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Original Contribution

LIPID PEROXIDATION CONTRIBUTES TO IMMUNE REACTIONS ASSOCIATED WITH ALCOHOLIC LIVER DISEASE

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Abstract—Increasing evidence indicates the involvement of immune reactions in the pathogenesis of alcoholic liver disease. We have investigated whether ethanol-induced oxidative stress might contribute to immune response in alcoholics. Antibodies against human serum albumin modified by reaction with malondialdehyde (MDA), 4-hydroxynonenal (HNE), 2-hexenal, acrolein, methylglyoxal, and oxidized arachidonic and linoleic acids were measured by ELISA in 78 patients with alcoholic cirrhosis and/or hepatitis, 50 patients with nonalcoholic cirrhosis, 23 heavy drinkers with fatty liver, and 80 controls. Titers of IgG-recognizing epitopes derived from MDA, HNE, and oxidized fatty acids were significantly higher in alcoholic as compared to nonalcoholic cirrhotics or healthy controls. No differences were instead observed in the titers of IgG-recognizing acrolein-, 2-hexenal-, and methylglyoxal-modified albumin. Alcoholics showing high IgG titers to one adduct tended to have high titers to all the others. However, competition experiments showed that the antigens recognized were structurally unrelated. Anti-MDA and anti-HNE antibodies were significantly higher in cirrhotics with more severe disease as well as in heavy drinkers with cirrhosis or extensive fibrosis than in those with fatty liver only. We conclude that antigens derived from lipid peroxidation contribute to the development of immune responses associated with alcoholic liver disease. © 2001 Elsevier Science Inc.

Keywords—Oxidative stress, Ethanol, Aldehyde adducts, Alcohol toxicity, Liver injury, Free radicals

INTRODUCTION

The etiology of alcoholic liver disease is poorly understood, but recent evidence points towards a possible contribution of immunological reactions against hepatic antigens [1,2]. The development of such reactions has been ascribed to the formation of new antigens as a result of the interaction between liver proteins and reactive metabolites originating from ethanol metabolism [3,4]. In particular, antibodies recognizing protein adducts of acetaldehyde and hydroxyethyl free radicals have been identified in the sera of alcohol-fed rats and in patients with alcoholic liver disease [5–8]. Nonetheless, the possible contribution of other antigens to immunological reactions in alcoholic liver disease cannot be excluded.

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Increasing evidence indicates that oxidative damage is an important feature of human alcoholic liver disease [9]. Protein modifications induced by oxidative damage, and particularly the formation of protein adducts with lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) can be detected in the liver of ethanol-fed rats [10,11]. Furthermore, Tuma and colleagues have demonstrated that the livers of ethanol-fed rats contain mixed adducts derived from the combined reaction of acetaldehyde and MDA, also known as malondialdehyde-acetaldehyde adducts [12].

Aldehyde-modified proteins are highly immunogenic and both monoclonal and polyclonal antibodies towards epitopes derived from different lipid peroxidation products have been produced following animal immunization with these adducts [13,14]. Recent studies have shown that chronic ethanol exposure in both experimental animals and humans is associated with the development of specific IgG-recognizing epitopes derived from MDA

and acetaldehyde interaction [15,16]. However, it is not known whether other lipid peroxidation-derived adducts might contribute to the humoral immune response in excessive drinkers. Thus, in the present study, we have investigated this possibility by determining the presence of antibodies directed towards proteins adducted to different oxidative products in patients with alcoholic liver disease.

