

blood

1993 81: 1255-1262

Beta 2-glycoprotein I is a requirement for anticardiolipin antibodies binding to activated platelets: differences with lupus anticoagulants

W Shi, BH Chong and CN Chesterman

Information about reproducing this article in parts or in its entirety may be found online at:
http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
<http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#reprints>

Information about subscriptions and ASH membership may be found online at:
<http://bloodjournal.hematologylibrary.org/site/subscriptions/index.xhtml>



β_2 -Glycoprotein I Is a Requirement for Anticardiolipin Antibodies Binding To Activated Platelets: Differences With Lupus Anticoagulants

By Wei Shi, Beng H. Chong, and Colin N. Chesterman

Antiphospholipid (aPL) antibodies are of major interest not only because the lupus anticoagulant (LA) causes an inhibition of *in vitro* blood coagulation, but also because the presence of aPL antibodies confers a risk of thrombosis. The inhibition of *in vitro* phospholipid-dependent coagulation (LA) is thought to be caused by the binding of LA to procoagulant phospholipid surfaces, thus impeding the clotting process. Another class of aPL antibodies are those originally described to be directed against negatively charged phospholipids, in particular cardiolipin (ACA). ACA are usually directed against a complex antigen consisting of negatively charged phospholipid and a plasma protein, β_2 -glycoprotein I (β_2 -GPI). Further, there is antibody heterogeneity even within individual patients so that ACA and LA are separable using physicochemical techniques such as ion exchange chromatography and chromatofocusing. Using such techniques we have enriched Ig fractions for LA and ACA from two patient plasmas. The majority of Ig

with LA activity had a pl of 7.2 to 7.3 whereas ACA had a pl of 5.0 to 5.2. Using these enriched fractions labeled with [125 I]-iodine we have shown that LA binds to platelets in a specific and saturable manner. Binding is dependent on thrombin activation. [125 I]-ACA behaves differently. Like LA, binding is specific and dependent on thrombin activation but in this case requires the presence of β_2 -GPI. ACA, in the presence of β_2 -GPI, competes for binding with LA suggesting the same or contiguous site. There is no cross-reactivity of these antibodies with GPIIb/IIIa and the most likely binding site is phospholipid. In neither case does LA nor ACA have an effect on thrombin-induced release of serotonin or β -thromboglobulin nor do they affect platelet aggregation induced by a number of agonists. This antibody binding may play an etiological role in thrombocytopenia associated with aPL, but does not explain thrombosis on the basis of hyperaggregability or increased platelet release. © 1993 by The American Society of Hematology.

IN PATIENTS with or without systemic erythematosus (SLE), the presence of antiphospholipid (aPL) antibodies, anticardiolipin antibody (ACA), and lupus anticoagulant (LA) is associated with thromboembolic complications (both venous and arterial), recurrent spontaneous abortion, and also thrombocytopenia.¹ The mechanisms responsible for thrombosis and thrombocytopenia in these patients remain unclear. With respect to aPL and platelets a number of observations have been made. For example, Firkin et al² showed that activated platelets resulted in bypassing of the LA phenomenon. They later showed this to be caused by a membrane component.³ aPL antibodies have been reported to be associated with antiplatelet antibodies in patients with SLE.^{4,5} Further, a strong correlation has been shown between levels of ACA and thrombocytopenia in a group of patients with SLE.⁶ Recently Hasselaar et al⁷ have reported cross-reactivity between antibodies against phospholipids, DNA, platelets, and endothelial cells. In another study washed, freeze/thawed platelets have been used to isolate aPL antibodies from patient sera.⁸ The binding of aPL antibody to platelets could, on the one hand, result in their premature removal from the circulation by the reticulo-endothelial system or, on the other hand, lead to functional changes, particularly hyperreactivity. Platelet hyperreactivity could in turn contribute to the reported thrombotic diathesis, although there is a body of evidence to implicate vascular endothelial cell dysfunction.¹ The two are not mutually exclusive.

A critical limitation of the majority of studies that have addressed, by *in vitro* experimentation, the role of aPL antibodies in the predisposition to thrombosis, is that whole serum or whole Ig fractions have been used.¹ Conflicting conclusions are likely to be caused by the coexistence of a number of autoantibodies in the sera so tested, particularly in patients with SLE and related disorders, a significant proportion of whom have ACA, LA, antiendothelial cell antibodies, antiplatelet antibodies, anti-DNA antibodies, and immune complexes. This confusion has been compounded by the unwarranted assumption that LA and ACA are one and the same and that "antiphospholipid antibodies" is a term that may be used without further qualification.

We^{9,10} and others¹¹ have shown that LA and ACA are discrete subgroups of aPL antibodies. They often coexist but are separable from plasma, one activity in the absence of the other, using cardiolipin affinity or physicochemical methods including ion exchange and size exclusion gel chromatography. These findings explain the previously noted discordance between the presence and plasma levels of LA and ACA in individual patients. After fractionation of ACA-containing plasma by ion exchange chromatography or after affinity purification on cardiolipin-polyacrylamide, purified autoimmune ACA fails to bind to immobilized anionic phospholipid antigens.^{12,13} Binding is only observed when normal human plasma or serum is present. The cofactor for ACA/phospholipid interaction has been isolated and characterized as β_2 -glycoprotein I (β_2 -GPI).^{12,13} β_2 -GPI has been reported to inhibit the contact phase of coagulation,¹⁴ inhibit the generation of platelet prothrombinase activity,¹⁵ and interfere with adenosine diphosphate (ADP)-induced platelet aggregation and release,¹⁶ all anticoagulant properties. Thus, an interaction of ACA with this plasma protein and anionic phospholipid could be relevant to the prothrombotic diathesis associated with aPL antibodies.

We have built on these recent findings and now report specific binding of both LA Ig and ACA Ig to platelets, both being dependent on thrombin activation but ACA being de-

From the Department of Haematology, Prince of Wales Hospital, School of Pathology, University of New South Wales, Sydney, Australia.

Submitted December 9, 1991; accepted October 27, 1992.

Supported by the National Health and Medical Research Council of Australia.

Address reprint requests to Colin N. Chesterman, MD, Department of Haematology, Prince of Wales Hospital, Cnr. High & Avoca Sts, Randwick NSW 2031, Australia.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

0006-4971/93/8105-0019\$3.00/0

pendent, in addition, on the presence of β_2 -GPI. In neither case did this specific antibody binding have any effect on platelet release induced by thrombin nor platelet aggregation stimulated by collagen or thrombin.

