

# Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor

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## Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor

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The binding of affinity-purified anticardiolipin antibodies (ACA) to liposomes that contained cardiolipin or phosphatidylserine was investigated. ACA bound to these liposomes only in the presence of plasma or serum, which indicated a requirement for a plasma component. This component—referred to as aca-cofactor—was purified; its activity to support ACA binding to liposomes that contained cardiolipin was not destroyed by heat (10 min at 90°C), but was greatly diminished on incubation with trypsin. aca-cofactor bound liposomes that contained negatively charged phospholipid but had no affinity for liposomes that contained neutral phospholipid (eg, phosphatidylcholine); this binding was independent of calcium ions. aca-cofactor was essential for ACA to bind to liposomes that contained cardiolipin or phosphatidylserine and, when coated on a microtitre plate in the absence of any phospholipid, aca-cofactor was an apparent antigen for ACA in an enzyme-linked immunosorbent assay. aca-cofactor is a single chain polypeptide with an apparent molecular weight of 50 kD (non-re-

duced), which increases to 70 kD upon reduction, and its properties closely resemble those of  $\beta_2$ -glycoprotein I (apolipoprotein H).

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### Introduction

Anticardiolipin antibodies (ACA) and lupus anticoagulants (LAC) are closely related autoantibodies, which may be found in the plasma of patients with systemic lupus erythematosus (SLE), other immunological, neoplastic, or infective disorders, and apparently normal people with no evidence of underlying disease.<sup>1</sup> Some 30% of people who

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have these antibodies suffer from arterial or venous thrombosis, thrombocytopenia, and repeated abortions.<sup>1</sup> ACA and LAC are detected because they react with negatively-charged phospholipids (eg, phosphatidic acid, cardiolipin, phosphatidylserine, and phosphatidylinositol) in immunoassays<sup>2,3</sup> and because they prolong phospholipid-dependent clotting tests *in vitro*.<sup>4</sup>

In 1959, Loeliger<sup>5</sup> observed that some plasma samples showed enhanced LAC activity when mixed with normal plasma; the concept of LAC cofactor was introduced, and prothrombin was suggested to represent this cofactor.<sup>5</sup> In 1965, LAC cofactor was localised in the gammaglobulin fraction of both normal and LAC plasma.<sup>6</sup> Rivard et al<sup>7</sup> showed that LAC cofactor was neither prothrombin nor a gammaglobulin but a different molecule with an apparent molecular weight of 200 kD<sup>7</sup>—although it has also been suggested that the cofactor is a laboratory artifact.<sup>8</sup> We have purified and characterised a protein, termed aca-cofactor, which is found in normal plasma, and is required for the expression of ACA activity.

### Materials and methods

We studied plasma from 2 men found to be seropositive for both ACA and LAC on routine investigation by enzyme-linked immunoassays (ELISAs). One patient, aged 39, had had repeated peripheral thrombosis; the other, aged 51, had had recurrent thrombosis of cerebral and renal arterioles. Neither had any other feature to suggest a diagnosis of SLE.<sup>9</sup> ACA were purified by a modification of the method described by Pengo et al.<sup>10</sup> Briefly, a mixture of cardiolipin, phosphatidylcholine, and cholesterol (molar ratio 2.5:10:4, respectively; all from Sigma, St Louis, Missouri, USA) in ethanol was dried under a stream of nitrogen. The lipids were resuspended directly in the plasma of the patients to a final cardiolipin concentration of 3 mg/ml. After incubation at 37°C for 1 h the mixture was diluted 1:4 in "tris"-buffered saline (TBS; 0.05 mol/l tris, 0.1 mol/l NaCl, pH 7.4) and centrifuged at 20 000 *g* for 15 min at 10°C. The precipitate was washed 3 times with TBS and the liposomal pellet was dissolved in a 2% (w/v) solution of *n*-octyl- $\beta$ -D-glucopyranoside (Sigma) in TBS so that the final concentration of cardiolipin was 3 mg/ml. This mixture was applied to a column of protein A-'sepharose CL 4B' (Pharmacia Fine, Uppsala, Sweden). After extensive washing with 2% octylglucoside to remove the lipids, followed by washing with TBS, the bound IgG was eluted with 1 mol/l acetic acid, and the eluates were immediately neutralised by 3 mol/l "tris". Fractions that contained IgG were pooled and dialysed against TBS before they were tested for ACA content.

