

Antiphospholipid antibodies associated with alcoholic liver disease specifically recognise oxidised phospholipids

R Rolla, D Vay, E Mottaran, M Parodi, M Vidali, M Sartori, C Rigamonti, G Bellomo, E Albano

Abstract

Background—Circulating antiphospholipid antibodies (aPL) are often detected in patients with alcoholic liver disease (ALD) but little is known about the causes of their formation.

Aims—We have evaluated whether ethanol mediated oxidative injury might promote the development of aPL in ALD.

Patients and methods—IgG against β_2 glycoprotein 1 (β_2 -GP1), cardiolipin, and human serum albumin (HSA) complexed with either oxidised arachidonic acid (HSA-APP) or malondialdehyde (HSA-MDA) were assayed by ELISA in heavy drinkers with or without ALD and in healthy subjects.

Results—Circulating IgG recognising cardiolipin were significantly higher in ALD patients than in controls. However, anti-cardiolipin reactivity of ALD sera was only evident using, as the antigen, oxidised cardiolipin but not oxidation protected cardiolipin. In ALD patients, individual values of IgG antioxidised cardiolipin were associated with the titres of antibodies against HSA-MDA and HSA-APP ($r=0.68$ and 0.72 , respectively; $p<0.0001$) used as markers of oxidative stress. ALD patients also displayed increased levels of antibodies against phospholipid binding protein β_2 -GP1, and individual reactivity towards oxidised cardiolipin and β_2 -GP1 were highly correlated ($r=0.85$; $p<0.0001$). IgG binding to oxidised cardiolipin, HSA-MDA, and HSA-APP was also significantly higher in β_2 -GP1 positive than in β_2 -GP1 negative sera. However, preadsorption of β_2 -GP1 positive sera on β_2 -GP1 coated ELISA plates reduced reactivity to oxidised cardiolipin by 80%, without affecting that to HSA-APP or HSA-MDA.

Conclusions—Ethanol induced oxidative injury is associated with the development of antibodies targeting complexes between oxidised cardiolipin and β_2 -GP1. These antibodies might account for high aPL titres observed in patients with severe ALD. (Gut 2001;49:852-859)

Keywords: oxidative stress; lipid peroxidation; β_2 glycoprotein 1; ethanol; autoantibodies

Antiphospholipid antibodies (aPL) belong to a heterogeneous group of autoantibodies with apparent specificity for negatively charged

phospholipids.^{1,2} The presence of these antibodies characterises primary antiphospholipid syndrome, or Hughes' syndrome, consisting of recurrent vascular thrombosis, fetal losses, and thrombocytopenia.³ aPL and antiphospholipid syndrome are often associated with rheumatic and autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and systemic sclerosis.¹⁻⁴ However, aPL are also present in patients with chronic infections (syphilis, malaria, human immunodeficiency virus 1), lymphoproliferative diseases, and sickle cell anaemia.¹⁻⁴

The molecular targets of aPL have not yet been characterised in detail. aPL are usually identified by their ability to react with solid phase bound cardiolipin but other anionic phospholipids—that is, phosphatidylserine and phosphatidylethanolamine—can be recognised also.^{1,2} Studies by McNeil and colleagues⁵ and Galli and colleagues⁶ have shown that aPL binding to phospholipids is largely mediated by β_2 glycoprotein 1 (β_2 -GP1) or apolipoprotein H, a plasmatic 50 kDa glycoprotein which readily binds to phospholipids as well as to other negatively charged substances.⁴ Conformational changes occurring in β_2 -GP1 following interaction with phospholipids render this protein antigenic to aPL.⁷ Indeed, β_2 -GP1 can be recognised by aPL also in the absence of phospholipids when attached to irradiated polystyrene or nitrocellulose membranes that are rich in negative charges.⁸ None the less, phospholipid complexes with prothrombin, protein C, and protein S can also be targets of aPL and this may contribute to the anticoagulant properties ascribed to aPL.⁴ Recently, Hörkkö *et al* have reported that epitopes originating during cardiolipin oxidation are recognised by aPL from women with primary antiphospholipid syndrome.⁹ They also suggested that the antigenic determinants might result from the reaction of β_2 -GP1 with products derived from the peroxidative breakdown of cardiolipin unsaturated fatty acids.^{9,10}

Recent studies have shown that aPL can be detected in patients with chronic liver disease,

Abbreviations used in this paper: ALD, alcoholic liver disease; aPL, antiphospholipid antibodies; β_2 -GP1, β_2 glycoprotein 1; DPPD, diphenylphenylendiamine; HCV, hepatitis C virus; HSA, human serum albumin; HSA-MDA, malondialdehyde adducts with human serum albumin; HSA-APP, adducts between human serum albumin and arachidonic acid oxidation products; HSA-LPP, adducts between human serum albumin and linoleic acid oxidation products; MDA, malondialdehyde; od, optical density; PBS, phosphate buffered saline.

Department of Medical Sciences and Medical Clinic, University "Amedeo Avodagro" of East Piedmont, Via Solaroli 17, 28100, Novara, Italy
R Rolla
D Vay
E Mottaran
M Parodi
M Vidali
M Sartori
C Rigamonti
G Bellomo
E Albano

Correspondence to: Professor E Albano, Department of Medical Science, University "Amedeo Avodagro" of East Piedmont, Via Solaroli 17, 28100 Novara, Italy. albano@med.unipmn.it

Accepted for publication 12 March 2001

particularly alcoholic liver disease (ALD) and hepatitis C.¹¹⁻¹⁶ High aPL titres are frequent in patients with alcoholic hepatitis or cirrhosis and the presence of aPL in these patients seems to reflect disease progression, showing significant correlation with disease severity.¹² However, aPL can also be detected in alcoholics without liver damage.^{12 17 18} Furthermore, aPL are often associated with venous and arterial thrombosis⁴; the presence of aPL in patients with chronic liver disease does not seem to be associated with thrombotic complications.¹⁶

Clinical and experimental evidence indicates that ethanol intoxication promotes free radical formation and oxidative liver injury.¹⁹ In particular, increased levels of lipid peroxidation products and protein carbonyls can be demonstrated in liver biopsies or in the sera obtained from patients with ALD.¹⁹⁻²³ Furthermore, the formation of hydroxyethyl free radicals during ethanol metabolism can also be documented in patients with alcohol abuse.²⁴

The aim of this work was to evaluate whether the development of aPL in patients with ALD might reflect ethanol mediated oxidative injury.

