Cofactor dependence and isotype distribution of anticardiolipin antibodies in viral infections

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Abstract

Background—Antibodies to cardiolipin (aCLs) are often detected in patients with autoimmune disorders or infectious diseases.

Objective—To investigate the distribution of aCL isotypes and requirement of protein cofactor in viral infections in order to establish the importance, if any, of these antibodies in these infectious diseases.

Patients and methods—The isotype distribution of aCLs in the sera from 160 patients with infection caused by HIV-1 (n=40), hepatitis A virus (n=40), hepatitis B virus (n=40), or hepatitis C virus (n=40) was studied by standardised enzyme linked immunosorbent assay (ELISA) in the presence and absence of protein cofactor (mainly β2-glycoprotein I). Serum samples from healthy volunteers and patients with syphilis and antiphospholipid syndrome were also included and served as negative and positive control groups respectively.

Results—The prevalence of one or more aCL isotypes in serum of patients with HIV-1, hepatitis A virus, hepatitis B virus, or hepatitis C virus infection was 47%, 92%, 42%, and 17% respectively (principally IgM and/or IgA). Most of these antibodies were mainly cofactor independent.

Conclusions—The presence of aCLs in viral infections is principally cofactor independent, suggesting that cofactor dependence of the aCLs should be assessed to distinguish subjects most likely to suffer from clinical symptoms observed in the presence of these antibodies.

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Lupus anticoagulant (LA) and antibodies to cardiolipin (aCLs) are acquired antibodies to phospholipid (aPLs) which may be present in a variety of conditions. Classically, they are found in patients with systemic lupus erythematosus (SLE) or related autoimmune disorders but they may also be detected in patients with syphilis and a variety of acute or chronic infections.[1 2] Certain people, however, have no identifiable underlying disorders and may be regarded as having primary antiphospholipid syndrome (APS).[3] Clinical signs linked to aPLs usually involve thrombosis, recurrent fetal loss, and thrombocytopenia which occur in about 30% of cases; less common conditions such as livedo reticularis, leg ulcers, chorea, epilepsy, and pulmonary hypertension are also involved.[4]

Matsuura *et al*[5] reported that a serum protein, β2-glycoprotein I (β2-GP I) with high affinity for anionic phospholipid, is required for binding of aCLs to cardiolipin in autoimmune disease, whereas this requirement is not found in the binding that occurs in association with various infections. In addition, Hunt *et al*[6] confirmed that binding of aCLs from patients with infections, unlike that of patients with SLE, is not dependent on β2-GP I. Classification of aCLs into β2-GP I dependent and β2-GP I independent has been applied to patients with different clinical manifestations and helped to distinguish between aCLs associated with autoimmune disease and those resulting from infections.[7] An enzyme linked immunosorbent assay (ELISA) using cardiolipin as antigen has been widely used for detection of aCLs, and the use of adult bovine serum as blocking agent and sample diluent was considered to be the source of β2-GP I. Moreover, it has been reported that aCLs detected with this assay include both β2-GP I dependent and β2-GP I independent forms.[5] Arvieux *et al*[8] reported that certain aCLs can bind directly to β2-GP I immobilised on irradiated microtitre plates in the absence of cardiolipin. In addition, an excellent correlation between aCL titres and β2-GP I antibody has been reported,[9 10] giving rise to the idea that autoimmune aCLs and β2-GP I antibodies are one and the same. It was recently reported that these antibodies provide only additional information and that the β2-GP I antibody test should not be considered a substitute for conventional LA or aCL assays.[11] Moreover, it was reported that β2-GP I antibodies have been closely associated with venous thrombosis but not with other clinical features of APS, such as arterial thrombosis and recurrent abortion. Unlike aCLs, which are often found in several clinical conditions other than APS,[1] β2-GP I antibodies are seldom found in patients with SLE and without APS and are rare in infectious conditions.[12]

A variety of infectious diseases, particularly viral, such as those caused by HIV-1, parvovirus B19, hepatitis A virus (HAV) and hepatitis C virus (HCV), have been reported to be associated with the presence of aCLs.[13–16] However, in some of these reports, aCL isotype distribution and cofactor dependence was not investigated.

In this study, we report the occurrence of β2-GP I dependent and β2-GP I independent aCL isotypes in a number of patients with infection caused by HIV-1, HAV, HCV, or hepatitis B virus (HBV), as well as an analysis of the relevance of one or more isotypes of aCL in these infectious diseases.

