

Immune mediated mechanism for thrombosis: antiphospholipid antibody binding to platelet membranes*

MUNTHER A KHAMASHTA,¹ E NIGEL HARRIS,²
AZZUDIN E GHARAVI,³ GENEVIEVE DERUE,¹ ANTONIO GIL,⁴
JUAN JOSÉ VÁZQUEZ,⁴ AND GRAHAM R V HUGHES¹

From the ¹Lupus Research Laboratory, The Rayne Institute, St Thomas's Hospital, London, UK; the ²Rheumatology Division, Department of Medicine, University of Louisville, Kentucky, USA; the ³Hospital for Special Surgery, Cornell University, New York, USA; and ⁴La Paz Hospital, Madrid, Spain

SUMMARY Because thrombocytopenia occurs frequently in patients with anticardiolipin (aCL) antibodies and thrombosis, some investigators have proposed that aCL antibodies may play a direct part in thrombosis by binding and activating platelets. To test this proposal experiments were performed to determine whether aCL antibodies can bind platelets. Preincubation of aCL positive sera with freeze-thawed platelets caused significant inhibition of aCL activity in four serum samples tested. Antibodies with cardiolipin binding activity were subsequently eluted from these platelets. Total phospholipids extracted from platelets inhibited aCL activity, and the specific phospholipids bound were shown to be phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. It is concluded that aCL antibodies can bind phospholipids in platelet membranes but perturbation of the membrane must first occur.

Key words: thrombocytopenia, lupus anticoagulant, anticardiolipin antibody.

Recent studies suggest that antiphospholipid (aPL) antibodies are associated with repeated episodes of venous or arterial thrombosis, or both, recurrent fetal loss, and thrombocytopenia.¹⁻³ Although the clinical association is strong, little is known of the mechanisms whereby the presence of aPL antibodies results in thrombosis.

As the phospholipid membrane of platelets has a role in the thrombotic process⁴ it has been proposed that aPL antibodies may bind platelet membranes, so causing their activation, aggregation, and thrombosis.¹⁻⁵ The occurrence of thrombocytopenia in some affected patients has also led to suggestions that aPL antibodies may directly mediate platelet destruction.⁵

The aims of this study were to determine whether

aPL antibodies can bind platelets *in vitro* and to identify the components of the platelet membranes bound by these antibodies.

Patients and methods

PATIENTS

Serum samples from four patients with high aCL antibody levels were selected for study. Two of these four patients fulfilled the revised American Rheumatism Association criteria for systemic lupus erythematosus,⁶ and two were classified as having the 'antiphospholipid syndrome'.^{7,8} Three of the four patients had a history of thrombosis, two had thrombocytopenia (platelet count $<100 \times 10^9/l$), and three had a positive Coombs' test. All four patients had a positive lupus anticoagulant test. One patient had IgG aCL antibodies, one patient IgM, and the remaining two patients had both IgG and IgM aCL antibodies.

PREPARATION OF PLATELETS AND ERYTHROCYTES

Recently expired units of platelet concentrates

Accepted for publication 19 May 1988.

Correspondence to Dr Munther A Khamashta, Lupus Research Laboratory, The Rayne Institute, St Thomas's Hospital, London SE1 7EH, UK.

*These data were presented in part at the American Rheumatism Association meeting in Washington DC, USA, June 1987.

collected in citrate phosphate dextrose (CPD) adenine 1 and units of whole blood collected in CPD anticoagulant were used for study.

Twenty millilitre aliquots of platelet concentrates were centrifuged at 1500 g for 20 minutes at room temperature. The supernatant was discarded and the pellets were washed three times in phosphate buffered saline (PBS) pH 7.4 by centrifugation at 1500 g for 10 minutes each time. The washing procedure was monitored by light microscopy. The final platelet preparations did not contain more than one erythrocyte or leucocyte per 50 high power fields.

Twenty millilitre aliquots from a whole blood sample were centrifuged at 300 g for 20 minutes, following which the supernatant plasma, leucocyte, and platelet layers were removed and discarded. The red cells were then washed three times by suspension in PBS.