Patients and methods

PATIENTS AND CONTROL SUBJECT RECRUITMENT

We enrolled 36 patients (27 men, nine women; mean age 51 years (range 31-73)) with alcoholic cirrhosis with or without hepatitis (ALD). The diagnosis was based on clinical, ecographycal, and laboratory criteria. Liver biopsy was available for 12 patients and showed the classical feature of micronodular cirrhosis. In three patients hepatocyte ballooning degeneration with Mallory's bodies and inflammatory infiltrates were also noted. Evaluation of the clinical severity of liver injury according to Maddrey's DF index or the Child Turcotte classification^{25 26} revealed that 72% of patients in the ALD group had moderate or severe liver damage (DF values above 60) and 58% belonged to Child group B or C. Fifteen heavy drinkers (11 men, four women; mean age 44 years (range 25-66)) with normal liver blood tests and no clinical or ecographycal evidence of liver disease were also investigated. All patients with alcohol abuse had an estimated mean daily ethanol intake over the previous 12 months above 100 g (table 1) and none had a previous history of co-exposure to drugs or toxins. Patients with alcohol abuse were negative for serum markers of hepatitis B virus or for the presence of antibodies against hepatitis C virus (HCV), measured by a second generation enzyme linked immunoadsorbent assay (Abbott Laboratories, Chicago, Illinois, USA).

A group of 40 patients (29 men, 11 women; mean age 54 years (range 32-76)) suffering

from HCV or hepatitis B virus chronic hepatitis or cirrhosis and without alcohol abuse were also investigated. In this group, 62% of patients were abstinent and the remaining had reported alcohol intake ranging from 10 to 50 g ethanol/day. The severity of liver injury in these patients was comparable with that in patients with alcoholic liver damage. Aspartate aminotransferase, alanine aminotransferase, gamma glutamyltransferase, bilirubin, and prothrombin time were assayed in all patients using routine laboratory procedures. The biological characteristics of the patients studied are reported in table 1.

Healthy controls were recruited among blood donors or university staff and consisted of 42 subjects (28 men, 14 women; mean age 47 years (range 33-66)). Alcohol intake among controls was below 20 g ethanol/day for women and was 40-60 g/day for men. At the time of blood testing, all subjects had abstained from alcohol for at least 48 hours. All subjects gave informed consent to the analysis and the study was planned according to the guidelines of the university ethics committee. Blood samples (5 ml) were taken after an overnight fast and serum was used for ELISA tests.

ANTI-PHOSPHOLIPID ANTIBODY DETERMINATION

IgG reacting with β_2 -GP1 and with cardiolipin were measured by commercial ELISA kits by, respectively, IMMCO Diagnostics Inc. (Buffalo, New York, USA) and IMTEC Immunodiagnostika GmbH (Berlin, Germany). Threshold values for anticardiolipin and anti- β_2 -GP1 antibodies were determined previously in a representative sample of healthy controls from the same population.

DETERMINATION OF IMMUNE REACTIVITY TOWARDS OXIDISED PHOSPHOLIPIDS

Phospholipid coated ELISA plates were prepared according to a standardised method proposed by Harris.¹ Briefly, 30 μ l of cardiolipin solution in ethanol (50 μ g/ml) were applied to each well and ethanol evaporated overnight at 4°C in air. After two washes with phosphate buffered saline (PBS), 0.3 ml of coating buffer containing 3% bovine serum albumin (BSA) in PBS, pH 7.4, were added and the plates were further incubated for one hour at 37°C to block non-specific binding sites. The coated wells were washed three times with PBS. To avoid cardiolipin auto-oxidation in some preparations, 0.1 mmol/l diphenylphenylendiamine (DPPD) was added to cardiolipin ethanol solution and coating of the plates was carried out in sealed plastic bags filled with nitrogen. Cardiolipin coated plates were used immediately after preparation. For the assay, sera from patients and controls were diluted 1:50

Table 1 Biological characteristics of patients admitted to the study

Group	n	Alcohol consumption (g/day)	AST (U/l)	ALT (U/l)	GGT (U/l)	Bilirubin (μ mol/l)	PT (%)
Alcoholic cirrhosis	36	146 (67)	74 (52)	50 (36)	143 (132)	1.68 (1.5)	72 (14)
Non-alcoholic cirrhosis	40	20 (14)	61 (36)	83 (58)	51 (24)	1.33 (0.6)	54 (4)
Heavy drinkers	15	190 (61)	28 (13)	22 (11)	37 (19)	0.62 (0.3)	101 (3)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyltransferase; PT, prothrombin time. Values are mean (SD).

with the coating buffer and 0.2 ml aliquots were added in duplicate to the appropriate wells and incubated for one hour at 37°C. The plates were then washed three times with PBS 0.25% and further incubated with peroxidase linked goat antihuman IgG (dilution 1:6000) for 60 minutes at 37°C. Antibody binding was revealed by addition of 0.15 ml of a reaction mixture containing 0.4 mg/ml of L-phenyldiamine, 0.4 µl/ml hydrogen peroxide (30%), 5.1 mg/ml citric acid, and 6.1 mg/ml anhydrous Na₂HPO₄, pH 5.0. After 15 minutes the reaction was stopped by adding 50 µl of 2 N H₂SO₄ and absorbance was measured at 490 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., Hercules, California, USA). The average optical densities (od) in duplicated wells were subtracted from non-specific background for each serum sample obtained by performing the test in plates coated with ethanol but lacking the phospholipid.