MATERIALS AND METHODS

Patients and control subject recruitment

For this study two distinct cohorts of patients were investigated. The first cohort included: (i) a group of 50 patients (39 men, 11 women; mean age 50 years, ranging from 31 to 73 years) with alcoholic cirrhosis (AlC); (ii) a group of 50 patients (37 men, 13 women; mean age 53 years, ranging from 32 to 76 years) suffering from HCV or HBV chronic hepatitis or cirrhosis without alcohol abuse (NAlC); (iii) 50 healthy controls (38 men, 12 women; mean age 37 years, range 23 to 60 years). The patients with liver disease were all admitted to the Medical Clinic of the University of East Piedmont in Novara, Italy, while the healthy controls were recruited from blood donors or University staff originating from the same geographical area. The diagnosis of liver cirrhosis was based on clinical and laboratory criteria and confirmed by biopsy in 12 AlC and 15 NAlC patients. Evaluation of the clinical severity of liver injury according to the Child-Turcotte classification [17] revealed that 54% of the AlC patients and 52% of the NalC patients were Child's Grade B or C. The patients with alcohol abuse were negative for serum markers for hepatitis B virus and for the presence of antibodies versus hepatitis C virus measured by a second-generation enzyme-linked immunoadsorbent assay (Abbott Laboratories, Chicago, IL, USA).

The second cohort of patients consisted of 51 heavy drinkers (39 men, 12 women; mean age 48 years, ranging from 29 to 69 years) with an alcohol consumption above 80 g ethanol/d for more than 10 years, recruited by the Centre for Liver Research at the University of Newcastle upon Tyne (UK) and 30 age- and sex-matched healthy controls. All drinkers and controls were Caucasoid and originate in the northeast of England. The severity of liver damage in all of the patients was determined by liver biopsy and on this basis they were sub-divided into two groups: 28 with advanced alcoholic liver disease, defined as the presence of extensive fibrosis or cirrhosis with or without alcoholic hepatitis, and 23 with fatty liver only.

The daily alcohol intake was assessed at the time of admission by a standardized questionnaire. All of the heavy drinkers had an estimated daily ethanol intake over the previous 12 months greater than 100 (158 ± 45) g/d. In the NAlC group 62% of the patients were abstinent and the remaining had a reported alcohol intake ranging from 10–50 g/d. The alcohol consumption in the controls was less than 20 g/d for women and between 20 and 60 g/d for men. At the time of the blood testing, all subjects had abstained from alcohol for at least 48 h.

All subjects gave informed consent to the analysis and the study was planned according to the guidelines of the local ethical committees. Blood samples (5 ml) were taken after an overnight fast and the serum was used for the ELISA tests.

Antigen preparation

Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) adducts with human serum albumin (MDA-HSA and HNE-HSA) were prepared by reacting 1 mg/ml HSA with, respectively, 50 mmol/l MDA and 3 mmol/l HNE for 2 h at 37°C. In some experiments, reduced MDA adducts were also prepared by reacting MDA (100 mmol/l) with HSA in the presence of 150 mmol/l sodium cyanoborohydride. Acrolein, methylglyoxal, 2-hexenal, and glutaraldehyde-modified HSA were obtained by incubating 2 mg/ml HSA solution in PBS pH 7.4 with 10 mmol/l of each aldehyde. The reaction was carried out for 2 h at 37°C for acrolein, 2-hexenal, and glutaraldehyde, and for 24 h at 37°C for methylglyoxal. The unbound aldehydes were removed by overnight dialysis at 4°C against PBS pH 7.4. HSA complexed with reactive products of fatty acid oxidation were generated by thermal auto-oxidation of arachidonic acid (AAOP-HSA) or linoleic acid (ALOP-HSA) according to Palinski et al. [13]. Briefly, 10 mg arachidonic acid or linoleic acid were transferred in a glass vial open to air and kept at 37°C for 72 h. The yellow-brown reaction products were dissolved in 50 μl methanol and suspended by vortexing in 1 ml PBS containing 10 μM EDTA pH 7.4. Aliquots containing 3 mg of oxidized fatty acid were added to 1 mg HSA and incubated overnight at 20°C. Modified HSA was dialyzed overnight at 4°C against PBS pH 7.4. Oxidized HSA was prepared by reacting HSA (1 mg/ml in PBS pH 7.4) for 2 h at 37° with free radicals originating from the thermal decomposition of 2,2′-azo-bis-(2-amidinopropane) hydrochloride. At the end of the reaction HSA was dialyzed overnight at 4°C against PBS pH 7.4. In order to reduce experimental variability the protein adducts with the different lipid peroxidation products were standardized by measuring the fluorescence intensity at 399/471 nm exc./em. wave length pairs for MDA adducts and 366/429 nm exc./em. wave length pairs for HNE adducts [18,19] or by assessing the degree of amino group derivatization with the

trinitrobenzenesulfonic acid reaction according to Habeeb [20]. Hydroxyethyl radical adducts with HSA were prepared by reacting HSA (1 mg/ml in PBS pH 7.4) for 30 min at 25°C with 1 mg of freshly prepared 1,1′-dihydroxyazoethane crystals, synthesized as described by Stoyanovsky et al. [21].

