxic T lymphocyte associated antigen-4 (CTLA-4) in combination with an immune response to HER increases by 23 times the risk of developing anti-CYP2E1 auto-reactivity. CTLA-4 is a membrane receptor protein expressed on $CD25^{+}CD4^{+}$ regulatory T lymphocytes as well as on activated T-cells that is involved in the down-modulation of T cell-mediated immune responses (Waterhouse et al. 1999). In humans the +49 A→G transition in the exon 1 of the CTLA-4 gene causes the substitution of threonine for alanine at position 17 in the leader peptide sequence and reduces the expression of CTLA-4 on the plasma membrane during T cell stimulation. Furthermore, CTLA-4 polymorphism has been associated with several autoimmune diseases and, in particular, with type-1 auto-immune hepatitis and in primary biliary cirrhosis (Agarwal et al. 2000a; Agarwal et al. 2000b). Recent observations indicate that the frequency of CTLA-4 polymorphism is significantly increased in the patients with advanced ALD, among whom 67% possess at least 1 copy of the G allele versus 49% in heavy drinkers without liver damage or with steatosis only and 46% in healthy controls (Day et al. 1999; Stewart et al. 2001). All together these data suggest that the presence of antigenic stimulation by HER-modified CYP2E1 peptides combined with an impaired Th cell regulation by the mutant CTLA-4 allele favour the expansion of auto-reactive Th cell clones. It would, therefore, appear that both genetic (CTLA-4) and epigenetic (immune response against CYP2E1-HER adducts) factors determine why some patients with ALD develop autoimmune

reactions directed against CYP2E1 and others do not. This represents the first demonstration of how heavy drinking might lead to the breaking of self-tolerance in the liver (Figure 1).

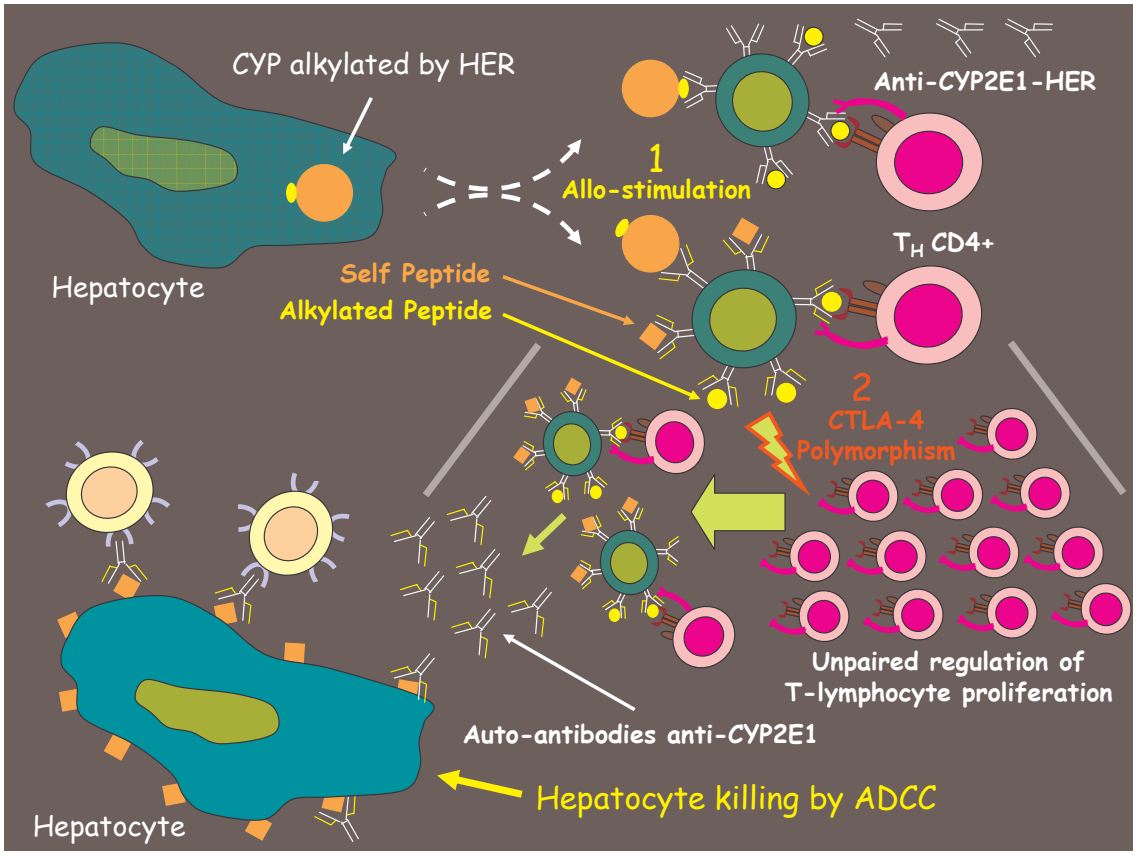

Figure 1: Mechanisms responsible for the development of auto-antibodies against cytochrome P4502E1 (CYP2E1) in ALD patients.