ASSESSMENT OF PHOSPHOLIPID OXIDATION

The occurrence of phospholipid peroxidation under the conditions used for ELISA plate coating was monitored by measuring conjugated diene absorbance. Briefly, 0.5 mg/ml of cardiolipin solution were placed in glass test tubes and allowed to evaporate from the solvent by overnight incubation at 4°C in air. Cardiolipin was then resolubilised in 2 ml hexane and conjugated diene absorbance was immediately measured at 234 nm using a Beckman DU650 UV visible spectrophotometer. Freshly prepared cardiolipin solution in hexane was used as reference.

PREPARATION OF PROTEIN ADDUCTS WITH LIPID PEROXIDATION PRODUCTS

Adducts between human serum albumin (HSA) and malondialdehyde (MDA) were prepared by reacting for two hours at 37°C, 1 mg/ml HSA with 50 mmol/l MDA, obtained by acid hydrolysis of malondialdehyde-bis-dimethylacetal. Unbound aldehyde was removed by overnight dialysis at 4°C against PBS, pH 7.4. The presence of MDA adducts was evaluated by measuring fluorescence intensity at 399/471nm exc/em wavelength pairs according to Cominacini and colleagues.²⁷ HSA complexed with reactive products of fatty acid oxidation was generated by thermal auto-oxidation of arachidonic acid (HSA-APP) and linoleic acid (HSA-LPP) according to Palinski and colleagues.²⁸ Briefly, 10 mg arachidonic acid or linoleic acid were transferred in a glass vial open to air and kept at 37°C for 72 hours. 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 oxidised fatty acids were added to 1 mg HSA and incubated overnight at 20°C. Modified HSA was dialysed overnight at 4°C against PBS, pH 7.4.

MEASUREMENT OF ANTIBODIES AGAINST LIPID PEROXIDATION DERIVED ANTIGENS

Polystyrene microwell plates for ELISA (Immunolon IV; Nunc, Fisher Scientific, St Louis, Missouri, USA) were coated for four hours at 37°C with 0.05 mg/ml of HSA conjugated with either MDA, arachidonic acid oxidation products (HSA-APP), or with the unmodified protein solubilised in 0.1 M bicarbonate buffer, pH 9.6. After incubation, solutions were removed and replaced by 0.3 ml of coating buffer containing 3% BSA in PBS, pH 7.4. The plates were further incubated for one hour at 37°C to block non-specific binding sites. The coated wells were washed three times with PBS containing 0.25% Triton X-100. The sera of 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 for one hour at 37°C. After washing three times with PBS-0.25% Triton X-100, IgG binding was revealed by peroxidase linked goat antihuman IgG as described above. The results were expressed by subtracting the spectrophotometric readings of each serum sample in the wells containing the unmodified HSA.

IMMUNOCOMPETITION EXPERIMENTS

Sera of ALD patients positive for anti-β₂-GP1 antibodies were diluted 1:50 with coating buffer and 300 µl aliquots placed in each well of microtitre plates coated with β₂-GP1 (IMTEC Immunodiagnostika GmbH, Berlin, Germany) or HSA. After overnight incubation at 4°C, 150 µl aliquots were added in duplicate to ELISA plates coated with oxidised cardiolipin, HSA-MDA, HSA-APP, or HSA-LPP, and antibody binding was assayed as described above.

DATA ANALYSIS AND STATISTICAL CALCULATIONS

Data are expressed as mean (SD). Statistical analysis was performed by InStat-3 statistical software (GraphPad Software Inc, San Diego, California, USA) using one way ANOVA with Bonferroni's correction for multiple comparisons when more than two groups were analysed. Pearson's *r* values were used for estimation of correlation. Distribution normality of all groups was preliminary verified by the Komolgorov-Smirnov test. Significance was taken at the 5% level.

MATERIALS

Arachidonic acid, linoleic acid, L-phenyldiamine, cardiolipin, and fatty acid free human serum albumin (fraction V) were from Sigma Chemical Co. (St Louis, Missouri, USA). DPPD and malondialdehyde-bis-dimethylacetal were supplied by Fluka Chemie AG (Buchs, Switzerland).

Results

The use of commercial kits for the aPL assay based on binding to cardiolipin coated ELISA plates revealed that 20 of 36 (55%) patients with alcoholic cirrhosis with or without alcoholic hepatitis (ALD) had anticardiolipin IgG levels above the threshold value (15 GPL units/ml) while one healthy control was positive for anticardiolipin IgG. Mean IgG reactivity

towards cardiolipin among ALD patients was 22.7 (14) U/ml (range 11.8–71.1), significantly higher ($p < 0.001$) than that in control subjects (11.8 (1.3) U/ml; range 10.9–17.6).

It has been shown that the unsaturated fatty acid component of cardiolipin rapidly auto-oxidises when exposed to air.⁹ Determination of conjugated diene absorbance at 234 nm, as an index of lipid peroxidation, revealed that overnight incubation at 4°C generally used for coating ELISA plates with phospholipids,¹ appreciably increased the absorbance ($od_{234\text{ nm}} 0.276$) of cardiolipin samples compared with freshly prepared solutions ($od_{234\text{ nm}} 0.110$). Cardiolipin auto-oxidation was however completely prevented ($od_{234\text{ nm}} 0.112$) by incubation under a nitrogen atmosphere in the presence of the antioxidant DPPD 100 $\mu\text{mol/l}$. When the antiphospholipid reactivity of sera was re-tested using ELISA plates coated with either oxidation protected cardiolipin or cardiolipin allowed to auto-oxidise in air, a dramatic difference was observed. As shown in fig 1, binding of IgG from ALD sera to oxidation protected cardiolipin ($od_{490\text{ nm}} 0.274$ (0.184)) was significantly lower ($p < 0.0001$) than that to oxidised cardiolipin ($od_{490\text{ nm}} 0.491$ (0.340)). Reactivity of control sera with either form of cardiolipin was unchanged ($od_{490\text{ nm}} 0.205$ (0.103) *v* 0.187 (0.111)) (fig 1). When tested with oxidised cardiolipin, 21 of 36 (58%) ALD sera showed IgG binding above the threshold value in controls given by mean $od_{490\text{ nm}}$ values +2 SD (0.408). However, only eight of 36 (19%) ALD sera still displayed increased reactivity when tested with oxidation protected cardiolipin (cut off value 0.387) (fig 1). Furthermore, reactivity of ALD and control sera to oxidation protected cardiolipin was not statistically different. This was not due to interference of DPPD with the antibody reaction as addition of the antioxidant to already oxidised cardiolipin preparations did not affect IgG binding (not shown). No