Patients and methods

PATIENTS

Stored non-heat-inactivated sera were obtained from the following groups of patients, none of whom were receiving antiviral treatment: group 1, 40 patients with acute HAV infections, confirmed by specific IgM antibodies detected using an ELISA from Abbott (Chicago, Illinois, USA); group 2, 40 patients who tested positive for antibody against HCV in the second generation ELISA 3.0 (Abbott), confirmed by the RIBA-3.0 assay (Ortho Diagnostic, Raritan, New York, USA); group 3, 40 patients with acute and chronic HBV who tested positive to hepatitis B surface antigen (HBsAg) in an ELISA (Abbott); 22 of these patients were positive and 18 were negative for anti-HBc IgM detected by ELISA (Abbott); group 4, 40 HIV-1 infected patients confirmed by western blot assay (Organon Teknika, Durham, North Carolina, USA). All the above patients were negative for fluorescent Treponema Pallidum antibody (FTA-Abs, Biocientifica, Argentina).

Serum samples from patients with long standing syphilis who were positive for the screening Venereal Disease Research Laboratory (VDRL) test were included in this study and confirmed by FTA-Abs, but none experienced clinical events suggesting APS and HAV, HIV-1, HBV or HCV infections. Other patients with primary and secondary APS (previously reported by our group[19]) were also included. The former group of patients provided a positive control for aCL cofactor independence and the second group a positive control for aCL cofactor dependence.

The normal control group consisted of 60 healthy subjects (36 women and 24 men) matched for age and sex. All showed normal coagulation assays and negative serological tests for syphilis VDRL and for the viral infections included in this study.

ASSAY OF ACL COFACTOR DEPENDENCE

The improvement of the aCL ELISA by the use of bovine serum based solutions has been documented previously, and is now considered to be due mainly to the presence of bovine β2-GP I.[17] To determine cofactor or β2-GP I dependence of aCL binding to cardiolipin, we used a standard ELISA[18] with minor modifications.[19] In brief, wells of ELISA plates (Linbro-Titertek; ICN-Biomedical, Horsham, Philadelphia, USA) were coated by the addition of 50 μl/well of 30 μg/ml cardiolipin (Sigma Chemical Co, St Louis, Missouri, USA) in ethanol, and the ethanol evaporated at 4°C overnight. Plates were washed three times with phosphate buffered saline (PBS) and blocked for one hour by the addition of 100 μl/well 10% (v/v) adult bovine serum (ABS) in PBS (10% ABS/PBS). After being blocked, plates were washed three times with PBS. The unknown samples and IgG, IgM and IgA aCL calibrators (Louisville APL Diagnostic Inc, Louisville, Kentucky, USA) and our own control sera diluted 1:50 in 10% ABS/PBS were added (50 μl) to the ELISA plate in duplicate wells. The diluted serum samples and standards were then incubated for three hours. The samples were then discarded and the plates rinsed three times with PBS. Then 50 μl/well alkaline phosphatase labelled, affinity purified, goat anti-human IgG, IgM, or IgA (Sigma Chemical Co) diluted 1:1000 in 10% ABS/PBS was added. After 90 minutes of incubation, the plates were washed three times with PBS, and 100 μl/well enzyme substrate (*p*-nitrophenyl phosphate/diethanolamine buffer) was added, and the plates were incubated in an oven at 37°C. The enzyme reaction was stopped with 3 M NaOH (50 μl/well) when the highest positive aCL calibrator for that isotype had reached an absorbance at 405 nm of about 1.0—for example, for aCL IgG the reaction was stopped when G1=90.1 GPL units reached an absorbance reading of 1.0. Plates were then read in an EL301 microwell strip reader (Bio-Tek Instrument Inc, Winooski, Vermont, USA). Results were expressed as antiphospholipid units (GPL, MPL, and APL for IgG, IgM, and IgA isotypes respectively) using a calibration curve constructed by plotting absorbance against concentration of a given aCL isotype in a log-log equation. A sample with known aCL concentrations was assayed on each occasion to confirm the reliability of the assay. Values higher than 10.2 GPL, 11.5 MPL, and 12.5 APL (mean of 60 healthy sera +2 SDs) were considered positive. Intra-assay and interassay coefficients of variation were 8% and 10% respectively.