Measurement of antibodies titers

Polystyrene microwell plates for enzyme-linked immunoabsorbent assay (ELISA) (Immunolon IV; Nunc, Fisher Scientific, St. Louis, MO, USA) were coated for 4 h at 37°C with 0.05 mg/ml of either modified HSA or native protein solubilized in 0.1 M bicarbonate buffer pH 9.6. After incubation, the solutions were removed and replaced by 0.3 ml of coating buffer containing 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) pH 7.4. The plates were further incubated for 1 h at 37°C to block nonspecific binding sites. The coated wells were washed three times with PBS containing 0.25% Triton X-100. The sera of the patients were diluted 1:50 with the coating buffer, and added in duplicate as aliquots of 0.20 ml to the appropriate wells and incubated 1 h at 37°C. After washing three times with PBS-0.25% Triton X-100, peroxidase-linked goat anti-human IgG (dilution 1:6,000), IgA (dilution 1:9,000), or IgM (dilution 1:5,000) (Dako S.P.A., Milano, Italy) were added and incubated for 60 min at 37°C. The antibody binding was revealed by the addition of 0.15 ml of a reaction mixture containing 0.4 mg/ml of 1-phenylendiamine, 0.4 μl/ml hydrogen peroxide (30%), 5.1 mg/ml citric acid, and 6.1 mg/ml anhydrous Na_2HPO_4 at pH 5.0. After 15 min the reaction was stopped by adding 50 μl 2N H_2SO_4 and absorbances were measured at 490 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results were corrected by subtracting the background reactivity with unmodified HSA.

Preadsorption experiments

For preadsorption experiments HSA modified with 50 mmol/l MDA, 3 mmol/l HNE, or 3 mg AAOP was conjugated with 0.6 g of CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer instructions and re-suspended in 2 ml of 3M NaCl. Native HSA was also conjugated with Sepharose beads 4B by the same procedure. Human sera (0.9 ml; dilution 1:100 in 3% w/v BSA solution) were added to 0.3 ml of bead suspension and incubated overnight at 4°C on a rotary plate. After incubation the beads were separated by centrifugation and the sera were tested on ELISA plates coated with either MDA-HSA, HNE-HAS, or AAOP-HSA. The results were expressed as percentage of antibody binding of nonpreadsorbed sera.

Data analysis and statistical calculations

The data were expressed as means ± standard deviation (SD). Statistical analysis were performed by Instat-3 statistical software (GraphPad Software Inc, San Diego, CA, USA) using one-way ANOVA test with Bonferroni's correction for multiple comparisons when more than two groups were analyzed. Pearson's *r* values were used for the estimation of correlation. Distribution normality of the groups considered was evaluated by the Kolmogorov and Smirnov test. Significance was taken at 5% level.

Materials

Malonildialdehyde-bis-dimethylacetal, methylglyoxal, 4-hydroxynonenal, 2-hexenal, glutaraldehyde, arachidonic acid, fatty acid free human serum albumin (fraction V), human trinitrobenzenesulfonic acid, and sodium cyanoborohydride were supplied by Sigma Chemical Co. (St. Louis, MO, USA). 2,2′-azo-bis-(2-amidinopropane) hydrochloride was obtained by Polyscience Inc. (Warrington, PA, USA). All other chemicals were of analytical grade and were supplied by Merck (Darmstad, Germany).