The alkylation of CYP2E1 by HER generated during ethanol oxidation results in the production of anti-HER antibodies and also favours the activation of normally quiescent auto-reactive lymphocytes, leading to the production of antibodies against the self CYP molecules. This latter process is favoured by an impaired control of T cell proliferation due to genetic polymorphism of immunoregulatory CTLA-4 molecule. The reaction of anti-CYP2E1 auto-antibodies with CYP2E1 expressed on the outer layer of hepatocyte plasmamembrane might trigger ADCC reactions toward liver parenchimal cells.

Possible role of immune response in the pathogenesis of Alcoholic Liver Disease

At the moment the role played by immune mechanisms in the pathogenesis of ALD is still poorly characterised. Circumstantial evidence suggests the occasional rapid exacerbation of the disease in abstinent patients who return to drink and the beneficial effect of corticosteroids in some patients. Moreover, an epidemiological prospective survey has associated the presence of antibodies toward alcohol-modified hepatocytes with an increased risk of developing alcoholic liver cirrhosis (Takase et al. 1993). We have observed that elevated titres of antibodies towards lipid peroxidation adducts are evident in patients with biopsy proven advanced alcoholic liver disease, but not in subjects without liver injury or with fatty liver only, irrespective of the magnitude and the duration of alcohol intake. Moreover, anti-MDA and anti-HNE IgG are higher in cirrhotics with Child's Grade B and C as compared to those with Child's Grade A. Accordingly, Viitala and co-workers (2000) have shown a significantly higher prevalence of antibodies toward oxidised and MDA-modified lipoproteins among alcoholics with more severe liver injury than both heavy drinkers without liver damage and controls. Since the immune stimulus represented by the extent of lipid peroxidation is not influenced by the severity of liver disease, as estimated by Child-Turcotte Score (Clot et al. 1994), our data suggest the possibility that an immune response involving lipid peroxidation antigens might have an important role in the progression of alcohol liver damage. On this respect, we have observed that moderate alcohol intake in patients with chronic hepatitis C stimulates the production of lipid peroxidation-related antibodies and that high titres of these antibodies are associated with four times increase in the frequency of diffuse piecemeal necrosis. Parallel studies aimed to investigate the cellular immunity in ALD have shown that a significant proportion (36%) of patients with advanced disease, but not heavy drinkers without liver injury, have peripheral blood CD4+ T-cell responses specific for the MDA-HSA adduct. In

the former a close association between T-cell and antibody responses to MDA-HSA is also evident. Thus, the presence of IgG against lipid peroxidation-derived antigens might represent an hallmark for a T cell-mediated reaction targeting hepatocytes undergoing oxidative stress. This hypothesis is supported by *in vitro* studies showing the cytotoxic potential against alcohol treated autologous hepatocytes of lymphocytes from ALD patients (Cochrane et al. 1977; Izumi et al. 1983). Moreover, histological analysis suggests the presence of activated T-cells with an effector phenotype in liver biopsies from ALD patients (Colombat et al. 2002). Interestingly, the proportion of patients showing lymphocyte infiltration is similar to the proportion of patients we have found to have an MDA-HSA specific peripheral blood T-cell response. These preliminary data argue in favour of a possible role of cell-mediated immune mechanisms in at least a subgroup of patients with ALD (Figure 2).