MODIFIED ELISA FOR COFACTOR INDEPENDENT ACL ISOTYPES

The modified ELISA for aCL cofactor independent isotypes was performed in the absence of ABS as β2-GP I source and at a 1:200 dilution of test samples and controls. We used 2% ovalbumin (Sigma Chemical Co) as blocking agent and sample diluent instead of bovine serum albumin to avoid possible contamination with bovine β2-GP I. Therefore the assay essentially measures β2-GP I independent binding to the phospholipid surface. The dilution was selected to prevent the cofactor effect of endogenous β2-GP I in serum samples, since no, or almost no, β2-GP I dependent aCL will be detected under these experimental conditions.[20] Results were expressed as the mean of two replicates; a result was regarded as positive when the absorbance was higher than 2 SDs of the mean for the control group. Sera from patients with syphilis or primary or secondary APS, and from selected normal controls were tested in each plate as positive and negative controls respectively. To avoid false positive results due to non-specific binding, a sample in the β2-GP I dependent assay had to show a 10% higher absorbance value than the β2-GP I independent assay performed in parallel to be considered positive, and vice versa.[20] The interassay and intra-assay coefficients of variation were lower than 12% (n=8) and 8% (n=8) respectively.

Table 1   Presence of IgG, IgM, and IgA cofactor dependent and independent antibodies to cardiolipin in patients with viral infections

| Antibody isotype | Cofactor dependent/cofactor independent | | | |
	HIV-1 (n=40)	HAV (n=40)	HBV (n=40)	HCV (n=40)
IgG alone	0/0	0/0	1/1	0/0
IgM alone	0/0	1/22	2/5	0/0
IgA alone	4/3	0/0	0/4	1/4
IgG + IgM	0/0	0/1	0/1	0/0
IgG + IgA	4/6	0/0	0/1	0/1
IgM + IgA	0/1	0/9	0/2	0/1
IgG + IgM + IgA	0/1	0/4	0/0	0/0
Total No with IgG	4/7	0/5	1/3	0/1
Total No with IgM	0/2	1/36	2/8	0/1
Total No with IgA	8/11	0/13	0/7	1/6

Cofactor dependence was considered positive when there was a ⩾10% increase in the absorbance reading in the presence of adult bovine serum above those for the same serum samples in the presence of ovalbumin. Cofactor independence was considered positive if there was a reduction in absorbance ⩽10%.
HAV = hepatitis A virus; HBV = hepatitis B virus; HCV = hepatitis C virus.

STATISTICAL ANALYSIS

All variables studied are expressed as the arithmetical mean (SEM). Differences in mean values were evaluated by unpaired Student's *t* test with $p<0.05$ considered to be significant. The relation between aCL levels and disease activity was studied by the χ^2 test.

Results

Serum samples from 160 patients with acute or chronic viral infection were screened for aCL isotypes, and the cofactor requirement was evaluated. Table 1 shows the prevalence of aCLs in the population studied. Positive samples for aCL (IgG, IgM, or IgA) were found in 19 of 40 patients with HIV-1 infection (47%), in 37 of 40 patients with acute HAV (92%), in 17 of 40 patients with acute and

chronic HBV (42%), and seven of 40 patients with HCV infection (17%). The isotype distribution of aCL reactivity also differed among the groups of patients studied (table 1). The total sum of aCLs was naturally higher than the positive samples because several patients exhibited positive titres for more than one isotype.

In the group of patients with HIV-1, the isotype spectra varied: IgA was present in seven of 19 patients, IgG + IgA aCL isotypes in 10 of 19 patients, and two other patients exhibited different combinations of aCL isotypes. Detailed analysis of the results showed that 64%, 100%, and 58% of the positive samples for IgG, IgM, and IgA aCL isotypes respectively were β2-GP I independent as defined by a reduction in binding to cardiolipin (expressed as a decrease in absorbance readings for the same serum samples in the presence of ovalbumin compared with ABS) ⩾10%.

On the other hand, in patients with acute HAV, the IgM isotype alone was present in 23 of 37 positive patients, combined with IgA in nine of 37 samples, and with IgG + IgA in four of the 37 patients. Interestingly, in this group of patients 100% of IgG and IgA aCL isotypes and 97% of the IgM class were β2-GP I or cofactor independent (table 1, fig 1).