The final concentrations of platelets and erythrocytes were estimated by Coulter Counter model S-plus IV and adjusted to 2×10^9 /ml. The washed platelets and erythrocytes were stored in small aliquots (1 ml) at -20°C until further use. Partially disrupted platelets and erythrocytes were obtained by freeze-thawing three times. Sonicated platelets were prepared by sonicating twice with a Soniprep 150 (MSE) sonicator, on ice, for three minutes each time with one minute intervals.

ISOLATION OF aCL ANTIBODIES USING PLATELETS

Ten millilitre aliquots of serum with high aCL binding activity were mixed with 5 ml of freeze-thawed platelets prepared as described previously. The mixture was incubated at 37°C for one hour and overnight at room temperature with constant mixing. The mixture was then centrifuged at 25 000 g for one hour and the supernatant removed. The pellet was resuspended in 10 ml of PBS and thoroughly washed. The mixture was again centrifuged at 25 000 g for 30 minutes and the supernatant removed. This washing procedure was repeated three times. After the third wash the pellet was resuspended in 2 ml of 1.5 M potassium iodide and incubated for one hour with constant mixing to elute antibodies bound to platelet membranes. The preparation was centrifuged at 25 000 g for 15 minutes and the pellet discarded. Five millilitres of chloroform was added to the supernatant, vortex mixed, allowed to stand for 10 minutes at room temperature, then centrifuged at 1500 g for 20 minutes. On this occasion the upper aqueous layer was carefully removed and dialysed overnight against PBS at 4°C . The final preparation was stored at -20°C until ready for use. This preparation was characterised by double immunodiffusion

(Ouchterlony technique) using antisera to human IgG, IgM, IgA, and human serum albumin (Sigma Chemical Company, UK) and by immunoelectrophoresis using anti-whole human serum (Sigma Chemical Company, UK). The isolated preparation was also tested for aCL binding activity by an enzyme linked immunosorbent assay (ELISA).

EXTRACTION OF PLATELET LIPIDS

Lipids were extracted from platelets according to the method outlined by Bligh and Dyer.⁹

THIN LAYER CHROMATOGRAPHY

Platelet phospholipids were separated using high performance thin layer chromatography plates (HPTLC precoated silica gel F₂₅₄ 10×10 cm purchased from Merck (Darmstadt, W Germany). The plate was developed in a solvent system containing chloroform:methanol:13.5 N ammonia water (70:30:4:1,v/v).¹⁰ Platelet phospholipids were identified by comparison with pure reference compounds (Sigma Chemical Company, UK). The platelet phospholipids separated on HPTLC plates were re-extracted with chloroform:methanol. An aliquot of each of the re-extracted phospholipid was rerun on HPTLC plates with appropriate reference compounds to confirm the purity of the extract and its identity. The remaining portions of the re-extracted phospholipids were used to prepare liposomes for inhibition studies.^{11 12}

ANTICARDIOLIPIN ELISA

Anticardiolipin activity was determined by the ELISA technique described by Gharavi *et al.*¹³

ANTITETANUS ELISA

Polyvinyl chloride plates (Flow Laboratories, UK) were coated with 50 μl /well of highly purified tetanus toxoid diluted in PBS at a concentration of 1/50. The plates were then placed in a chamber and incubated for one hour at 37°C and overnight at 4°C . Antitetanus activity was determined by a protocol similar to that used for the aCL antibody.¹³

INHIBITION STUDIES

Inhibition studies of antibody to cardiolipin coated ELISA plates were performed by preincubating serum at 50% maximum binding activity with platelets, erythrocytes, or phospholipid liposomes at 37°C for one hour and overnight at room temperature, with constant mixing. Control samples were prepared by preincubating each serum at 50% activity with PBS under conditions described above. The percentage inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{\text{OD control sample} - \text{OD inhibited sample}}{\text{OD control sample}} \times 100$$

where OD is the optical density.

Results

INHIBITION OF CARDIOLIPIN BINDING ACTIVITY BY PLATELETS AND ERYTHROCYTES

Preincubation of the four aCL positive sera with intact platelets caused little inhibition of cardiolipin binding activity. On the other hand, preincubation of these sera with freeze-thawed or sonicated platelets caused significant inhibition of cardiolipin binding activity. It was found too, that preincubation of the four aCL positive sera with freeze-thawed erythrocytes also caused marked inhibition of cardiolipin binding activity, but there was no inhibition by intact erythrocytes (Fig. 1). Freeze-thawed platelets caused no inhibition of binding activity of the positive antitetanus serum used as control (Fig. 2).