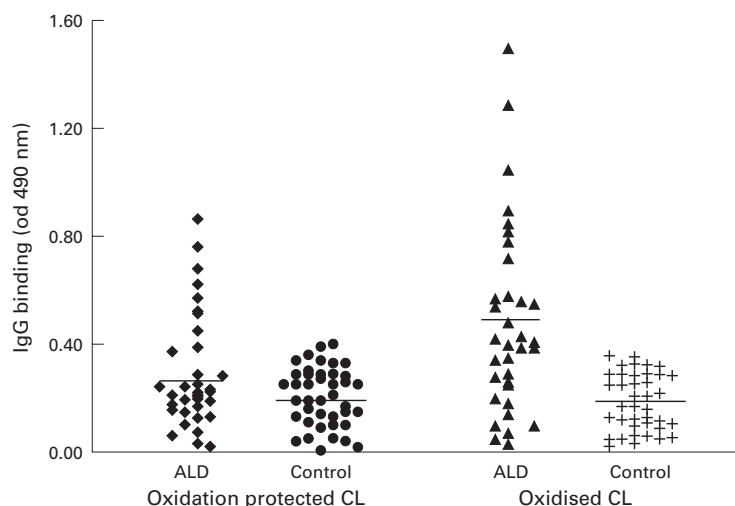


Figure 1 Immune reactivity against native or oxidised cardiolipin (CL) of sera from 36 patients with alcoholic cirrhosis with or without alcoholic hepatitis (ALD) and from 42 healthy control subjects. IgG binding to ELISA plates coated with CL allowed to auto-oxidise in air or oxidation protected CL was evaluated, as reported in the patients and methods section. The horizontal bars represent median values in each group. Values in each group were normally distributed, as evaluated by the Kolmogorov-Smirnov test.

increase in reactivity towards oxidised cardiolipin was apparent in a group of 15 heavy drinkers without clinical or biochemical evidence of liver damage (0.153 (0.09)) or in 40 patients with cirrhosis unrelated to alcohol abuse (0.284 (0.10)).

Lipid hydroperoxides and aldehyde generated during lipid peroxidation are known to interact with proteins forming adducts that are immunogenic.²⁸ We observed that ALD patients had enhanced level of circulating IgG against HSA modified by the reaction with malondialdehyde (HSA-MDA) or with oxidation products derived from arachidonic acid (HSA-APP) ($od_{490\text{ nm}} 0.79$ (0.27) and 0.47 (0.25), respectively, *v* 0.45 (0.14) and 0.11 (0.08) in healthy controls; $p < 0.001$). Individual levels of antibodies against oxidised cardiolipin were correlated with those of IgG directed against HSA-APP or HSA-MDA ($r = 0.67$ and 0.60, respectively; $p < 0.0001$). Moreover, among ALD patients, levels of antibodies against lipid peroxidation products were significantly higher in subjects positive for anti-oxidised cardiolipin antibodies than in the negative group (fig 2), indicating a relationship between the presence of an immune response against phospholipids and ethanol induced oxidative injury. On the other hand, titres of IgG against HSA-MDA and HSA-APP were not significantly increased in heavy drinkers without liver damage ($od_{490\text{ nm}} 0.450$ (0.140) and 0.039 (0.030), respectively) or in non-alcoholic cirrhotics ($od_{490\text{ nm}} 0.590$ (0.220) and 0.196 (0.151), respectively).

Antiphospholipid antibodies can also be detected by their ability to recognise complexes formed by β_2 -GP1 and phospholipids.⁴ To avoid the confounding factor associated with cardiolipin oxidation state, antiphospholipid reactivity associated with ALD was re-evaluated by assaying anti- β_2 -GP1 antibodies. IgG reactivity towards β_2 -GP1 (46.1 (29.5) U/ml (range 13–150)) was significantly higher ($p < 0.001$) in ALD patients than in control

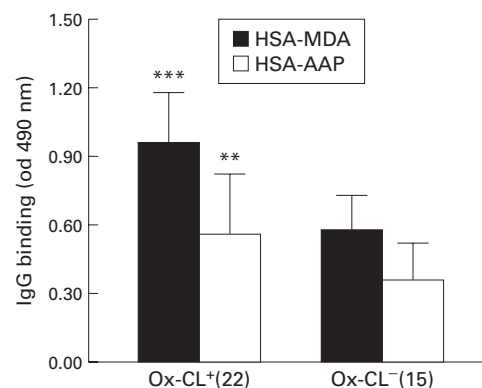


Figure 2 Reactivity with antigens derived from the reaction of proteins with lipid peroxidation products of sera from patients with alcoholic liver disease positive (Ox-CL⁺) or negative (Ox-CL⁻) for the presence of antibodies against oxidised cardiolipin. Human serum albumin (HSA) modified by the reaction with either malondialdehyde (HSA-MDA) or products from arachidonic acid peroxidation (HSA-APP) were used as antigens, as reported in the patients and methods section. Results are mean (SD) ELISA values. ** $p < 0.01$, *** $p < 0.0001$ versus Ox-CL⁻ subjects.