RESULTS

The possible contribution of lipid peroxidation-derived antigens to the immune response seen in patients with alcoholic liver disease was first investigated by measuring the titers of antibodies toward the antigens generated in commercial preparations of human serum albumin (HSA) modified by the reaction with malondialdehyde (MDA-HSA) and 4-hydroxynonenal (HNE-HSA). Two groups of patients with either alcoholic or nonalcoholic liver cirrhosis matched for sex, age, and disease severity were investigated together with the same number of healthy controls. Figure 1 shows that IgG against MDA and HNE adducts were significantly ($p < .001$) increased in patients with alcoholic cirrhosis ($abs._{490\ nm}$ 0.73 ± 0.25 for anti-MDA-HSA IgG; 0.10 ± 0.09 for anti-HNE-HSA IgG) as compared to those with nonalcoholic 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). Conversely, the titers of the same antibodies were not statistically different between nonalcoholic cirrhotics and healthy

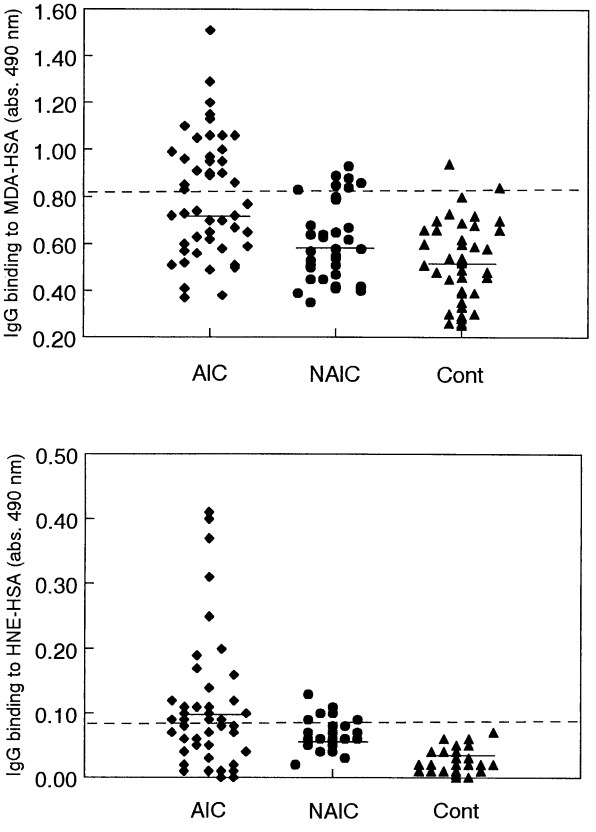

Fig. 1. Immune reactivity against malondialdehyde (MDA-HSA) (top panel) and 4-hydroxynonenal (HNE-HSA) (bottom panel) adducts with human serum albumin in the sera of 50 patients with alcoholic cirrhosis (AlC), 50 patients with HBV- or HCV-induced cirrhosis without alcohol abuse (NAlC), and 50 healthy control subjects (Cont). HSA was modified by 2 h reaction with either 50 mmol/l MDA or 3 mmol/l HNE. Human sera were tested at 1:50 dilution in microplate ELISA plates coated with aldehyde-modified HSA or native HSA, and the IgG binding was revealed with peroxidase-linked goat anti-human IgG anti-serum. The results are expressed as absorbance (abs.) at 490 nm after the subtraction of the individual reactivity to unmodified HSA. The horizontal bars represent the median values in each group. The dotted lines show cut-off values calculated on the 95th percentile of the control population. The values in each group were normally distributed as evaluated by Kolmogorov and Smirnov test.