PATIENTS AND METHODS

Patient Plasmas

Two patients' plasmas have been studied. Both exhibited strong LA activity and high levels of ACA. Patient F.M. was a 45-year-old man with a history of aortic thrombosis and myocardial infarction. Patient C.C. was a 24-year-old woman suffering multiple arterial thromboses and more recently pulmonary embolism. Thus, both patients fulfilled the criteria for a diagnosis of antiphospholipid syndrome¹⁷ but not for SLE. Relevant laboratory data are presented in Table 1.

Patient plasma was collected into nine volumes of 0.11 mol/L sodium citrate and centrifuged at 2,500g for 15 minutes followed by filtration through 0.22- μ m filters (Millipore Australia, Sydney; type GS).¹⁷ The platelet-free plasma (PFP) was frozen immediately and stored at -80°C until use.

Detection of Antiphospholipid Antibodies

Lupus anticoagulant. The kaolin clotting time (KCT) was performed as described by Exner et al¹⁸ with a mixture of test sample and normal PFP. The prolongation (dKCT) of the control clotting time, which was with normal PFP or with sample buffer because of the addition of 1/5 vol test sample, was used to define LA activity. A linear relationship was demonstrated between the concentration of added purified LA IgG in normal plasma (0.014 to 1.8 mg/mL) and the dKCT.

Normal PFP used for KCT was a single collection, prepared as described for the patients' plasma and stored in lots at -20°C .

Anticardiolipin antibodies. Enzyme-linked immunoassay (ELISA) for IgG and IgM ACA was performed as previously described.¹⁹ Plasma samples were assayed at 1/50 dilution and purified fractions at 1/10 or 1/5 dilution. The IgG and IgM ACA level of the samples was read from a standard curve constructed from a secondary standard plasma and expressed in IgG phospholipid (GPL) or IgM phospholipid (MPL) units per milliliter, respectively.

Purification of Ig With LA or ACA Activity

PFP was subjected to cardiolipin affinity, cation exchange, anti-human IgG or IgM affinity chromatography, and chromatofocusing sequentially.

Cardiolipin affinity chromatography. Cardiolipin (Sigma Chemical Co, St Louis, MO) was immobilized in polyacrylamide gel using the method described by McNeil et al¹⁹ with minor modifications. This method was a hybrid of immobilizing glycolipids²⁰ and the liposome technique for isolating aPL devised by Pengo et al.²¹ The molar ratio of cardiolipin:cholesterol:dicetylphosphate was 10:5:2. The plasma was diluted 1:2 in buffer (0.01 mol/L phosphate, 0.01 mol/L NaCl, pH 7.3) and applied to the column; following extensive washing the bound protein was eluted with 0.01 mol/L phosphate, 1 mol/L NaCl. The fractions were pooled and dialyzed against phosphate-buffered saline (PBS), pH 7.3.

Cation exchange chromatography. The unbound fraction from cardiolipin affinity was subjected to cation exchange chromatography using a 26 mm \times 60 cm column packed with 300 mL S-Sepharose fast flow (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was operated with a computer controlled Pharmacia fast protein liquid chromatography (FPLC) system.¹⁰

Anti-IgG and anti-IgM affinity chromatography. Anti-IgG affinity was performed using 25 mL goat anti-human IgG agarose (Sigma). For anti-IgM affinity, 8 mg anti-human IgM raised in sheep (Silenus

Table 1. Laboratory Characteristics of Patients F.M. and C.C.

| | | Patient F.M. | Patient C.C. |
|----------|------|--------------|----------------------------|
| ACA | IgG | 84 GPL U | 107 GPL U |
| | IgM | 42 MPL U | 70 MPL U |
| LA | dKCT | 377 s | 122 s |
| Platelet | | 294 | $350 \times 10^9/\text{L}$ |
| aPTT | | 78 s | 46 s (NR: 20-32 s) |
| ANA | | +ve (1:320 | +ve (1:80 |
| | | homogeneous) | homogeneous) |
| dsDNA | | -ve | 4 (NR: 0-7) |
| INR | | 2.47 | 3.6 |

Laboratories, Melbourne, Australia) was coupled to 3 mL affigel-10 (Bio-Rad Laboratories, Sydney, Australia).

Cation exchange fractions were dialyzed against PBS and applied to the anti-IgG column at 12 mL/h. After washing with PBS, the bound IgG was eluted with 0.1 mol/L glycine-HCL/0.15 mol/L NaCl, pH 2.5 at a flow rate of 45 ml/h and neutralized with 80 μ L 1.5 mol/L Tris-HCL, pH 8.0 immediately.

Chromatofocusing. IgG LA and IgG ACA were further fractionated using a Pharmacia "Mono-P" column with the FPLC system. Samples were dialyzed against two changes of starting buffer, (5 mmol/L Tris-HCL, pH 8.0) over 24 hours. A maximum of 2 mL sample containing less than 25 mg protein was applied to the column at a flow rate of 0.9 mL/min. A linear pH gradient from pH 8.0 to pH 5.0 was generated by running the column with 1:50 diluted Buffalyte/pH 4 to 8 (Pierce, Rockford, IL) over 60 minutes. The absorbance at 280 nm was monitored and 900- μ L fractions collected. Fractions were dialyzed against PBS for 50 hours with three exchanges and assayed for LA and ACA.

IgM LA

IgM LA was purified from patient C.C. using the following steps.

Gel filtration chromatography. Cation exchange fractions with LA activity were pooled and subjected to a Pharmacia Superose 12 HR 10/30 gel filtration column. The column was operated at 0.4 mL/min in PBS, 0.8-mL fractions collected and tested for LA activity and ACA. The void volume possessing LA activity was further purified using anti-IgM affinity.

Anti-IgM affinity chromatography. The procedure for anti-IgM affinity chromatography was as described for IgG.

Control Igs

Control Igs were prepared from normal plasma using a procedure identical to that used to prepare patients' LA and ACA fractions after the cardiolipin affinity procedure. The same fractions as those with the two activities derived from the patient plasmas were collected at each step. They were subsequently used in the same concentrations as their active counterparts in each experiment.

