Antiphospholipid antibodies associated with alcoholic liver disease specifically recognise oxidised phospholipids

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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 β2-glycoprotein 1 (β2-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 targeting complexes between HSA and oxidised phospholipids (HSA-APP) and HSA-MDA (r = 0.68 and 0.72, respectively; p < 0.0001) used as markers of oxidative stress. APL patients also displayed increased levels of antibodies against phospholipid binding protein β2-GP1, and individual reactivity towards oxidised cardiolipin and β2-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 β2-GP1 positive than in β2-GP1 negative sera. However, preadsorption of β2-GP1 positive sera on β2-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 β2-GP1. These antibodies might account for high aPL titres observed in patients with severe ALD.

Keywords: oxidative stress; lipid peroxidation; β2-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.3,4 aPL and antiphospholipid syndrome are often associated with rheumatic and autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and systemic sclerosis.3,4 However, aPL are also present in patients with chronic infections (syphilis, malaria, human immunodeficiency virus 1), lymphoproliferative diseases, and sickle cell anaemia.3,4

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.1–5 Studies by McNeil and colleagues4 and Galli and colleagues5 have shown that aPL binding to phospholipids is largely mediated by β2-glycoprotein 1 (β2-GP1) or apolipoprotein H, a plasmatic 50 kDa glycoprotein which readily binds to phospholipids as well as to other negatively charged substances.6 Conformational changes occurring in β2-GP1 following interaction with phospholipids render this protein antigenic to aPL. Indeed, β2-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.6 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.7 They also suggested that the antigenic determinants might result from the reaction of β2-GP1 with products derived from the peroxidative breakdown of cardiolipin unsaturated fatty acids.8,9

Recent studies have shown that aPL can be detected in patients with chronic liver disease, and that certain aPL levels correlate with the degree of histological liver injury.10–12 The presence of these antibodies in patients with alcoholic liver disease suggests that aPL might be involved in the development of thrombocytopenia.2–4 It has been hypothesised that chronic ethanol ingestion might lead to aPL production through oxidative injury, and that oxidative stress might be responsible for both aPL production and the anticoagulant properties ascribed to aPL.13–15 Our previous study showed that ethanol induced oxidative stress might promote the development of aPL in heavy drinkers without alcoholic liver disease.16 The present study was undertaken to evaluate whether ethanol induced oxidative injury might promote the development of aPL in patients with alcoholic liver disease.

Methods

Patients

Patients were recruited from heavy drinkers attending the Liver Unit of the Amedeo Avogadro’ University Hospital and the General Hospital, Novara, Italy. All patients were referred to the Liver Unit because of abnormal hepatic function tests. The diagnosis of alcoholic liver disease (ALD) was based on the history and the presence of at least two of the following: (i) a minimum of 2 weeks with an average consumption of 60 g alcohol per day for at least 10 years; (ii) abnormal hepatic function tests; (iii) normal or increased serum concentrations of γ-glutamyl transpeptidase; (iv) absence of clinical evidence of chronic active hepatitis, cryoglobulinaemia, and chronic viral infection. Patients who were positive for hepatitis C virus (HCV) or human immunodeficiency virus (HIV) were excluded. Controls were 12 heavy drinkers without ALD and 20 healthy subjects. All patients and controls gave informed consent.

Biochemical tests

The following tests were performed on all patients and controls: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transferase, total bilirubin, direct bilirubin, serum albumin, cholesterol, triglycerides, uric acid, lactate dehydrogenase, and γ-glutamyl transferase. In 12 patients and controls with persistently abnormal liver function tests, a liver biopsy was performed. In 20 patients with alcoholic hepatitis, the degree of histological liver injury was scored according to the system of Knodell et al.17

Immunoassays

Immunoglobulin G (IgG) against β2-glycoprotein 1 (β2-GP1), cardiolipin, and human serum albumin (HSA) complexed with either oxidised arachidonic acid (HSA-APP) or malondialdehyde (HSA-MDA) were assayed by ELISA (Boehringer Mannheim, Germany). The IgG titre was expressed as the reciprocal of the highest dilution showing a positive reaction.