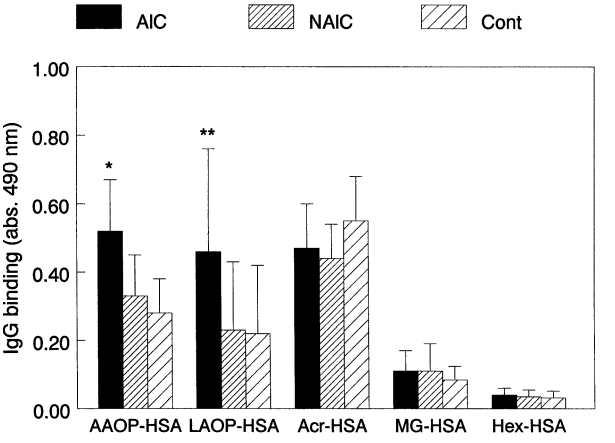

Fig. 2. Immune reactivity against human serum albumin (HSA) modified by the reaction with 3 mg of oxidation products derived from arachidonic acid (AAOP-HSA) or linoleic acid (LAOP-HSA) or with 10 mmol/l acrolein (Acr-HSA), 2-hexenal (Hex-HSA), and methylglyoxal (Mg-HSA) in the sera from 50 patients with alcoholic cirrhosis (AlC), 50 patients with HBV- or HCV-induced cirrhosis without alcohol abuse (NAlC), and 50 healthy control subjects (Cont). HSA was modified as reported in the Materials and Methods section. Human sera were tested at 1:50 dilution in microplate ELISA plates coated with modified HSA or native HSA and the IgG binding was revealed with peroxidase-linked goat anti-human IgG anti-serum. The results are expressed as means ± SD of the absorbances (abs.) at 490 nm obtained after the subtraction of the individual reactivity to unmodified HSA. The values in each group were normally distributed as evaluated by Kolmogorov and Smirnov test. Statistical significance: $*p < .001$ and $**p < .01$ vs. NAlC and control groups.

controls (Fig. 1). Cirrhotics with alcohol abuse, but not patients with nonalcoholic cirrhosis also displayed an increase in IgG-recognizing HSA complexed with oxidation products derived from arachidonic acid (AAOP-HSA) or linoleic acid (LAOP-HSA) (Fig. 2). No difference was observed between the different groups when HSA complexed with other lipid peroxidation products such as acrolein, 2-hexenal, and methylglyoxal was used as the antigen (Fig. 2). Similarly, the three groups did not differ in their reactivity towards HSA oxidized by free radicals originating from the thermal decomposition of 2,2′-azo-bis-(2-amidinopropane) hydrochloride (not shown). The antibodies recognizing lipid peroxidation-derived antigens were mostly present in the IgG fraction with little contribution from IgA and IgM (not shown).

Frequency distribution analysis revealed that the majority (55–72%) of the patients with alcoholic cirrhosis had IgG against the different lipid peroxidation-derived epitopes above the 95 percentile in the control population. Antibody levels above this threshold were found only in a small fraction (8–13%) of nonalcoholic cirrhotics.

Among the patients with alcoholic cirrhosis, the presence of IgG against lipid peroxidation-derived antigens was unrelated to age, disease duration, or alcohol intake. However, by sub-grouping the alcoholic patients according to the Child-Turcotte Score, the subjects with severe liver injury (Child's Grade B and C) had antibodies titers against MDA and HNE adducts significantly higher ($p < .01$) than those with moderate liver disease (Child's Grade A) (Fig. 3). In order to better characterize the relationship between the presence of antibodies against lipid peroxidation products and the severity of alcohol-related liver damage, a group of long-term heavy drinkers with biopsy-proven fatty liver or advanced liver disease (cirrhosis or extensive fibrosis with or without hepatitis) was investigated. Figure 4 shows that IgG against MDA, HNE, and AAOP adducts were lower in patients with fatty liver compared to those in patients