Purification of β_2 -GPI

β_2 -GPI was purified from normal human serum following heparin-sepharose affinity, gel filtration, and cation exchange chromatography as described by McNeil et al.¹² The preparation resulted in a single band of molecular weight (MW) 50,000 when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Platelet Antibody Binding Studies

Platelet preparation. Blood was obtained from healthy volunteers in 14% ACD (85 mmol/L trisodium citrate, 71 mmol/L citrate acid, 101 mmol/L dextrose, pH 4.5) and centrifuged at room temperature

Table 2. Patient F.M.: Sequential Purification Schedule to Enrich Ig for LA or ACA

| | LA-Enriched Fractions | | ACA-Enriched Fractions | |
|-------------------------|------------------------|-----------------------|------------------------|------------------------|
| | dKCT (s/mg protein) | ACA (U/mg protein) | ACA (U/mg protein) | dKCT (s/mg protein) |
| Starting PFP | 7.8 | 1.37 | 1.37 | 7.8 |
| Cardiolipin affinity | | | 90 | 0.18 |
| Cation exchange | 26 | 0.79 | | |
| Anti-IgG chromatography | 91 | 0.36 | 392 | NA |
| Chromatofocusing | 512 | 0.08 | 450 | ND |

Abbreviations: NA, not available; ND, not detectable.

at 180g for 15 minutes. The platelet-rich plasma (PRP) was overlaid onto 1 mL 40% bovine serum albumin (BSA) and further centrifuged at 700g for 15 minutes. The layer of platelets was pooled and applied to sepharose 2B (Pharmacia) gel (column size 1.5 × 5 cm) equilibrated with Tyrode buffer (140 mmol/L NaCl, 1.05 mmol/L MgCl₂, 11.9 mmol/L NaHCO₃, 0.42 mmol/L NaH₂PO₄, 10 mmol/L glucose, 0.35% BSA, pH 7.4). The gel-filtered platelets (GFP) were eluted in the void volume. GFP released approximately 75% of the α -granule protein β TG and 70% of [¹⁴C]-5HT when stimulated with 0.05 U/mL thrombin as described below and reported previously.²²

Radiolabeling of LA and ACA. Five micrograms of purified patient LA and ACA and appropriate controls were labeled with [¹²⁵I] using a modification of the chloramine-T method.²³ The amount of chloramine-T added was reduced to 30 μ g as was the sodium metabisulfate. The carrier potassium iodide was reduced to 400 μ g.

Antibody binding protocol. Fifty-microliter GFP suspension in Tyrode buffer at a platelet concentration of 2.5 to 3 × 10⁸/mL was overlaid onto 100 μ L phthalate oil (dibutyl phthalate: di-iso-octyl-phthalate, 1:1; SG1.015) in 250 μ L polypropylene tubes and incubated at room temperature for 30 minutes with human IgG (Sigma) 1.3 μ mol/L (at least three orders of magnitude excess over [¹²⁵I]-labeled Ig). After the addition of [¹²⁵I]-labeled Ig (LA, ACA, or control Ig), the GFP was incubated in the presence of dilutions of 0 to 0.05 U/mL bovine thrombin (Parke Davis) at 37°C, without stirring, for a further 60 minutes.²² It had previously been shown that maximum binding was reached within this time period. Specificity of binding of Ig and other competition experiments were performed using the same protocol but with the addition of unlabeled Ig preparations. The unbound [¹²⁵I]-Ig was separated by centrifugation of the platelets through the phthalate oil at 12,300g for 1 minute (Sorvall, microspin 24). The radioactivity of the platelet pellet was counted in a γ -counter (Packard Crystal II, Groningen, The Netherlands).

Monoclonal antibody-specific immobilization of platelet antigens (MAIPA). To test for possible specificity of enriched Ig fractions for platelet GP IIb/IIIa MAIPA was performed out as previously described.²⁴ LA IgG (patient F.M.), LA IgM (patient C.C.), ACA IgG (patient C.C.), the last with β_2 -GPI, in final concentrations of 66 nmol/L, 11 nmol/L, 66 nmol/L, and 4 μ mol/L, respectively were assayed using MoAbs AP2, SZ22, PA15C419 directed against GPIIb/GPIIIa.

Assays for Platelet Granule Release

PRP was prepared as described above. Before application to the Sepharose 2B column, the PRP, at a concentration of 1 × 10⁹/mL, was incubated with 20 mmol/L, final concentration, of 5HT-[¹⁴C]-creatinine sulfate (57 mCi/mmol; Amersham, Sydney, Australia) at 37°C for 45 minutes. Free [¹⁴C]-5HT and plasma proteins were resolved by gel filtration of the platelets on the Sepharose 2B column.

The antibody binding protocol described above was followed. LA or ACA, or control Ig at equivalent concentration, was added to 100 μ L [¹⁴C]-5HT labeled GFP in the presence of serial dilutions of thrombin. After incubation at 37°C for 60 minutes, the platelets

were centrifuged through phthalate oil at 12,300g for 1 minute. The supernatant was divided into lots and 15 μ L in 4 mL scintillant, and counted for [¹⁴C] in a Beckman LS5801 (Gladesville, NSW, Australia). β TG in the supernatant was measured in a solid-phase radioimmunoassay (RIA) as previously described.²⁵

Platelet aggregation. PRP was prepared from normal citrated blood as described above and 250 μ L (3 × 10⁸/mL) incubated with 15 μ L of purified LA, ACA, or control Ig for 30 minutes at 37°C. Platelet aggregation was induced by 25 μ L of thrombin (0.1 and 0.25 U/mL), collagen (0.8 μ g/mL), and ADP (1.25 and 2.5 μ g/mL) in a Chronolog four-channel aggregometer Model 680 (Haverton, PA).

RESULTS

Purification of LA and ACA

The LA and ACA activities and protein concentration were monitored after each step of purification and expressed as dKCT seconds per milligram of protein and units per milligram of protein, respectively. From the plasma of patient F.M. the resulting IgG was 66-fold and 328-fold enriched for LA and ACA activities, respectively, compared with the starting plasma (Table 2).

From the plasma of patient C.C., the LA activity was concentrated predominantly in the IgM fraction. When pooled, LA-positive cation exchange fractions were separated on a superose 12 HR 10/30 column, LA activity eluted primarily in the void volume. The pooled IgM peak was purified further by anti-IgM affinity chromatography. From this plasma, the ACA bound poorly to the cardiolipin affinity column and thus this step was omitted from the purification schedule. The activities of IgM LA and IgG ACA separated from patient C.C. were increased 113-fold and 60-fold, respectively (Table 3).

When IgG fractions from either plasma with LA or ACA activity were fractionated on a "mono-P" chromatofocusing column, the majority of LA activity was resolved at a pI of 7.2 to 7.3 whereas ACA had a pI of 5.0 to 5.2 (Fig 1). Only the peak of LA and the peak of ACA with the highest activity (which corresponded to highest specific activity) were used in the binding experiments.

The purified LA Ig or ACA Ig yielded a single band at MW 150 Kd on SDS-PAGE, which reduced to two bands at MW 50 and 23 Kd, consistent with the heavy and light chains of IgG. IgM LA did not enter the running gel but yielded two bands corresponding to the heavy and light chains of IgM when electrophoresed under reducing conditions.