The ELISA for ACA essentially followed the procedure of Loizou et al.<sup>3</sup> Briefly, microtitre plates were coated with cardiolipin (30  $\mu$ l of a 50  $\mu$ g/ml solution of cardiolipin in ethanol per well). After evaporation of the solvent, followed by washing with PBS, non-specific binding sites were blocked with 10% bovine serum albumin (Sigma) in PBS for 1 h. Samples that contained ACA were diluted in 10% bovine serum in PBS and subsequently applied to the wells. The amount of ACA bound was assessed by incubation with peroxidase-conjugated goat anti-human IgG. After reaction of peroxidase with chromogenic substrate (tetramethylbenzidine) the optical density at 450 nm (OD 450) was measured.

#### ACA BINDING TO LIPOSOMES IN PRESENCE OF PLASMA OR PURIFIED ACA-COFACTOR

Liposome composition (molar ratios)	Control (TBS)	Plasma	aca-cofactor
CL:PC:Chol (2.5:10:4)	4%	96%	94%
PS:PC:Chol (5:10:4)	2%	89%	93%
PC:Chol (15:4)	0%	0%	2%

CL = cardiolipin; PC = phosphatidylcholine; Chol = cholesterol, PS = phosphatidylserine

To determine binding affinity to liposomes, 25  $\mu$ l ACA were mixed with 50  $\mu$ l cardiolipin:phosphatidylcholine:cholesterol (molar ratio 2.5:10:4) liposomes in the presence of 125  $\mu$ l plasma, TBS, or other test materials. Final concentrations were 125  $\mu$ g/ml and 750  $\mu$ g/ml for ACA and cardiolipin, respectively; aca-cofactor was used at a final concentration of 300  $\mu$ g/ml. After 30 min incubation at 37°C mixtures were centrifuged at 100 000 *g* for 15 min and supernatants were evaluated for residual ACA activity by the standard ELISA described above. Data are calculated according to the formula:

$$\frac{ACA_{total} - ACA_{supernatant}}{ACA_{total}} \times 100\%$$

In some experiments liposomes were used in which cardiolipin was replaced by phosphatidylserine at a molar ratio for phosphatidylserine:phosphatidylcholine:cholesterol of 5:10:4 to correct for the difference in net charge between cardiolipin and phosphatidylserine or by phosphatidylcholine at a molar ratio for phosphatidylcholine:cholesterol of 15:4.

aca-cofactor was purified from plasma by ammonium sulphate precipitation (50%–90% saturation) followed by ion-exchange chromatography on 'QAE-Sephadex' in 50 mmol/l "tris" buffer at pH 7.4. The column was eluted by a salt-gradient and aca-cofactor activity was recovered at 100 mmol/l NaCl. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done on a 7.5% polyacrylamide gel with a 4% polyacrylamide stacking gel; after electrophoresis the gel was stained with coomassie brilliant blue. Protein content of samples was assayed according to Sedmak and Grossberg.<sup>11</sup>

### Results and discussion

Affinity-purified ACA from the plasma of both patients reacted strongly with cardiolipin in a lipid-based ELISA. Binding was little changed by substitution of cardiolipin by phosphatidylserine, whereas binding of affinity-purified ACA was abolished with phosphatidylcholine-coated wells (data not shown). In direct binding experiments, in which ACA were incubated with liposomes composed of a mixture of cardiolipin, phosphatidylcholine, and cholesterol in TBS, the 100 000 *g* supernatant of this incubation still contained 100% ACA activity—which indicated no ACA binding to liposomes. Replacement of TBS by normal plasma reduced ACA activity in the supernatant to less than 5% (table),