Antiphospholipid antibodies

Antiphospholipid antibodies (aPL) were defined as the presence of IgG against β2-GP1, specific for negatively charged phospholipids, and IgG against cardiolipin, a highly negatively charged lipid. The diagnosis of aPL syndrome was made according to the criteria of the International Society of Thrombosis and Haemostasis.18

Statistical analysis

The level of significance was set at p < 0.05. Statistical analysis was performed using the Stat View statistical software (SAS Institute, Cary, NC, USA). The Student’s t test for unpaired data was used. The correlation of the IgG titres to aPL with the biochemical tests was performed using the Pearson correlation coefficient.

Results

As shown in table 1, the biochemical tests were normal in 12 heavy drinkers without alcoholic liver disease and in 20 healthy subjects. In contrast, all patients with alcoholic liver disease had an abnormal transaminase level and only half of the patients were hyperbilirubinaemic. In 20 patients with alcoholic hepatitis, the degree of histological liver injury was as follows: mild, 1; moderate, 6; severe, 13. In a PL positive patient, the degree of histological liver injury was mild.

As shown in table 1, aPL were present in 11 (50%) of 22 patients with alcoholic liver disease and in none of the 12 controls or 20 healthy subjects. The IgG titre against β2-GP1 was 1:40 in one patient and 1:100 in 10 other patients. The IgG titre against cardiolipin was 1:80 in two patients and 1:160 in nine other patients. In all patients with alcoholic liver disease, IgG against β2-GP1 and cardiolipin was stronger than IgG against HSA-MDA or HSA-APP. However, the IgG titre against HSA-MDA was significantly higher in patients with ALD than in controls (p = 0.0008). As shown in table 1, patients with alcoholic liver disease had higher aPL titres than controls. However, aPL were also present in patients with chronic infections (syphilis, malaria, human immunodeficiency virus 1), lymphoproliferative diseases, and sickle cell anaemia. The aPL titres observed in patients with severe ALD were even higher than those of patients with alcoholic hepatitis without aPL.

As shown in table 2, patients with alcoholic liver disease had higher levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transferase, total bilirubin, direct bilirubin, serum albumin, cholesterol, triglycerides, uric acid, lactate dehydrogenase, and γ-glutamyl transferase than controls. As shown in figure 1, the IgG titre against β2-GP1 and cardiolipin was highly correlated with the degree of histological liver injury (r = 0.72 and 0.74, respectively; p < 0.0001). The IgG titre against HSA-MDA and HSA-APP was also highly correlated with the degree of histological liver injury (r = 0.72 and 0.74, respectively; p < 0.0001).

The data shown in table 2 indicate that the aPL titres observed in patients with severe ALD were even higher than those of patients with alcoholic hepatitis without aPL. As shown in table 2, patients with alcoholic liver disease had higher levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transferase, total bilirubin, direct bilirubin, serum albumin, cholesterol, triglycerides, uric acid, lactate dehydrogenase, and γ-glutamyl transferase than controls. As shown in figure 1, the IgG titre against β2-GP1 and cardiolipin was highly correlated with the degree of histological liver injury (r = 0.72 and 0.74, respectively; p < 0.0001). The IgG titre against HSA-MDA and HSA-APP was also highly correlated with the degree of histological liver injury (r = 0.72 and 0.74, respectively; p < 0.0001).

Discussion

In conclusion, ethanol induced oxidative stress might promote the development of aPL in patients with alcoholic liver disease. The presence of these antibodies was highly correlated with the degree of histological liver injury.

Abbreviations used in this paper: ALD, alcoholic liver disease; aPL, antiphospholipid antibodies; β2-GP1, β2-glycoprotein 1; DPPD, diphenylphenylendiamine; HCV, hepatitis C virus; HSA, human serum albumin; HSA-APP, adducts between human serum albumin and arachidonic acid oxidation products; HSA-MDA, malondialdehyde adducts with human serum albumin; HSA-APP, adducts between human serum albumin and arachidonic acid oxidation products; MDA, malondialdehyde; od, optical density; PBS, phosphate buffered saline.
particularly alcoholic liver disease (ALD) and hepatitis C. 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. However, aPL can also be detected in alcoholics without liver damage. Furthermore, aPL are often associated with venous and arterial thrombosis: the presence of aPL in patients with chronic liver disease does not seem to be associated with thrombotic complications.

