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, β2-glycoprotein I (β2-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 [125I]-iodine we have shown that LA binds to platelets in a specific and saturable manner. Binding is dependent on thrombin activation. [125I]-ACA behaves differently. Like LA, binding is specific and dependent on thrombin activation but in this case requires the presence of β2-GPI. ACA, in the presence of β2-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.

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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 woman suffering multiple arterial thromboses and more recently pulmonary embolism. Both patients fulfilled the criteria for a diagnosis of antiphospholipid syndrome but not for SLE. Relevant laboratory data are presented in Table 1.

Patient C.C. was a 24-year-old woman suffering multiple arterial thromboses and more recently pulmonary embolism. Both patients fulfilled the criteria for a diagnosis of antiphospholipid syndrome but not for SLE. Relevant laboratory data are presented in Table 1.

Detection of Antiphospholipid Antibodies

Lupus anticoagulant. The kaolin clotting time (KCT) was performed as described by Exner et al 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 until use.

Platelet Antibody Binding Studies

Cardiolipin affinity chromatography. Cardiolipin (Sigma Chemical Co, St Louis, MO) was immobilized in polyacrylamide gel using the method described by McNeil et al with minor modifications. This method was a hybrid of immobilizing glycolipids and the liposome technique for isolating aPL devised by Pengo et al. The molar ratio of cardiolipin:cholesterol:dicetylphosphosphate 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 × 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.

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 Laboratories, Melbourne, Australia) was coupled to 3 mL affinity-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 μL 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 Buffalaye/pH 4 to 8 (Pierce, Rockford, IL) over 60 minutes. The absorbance at 280 nm was monitored and 900-μL 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 β2-GPI

β2-GPI was purified from normal human serum following heparin-Sepharose affinity, gel filtration, and cation exchange chromatography as described by McNeil et al. 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.
ANTICARDIOLIPIN ANTIBODIES, LA, AND PLATELETS

thrombin. After incubation at 37°C for 10 minutes, the platelets were centrifuged at 300g for 15 minutes. The supernatant was divided into lots and 15 mL in 4 mL scintillant, and counted for [125I] in a Beckman LS5801 (Gladesville, NSW, Australia). βTG in the supernatant was measured in a solid-phase radioimmunooassay (RIA) as previously described. Platelet aggregation. PRP was prepared from normal citrated blood as described above and 250 mL (3 x 10^9/mL) incubated with 15 mL of mixed LA, ACA, or control Ig for 30 minutes at 37°C. Platelet aggregation was induced by 25 mL of thrombin (0.1 and 0.25 U/mL), collagen (0.8 μg/mL), and ADP (1.25 and 2.5 μg/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 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 PP | 7.8 | 1.37 | 1.37 | 7.8 |
| Cardiolipin affinity | | | |
| Cation exchange | 60 | 0.79 | 90 | 0.18 |
| Anti-IgG chromatography | 91 | 0.36 | 392 | NA |
| Chromatofocusing | 512 | 0.08 | 450 | ND |

Abbreviations: NA, not available; ND, not detectable.
Table 3. Patient C.C.: Sequential Purification Schedule To Enrich Ig for LA or ACA

<table>
<thead>
<tr>
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<th>LA-Enriched Fractions</th>
<th>ACA-Enriched Fractions</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>dkCT (s/mg protein)</td>
<td>ACA (U/mg protein)</td>
</tr>
<tr>
<td>Starting PFP</td>
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<td>Anti-IgM chromatography</td>
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<td>0.36</td>
</tr>
<tr>
<td>Chromatofocusing</td>
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Abbreviations: NA, not available; ND, not detectable.

was dependent on platelet numbers and on incubation time at 37°C. Between platelet concentrations of 8 × 10⁷/mL and 1.3 × 10⁹/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 × 10⁸/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 regularly 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 pl 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 pl 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.
ANCARDIOLIPIN ANTIBODIES, LA, AND PLATELETS

There was no specific binding of [\(^{125}\)I]-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 \(\mu\)mol/L \(\beta_2\)-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 \(\beta_2\)-GPI and ACA IgG on LA IgG Binding to Platelets

\(\beta_2\)-GPI (4 \(\mu\)mol/L) had no additive effect when incubated with [\(^{125}\)I]-LA IgG, thrombin, and platelets. However, the combination of increasing concentrations of ACA IgG in the presence of 4 \(\mu\)mol/L \(\beta_2\)-GPI resulted in competition for binding of [\(^{125}\)I]-LA IgG to thrombin-activated GFP (Fig 7a).

In the converse experiment unlabeled LA IgG competed for binding [\(^{125}\)I]-ACA IgG to thrombin-activated GFP in the presence of 4 \(\mu\)mol/L \(\beta_2\)-GPI (Fig 7b).

Effects of LA Ig, ACA Ig, and \(\beta_2\)-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 \(\mu\)mol/L \(\beta_2\)-GPI, was added to [\(^{14}\)C]-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 [\(^{14}\)C]-5HT and \(\beta\)TG was measured. None of the Ig preparations had any discernible effect on the thrombin-induced release of either 5HT or \(\beta\)TG 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 \(\mu\)g/mL), thrombin (0.1 and 0.25 U/mL), or ADP (1.25 and 2.5 \(\mu\)g/mL).
Fig 5. Effect of \( \beta_2 \)-GPI on ACA binding to thrombin-activated platelets. In the presence of 4 \( \mu \)mol/L \( \beta_2 \)-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 ± 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. 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. Binding of LA Ig to activated platelets is completely independent of added lipid binding plasma protein \( \beta_2 \)-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 \( \beta_2 \)-GPI is added and again the binding is activation dependent. This confirms and extends original observations that ACA bind a complex epitope consisting of \( \beta_2 \)-GPI and anionic phospholipid immobilized on plastic or in a liposome entrapped in acrylamide gel. Thus, the same \( \beta_2 \)-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 collagen and with aggregation induced by stirring. The latter was not used in these experiments to avoid aggregation that might result in nonspecific 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 \( \beta_2 \)-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/\( \beta_2 \)-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. 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. No cross-reactivity was demonstrable. Fc receptors are reported to increase by up to 50%
after platelet activation. 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/β2-GPI or LA binding to the platelets, especially because β2-GPI has been reported to bind platelet phospholipid independently and to interfere with ADP-induced aggregation. 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 binding requirement, altered the release of dense granule (5HT) or α-granule (βTG) 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.

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

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