Binding of LA Ig to Platelets

Preliminary experiments demonstrated binding of [¹²⁵I]-LA IgG (patient F.M.) to thrombin-activated GFP, which

Table 3. Patient C.C.: Sequential Purification Schedule To Enrich Ig for LA or ACA

| | LA-Enriched Fractions | | ACA-Enriched Fractions | |
|-------------------------|-----------------------|--------------------|------------------------|---------------------|
| | dKCT (s/mg protein) | ACA (U/mg protein) | ACA (U/mg protein) | dKCT (s/mg protein) |
| Starting PFP | 1.65 | 1.45 | 1.45 | 1.65 |
| Cation exchange | 13.7 | 0.09 | 3.3 | NA |
| Anti-IgG chromatography | | | 96 | NA |
| Gel filtration | 168 | 0.36 | | |
| Anti-IgM chromatography | 186 | 0.36 | | |
| Chromatofocusing | | | 87 | ND |

Abbreviations: NA, not available; ND, not detectable.

was dependent on platelet numbers and on incubation time at 37°C. Between platelet concentrations of 8×10^7 /mL and 1.3×10^9 /mL the bound radioactive counts increased from a mean of 1.2% to 6.6% and there was no suggestion that this binding had reached a plateau (data not shown). Maximum binding was achieved after 30 to 60 minutes of incubation. As the platelet count was limited by availability and the gel-filtering procedure, further experiments were performed using a concentration of 2.5 to 3.0×10^8 /mL and [¹²⁵I]-LA IgG binding was of the order of 2.2% under these conditions. When increasing concentrations of unlabeled LA IgG were coincubated with [¹²⁵I]-LA IgG, thrombin 0.05 U/mL and GFP, the [¹²⁵I] associated with the platelets was reg-

ularly maximally competed (to approximately 50% of total) at 20 nmol/L LA IgG, indicating saturability and specificity of binding (Fig 2a). That the binding was dependent on activation of the platelets was demonstrated by incubating [¹²⁵I]-LA IgG in the presence and absence of an excess unlabeled LA IgG (417 nmol/L) with increasing concentrations of

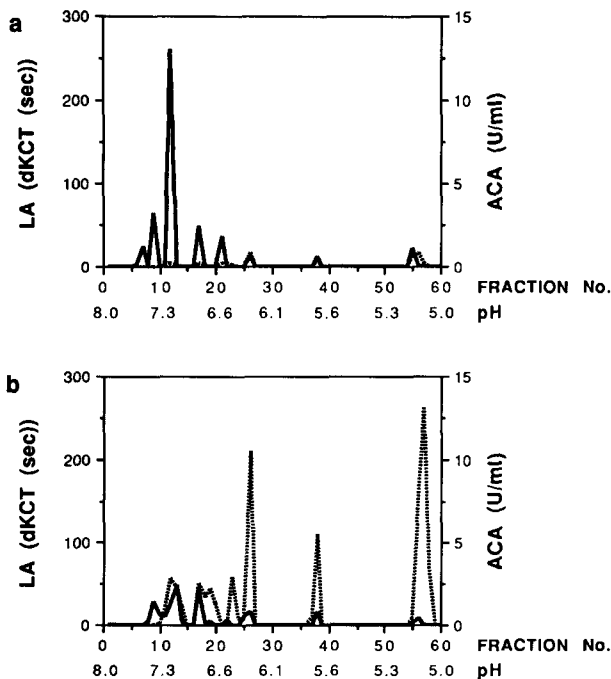


Fig 1. Chromatofocusing of two enriched Ig preparations from patient F.M. (a) The profile of the LA Ig after affinity chromatography on the anti-IgG column. The majority of the LA activity (—) is concentrated at a pI of 7.2 to 7.3. A small residual ACA (· · ·) is detectable in these fractions. **(b)** The chromatofocusing profile of the ACA IgG preparation after affinity chromatography on the anti-IgG column. The majority of the ACA (· · ·) had a pI of 5.0 to 5.2. Fractions with ACA were virtually devoid of LA activity (—).

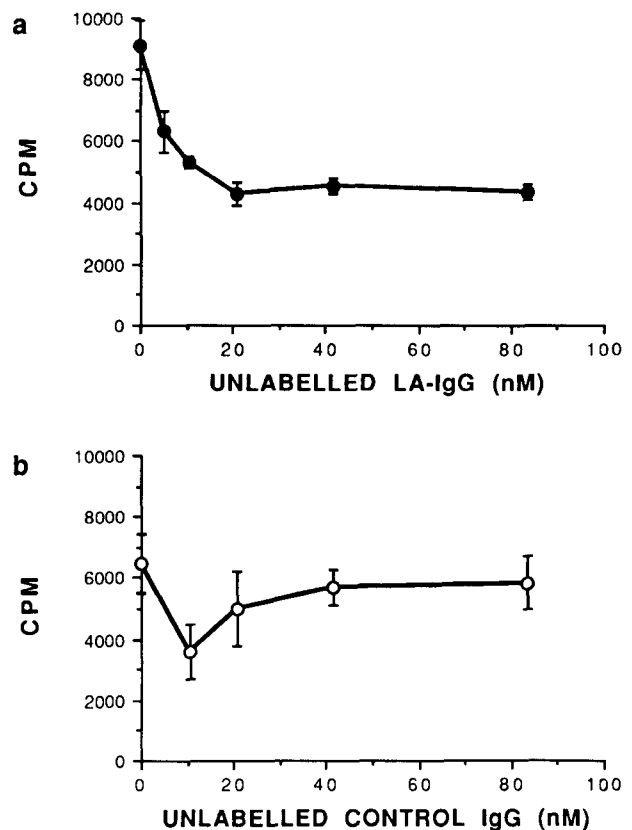


Fig 2. Binding of LA IgG to thrombin-activated platelets. (a) Incubation of GFP with [¹²⁵I]-LA IgG (● — ●) in the presence of 0.05 U/mL thrombin at 37°C for 60 minutes. Increasing concentrations of unlabeled LA IgG resulted in approximately 50% competition of platelet-associated [¹²⁵I]-LA IgG at a concentration of 20 nmol/L. Mean ± SEM (n = 3). This experiment is representative of five similar experiments. **In (b)** a similar assay for binding of [¹²⁵I]-control IgG (○ — ○) competed by unlabeled control IgG was performed under the same conditions. It can be seen that there is no significant competition for the [¹²⁵I]-labeled control IgG. Mean ± SEM (n = 5). Three other experiments showed similar results.

thrombin. There was no specific binding of [¹²⁵I]-LA IgG in the absence of thrombin (bound radioactive counts not displaced by an excess of unlabeled LA IgG) and specific binding showed dependency on the concentration of the thrombin (Fig 3). LA IgM (patient C.C.) behaved identically (Fig 4a).