ANTIBODY ELUTED FROM PLATELET MEMBRANES

A second series of experiments (see 'Patients and methods') was performed to demonstrate aCL binding to platelets. Essentially, each of the four

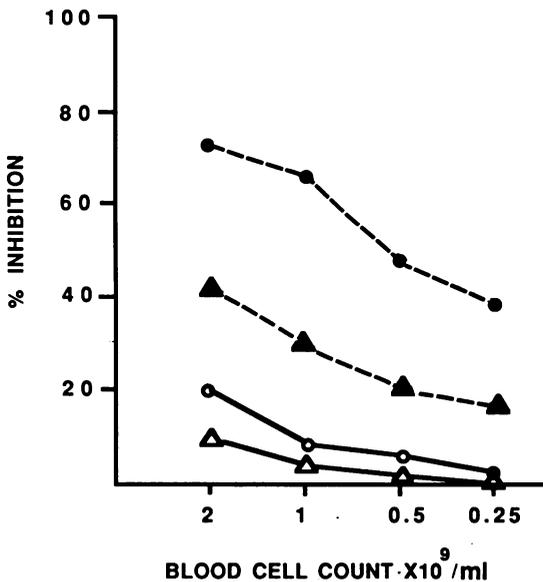


Fig. 1 Inhibition of IgM anticardiolipin activity in one serum by varying concentrations of freeze thawed (●) and intact (○) platelets and freeze-thawed (▲) and intact (△) erythrocytes.

serum samples studied was incubated with freeze-thawed platelets and antibodies bound to platelets eluted by potassium iodide.

All four eluted preparations contained immunoglobulins. In every instance the predominant isotype of the eluted preparation corresponded to the aCL antibody isotype of the serum from which the preparation was obtained. Thus platelet eluates from the serum of the patient with IgG aCL activity alone contained immunoglobulins only of IgG isotype (Fig. 3). The eluate obtained from the serum of the patients with IgM aCL activity had only IgM,

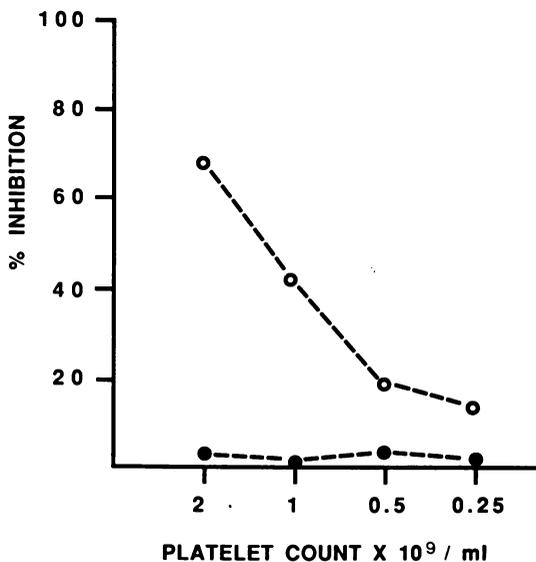


Fig. 2 Inhibition of IgG anticardiolipin activity (○) and of IgG antitetanus activity (●) by varying concentrations of freeze-thawed platelets.

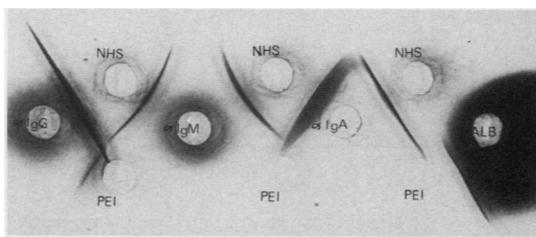


Fig. 3 Ouchterlony pattern of immunoglobulins eluted from platelets (PEI) after preincubation with patient serum. The patient serum had only IgG aCL activity and after preincubation with platelets only immunoglobulins of the IgG isotype were eluted. Normal human serum (NHS) was used as control. ALB=albumin.

and the eluates from the two serum samples with IgG and IgM aCL activities had both IgG and IgM immunoglobulins. Eluted preparations from platelets also contained significant quantities of albumin. Each of the four eluted preparations had cardiolipin binding activity and the binding activity of each preparation was confined to the same isotype as that of the serum from which the preparation was obtained.