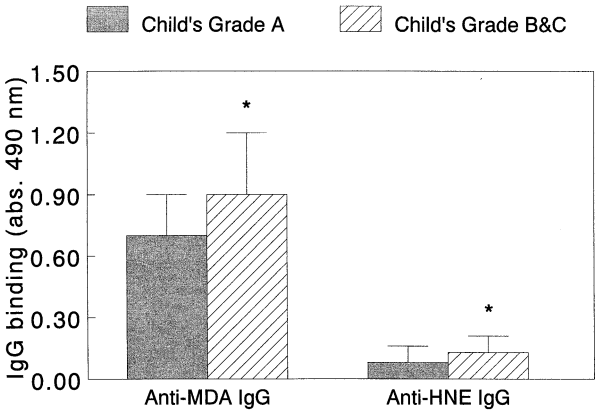

Fig. 3. IgG reactivity against MDA- and HNE-derived antigens in relation to the severity of alcohol liver injury. Estimation of the clinical severity of hepatic damage according to the Child-Turcotte classification revealed 27 patients with Child's B or C Grades and 23 patients with Child's A Grade. The results are expressed as means ± SD of the absorbance (abs.) at 490 nm obtained after the subtraction of the individual reactivity to unmodified HSA. The values in each group were normally distributed as evaluated by Kolmogorov and Smirnov test. Statistical significance: $*p < .01$ vs Child's Grade A group.

with advanced liver injury. Furthermore, the titers of antibodies towards HNE- and AAOP-derived epitopes in the fatty liver group were not different from those in the control group (Fig. 4).

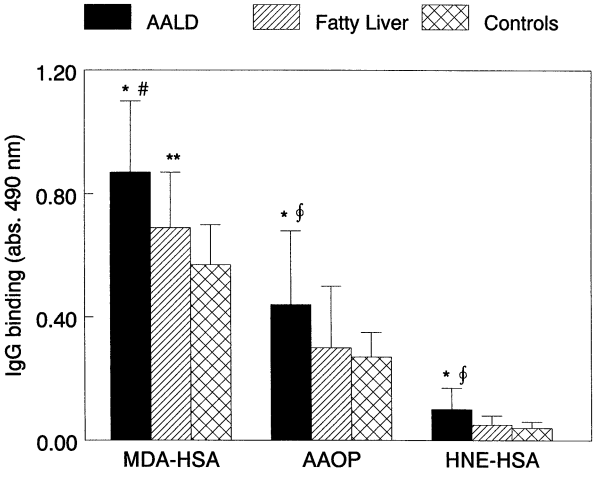

Fig. 4. IgG reactivity against human serum albumin (HSA) modified by the reaction with malondialdehyde (MDA-HSA), 4-hydroxynonenl (HNE-HSA), and oxidation products derived from arachidonic acid (AAOP-HSA) in the sera from 51 heavy drinkers with biopsy-proven advanced alcoholic liver disease (AALD) ($n = 28$) or with fatty liver only ($n = 23$) and in 30 healthy control subjects (Cont). Human sera were tested at 1:50 dilution in microplate ELISA plates coated with aldehyde-modified HSA or native HSA and the IgG binding was revealed with peroxidase-linked goat anti-human IgG anti-serum. The results are expressed as means ± SD of the absorbance (abs.) at 490 nm obtained after the subtraction of the individual reactivity to unmodified HSA. The values in each group were normally distributed as evaluated by Kolmogorov and Smirnov test. Statistical significance: $*p < .01$ and $**p < .05$ vs. control group; $^{\S}p < .02$ and $^{\#}p < .05$ vs. fatty liver group.

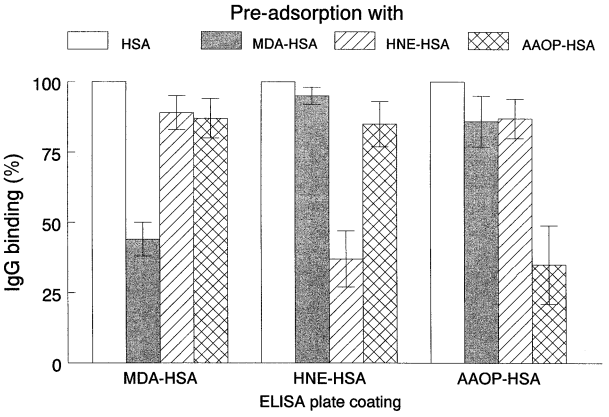

Fig. 5. Effect of preadsorption with differently modified human serum albumin (HSA) on the reactivity of sera from patients with alcoholic cirrhosis to lipid peroxidation-derived antigens. CNBr-activated Sepharose 4B beads were complexed with native HSA or HSA modified with malondialdehyde (MDA-HSA), 4-hydroxynonenal (HNE-HSA), and arachidonic acid oxidation products (AAOP-HSA) as reported in the Materials and Methods section. Preadsorption experiments were performed by incubating overnight at 4°C 10 human sera (1:100 dilution) with the different Sepharose bead preparations. At the end of the incubation the beads were removed by centrifugation. Aliquots of the preadsorbed sera were then added to ELISA plates coated with either MDA-HSA, HNE-HSA, or AAOP-HSA, and IgG binding was revealed with peroxidase-linked goat anti-human IgG anti-serum. The results are expressed as percent of the absorbance at 490 nm in the sera preadsorbed with native HSA.