Neither the control IgG nor control IgM (prepared in an identical fashion from normal plasma excluding the phospholipid affinity step) showed specific binding under any of the conditions tested (Fig 2b, Fig 4b). This was evidenced by a lack of competition by an increasing excess of its unlabeled counterpart.

Binding of ACA Ig to Platelets

ACA Ig from neither patient bound to platelets, thrombin-stimulated or not. However, when 4 μmol/L β₂-GPI was included in the incubation, a similar pattern of binding to that seen in LA Ig was observed, both in saturability (Fig 5) and in thrombin dependency (Fig 6), although there was minimal binding to resting platelets. A control preparation of IgG bound nonspecifically to platelets under all of the conditions tested. There was no competition for binding by an increasing excess of unlabeled control IgG (data not shown).

Effects of β₂-GPI and ACA IgG on LA IgG Binding to Platelets

β₂-GPI (4 μmol/L) had no additive effect when incubated with [¹²⁵I]-LA IgG, thrombin, and platelets. However, the combination of increasing concentrations of ACA IgG in the presence of 4 μmol/L β₂-GPI resulted in competition for binding of [¹²⁵I]-LA IgG to thrombin-activated GFP (Fig 7a).

In the converse experiment unlabeled LA IgG competed for binding [¹²⁵I]-ACA IgG to thrombin-activated GFP in the presence of 4 μmol/L β₂-GPI (Fig 7b).

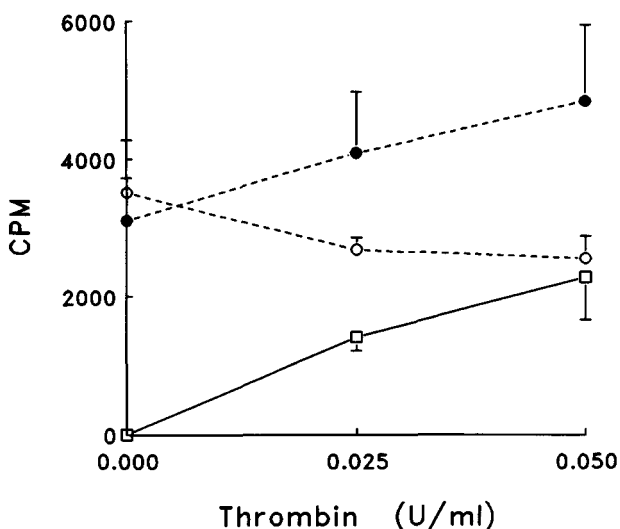


Fig 3. The effect of thrombin on LA IgG binding to platelets. GFP was incubated with [¹²⁵I]-LA IgG at 37°C for 60 minutes in the absence and the presence of two concentrations of thrombin. The cpm bound to the platelets is shown (● --- ●). To determine nonspecific binding an identical experiment was performed with an excess (417 nmol/L) of unlabeled IgG (○ --- ○). Specific binding (total minus nonspecific) is shown (□ --- □). Mean ± SEM (n = 5) combined from two separate experiments.

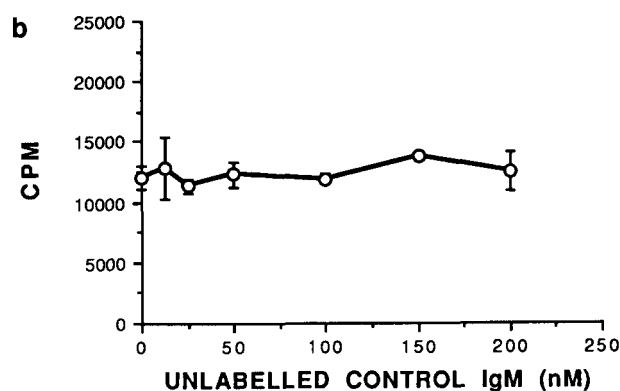
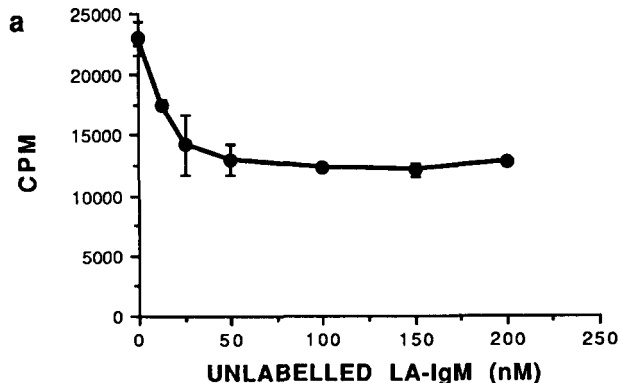


Fig 4. Binding of LA IgM to thrombin-activated platelets. (a) Incubation of GFP with [¹²⁵I]-LA IgM (● — ●) in the presence of 0.05 U/mL thrombin at 37°C for 60 minutes. Increasing concentrations of unlabeled LA IgM resulted in approximately 50% competition of platelet-associated [¹²⁵I]-LA IgM at a concentration of 50 nmol/L. Mean ± SEM (n = 2). This experiment is representative of three similar experiments. In (b) a similar assay for binding of [¹²⁵I]-control IgM (○ — ○) competed by unlabeled control IgM was performed under the same conditions. It can be seen that there is no significant competition for the [¹²⁵I]-labeled control IgM. Mean ± SEM (n = 2). Three other experiments showed similar results.

MAIPA Studies

None of the LA Ig or ACA Ig showed evidence of cross-reactivity with GPIIb/IIIa.

Effects of LA Ig, ACA Ig, and β₂-GPI on Thrombin-Induced Release Reaction and Platelet Aggregation in PRP

An excess of either LA IgG (66 nmol/L), LA IgM (11 nmol/L), ACA IgG (66 nmol/L), or control Ig preparations, with or without 4 μmol/L β₂-GPI, was added to [¹⁴C]-5HT-labeled GFP followed by the addition of increasing concentrations of thrombin. At the completion of a 60-minute incubation at 37°C, the release of [¹⁴C]-5HT and βTG was measured. None of the Ig preparations had any discernible effect on the thrombin-induced release of either 5HT or βTG that showed the expected dose dependency (Fig 8).

In a similar series of experiments, the addition of LA, ACA, and control Ig had no effect on aggregation patterns of normal PRP to collagen (0.8 μg/mL), thrombin (0.1 and 0.25 U/mL), or ADP (1.25 and 2.5 μg/mL).