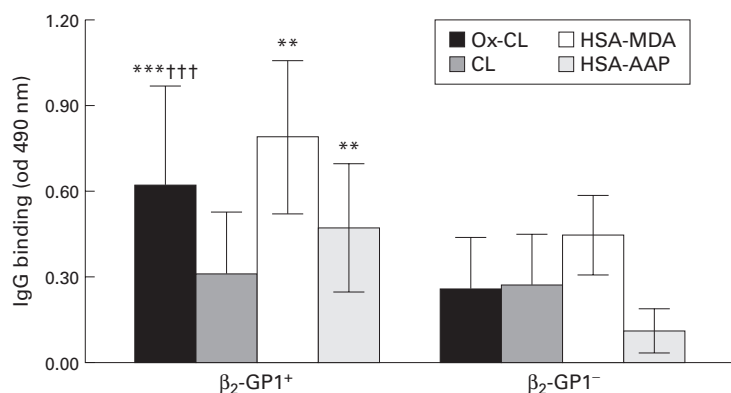


Figure 3 Reactivity with oxidised cardiolipin or with antigens derived from the reaction of proteins with lipid peroxidation products of sera from patients with alcoholic liver disease positive (β_2 -GP1⁺; n=23) or negative (β_2 -GP1⁻; n=13) for the presence of antibodies against β_2 -glycoprotein 1. ELISA plates coated with oxidation protected (CL) or oxidised (Ox-CL) cardiolipin, or with malondialdehyde (HSA-MDA) or products from arachidonic acid peroxidation (HSA-APP) were used to evaluate IgG binding. Results are mean (SD) ELISA values. ** $p < 0.005$, *** $p < 0.001$ versus β_2 -GP1 negative subjects; ††† $p < 0.001$ versus native cardiolipin.

subjects (22.5 (5.8) U/ml (range 10–32)) and 23 of 36 (64%) ALD patients were positive for anti- β_2 -GP1 IgG. β_2 -GP1 positive ALD sera showed titres of IgG recognising oxidised cardiolipin significantly higher ($p < 0.001$) than β_2 -GP1 negative sera (fig 3), despite the fact that total circulating IgG levels were not significantly different between the two groups (15.89 (7.37) g/l v 13.26 (3.01) g/l). Furthermore, a strong positive correlation ($r = 0.85$; $p < 0.0001$) was evident when individual values of anti- β_2 -GP1 IgG were compared with those of antibodies directed against oxidised cardiolipin. Thus we postulated that aPL detected in ALD sera might recognise oxidatively modified cardiolipin complexed with β_2 -GP1. Indeed, preadsorption of ALD sera displaying both anticardiolipin and anti- β_2 -GP1 reactivity on ELISA plates coated with β_2 -GP1 reduced IgG binding to oxidised cardiolipin by about 80% (fig 4). ALD patients positive for β_2 -GP1 also showed reactivity towards HSA-MDA and HSA-AAP significantly higher ($p < 0.005$) than that of β_2 -GP1 negative subjects (fig 3). To exclude the possibility that antibodies directed towards MDA or APP derived epitopes might account for antiphospholipid reactivity by recognising complexes between β_2 -GP1 and products of phospholipid oxidation, further experiments were performed. As shown in fig 4, preadsorption on ELISA plates coated with β_2 -GP1 that almost abolished recognition of oxidised cardiolipin by aPL positive sera did not affect IgG binding to HSA-APP or HSA-MDA. Similarly, preadsorption of ALD sera on plates coated with oxidised cardiolipin also did not interfere with the recognition of antigens formed by the reaction of HSA with oxidation products originating from auto-oxidation of linoleic acid (HSA-LPP), the main unsaturated fatty acid of cardiolipin (od_{490 nm} 0.280 (0.09) in preadsorbed sera v od_{490 nm} 0.272 (0.120) in non-preadsorbed sera). However, in the same experiments, preadsorption with HSA-LPP decreased (od_{490 nm} 0.049 (0.07)) binding of ALD sera to the same antigen by 82%. Therefore, antibodies towards

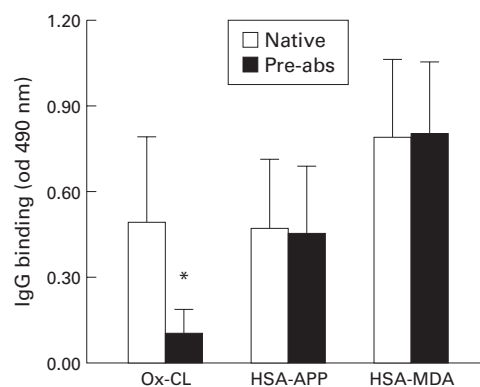


Figure 4 Reactivity with oxidised cardiolipin or human serum albumin (HSA) complexed with lipid peroxidation products following preabsorption with the β_2 -glycoprotein 1 (β_2 -GP1) of sera from alcoholic patients. Fifteen sera from patients with alcoholic liver disease displaying high titres of IgG against both cardiolipin and β_2 -GP1 were incubated overnight at 4°C on irradiated ELISA plates coated with β_2 -GP1 and tested for IgG binding to oxidised cardiolipin (Ox-CL) or HSA modified by reaction with malondialdehyde (HSA-MDA) or products of arachidonic acid peroxidation (HSA-APP). Results are mean (SD) ELISA values obtained with native sera or β_2 -GP1 preadsorbed sera. * $p < 0.0001$ versus non-pre-absorbed sera.

antigens derived from lipid peroxidation products did not account for the reactivity against oxidised phospholipids detected in the sera of ALD patients.

Previous studies have shown that anti-phospholipid immune reactivity was prevalent in patients with alcoholic hepatitis or cirrhosis^{11 12 14} and was particularly higher in non-abstaining subjects or in cirrhotics with severe liver damage (Child grades B and C).¹⁴ We have observed that individual reactivity against oxidised cardiolipin was positively correlated ($r = 0.65$; $p < 0.0001$) with Maddrey's DF values. Furthermore, circulating levels of IgG against oxidised cardiolipin were significantly ($p < 0.0002$) higher in ALD patients with severe liver injury (Maddrey's DF index >90) compared with patients with moderate or mild liver damage (Maddrey's DF index <90) (fig 5). A statistically significant difference ($p < 0.01$) was also observed when the same patients were subgrouped according to the Child-Turcotte classification (fig 5). This indicated that the presence of antibodies against oxidised phospholipids might actually account for high aPL titres observed in association with severe ALD.