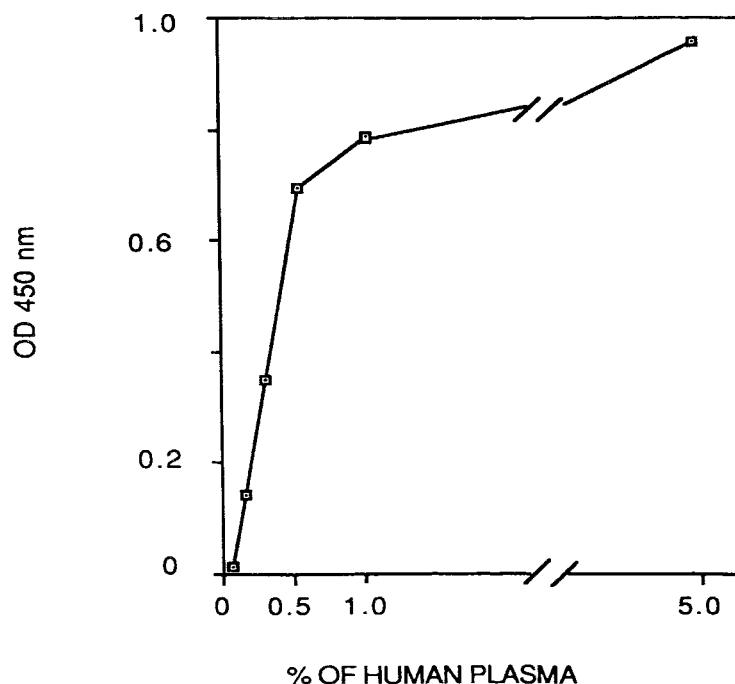
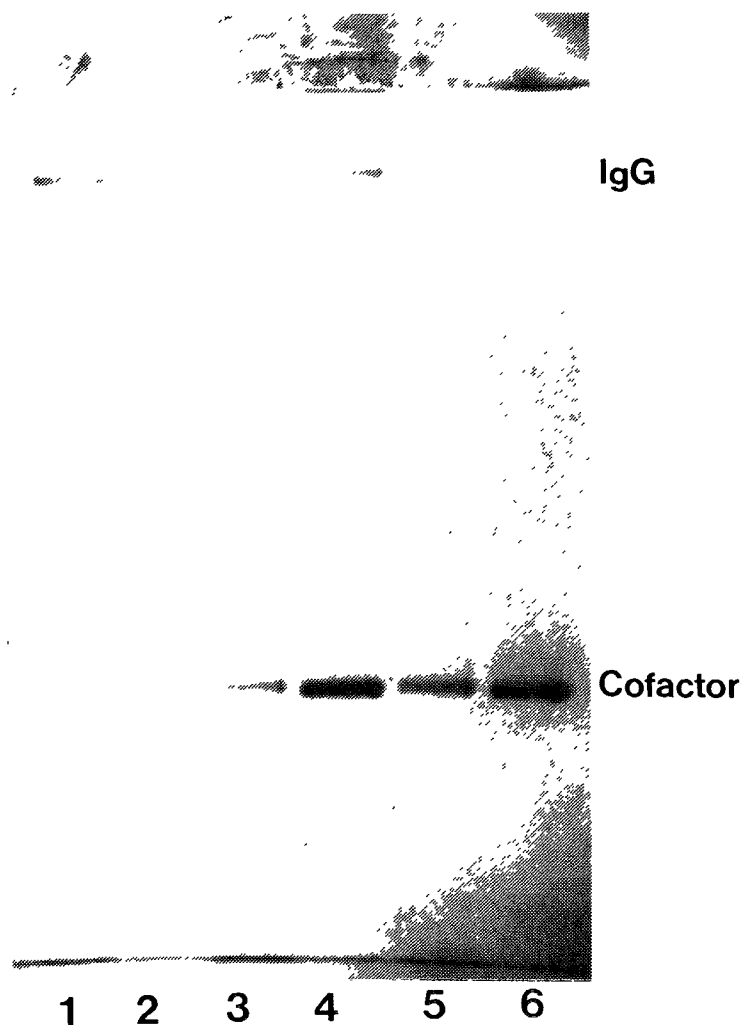


Fig 1—ACA binding to immobilised cardiolipin at different concentrations of normal plasma.