IDENTIFICATION OF COMPONENTS OF PLATELET MEMBRANE BOUND BY aCL ANTIBODIES

Two aCL positive sera were preincubated with total phospholipids extracted from platelets and inhibition of cardiolipin binding activity determined. In both instances total platelet phospholipids inhibited cardiolipin binding activity to an extent similar to that observed using freeze thawed platelets.

The total platelet phospholipid extract was

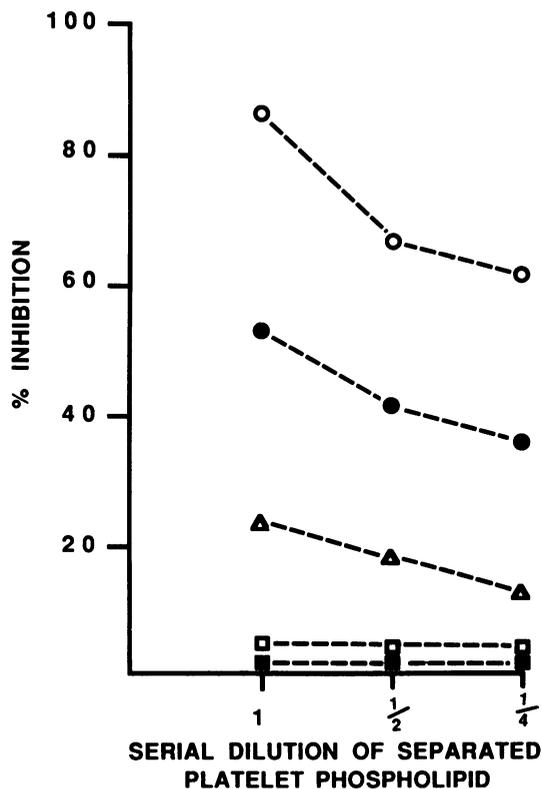


Fig. 4 Inhibition of IgM anticardiolipin activity in one serum by individual phospholipids extracted from platelets. ○=phosphatidylethanolamine; ●=phosphatidylserine; △=phosphatidylinositol; □=sphingomyelin; ■=phosphatidylcholine.

separated into its individual components by HPTLC and inhibition of cardiolipin binding activity by each of the separated components examined. The phospholipid components identified were phosphatidylethanolamine (PE), sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI). PE, PS, and PI all inhibited cardiolipin binding activity, but SPH and PC caused little or no inhibition (Fig. 4). It was noteworthy that although the extracted platelet phospholipid identified as PE caused most marked inhibition of cardiolipin binding activity, a similar preparation obtained commercially and with the same R_f value on HPTLC plates did not inhibit cardiolipin binding activity.

Discussion

Recent studies have shown that patients with high levels of aPL antibodies have an increased risk for venous or arterial thrombosis or both.^{14, 15} Because some of these patients also have episodes of thrombocytopenia,⁵ and because platelets have an important role in the clotting process,¹⁶ we have proposed that aPL antibodies may bind platelet membrane phospholipids so causing their activation, aggregation, and thrombosis.^{1, 5} On the other hand, it may be argued that platelet phospholipids are not accessible to aPL antibodies, either because of steric hindrance by platelet membrane glycoproteins,¹⁷ or because anionic phospholipids (PS, PI) bound by aCL antibodies^{11, 12} are located on the inner leaflet of the lipid bimolecular membrane away from the extracellular milieu.¹⁶

The present study was designed to determine whether aCL antibodies bind platelet membrane and to identify the membrane components bound by these antibodies. We found that preincubation of aCL positive sera with 'intact' platelets caused little inhibition of cardiolipin binding activity, but preincubation with freeze-thawed or sonicated platelets resulted in greater than 50% inhibition of cardiolipin binding activity. Similar results were obtained with intact and freeze-thawed red blood cells. The similarity between results obtained with platelets and with red cells may be explained by the fact that the composition and probably the distribution of phospholipids in platelet and red blood cell membranes are similar.¹⁰ Hence freeze-thawing probably results in exposure in both types of cells of phospholipids that can be bound by aCL antibodies. As far as is known, however, only platelet membranes undergo molecular rearrangements during the clotting process,⁴ therefore the present study was confined to platelets.