In general, alcohol abusers with high IgG titers to one adduct tended to have high titers to all others (intergroup correlation ranging from $r = 0.54$ to $r = 0.78$; $p < .001$). Furthermore a positive correlation was also observed between the levels of antibodies recognizing HSA adducts with hydroxyethyl free radicals and those towards the different lipid peroxidation products ($r = 0.50-0.76$; $p < .001$). Although in patients with alcoholic liver disease the individual IgG titers against the different lipid peroxidation adducts were positively correlated, preadsorption experiments using Sepharose beads coated with differently modified HSA showed that the antigens recognized in the different adducts were structurally unrelated (Fig. 5). In particular, sera preadsorption with MDA-HSA and HNE-HSA did not appreciably affect the IgG binding to AAOP-HSA (Fig. 5), indicating that the epitopes originating from arachidonic acid oxidation were structurally different from those generated by the binding of the two aldehydes.

In both British and Italian alcoholics the titers of anti-MDA antibodies were much higher than those of the antibodies recognizing other lipid peroxidation products. Since previous studies have shown that healthy human sera contain appreciable titers of IgG interacting with proteins cross-linked by di-aldehydes, such as MDA or glutaraldehyde, further experiments were performed to assess whether the increase in anti-MDA reactivity in the

patients with alcohol abuse involved or not these antibodies. ELISA tests performed using as antigen HSA reacted with 100 mmol/l glutaraldehyde showed no difference between control sera and sera from patients with alcoholic cirrhosis ($abs._{490\ nm}$ 0.878 ± 0.164 vs. 0.990 ± 0.329). The preparation of MDA-HSA adducts in the presence of 150 mmol/l sodium cyanoborohydride to prevent the formation of protein cross-links [22] lowered by more than 80% the recognition by human IgG. However, sera from alcoholic cirrhotics still displayed a reactivity towards reduced MDA-HSA ($abs._{490\ nm}$ 0.272 ± 0.167) significantly higher $p < .001$) than the sera of patients with nonalcoholic cirrhosis or healthy controls ($abs._{490\ nm}$ 0.102 ± 0.045 and 0.101 ± 0.047, respectively). By subtracting the reactivity towards reduced MDA-HSA from that against nonreduced MDA-HSA antigens no difference was observed between alcoholics and controls ($abs._{490\ nm}$ 0.452 ± 0.115 vs. 0.437 ± 0.1479). This suggested that the increase in anti-MDA antibodies observed in patients with alcoholic liver disease is not due to IgG recognizing MDA-cross linked lysine epitopes, but to antibodies directed against newly developed antigens.

DISCUSSION

Substantial evidence indicates that during oxidative damage reactive lipid peroxidation products can form adducts with the free amino groups of lysine and of other amino acids [19]. These modified proteins are highly immunogenic, and antibodies towards epitopes generated by several lipid peroxidation products have been characterized in recent years [13,23–25]. A number of studies have also demonstrated that antibodies against oxidized and MDA-modified human low density lipoproteins can be found in the plasma of patients with overt atherosclerotic disease, non-insulin-dependent diabetes mellitus, and renal failure [26–28].