In the group with acute and chronic HBV infection, the aCL positive samples for one or more isotype showed a randomised distribution pattern. Three of four positive samples for IgG aCL, eight of 10 positive samples for IgM aCL, and seven of seven IgA aCL isotypes were found to be β2-GP I independent in the modified ELISA. No trend or particular association with the spectra of aCL isotype distribution was found in patients with acute or chronic HBV infection.

Finally, in the patients with HCV, the IgA isotype alone was present in five of the seven positive samples and combined with IgG aCL (one patient) and with IgM aCl isotype in the other patient. All samples with IgG and IgM isotypes (100%) and six of seven samples (86%) with IgA aCL were tested as β2-GP I independent.

The complete clinical records were analysed with a specific focus on aCL related involvement such as venous and/or arterial thrombosis and spontaneous abortion. The mean titre of cofactor dependent IgG aCLs was low in all groups but tended to be significantly higher in patients with HIV-1 than in the patient with HBV (28.2 (1.8) *v* 17.6; $p<0.05$). For IgM aCLs, the titres were low and there were no differences between patients with HAV and those with HBV (11.6 (0.9) *v* 12.2 (1.4); NS) and the isotype IgA, β2-GP I dependence was higher (38.2 (2.1) APL) in patients with HIV-1 than in the patient with HCV (14.8 APL, $p<0.05$).

On the other hand, the aCL values were related to the clinical status at the time the sample was drawn from patients with HBV, but there was no relation between disease activity (acute or chronic) and increased aCL levels.
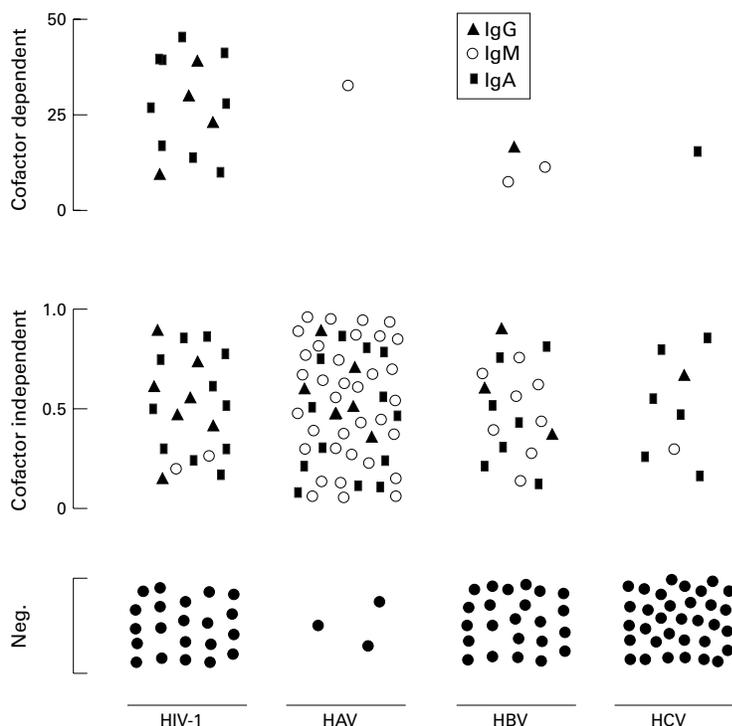

Figure 1   Distribution and cofactor dependence of the cardiolipin antibody isotypes in the population studied.

Discussion

Although raised levels of aPLs were first reported in patients with autoimmune diseases, their presence is now known to be more widespread.[1] Elevated serum levels of all three aCL isotypes, IgG, IgM, and IgA, have been reported in human infections caused by bacteria, viruses, spirochetes, and parasites; a higher prevalence of the IgG aCL isotype has been found in syphilis, HIV, tuberculosis, Q fever, Lyme disease, and cytomegalovirus infections.[15] The mechanism of the aCL response to chronic infection such as that caused by HIV-1 and HCV is unclear. Infection induced aCLs have been shown to differ from autoimmune aCLs. The former tend to be transient, of low titre, and more often of the IgM isotype; in contrast, autoimmune aCLs often have highly sustained rather than transient titres, and are more often of the IgG isotype.[15 21] Prieto *et al*[13] reported the presence of IgG aCLs in 22% of HCV infected patients. This contrasts with a previous report by Gotoh and Matsuda,[22] who found significantly higher titres of serum aCLs in patients with HIV-1 than in those with HCV. The different results of viral infection associated with aCLs may be related to the immunoassay system used to detect aCLs in the two laboratories. The former group screened for β2-GP I dependent aCLs and the latter group analysed β2-GP I independent aCLs.