The observed inhibition of aCL activity by freeze

thawed platelets might have occurred because these immunoglobulins might have become attached to platelet membranes non-specifically or by F_c receptors. Two experiments excluded this possibility. Firstly, preincubation of an antitetanus positive serum with freeze-thawed platelets caused little or no inhibition of antitetanus activity. Secondly, when serum samples with high cardiolipin binding activity were preincubated with platelets, immunoglobulins with aCL activity were eluted from these platelets. In addition, the eluted antibodies were restricted to the same isotype as that of the aCL positive sera. If binding were non-specific then one might have expected that both IgG and IgM immunoglobulins would have been eluted from platelet membranes in all serum samples tested. On the other hand, if binding occurred via F_c receptors then only IgG should have been eluted.

This study showed that it is the lipid component of the platelet membrane that is bound by aCL antibodies as demonstrated by the fact that aCL activity was inhibited by total phospholipids extracted from platelets to an extent similar to that obtained with freeze-thawed platelets. The zwitterionic phospholipids PC and SPH were not bound by aCL antibodies. PC and SPH are the main phospholipids located on the outer leaflet of the bimolecular membrane and this may explain why aCL antibodies may not bind intact platelets. The anionic phospholipids PS and PI as well as the zwitterionic PE were bound by aCL antibodies. Of interest is the fact that all three platelet phospholipids bound by aCL antibodies are located primarily on the inner leaflet of the bimolecular lipid membrane^{4 16} and presumably become exposed and accessible to aCL antibodies by freeze thawing. Anticardiolipin binding to platelet PE was unexpected. We have found previously, and confirmed in the present series of experiments, that preincubation of aCL positive sera with commercially obtained PE (bovine brain) does not result in inhibition of cardiolipin binding activity.¹¹ It is possible that there may be subtle differences in the conformation of individual phospholipid molecules depending on their source and on the phospholipids with which they are mixed.^{18 19} Recently, Rauch and colleagues using a monoclonal aPL antibody with lupus anticoagulant activity found that this antibody bound PE in one configuration but not another.²⁰

It is well known that the lupus anticoagulant test can be 'bypassed' by addition of freeze-thawed or sonicated platelets, but the mechanism of this bypass phenomenon is not established.^{21 22} One possibility is that the sonicated platelets provide an additional phospholipid template on which the prothrombin activator complex can form and this

would enable bypass of the lupus anticoagulant activity, whatever the mechanism that accounts for this activity. An alternative explanation is that platelet membrane phospholipids exposed on sonication absorb aPL antibodies, so preventing these antibodies from blocking the prothrombin activator complex-phospholipid interaction. The findings of the present series of experiments show that the neutralisation reaction probably occurs because of the latter mechanism whereby aPL antibodies are absorbed out of plasma by platelet phospholipids.

The absence of binding of aPL antibodies to intact platelets in this study confirms a number of abstracted studies which show that lupus anticoagulant or aCL positive plasma have no effect on platelet function.²³ On the other hand, demonstration of binding to sonicated platelets suggests that if aPL antibodies cause thrombosis through a platelet mediated mechanism one must propose that partial activation of the platelet and exposure of target phospholipids must take place before antibody binding. Hence, owing to the observations reported here, we are currently performing experiments in our laboratories using partially activated rather than inactivated platelets.

We thank Dr K L I Rogers and Ms P Tannenbaum of the South London Transfusion Centre for the donation of blood cells and Mr Thomas Patterson for technical assistance. This work was supported by grants from the British SLE Aid Group and by the Arthritis and Rheumatism Council.