Discussion

In recent years several reports have highlighted the association between alcoholic intoxication and the presence of circulating antiphospholipid antibodies (aPL).^{11 12 17 18} In these studies, the prevalence of aPL among patients with ALD ranged from 48% up to 81%. These values are in agreement with the prevalence of aPL (about 55%) observed in our group of patients with alcoholic liver cirrhosis. However, aPL have also been found in 31% of alcoholics with abnormal liver function but without cirrhosis¹² and in 15–17% of alcoholics without clinical or biochemical signs of liver injury.^{17 18} Anticardiolipin antibodies can also be detected in patients with HCV and are more frequent in subjects with advanced cirrhosis.^{13–15} None the

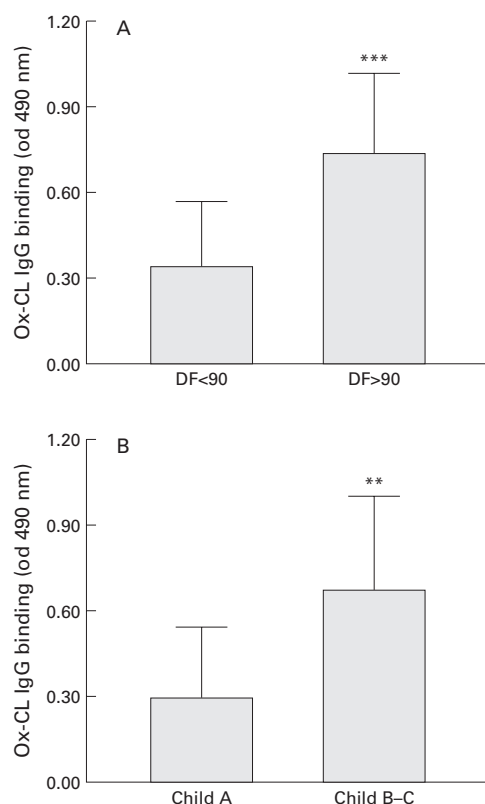


Figure 5 Reactivity against oxidised cardiolipin (Ox-CL) in relation to the severity of alcohol liver injury. The clinical severity of hepatic damage was estimated according to Maddrey's DF classification²³ (A) or Child-Turcotte classification (B). Patients with DF values less than 90 ($n=26$) were considered to have mild or moderate hepatic injury and patients with DF values greater than 90 ($n=10$) represented the group with severe damage. Child A group consisted of 15 patients, while 21 subjects belonged to Child B-C groups. The optical density (od) values in each group were normally distributed, as evaluated by the Kolmogorov-Smirnov test. *** $p<0.0002$ versus DF <90; ** $p<0.01$ versus Child A group.

less, the prevalence of anticardiolipin antibodies associated with HCV infection is much lower than in patients with ALD.^{14,15} Coexistence of HCV infection with alcohol abuse was taken into consideration in the present study as well as in previous reports, by excluding patients with evidence of HCV infection.

Little is known about the nature of the antigens recognised by aPL associated with ALD. These antibodies have been detected by binding to cardiolipin immobilised on the surface of ELISA plates, but immune reactivity with other classes of phospholipids (that is, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol) have also been reported.¹² An elegant study by Hörkkö *et al* demonstrated that selected sera from women with antiphospholipid syndrome bind to neo-epitopes generated when cardiolipin undergoes oxidation.⁹ The fatty acid content of cardiolipin largely consists (about 90%) of linoleic acid, and peroxidation of these fatty acids readily occurs when microtitre ELISA plates are exposed to air during the coating procedure.⁹ We now report that anticardiolipin antibodies detected in sera of patients with ALD weakly react with native

cardiolipin when it is protected from spontaneous auto-oxidation, but recognise instead antigens present in oxidised cardiolipin.

Several lines of evidence indicate the involvement of oxidative damage in alcoholic liver damage. Liver biopsies or sera from patients with ALD contain higher amounts of lipid peroxidation products (conjugated dienes, malondialdehyde, 4-hydroxynonenal, F₂-isoprostanes) and protein carbonyls than similar specimens from non-drinking subjects or patients with liver diseases unrelated to alcohol.¹⁹⁻²³ By reacting with proteins, free radicals, lipid hydroperoxides, and aldehydes generated from lipid peroxidation can produce a variety of adducts most of which are antigenic and can stimulate the production of specific antibodies.^{28,29} Increased levels of antibodies against hydroxyethyl free radicals and MDA-protein adducts have previously been detected in patients with ALD.^{24,30-32} We have observed that individual levels of IgG directed against adducts between serum albumin and either malondialdehyde (HSA-MDA) or arachidonic acid oxidation (HSA-APP) products are closely associated with those of antioxidant cardiolipin antibodies. Furthermore, among patients with ALD, levels of antibodies against lipid peroxidation products are significantly higher in groups positive for antioxidant cardiolipin antibodies than in negative subjects. Conversely, titres of IgG antioxidant cardiolipin in heavy drinkers without liver damage or in non-alcoholic cirrhotics that do not display evidence of oxidative injury are not significantly different from those in healthy controls. Thus we propose that the development of antiphospholipid autoimmunity associated with ALD might be related to the pro-oxidant conditions induced by ethanol. Such a hypothesis is consistent with experimental observations suggesting that oxidative modifications of phospholipids triggers the development of the antiphospholipid immune response.^{10,33} Furthermore, Iuliano *et al* have shown that among patients affected by systemic lupus erythematosus, those positive for aPL have higher urinary excretion of the lipid peroxidation product F₂-isoprostane than similar patients not displaying aPL reactivity.³⁴