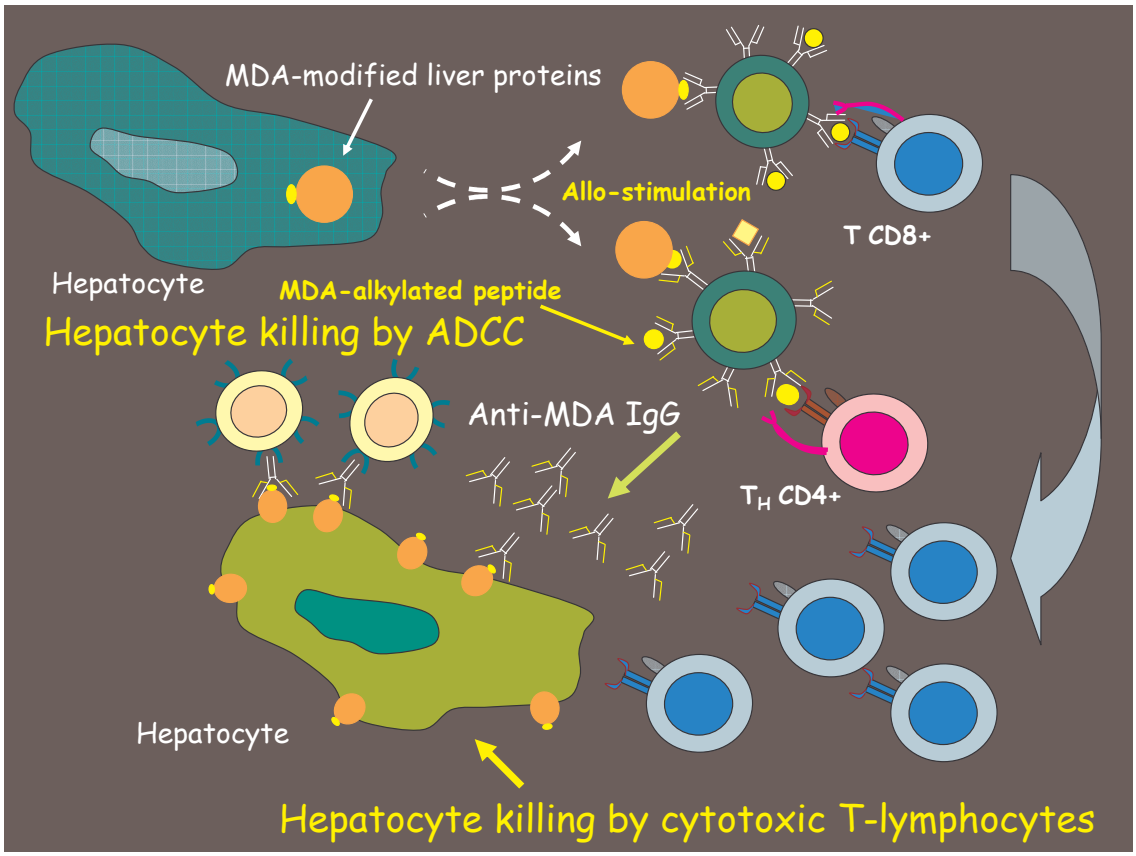

Figure 2: Possible role of lipid peroxidation-derived protein adducts in Alcoholic Liver Disease. The alkylation of liver proteins by lipid peroxidation-derived products might result in the hepatocyte killing by both an ADCC response anti-MDA IgG-mediated and by the activation of cytotoxic T-lymphocytes.

According to previous surveys, anti-phospholipid antibodies targeting oxidized phospholipids are also prevalent among non-abstaining patients with alcoholic hepatitis or cirrhosis, particularly cirrhotics with severe liver damage (Child grade B and C) (Biron et al. 1998). The contribution of aPL to the clinical history of ALD is at the moment poorly understood. Alcoholic liver injury is associated with stimulation of hepatocyte apoptosis and oxidative events have been proposed to play a role in triggering apoptotic changes in hepatocytes exposed to ethanol. On this respect, studies in progress in our laboratory indicate that anti-phospholipid antibodies present in ALD patients selectively bind to apoptotic, but not to living cells, by recognising oxidised phosphatidylserine exposed on cell surface (Vay et al. 2002). The presence of phosphatidylserine on the plasma membrane of apoptotic cells represents a key signal for their recognition by phagocytes (Fadok and Chimini 2001). According to recent evidence the phagocytosis of apoptotic cells stimulates macrophages to secrete anti-inflammatory cytokine TGF-β (Huynh et al. 2002). The binding of anti-phospholipid antibodies to phosphatidylserine not only would affect such a scavenging pathway, but would also favour the recognition of apoptotic bodies by the IgG-Fc receptors and the consequent pro-inflammatory activation of phagocytes (Manfredi et al. 1998). Thus, in the presence of anti-phospholipid antibodies, ethanol-induced hepatocyte apoptosis might represent a fuel for the continuous release of cytokines by Kupffer cells and other phagocytes infiltrating the liver (Figure 3).