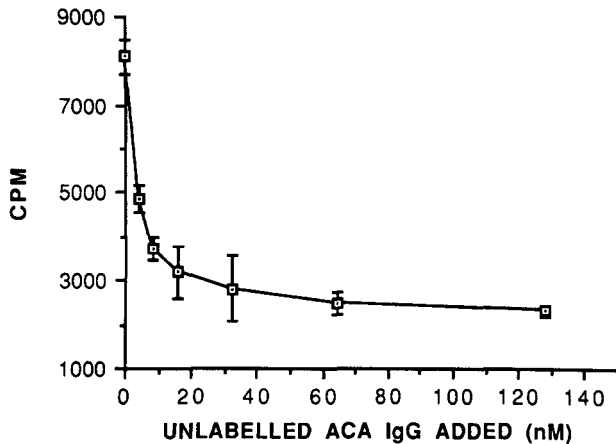


Fig 5. Effect of β_2 -GPI on ACA binding to thrombin-activated platelets. In the presence of 4 μ mol/L β_2 -GPI, [125 I]-ACA IgG from patient C.C. bound to thrombin-stimulated GFP and was competed with increasing concentrations of unlabeled ACA IgG. The inhibition was maximal at 16 nmol/L unlabeled ACA IgG. Data show the mean \pm SEM (n = 2). This experiment is representative of three separate experiments.

DISCUSSION

This study emphasizes the importance of resolving autoantibody activities before investigating their properties in experimental systems. It points to differences between the aPL antibody subclasses LA and ACA derived from plasmas containing both activities. The plasmas came from two patients with thrombotic diathesis and a clinical diagnosis of primary antiphospholipid antibody syndrome.¹⁷ We have shown that while both LA Ig and ACA Ig bind to GFP, the conditions that favor binding differ significantly.

We have shown that Ig fractions enriched for LA and essentially devoid of ACA do not bind to resting platelets but do bind to thrombin-activated platelets, the binding being specific, saturable, and thrombin dose-dependent. The data complements and extends that of a number of previous studies.^{8,26,27} Binding of LA Ig to activated platelets is completely independent of added lipid binding plasma protein β_2 -GPI. Further, LA of both IgG and IgM isotypes behave similarly.

ACA IgG, essentially devoid of LA activity and from the same two patients, does not bind to platelets unless β_2 -GPI is added and again the binding is activation dependent. This confirms and extends original observations that ACA bind a complex epitope consisting of β_2 -GPI and anionic phospholipid immobilized on plastic or in a liposome entrapped in acrylamide gel.^{12,13} Thus, the same β_2 -GPI requirement is demonstrated for the platelet membrane.

Candidates for the platelet binding site are in favor of phospholipids such as phosphatidylserine and phosphatidylethanolamine, although a complex requiring protein is not excluded. A number of investigators have shown that up to 35% phospholipid is exposed by thrombin activation and this is augmented by the addition of collagen²⁸⁻³⁰ and with aggregation induced by stirring. The latter was not used in these experiments to avoid aggregation that might result in non-specific trapping of Ig.

The antibodies both bind to the same site or at least a contiguous one as we demonstrated, quite clearly, competition between the LA and ACA as long as β_2 -GPI was present. Apparent differences in affinity between the two antibodies are probably not relevant, given that the antibody preparations are not monospecific. Interpretation of the competition between LA and ACA/ β_2 -GPI is further complicated by the recent report that a proportion of LA are probably directed to an epitope that becomes exposed on Ca^{2+} -mediated binding of prothrombin to phospholipids.³¹ It is unlikely that a significant concentration of prothrombin could be associated with the GFP used in our experiments and the added thrombin would not bind to membrane phospholipid. It is possible that the LA used in our studies fall into the subgroup that do not require prothrombin to bind phospholipid. Other possible binding sites might be GPIIb/IIIa, which become conformationally altered on activation of platelets. In view of the activation dependency of the LA and ACA binding and reports of cross-reactivity between aPL and antiplatelet antibodies, we specifically investigated this possibility using a highly sensitive MAIPA.²⁴ No cross-reactivity was demonstrable. Fc receptors are reported to increase by up to 50%

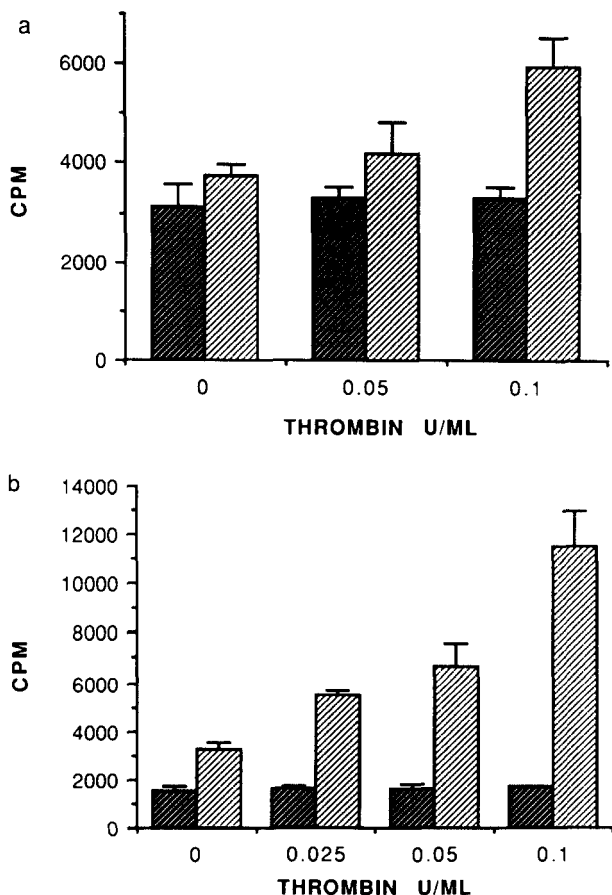


Fig 6. The requirement of β_2 -GPI for ACA to bind to GFP. (a) The binding of ACA IgG from patient F.M. and (b) patient C.C. to GFP was observed only if β_2 -GPI (4 μ mol/L) was present. The binding was thrombin dependent. Mean \pm SD of four experimental points (F.M.) and three experimental points (C.C.) (■) ACA IgG; (▨) ACA IgG/B₂-GPI.

after platelet activation.³² However, the excess monomeric Ig present in the system, the total dependency for platelet activation, and the lack of binding of control Ig with the same physicochemical characteristics, including isoelectric point, all militate against the LA and ACA association being nonspecific Fc binding. A final possible contender for binding might be membrane glycolipids, which are exposed by platelet activation. While this possibility has not been excluded, aPL did not bind specifically to a range of glycolipids in solid-phase ELISA (W.S., unpublished observations, 1989).