It is now largely accepted that the plasma protein β_2 -GP1 or apolipoprotein H plays a key role in aPL reactivity, serving as a cofactor for antibody binding to phospholipids.^{3,4} In the liquid phase, β_2 -GP1 is not recognised by aPL but conformational changes occurring following interaction with phospholipids render β_2 -GP1 antigenic for aPL.⁷ We have observed that a high proportion (64%) of sera from patients with ALD displayed reactivity with β_2 -GP1. A close correlation ($r=0.85$; $p<0.0001$) was also seen between individual levels of anti- β_2 -GP1 IgG and those of antibodies directed against oxidised cardiolipin. Interestingly, only β_2 -GP1 positive sera recognised antigens in oxidised cardiolipin and preadsorption of β_2 -GP1 positive sera with β_2 -GP1 attached to ELISA microtitre plates decreased antibody binding to oxidised cardiolipin antigens by 80%. This indicates that aPL

associated with alcoholic damage are directed against epitopes formed by the interaction of β_2 -GP1 with oxidised phospholipids. Hörrkö *et al* have suggested that the neo-epitopes recognised by some aPL might consist of adducts formed between breakdown products of oxidised phospholipids and β_2 -GP1.^{9,10} As mentioned above, circulating levels of antibodies directed towards lipid peroxidation related epitopes are increased in ALD patients. Furthermore, ALD patients displaying β_2 -GP1 reactivity have higher serum levels of IgG recognising HSA-MDA or HSA-APP than β_2 -GP1 negative subjects. However, preadsorption of ALD sera with β_2 -GP1 that almost abolishes antibody binding to oxidised cardiolipin does not interfere with binding to epitopes in HSA complexed with MDA or with oxidised arachidonic or linoleic acids. We conclude that antibodies directed against protein adducts with lipid peroxidation products, such as MDA or lipid hydroperoxides, do not account for the immune reactivity towards oxidised cardiolipin. We propose that antiphospholipid antibodies detected in patients with ALD rather recognise complexes between β_2 -GP1 and oxidised cardiolipin.

Few data are available concerning the possible role of aPL in alcohol mediated liver injury. Antiphospholipid immune reactivity can be detected in patients with alcoholic hepatitis or cirrhosis^{11,12,14} and particularly in non-abstaining patients and in cirrhotics with severe liver damage (Child grades B and C).¹⁴ We have detected higher values of IgG against oxidised cardiolipin in ALD patients with severe liver damage. Moreover, a positive correlation (0.65; $p < 0.0001$) was evident between individual antioxidant cardiolipin IgG and Maddrey's DF values. This indicates that the high prevalence of aPL in association with severe ALD can be ascribed to the presence of antibodies targeting oxidised phospholipids. The clinical significance of aPL associated with alcoholic liver damage is at the moment unknown. The presence of aPL in patients with liver disease is not associated with an increased risk of thrombotic complications.¹⁶ Recent studies have proposed a possible link between aPL and cell death by apoptosis by showing that mice injected intravenously with syngenic apoptotic thymocytes develop anticardiolipin antibodies.³⁵ Consistently, sera of patients with antiphospholipid syndrome bind specifically to apoptotic thymocytes but not to viable cells by recognising epitopes generated by the interaction of β_2 -GP1 with plasma membrane cardiolipin.^{36,37} Interestingly, plasma membrane antigens in apoptotic cells are also targets for monoclonal antibodies recognising oxidised phospholipids.³⁸ Alcoholic liver injury is associated with stimulation of hepatocyte apoptosis³⁹ and oxidative events have been proposed to play a role in triggering apoptotic changes in hepatocytes exposed to ethanol.⁴⁰ Thus it is possible that oxidised phospholipids in the membranes of apoptotic hepatocytes might act as a stimulus as well as a target for aPL detected in ALD patients.

In conclusion, our results indicate that aPL detected in patients with ALD have a strict relationship with ethanol induced oxidative damage and preferentially target oxidised phospholipids complexed with β_2 -GP1. These findings suggest a possible role for oxidative mechanisms in the development of aPL associated with alcoholic liver injury.

This work was supported by grants from the Italian Ministry for University and Scientific and Technological Research (Research Program: Redox Regulation of Cellular Processes) and by the National Research Council (contribution No 96.00073.CT04).