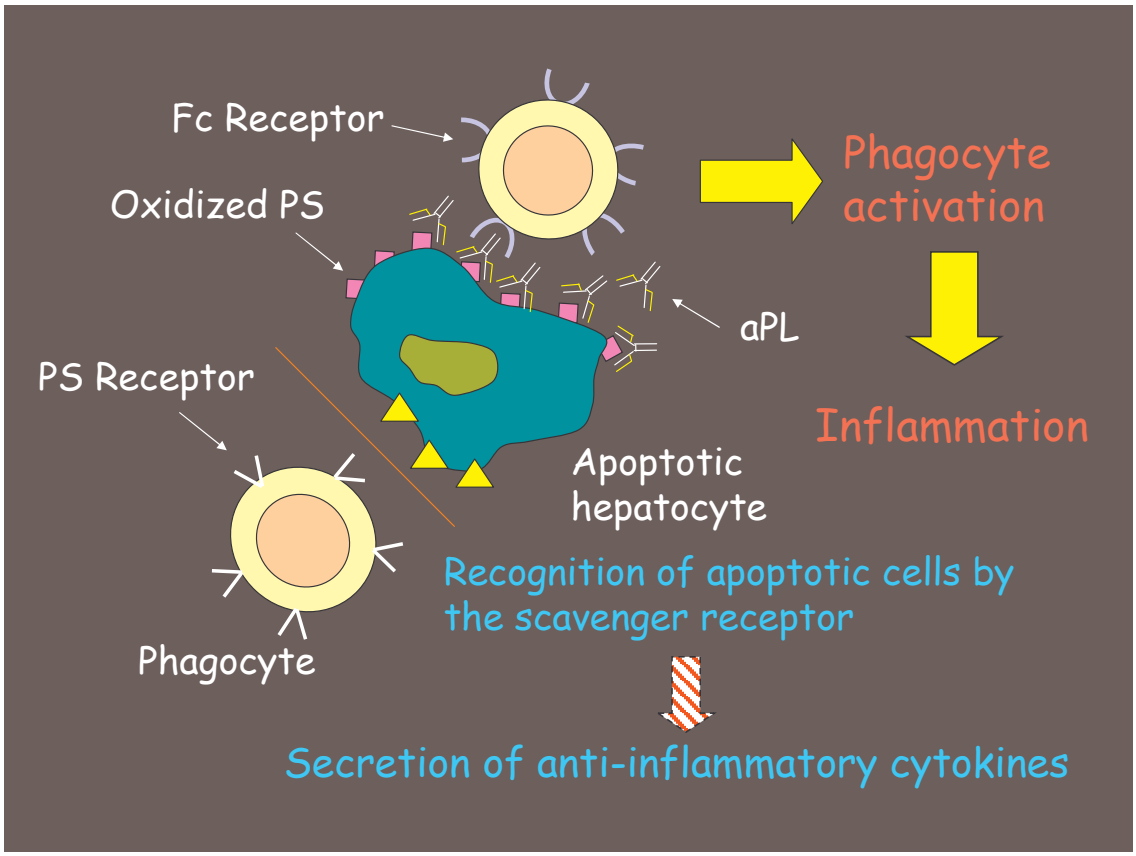

Figure 3: Possible role of aPL associated with Alcoholic Liver Disease in hepatic inflammatory reactions. Circulating aPL detected in patients with ALD are able to selectively bind to apoptotic cells by recognizing oxidised PS exposed on the cell surface. Such an event can interfere with the removal of apoptotic cells and induce the release of ROS, nitric oxide and inflammatory cytokines.

Previous studies using immunofluorescence detection by confocal microscopy have revealed that HER-CYP2E1 adducts can be traced on the outer layer of the plasma membrane of ethanol-treated hepatocytes, where are recognised by anti-HER allo-antibodies and activate antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of normal human blood mononuclear cells (Clot et al. 1997). Similar cytotoxic reactions can also result from the recognition of CYP2E1 present on the hepatocytes surface by anti-CYP2E1 auto-antibodies (Neve and Ingelman-Sundberg 2000) (Figure 1).

Indeed, the orientation of the two conformational epitopes, at the juxtaposition of the J' and K" helices and in the G helix at CYP2E1 surface in relation to their position with respect to cell membrane shows that these epitopes are both on the

outer portion of the molecule and well accessible to antibody recognition. These findings along with the correlation between the magnitude of the humoral responses to CYP2E1 and the degree of lymphocyte infiltration in ALD biopsies suggest that autoimmune responses might also play a significant role in the pathogenesis of ALD.

Conclusions

In conclusion, the results obtained indicate that the modifications of hepatic constituents consequent to alcohol-induced oxidative triggers promotes both humoral and cellular immune response. Furthermore, in combination with genetic predisposition, they can favour the breaking of the self-tolerance in the liver. These immune responses might represent one of the mechanisms by which alcohol abuse promotes and maintains inflammatory processes during the evolution of ALD.

Main Findings

From the results obtained it is possible to draw the following conclusions:

1. Alcoholic Liver Disease is associated with a specific antibody response against lipid peroxidation-derived antigens;
2. aPL detected in sera of ALD patients do not target native phospholipids but complexes between oxidised phospholipids and β_2-GP1;
3. ethanol-induced oxidative stress may represent, at least in a subgroup of ALD patients, an important stimulus for the development of cellular immune response;
4. oxidative stress, in combination with an impaired control of T-cell proliferation by CTLA-4 polymorphism, promotes the development of an anti-CYP2E1 auto-immune response in advanced alcoholic liver disease;
5. anti-CYP2E1 auto-antibodies in ALD patients, recognized mainly conformational epitopes, located in two different and opposite side areas, at the outer surface of the hepatocyte plasma membrane.

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