The model of nonstirred thrombin activation of GFP was also chosen to test possible functional associations with ACA/ β_2 -GPI or LA binding to the platelets, especially because β_2 -GPI has been reported to bind platelet phospholipid independently³³ and to interfere with ADP-induced aggregation.¹⁶ The present experiments allowed us to identify either inhibition or synergism in the release reaction induced by increasing concentrations of thrombin. None of the antibodies, in concentrations exceeding the calculated half-maximal

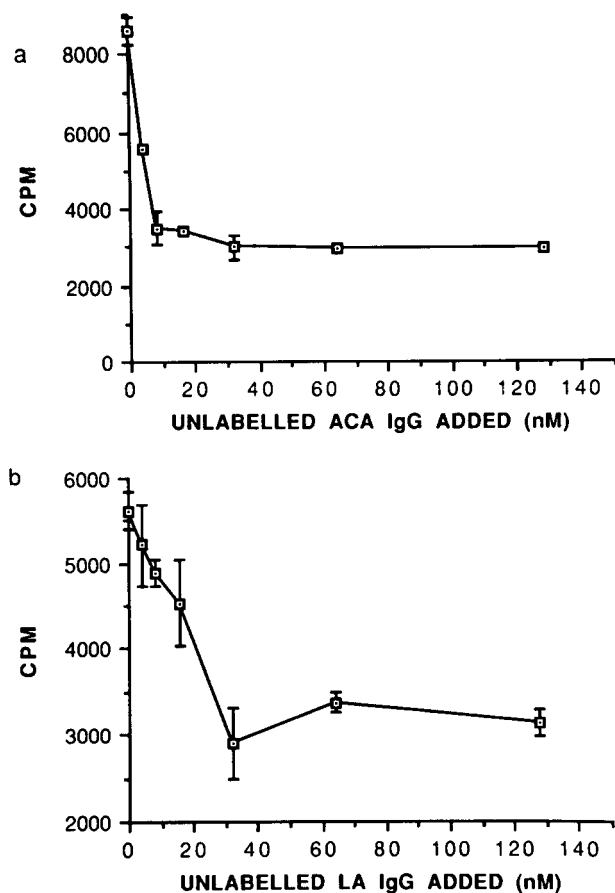


Fig 7. Competition between LA IgG and ACA IgG for binding to GFP. (a) Increasing concentrations of unlabeled ACA IgG in the presence of β_2 -GPI competed for the binding of [¹²⁵I]-LA IgG to thrombin-activated GFP. The effect was maximal at a concentration of 16 nmol/L unlabeled ACA IgG. (b) Bound [¹²⁵I]-ACA IgG to thrombin-activated platelets in the presence of β_2 -GPI (4 μ mol/L) was displaced by addition of increasing concentrations of unlabeled LA IgG and was maximal at 32 nmol/L LA IgG. Mean \pm SEM (n = 2).

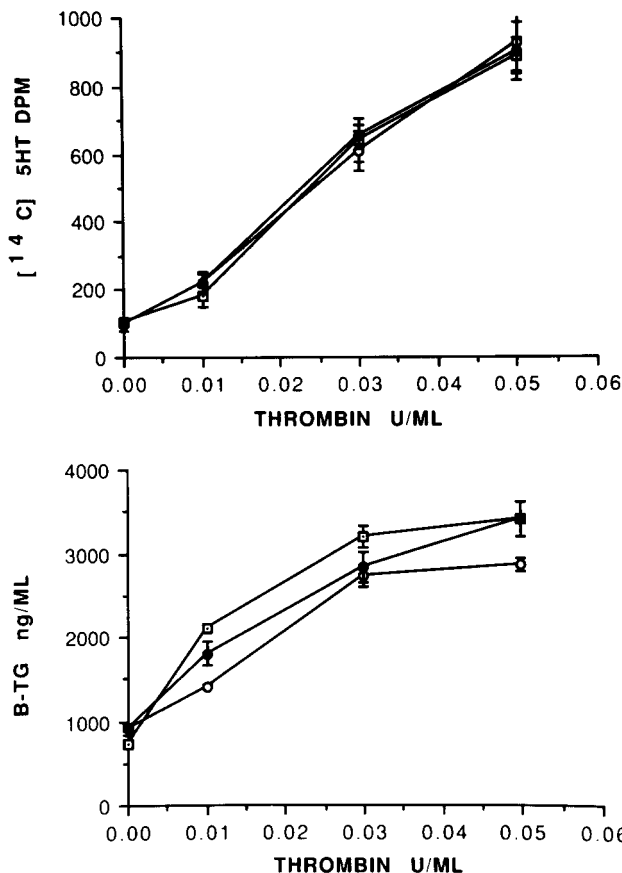


Fig 8. Experiments to determine the effects of LA, ACA, and β_2 -GPI on the platelet release reaction induced by thrombin. The top graph demonstrates that when LA IgG from patient F.M. incubated with [¹⁴C]-5HT-labeled GFP, the release of [¹⁴C]-5HT (● — ●) on stimulation with increasing concentrations of thrombin (0 to 0.05 U/mL) was not affected in comparison with buffer control (□ — □) and control IgG (○ — ○) (bottom). The release of β TG in the same experiment measured by RIA showed similar results. Mean \pm SD (n = 4).

binding requirement, altered the release of dense granule (5HT) or α -granule (β TG) components in any way. In succeeding experiments, similar concentrations of LA and ACA had no effect on platelet aggregation using ADP, collagen, and thrombin as agonists.

The quite specific binding of these antibodies to platelets during the process of activation may well provide an explanation for accelerated removal of platelets from the circulation via Fc receptor-mediated phagocytosis in the reticuloendothelial system. This chain of events implies the ongoing activation of platelets, a process, however, that is consistent with the tendency to thrombosis. Thus, the present studies, while not providing an explanation for the thrombotic propensity associated with the antibodies, do provide a possible mechanism for thrombocytopenia frequently associated with aPL antibodies.

ACKNOWLEDGMENT

The authors acknowledge useful discussions with Drs S. Krilis and P. Hogg. We thank Dr Y.L. Kwan for allowing us to study the blood

of one of his patients. Barbara Murray, Sue Evans, and Georgina Joulianos assisted with assay procedures and purifications. Gael Campbell prepared the manuscript. Drs C. Ruan (Suzhou Medical College, Suzhou, China) SZ22, T.J. Kunicki (The Blood Centre of SE Wisconsin, Milwaukee) AP2, and P. Thurlow (Austin Hospital, Melbourne, Australia) PA15C419, generously donated monoclonal antibodies.