References

- Harris E N, Gharavi A E, Hughes G R V. Anti-phospholipid antibodies. *Clin Rheum Dis* 1985; **11**: 591-609.
- Vermynen J, Blockmans D, Spitz B, Deckmyn H. Thrombosis and immune disorders. *Clin Haematol* 1986; **15**: 393-412.
- Hughes G R V. Thrombosis, abortion, cerebral disease, and the lupus anticoagulant. *Br Med J* 1983; **287**: 1088-9.
- Schick P K, Kurica K B, Chacko G K. Location of phosphatidyl ethanolamine and phosphatidyl serine in the human platelet plasma membrane. *J Clin Invest* 1976; **57**: 1221-6.
- Harris E N, Asherson R A, Gharavi A E, Morgan S H, Derue G, Hughes G R V. Thrombocytopenia in SLE and related autoimmune disorders: association with anticardiolipin antibodies. *Br J Haematol* 1985; **59**: 227-30.
- Tan E M, Cohen A S, Fries J F, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271-7.
- Hughes G R V, Harris E N, Gharavi A E. The anticardiolipin syndrome. *J Rheumatol* 1986; **13**: 486-9.
- Harris E N. Syndrome of the black swan. *Br J Rheumatol* 1987; **26**: 324-6.
- Bligh E G, Dyer W J. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 1959; **37**: 911-7.
- Cohen P, Derksen A. Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets. *Br J Haematol* 1969; **17**: 359-71.
- Harris E N, Gharavi A E, Loizou S, et al. Crossreactivity of antiphospholipid antibodies. *J Clin Lab Immunol* 1985; **16**: 1-6.

- 12 Harris E N, Gharavi A E, Tincani A, *et al.* Affinity purified anti-cardiolipin and anti-DNA antibodies. *J Clin Lab Immunol* 1985; **17**: 155–62.
- 13 Gharavi A E, Harris EN, Asherson R A, Hughes G R V. Anticardiolipin antibodies: isotype distribution and phospholipid specificity. *Ann Rheum Dis* 1987; **46**: 1–6.
- 14 Harris E N, Chan J K H, Asherson R A, Aber V R, Gharavi A E, Hughes G R V. Thrombosis, recurrent fetal loss, and thrombocytopenia. Predictive value of the anticardiolipin antibody test. *Arch Intern Med* 1986; **146**: 2153–6.
- 15 Lockshin M D, Druzin M L, Goei S, *et al.* Antibody to cardiolipin as a predictor of fetal distress or death in pregnant patients with systemic lupus erythematosus. *N Engl J Med* 1985; **313**: 152–6.
- 16 Marcus A J, Ullman H L, Safier L B. Lipid composition of subcellular particles of human platelets. *J Lipid Res* 1969; **10**: 108–14.
- 17 Thiagarajan P, Shapiro S S, DeMarco L. Monoclonal immunoglobulin M coagulation inhibitor with phospholipid specificity—mechanism of a lupus anticoagulant. *J Clin Invest* 1980; **66**: 397–405.
- 18 Alving C R. Antibodies to liposomes, phospholipids and phosphate esters. *Chem Phys Lipids* 1986; **40**: 303–14.
- 19 Lockshin M D. Anticardiolipin antibody. *Arthritis Rheum* 1987; **30**: 471–2.
- 20 Rauch J, Tannebaum M, Tannebaum H, *et al.* Human hybridoma lupus anticoagulant distinguish between lamellar and hexagonal phase lipid system. *J Biol Chem* 1986; **261**: 9672–7.
- 21 Howard M A, Firkin B G. Investigations of the lupus-like inhibitor by-passing activity of platelets. *Thromb Haemost* 1983; **50**: 775–9.
- 22 Triplett D A, Brandt J T, Kaczor D, Schaeffer J. Laboratory diagnosis of lupus inhibitors: a comparison of the tissue thromboplastin inhibition procedure with a new platelet neutralization procedure. *Am J Clin Pathol* 1983; **79**: 678–82.
- 23 Derksen R H W M, Hasselaar P, Blokzijl L, de Groot Ph G. Thrombosis associated with antiphospholipid antibodies cannot be explained by effects on endothelial and platelet prostanoid synthesis. Proceeding of the IIIrd International symposium on antiphospholipid antibodies. Kingston, Jamaica, 1988. *Clinical and Experimental Rheumatology Supplement* (in press).