- Harris NE. Antiphospholipid antibodies. *Br J Haematol* 1990;74:1-9.
- Mc Neil HP, Chesterman CN, Krilis SA. Immunological and clinical importance of antiphospholipid antibodies. *Adv Immunol* 1991;49:193-280.
- Hughes GRV. The antiphospholipid syndrome: ten years on. *Lancet* 1993;342:341-4.
- Greaves M. Antiphospholipid antibodies and thrombosis. *Lancet* 1999;352:1384-53.
- McNeil HP, Simpson J, Chesterman CN, *et al*. Antiphospholipid antibodies are directed against a complex antigen that include a lipid binding inhibitor of coagulation β_2 -glycoprotein 1 (apolipoprotein H). *Proc Natl Acad Sci USA* 1990;87:4120-4.
- Galli M, Comfurius P, Maassen C, *et al*. Anticardiolipin antibodies directed not to cardiolipin but to a plasma cofactor. *Lancet* 1990;355:1544-7.
- Chamley LW, Duncalf AM, Konarkowska B, *et al*. Conformational altered β_2 -glycoprotein 1 is the antigen of anti-cardiolipin autoantibodies. *Clin Exp Immunol* 1999;115:571-6.
- Matsuura E, Igarashi Y, Yasuda T, *et al*. Anticardiolipin antibodies recognise β_2 -glycoprotein 1 structure altered by oxygen modified solid phase surfaces. *J Exp Med* 1994;179:457-62.
- Hörrkö S, Miller E, Dudl E, *et al*. Antiphospholipid antibodies are directed against epitopes of oxidized phospholipids. Recognition of cardiolipin by monoclonal antibodies to epitopes of oxidized low density lipoprotein. *J Clin Invest* 1996;98:815-25.
- Hörrkö S, Miller E, Branch DW, *et al*. The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and β_2 -glycoprotein 1 (and other proteins). *Proc Natl Acad Sci USA* 1997;94:10356-61.
- Bird G, Millis P, Smith D, *et al*. Antibodies to phospholipid in alcoholic liver disease. *BMJ* 1994;309:1161.
- Chedid A, Chadawada KR, Morgan TR, *et al*. Phospholipid antibodies in alcoholic liver disease. *Hepatology* 1994;20:1465-71.
- Prieto J, Yuste JR, Beloqui O, *et al*. Anticardiolipin antibodies in chronic hepatitis C. Implication of hepatitis C virus as the cause of the antiphospholipid syndrome. *Hepatology* 1996;23:204-11.
- Biron C, Andreani H, Blanc P, *et al*. Prevalence of antiphospholipid antibodies in patients with chronic liver disease related to alcohol or hepatitis C virus: correlation with liver injury. *J Lab Clin Med* 1998;131:243-50.
- Leroy V, Arvieux J, Jacob MC, *et al*. Prevalence of anticardiolipin, anti- β_2 -glycoprotein 1 and anti-prothrombin antibodies in chronic hepatitis C. *Br J Haematol* 1998;101:468-74.
- Maggia A, Maraglione M, Cascavilla I, *et al*. Anticardiolipin antibodies in patients with liver disease. *Am J Gastroenterol* 1999;94:2983-7.
- Biron C, Lalloyer N, Tonnelot JL, *et al*. Anticardiolipin antibodies and acute alcoholic intoxication. *Lupus* 1995;4:486-90.
- Zima T, Fialova L, Mikulikova L, *et al*. Antibodies against phospholipids and oxidized LDL in alcoholic patients. *Physiol Res* 1998;47:351-5.
- Nordmann R, Ribière C, Rouach H. Implication of free radical mechanisms in ethanol induced cellular injury. *Free Radic Biol Med* 1992;12:219-40.
- Clot P, Tabone M, Aricò S, *et al*. Monitoring oxidative damage in patients with liver cirrhosis and different daily alcohol intake. *Gut* 1994;35:1637-43.
- Grattagliano I, Vendemiale G, Sabbà G, *et al*. Oxidation of circulating proteins in alcoholics: role of acetaldehyde and xanthine oxidase. *J Hepatol* 1996;25:28-36.
- Aleynik SI, Leo MA, Aleynik MK, *et al*. Increased circulating products of lipid peroxidation in patients with alcoholic liver disease. *Alcohol Clin Exp Res* 1998;22:192-6.
- Hill DB, Awad JA. Increased urinary F_2 -isoprostane excretion in alcoholic liver disease. *Free Radic Biol Med* 1999;26:656-60.
- Clot P, Bellomo G, Tabone M, *et al*. Detection of antibodies against proteins modified by hydroxyethyl free radicals in patients with alcoholic cirrhosis. *Gastroenterology* 1995;108:201-7.
- Maddrey WC, Boitnott JK, Bedine MS, *et al*. Corticosteroid therapy of alcoholic hepatitis. *Gastroenterology* 1978;75:193-9.
- Conn HO. A peek at the Child-Turcotte classification. *Hepatology* 1981;1:673-6.

- 27 Cominacini M, Garbin U, Davoli A, *et al.* A simple test for predisposition to LDL oxidation based on the fluorescence development during copper-catalysed oxidative modification. *J Lipid Res* 1991;**32**:349–58.
- 28 Palinski W, Hökkö S, Miller E, *et al.* Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoproteins in human plasma. *J Clin Invest* 1996;**98**:800–14.
- 29 Palinski W, Ylä-Herttuala S, Rosenfeld ME, *et al.* Antisera and monoclonal antibodies specific for epitopes generated during the oxidative modification of low density lipoprotein. *Atherosclerosis* 1990;**10**:325–35.
- 30 Lecomte E, Herberth B, Pirrolet P, *et al.* Effect of alcohol consumption on blood antioxidant nutrients and oxidative stress indicators. *Am J Clin Nutr* 1994;**60**:255–61.
- 31 Dupont I, Lucas D, Clot P, *et al.* Cytochrome P450E1 inducibility and hydroxyethyl radical formation among alcoholics. *J Hepatol* 1998;**28**:564–71.
- 32 Rolla R, Vay D, Mottaran E, *et al.* Detection of circulating antibodies against malondialdehyde-acetaldehyde adducts in patients with alcoholic liver disease. *Hepatology* 2000;**31**:878–84.
- 33 Itabe H, Yamamoto H, Suzuki M, *et al.* Oxidized phosphatidylcholine that modify proteins. Analysis by monoclonal antibodies against oxidized low density lipoprotein. *J Biol Chem* 1996;**271**:33208–17.
- 34 Iuliano L, Praticò D, Ferro D, *et al.* Enhanced lipid peroxidation in patients positive for antiphospholipid antibodies. *Blood* 1997;**90**:3931–5.
- 35 Mevorach D, Zhou JL, Song X, *et al.* Systemic exposure to irradiated cells induces autoantibody production. *J Exp Med* 1998; **188**:387–92.
- 36 Prince BE, Rauch J, Shia MA, *et al.* Anti-phospholipid autoantibodies bind to apoptotic, but not viable thymocytes in a β_2 -glycoprotein 1-dependent manner. *J Immunol* 1996; **157**:2201–8.
- 37 Sorice M, Circella A, Misasi R, *et al.* Cardiolipin on the surface of apoptotic cells as a possible trigger for antiphospholipid antibodies. *Clin Exp Immunol* 2000;**122**:277–84.
- 38 Chang MK, Bergmark C, Laurila A, *et al.* Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: Evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci USA* 1999;**96**:6353–8.
- 39 Nanji AA. Apoptosis and alcoholic liver disease. *Semin Liver Dis* 1998;**18**:187–90.
- 40 Kurose I, Higuchi H, Miura S, *et al.* Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication. *Hepatology* 1997; **25**:368–78.