REFERENCES

1. McNeil HP, Chesterman CN, Krilis SA: Immunology and clinical importance of antiphospholipid antibodies. *Adv Immunol* 49: 193, 1991
2. Firkin BG, Booth P, Hendrix L, Howard MA: Demonstration of a platelet bypass mechanism in the clotting system using an acquired anticoagulant. *Am J Hematol* 5:81, 1978
3. Howard MA, Firkin BG: Investigation of the lupus-like inhibitor bypassing activity of platelets. *Thromb Haemost* 50:755, 1983
4. Mueller-Eckhardt C, Kaysr W, Mersch-Baumert K, Mueller-Eckhardt G, Breindenbach M, Kugel HG, Graubner M: The clinical significance of platelet-associated IgG: A study of 298 patients with various disorders. *Br J Haematol* 46:123, 1980
5. Hegde VM, Williams K, Devereus S, Bowes A, Powell D, Fisher D: Platelet associated IgG and immune thrombocytopenia in lymphoproliferative and autoimmune disorders. *Clin Lab Haematol* 5: 9, 1983
6. Harris EN, Asherson RA, Gharavi AE, Morgan SH, Derue G, Hughes GRV: Thrombocytopenia in SLE and related autoimmune disorders: Association with anticardiolipin antibodies. *Br J Haematol* 59:227, 1985
7. Hasselaar P, Derksen RHWM, Blokzijl L, de Groot PG: Cross-reactivity of antibodies directed against cardiolipin, DNA, endothelial cells and blood platelets. *Thromb Haemost* 63:169, 1990
8. Khamashta MA, Harris EN, Gharavi ARE, Derue G, Gil A, Vazquez JJ, Hughes GRV: Immune mediated mechanism for thrombosis: Antiphospholipid antibody binding to platelet membranes. *Ann Rheum Dis* 47:849, 1988
9. Shi W, Krilis SA, Chong BH, Gordon S, Chesterman CN: Prevalence of lupus anticoagulant and anticardiolipin antibodies in a healthy population. *Aust NZ J Med* 20:231, 1990
10. McNeil HP, Chesterman CN, Krilis SA: Anticardiolipin antibodies and lupus anticoagulants comprise separate antibody subgroups with different phospholipid binding characteristics. *Br J Haematol* 73:506, 1989
11. Exner T, Sahman N, Trudinger B: Separation of anticardiolipin antibodies from lupus anticoagulant on a phospholipid-coated polystyrene column. *Biochem Biophys Res Commun* 155:1001, 1988
12. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA: Antiphospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: β_2 -glycoprotein I (apolipoproteinH). *Proc Natl Acad Sci USA* 87:4120, 1990
13. Galli M, Comfurius P, Maassen C, Hemker HC, De Baets MH, Van Breda-Vriesman PJC, Barbui T, Zwaal RFA, Bevers EM: Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet* 335:1544, 1990
14. Schousboe I: β_2 -glycoprotein I: A plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. *Blood* 66: 1086, 1985
15. Nimpf J, Bevers EM, Bomans PHH, Till U, Wurm H, Kostner GM, Zwaal RFA: Prothrombinase activity of human platelets is inhibited by β_2 -glycoprotein I. *Biochem Biophys Acta* 884:142, 1986
16. Nimpf J, Wurm H, Kostner GM: β_2 -glycoprotein I (apo H) inhibits the release reaction of human platelets during ADP-induced aggregation. *Atherosclerosis* 63:109, 1987
17. Hughes GRV: The anticardiolipin syndrome. *Clin Exp Rheumatol* 3:285, 1985
18. Exner T, Richard KA, Kronenberg H: A sensitive test demonstrating lupus anticoagulant and its behavioural patterns. *Br J Haematol* 40:143, 1978
19. McNeil HP, Krilis SA, Chesterman CN: Purification of antiphospholipid antibodies using a new affinity method. *Thromb Res* 52:641, 1988
20. Uchida T, Filburn CR: Affinity chromatography of protein kinase C-phorbol ester receptor on polyacrylamide-immobilised phosphatidyl serine. *J Biol Chem* 259:12311, 1984
21. Pengo V, Thiagarajan P, Shapiro SS, Heine MJ: Immunological specificity and mechanism of action of IgG lupus anticoagulants. *Blood* 70:69, 1987
22. Wiley JS, Chesterman CN, Morgan FJ, Castaldi PA: The effect of sulphinyprazole on the aggregation and release reactions of platelets. *Thromb Res* 14:23, 1979
23. Hunter WM, Greenwood FC: Preparation of iodine-131 labelled human growth factor of higher specific activity. *Nature* 194: 495, 1962
24. Chong BH, Du X, Berndt MC, Horn S, Chesterman CN: Characterization of the binding domains on platelet glycoproteins Ib-IX and IIb/IIIa complexes for the quinine/quinidine-dependent antibodies. *Blood* 77:2190, 1991
25. Gavaghan TP, Hickie JB, Krilis SA, Baron DW, GebSKI V, Low, Chesterman CN: Increased plasma beta-thromboglobulin in patients with coronary artery vein graft occlusion: Response to low dose aspirin. *J Am Coll Cardiol* 15:1250, 1990
26. Ichikawa Y, Kobayashi N, Kawada T, Shimizu H, Morichi J, Ono H, Watanabe K, Arimori S: Reactivities of antiphospholipid antibodies to blood cells and their effects on platelet aggregations *in vitro*. *Clin Exp Rheumatol* 8:461, 1990
27. Mikhail MH, Szczech LAM, Shapiro SS: The binding of lupus anticoagulants (LAC's) to human platelets. *Blood* 72:333a, 1988 (abstr, suppl 1)
28. Schick PK, Kurica KB, Chacko GK: Location of phosphatidylethanolamine and phosphatidylserine in the human platelet plasma membrane. *J Clin Invest* 57:1221, 1976
29. Zwaal RFA, Bevers EM: Platelet phospholipid asymmetry and its significance in hemostasis. *Subcell Biochem* 9:299, 1983
30. Bevers EM, Comfurius P, Van Rijn JLML, Hemker HC, Zwaal RFA: Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outersurface of platelets. *Eur J Biochem* 122:429, 1982
31. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RFA: Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thromb Haemost* 66:629, 1991
32. McCrae KR, Shattil SJ, Cines DB: Platelet activation induces increased Fc α receptor expression. *J Immunol* 144:3920, 1990
33. Schousboe I: Binding of β_2 -glycoprotein I to platelets: Effect on adenylate cyclase activity. *Thromb Res* 19:225, 1980