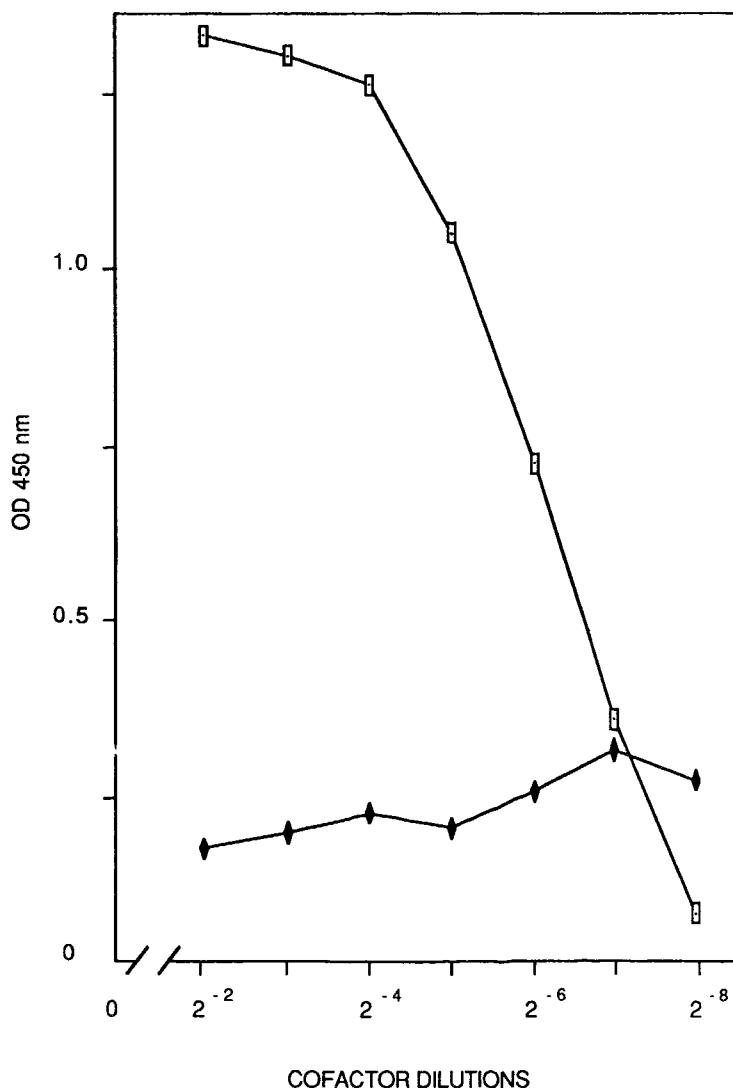
**Fig 2—Binding of ACA and/or aca-cofactor to liposomes that contain cardiolipin.**

Lanes 1 & 2: ACA with liposomes; lanes 3 & 4: ACA and aca-cofactor with liposomes; lanes 5 & 6: aca-cofactor with liposomes.

Lanes 1, 3, and 5 represent proteins in the supernatant, lanes 2, 4, and 6 represent proteins in the liposomal pellet.

and a similar result was obtained when phosphatidylserine was substituted for cardiolipin. No ACA binding was found to liposomes composed of phosphatidylcholine and cholesterol. These findings indicate that a plasma component is involved in the binding of ACA to liposomes that contain negatively charged phospholipids, as suggested by McNeil et al,<sup>12</sup> who also observed that binding of purified antiphospholipid antibodies to immobilised phospholipid required the presence of plasma.

In the standard ELISA for ACA, different dilutions of the antibody-containing samples are routinely made in 10% bovine serum in TBS; the same medium is also used during the binding-step of ACA to the cardiolipin-coated wells of the microtitre plates. We found that bovine serum could be replaced by bovine plasma, human serum, or human plasma, but not by purified bovine or human serum albumin. To further investigate the role of plasma in ACA binding to cardiolipin, the standard ELISA was modified. Instead of dilution in 10% bovine serum, constant amounts of ACA were mixed with dilutions of normal pooled plasma and applied to the well. Microtitre plates were further processed as described for the standard ELISA. Fig 1 shows the OD 450 as a function of the plasma concentration: a linear correlation between OD and plasma concentrations is observed from 0 to 0.5%, where a plateau is reached; similar findings were obtained with human serum and bovine plasma or serum. Thus a plasma component



**Fig 3—IgG binding to microtitre plates coated with aca-cofactor in absence of phospholipid.**

□ = affinity purified anticardiolipin IgG (17 µg/ml).

◆ = normal (non-specific) IgG (133 µg/ml).

Concentration of undiluted aca-cofactor was 300 µg/ml.

is necessary for ACA binding to immobilised cardiolipin. This plasma component, termed aca-cofactor, can be detected and measured by this modified ELISA by comparison with a calibration curve as shown in fig 1.

The final step in the purification of aca-cofactor involves heat treatment (10 min at 90°C) followed by centrifugation to remove denatured protein material. No loss of aca-cofactor activity occurred in this step, but when the preparation obtained was incubated with trypsin, aca-cofactor became inactive. SDS-PAGE showed aca-cofactor to be a single-chain polypeptide with an apparent molecular weight of 50 kD (non-reduced) which increased to 70 kD upon reduction. The specific activity of the cofactor preparation was 350 times that of crude plasma; if 100% purity of this preparation was assumed the plasma concentration of aca-cofactor would be approximately 0.2 mg/ml.