Clinical and experimental evidence indicates that ethanol intoxication promotes free radical formation and oxidative liver injury. 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. Furthermore, the formation of hydroxethyl free radicals during ethanol metabolism can also be documented in patients with alcohol abuse.

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, ecographical, 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-Pugh classification 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 ecographical 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 immunosorbent 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 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.

**DETERMINATION OF IMMUNE REACTIVITY TOWARDS OXIDISED PHOSPHOLIPIDS**

Phospholipid coated ELISA plates were prepared according to a standardised method proposed by Harris. Briefly, 30 µl of cardiolipin 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**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Alcohol consumption (g/day)</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>GGT (U/l)</th>
<th>Bilirubin (µmol/l)</th>
<th>Bilirubin (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol cirrhosis</td>
<td>36</td>
<td>146 (67)</td>
<td>74 (52)</td>
<td>50 (36)</td>
<td>143 (132)</td>
<td>1.68 (1.5)</td>
<td>72 (14)</td>
</tr>
<tr>
<td>Non-alcoholic cirrhos</td>
<td>40</td>
<td>20 (14)</td>
<td>83 (58)</td>
<td>51 (24)</td>
<td>37 (19)</td>
<td>1.33 (0.6)</td>
<td>54 (4)</td>
</tr>
<tr>
<td>Heavy drinkers</td>
<td>15</td>
<td>190 (61)</td>
<td>28 (13)</td>
<td>22 (11)</td>
<td>37 (19)</td>
<td>0.62 (0.3)</td>
<td>101 (3)</td>
</tr>
</tbody>
</table>

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 1-phenylendiamine, 0.4 µl/ml hydrogen peroxide (30%), 5.1 mg/ml citric acid, and 6.1 mg/ml anhydrous NaHPO₄, 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. 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.

**MATERIALS**

Arachidonic acid, linoleic acid, 1-phenylendiamine, 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 antecardiolipin IgG levels above the threshold value (15 GPL units/ml) while one healthy control was positive for anticardiolipin IgG. Mean IgG reactivity was 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.
It has been shown that the unsaturated fatty acid component of cardiolipin rapidly auto-oxidises when exposed to air.\(^1\) 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 (\(\text{OD}_{234\text{ nm}} 0.276\)) of cardiolipin samples compared with freshly prepared solutions (\(\text{OD}_{234\text{ nm}} 0.110\)). Cardiolipin auto-oxidation was however completely prevented (\(\text{OD}_{234\text{ nm}} 0.112\)) 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 (\(\text{OD}_{490\text{ nm}} 0.274 (0.184)\)) was significantly lower (\(p<0.0001\)) than that to oxidised cardiolipin (\(\text{OD}_{490\text{ nm}} 0.491 (0.340)\)). Reactivity of control sera with either form of cardiolipin was unchanged (\(\text{OD}_{490\text{ nm}} 0.205 (0.103)\) vs 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 \(\text{OD}_{490\text{ nm}}\) 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 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.\(^{23}\) 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) (\(\text{OD}_{490\text{ nm}} 0.79 (0.27)\) and 0.47 (0.25), respectively; \(p<0.001\)) in healthy controls; \(p<0.0001\). 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 (\(\text{OD}_{490\text{ nm}} 0.450 (0.140)\) and 0.039 (0.030), respectively) or in non-alcoholic cirrhosis (\(\text{OD}_{490\text{ nm}} 0.590 (0.220)\) and 0.196 (0.151), respectively).