An accumulating body of evidence supports a role for oxidative damage in the pathogenesis of human alcoholic liver disease. Recent clinical studies have shown that lipid peroxidation products (conjugated dienes, malondialdehyde, 4-hydroxynonenal, F_2-isoprostanes) and protein carbonyls are higher in liver biopsies or in the sera of patients with alcoholic liver disease as compared to samples from nondrinking controls or from patients with nonalcoholic liver disease [29–32]. Furthermore, morphological studies using immunohistochemistry have shown the presence of lipid peroxidation products in the areas of liver fatty infiltration, focal necrosis, and fibrosis [10,11,33]. Previous experiments using intragastric alcohol-fed rats showed that MDA accumulation within the liver is associated with the development of antibodies recognizing protein-MDA adducts [6]. The present results in humans are consistent with these animal studies, demonstrating that several products originating during lipid peroxidation contribute to antibody formation in patients with alcoholic liver disease. In particular, IgG against epitopes derived from the binding of MDA, HNE, and oxidized fatty acids are increased in patients with alcoholic cirrhosis, but not in those with cirrhosis of nonalcoholic etiology. Such an effect is relatively specific, since acrolein, 2-hexenal, and methylglyoxal, that similarly originate from peroxidative processes and are capable of generating immunogenic adducts [25,34], do not contribute to the enhanced immune response seen in alcoholic cirrhotics.

The chemical structure of the adducts derived from aldehydic products of lipid peroxidation has been characterized to some extent [19,35]. However, 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 [19,35,36]. Moreover, the combination with arginine gives rise to N^{δ} -(2-pyrimidyl)-L-ornithine adducts [36]. Human sera from healthy controls show extensive immune reactivity toward MDA-modified proteins. This reactivity is attributable largely to the presence of IgG recognizing amino-3-imino-propene bridges between lysine residues [22]. However, our data demonstrate that the increase in anti-MDA IgG observed in patients with alcoholic liver disease depends upon newly developed antibodies recognizing MDA-derived epitopes produced as a result of ethanol-induced oxidative stress. Among these epitopes, N-lysyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde is likely to play a major role, since this compound can be generated by the combined reaction of MDA and acetaldehyde with lysine, and is immunogenic in both alcohol-fed animals and human alcoholics [15,16]. Similarly, the structure of the epitopes responsible for the development of anti-HNE reactivity in patients with alcoholic liver disease remains unclear. HNE is known to generate immunogenic adducts with both lysine and histidine [35]. However, preliminary experiments using a monoclonal IgG against HNE-histidine hemiacetal largely exclude the recognition of this epitope by the human anti-HNE antibodies. Patients with alcoholic liver disease have appreciable titers of IgG against protein complexed with oxidized arachidonic acid and linoleic acid. Our preabsorption 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. In support, Kim and co-workers [37] have reported

the preparation of rabbit polyclonal antibodies specifically directed against lipid hydroperoxide-modified albumin that do not cross-react with aldehyde-bound proteins.

The actual role of immune reactions induced by alcohol in the pathogenesis of liver injury is still largely unknown [1,2]. In the present study, elevated titers of antibodies towards lipid peroxidation adducts are evident in patients with biopsy-proven advanced alcoholic liver disease, but not in subjects 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. Since the immune stimulus represented by the extend of lipid peroxidation is not influenced by the severity of liver disease, as estimated by Child-Turcotte Score [30], we suggest the possibility that an immune response involving lipid peroxidation antigens might have a role in the progression of alcohol liver damage. Consistently, Viitala and co-workers have shown the prevalence of antibodies toward oxidized and MDA-modified lipoproteins among alcoholics with more severe liver injury [38].

In conclusion, the present results demonstrate that alcoholic liver disease is associated with a specific antibody response against lipid peroxidation products, and suggest the possible contribution of immune reactions triggered by lipid peroxidation-derived antigens in the development of alcohol-mediated liver damage.

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ABBREVIATIONS

AAOP-HSA—adducts between human serum albumin and arachidonic acid oxidation products
ELISA—enzyme-linked immunoabsorbent assay
HSA—human serum albumin
HNE—4-hydroxynonenal
HNE-HSA—4-hydroxynonenal adducts with human serum albumin
LAOP-HSA—adducts between human serum albumin and linolenic acid oxidation products
MDA-HSA—malonildialdehyde adducts with human serum albumin
MDA—malonildialdehyde
PBS—phosphate-buffered saline