Purified aca-cofactor was used to study the interaction of affinity-purified ACA with liposomes (see table). aca-cofactor can be substituted for plasma without loss of binding of ACA to liposomes that contained negatively-charged phospholipid. Both supernatant and pellet of the binding experiments with purified aca-cofactor and/or ACA were analysed by SDS-PAGE as shown in fig 2 for samples

obtained after incubations with cardiolipin-containing liposomes; similar results are obtained with liposomes that contained phosphatidylserine. No binding of either of the proteins was observed with liposomes without negatively-charged lipids. Without cofactor, no IgG was detectable in the 100 000 g pellet of the incubations (lanes 1, 2); with cofactor, approximately 60–70% of the IgG co-sediments with the liposomes (lanes 3, 4). Incomplete binding of IgG was not due to a limiting amount of aca-cofactor and the unbound fraction of IgG in the 100 000 g supernatant no longer contained any ACA activity, so the IgG fraction isolated from the plasma presumably contains antibodies other than ACA with non-specific binding to the liposomes used in the affinity purification procedure, or antibodies directed against other lipid-binding proteins in plasma.

Fig 2 shows that aca-cofactor also binds to liposomes that contain cardiolipin in the absence of IgG (lanes 5, 6). Binding of aca-cofactor is not dependent on or inhibited by the presence of calcium ions, so it is unlikely that binding is mediated by  $\gamma$ -carboxyglutamic acid residues on the cofactor. We investigated the direct interaction between aca-cofactor and affinity-purified ACA in an ELISA in which the microtitre plates were coated with different amounts of cofactor. After coating, a fixed amount of ACA or normal IgG was applied to the wells in the absence of phospholipid. ACA binding was proportional to the amount of aca-cofactor in the wells (fig 3) and no binding was found with normal human IgG. Thus anticardiolipin antibodies of at least these two patients are not directed against cardiolipin but against a protein with a high affinity for negatively-charged phospholipid surfaces.

It is remarkable that the cofactor is not co-purified during the affinity purification of ACA via cardiolipin-containing liposomes. Two possible explanations might account for this: ACA only recognises aca-cofactor when bound to lipid or absorbed on a microtitre plate; or aca-cofactor undergoes structural modification upon addition of octylglucoside during ACA purification. Although aca-cofactor is present in normal human (and bovine) plasma in concentrations of up to 0.2 mg/ml, its identity is still obscure. The heat stability and behaviour on SDS-PAGE (increase in apparent molecular weight upon reduction) suggest the presence of several disulphide bridges. Preliminary experiments with purified coagulation factors indicate that binding of aca-cofactor to negatively-charged phospholipid surfaces interferes with binding of the prothrombin activating complex factor Xa-factor Va, leading to a diminished rate of thrombin formation. Thus ACA may be directed against a naturally circulating anticoagulant with high affinity towards negatively-charged phospholipid surfaces.

The high purity of the aca-cofactor allows analysis of aminoacid composition, which is under investigation. However the properties so far identified (molecular weight 50 kD [non-reduced], 70 kD [reduced with 5%  $\beta$ -mercaptoethanol]; estimated plasma concentration 200  $\mu$ g/ml; heat stability; calcium-independent binding to anionic phospholipids; support of ACA binding to anionic phospholipids; and inhibition of phospholipid-dependent coagulation reactions) are very similar to those of  $\beta_2$ -glycoprotein I (apolipoprotein H), the anticoagulant properties of which we have previously described.<sup>13</sup>

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## From The Lancet

### Medical examinations

The qualified practitioners of the present day are all members of medical corporations. Are they to have anything to do with the management of those corporations? Are twenty-one, or any other number, of self-elected, irresponsible gentlemen in London, Dublin, or Edinburgh, to appropriate the public money, expend it as they please, dispose of the property, power, patronage, and interests of twenty or thirty thousand enlightened men scattered over the United Kingdom? Have the physicians, surgeons, general practitioners of the United Kingdom no fair claim to the franchise? Are they to be mocked for ever by the Councils of their own Institutions, and to be trampled under foot by the Poor-Law Commissioners, or by any other class of men, from the want of the energy and protection which a united, well-organised, representative corporation would give? Does any one mean to assert, that the present generation of practitioners is unworthy of a free constitution; that we do not understand our own interests and rights; or are so besotted as to be incapable of selecting representatives who would promote science, study the public good, and uphold the honour of the profession? Are we to sow all for the next generation, and to reap nothing? Are our successors to surpass us so immeasurably, because they undergo a uniform examination? Our faith in the qualifications of the present race of practitioners is great. They have become what they are, intelligent, efficient, high-minded, in spite of the worst examinations, and the worst means of education that were ever devised by the ingenuity or wickedness of man.

(8 August 1840)