Antiphospholipid antibodies can also be detected by their ability to recognise complexes formed by \(\beta\)-GP1 and phospholipids.\(^1\) To avoid the confounding factor associated with cardiolipin oxidation state, antiphospholipid reactivity associated with ALD was re-evaluated by assaying anti-\(\beta\)-GP1 antibodies. IgG reactivity towards \(\beta\)-GP1 (46.1 (29.5) \(\text{U/ml}\) (range 13–150)) was significantly higher \((p<0.001)\) in ALD patients than in control patients with alcoholic liver disease positive (Ox-CL\((22)\)) or negative (Ox-CL\((15)\)) 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. \(*p<0.01, **p<0.001\) versus Ox-CL\((15)\) subjects.
subjects (22.5 (5.8) U/ml (range 10–32)) and 23 of 36 (64%) ALD patients were positive for anti-β2-GP1 IgG. β2-GP1 positive ALD sera showed titres of IgG recognising oxidised cardiolipin significantly higher (p<0.001) than β2-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-β2-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 β2-GP1. Indeed, preadsorption of ALD sera displaying both anticardiolipin and anti-β2-GP1 reactivity on ELISA plates coated with β2-GP1 reduced IgG binding to oxidised cardiolipin by about 80% (fig 4). ALD patients positive for β2-GP1 also showed reactivity towards HSA-MDA and HSA-AAP significantly higher (p<0.005) than that of β2-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 β2-GP1 and products of phospholipid oxidation, further experiments were performed. As shown in fig 4, preadsorption on ELISA plates coated with β2-GP1 that almost abolished recognition of oxidised cardiolipin by aPL positive sera did not affect IgG binding to HSA-AAP 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 preabsorbed sera v 0.272 (0.120) in non-preabsorbed 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 antigens derived from lipid peroxidation products did not account for the reactivity against oxidised phospholipids detected in the sera of ALD patients.

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 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.15 16 None the
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. 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. Increased levels of antibodies against hydroxyethyl free radicals and MDA-protein adducts have previously been detected in patients with ALD. We have observed that individual levels of IgG directed against adducts between serum albumin and either malonildialdehyde (HSA-MDA) or arachidonic acid oxidation (HSA-APP) products are closely associated with those of oxidised cardiolipin antibodies. Furthermore, among patients with ALD, levels of antibodies against lipid peroxidation products are significantly higher in groups positive for oxidised cardiolipin antibodies than in negative subjects. Conversely, titres of IgG oxidised cardiolipin in heavy drinkers without liver damage or in non-alcoholic cirrhosis 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. 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. It is now largely accepted that the plasma protein β2-GP1 or apolipoprotein H plays a key role in aPL reactivity, serving as a cofactor for antibody binding to phospholipids. In the liquid phase, β2-GP1 is not recognised by aPL, but conformational changes occurring following interaction with phospholipids render β2-GP1 antigenic for aPL. We have observed that a high proportion (64%) of sera from patients with ALD displayed reactivity with β2-GP1. A close correlation (r=0.85; p<0.0001) was also seen between individual levels of anti-β2-GP1 IgG and those of antibodies directed against oxidised cardiolipin. Interestingly, only β2-GP1 positive sera recognised antigens in oxidised cardiolipin and preadsorption of β2-GP1 positive sera with β2-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 β2-GP1 with oxidised phospholipids. Hörthók et al. have suggested that the neo-epitopes recognised by some aPL might consist of adducts formed between breakdown products of oxidised phospholipids and β2-GP1. As mentioned above, circulating levels of antibodies directed towards lipid peroxidation related epitopes are increased in ALD patients. Furthermore, ALD patients displaying β2-GP1 reactivity have higher serum levels of IgG recognising HSA-MDA or HSA-APP than β2-GP1 negative subjects. However, preadsorption of ALD sera with β2-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 β2-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 and particularly in non-alcohol abusers and in cirrhotics with severe liver damage (Child grades B and C). 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 antioxidised 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. 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 antiphospholipid antibodies. 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 β2-GP1 with plasma membrane cardiolipin. Interestingly, plasma membrane antigens in apoptotic cells are also targets for monoclonal antibodies recognising oxidised phospholipids. 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. 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 β2-GP1. These findings suggest a possible role for oxidative mechanisms in the development of aPL associated with alcoholic liver injury.

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5 Consistently, sera of patients with alcoholic liver disease.