In HBV infection, it has been shown that the cell receptors for lipid components of the HBV envelope include both annexin V and β2-GP I, which may act as a trigger mechanism to a possible aCL response.[23] In turn, for patients with HAV, a response to IgM aCLs has been found but no differentiation with regard to requirement of β2-GP I as cofactor has yet been observed.[21]

In this study, we have assayed cofactor dependent and independent aCLs of the IgG, IgM, and IgA isotypes in a series of 160 sera from patients with a variety of acute and chronic infections verified by the presence of specific antibody levels, in order to establish the importance, if any, of these antibodies in viral infections.

The prevalence of aCLs in patients with viral infections indicated in table 1 is in agreement with previous reports.[13 21] In our study, we found that the prevalence of IgG aCL cofactor dependence was randomly distributed among the groups of patients analysed. The association of aCLs, usually of the IgG isotype, with thrombosis, recurrent fetal losses, and thrombocytopenia has been reported.[1 4] It has been reported that IgG aCL levels higher than 40 GPL are closely associated with venous thrombosis.[24] Our results show that, in patients with HIV-1 or HAV, such levels are unusual and may explain why none of our patients developed any of the complications related to aPLs. Furthermore, in all our patients, except four with HIV-1 and one with HBV infection, these antibodies were β2-GP I independent (table 1, fig 1).

In our study, we found a significantly higher prevalence of IgM aCLs that was virtually confined to patients with HAV infection and to a minor extent to those with acute and chronic HBV infection. Elevated levels of IgM aCLs have been reported in sera of patients with other infectious diseases,[25] and their pathogenic significance is uncertain. We observed significantly higher levels of binding to cardiolipin of this isotype, and a high percentage of these antibodies were β2-GP I independent (table 1, fig 1). Cross reactivity between raised levels of IgM-type HAV and cardiolipin antibodies, an increase in non-specific binding in the ELISA, or the presence of low affinity antibodies that may have broad epitope specificity are all plausible explanations for this finding.

Raised levels of IgA type aCLs were found in all the groups of patients studied. Elevated levels of IgA aCLs have also been found in patients with mumps.[25] Unlike the other two isotypes, IgA aCL positivity was higher in patients with HBV and HCV infections than in those with HIV-1 and HAV infections. Like the other isotypes, these antibodies were principally β2-GP I independent (table 1). Antibodies of the IgA isotype are excreted from mucosa associated lymphoid tissue found in various organs such as gastrointestinal, respiratory, and urogenital tracts, and probably participate in protective mechanisms against infectious microorganisms. Our results show that most of the positive IgA aCLs were cofactor independent, suggesting that autoantibodies of the IgA isotype are part of a non-specific response to viral infections or the consequence of recent mucosal infections caused by an unidentified pathogen.

Previous studies concluded that aCLs in patients with infections such as malaria, tuberculosis, infectious mononucleosis, and syphilis are mainly cofactor independent.[13–16 21] In our study, we extend such conclusions to patients with viral infections caused by HIV-1, HAV, HBV, and HCV. Similar results were published by Biron *et al*,[26] who found that a high proportion of patients with HCV and positive aCLs was cofactor independent. Unfortunately, we were unable to perform follow up studies in the patients with HIV-1 or HCV infections because they were included in a protocol treatment with antiviral drugs. Futhermore, a significant increase in the rate of aCL positivity during α-interferon treatment has recently been reported.[27] On the other hand, we were unable to find significant differences in levels and distribution of aCL isotypes in patients with acute or chronic HBV infections. These results clearly indicate that the early IgM results do not represent a "fixed" antibody response to an ultimate "switch" to IgG aCL isotype. Furthermore, these results may be extended to other viral infections. We found that IgM and IgA class antibodies are the most prevalent isotypes in the population studied. Several different mechanisms, which are still only attractive hypotheses, may be advanced. There may be cross reactivity between viral antigens and phospholipids during the immune response to

infection, leading to the development of antibodies, or it may simply be an epiphenomenon of a pathological process that occurs as a secondary consequence of an infection.

In short, these results suggest that, in patients with viral infection and positive aCLs, assessment of the cofactor dependence appears to be a useful tool for estimating the risk of the clinical manifestations observed in the presence of these antibodies. This assay may be regarded as a useful complement to the highly specific β2-GP I antibody assay in the study of the very heterogeneous syndrome called APS.

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