DETECTION OF ANTIBODY-MEDIATED REDUCTION OF ANNEXIN A5 ANTICOAGULANT ACTIVITY IN PLASMAS OF PATIENTS WITH THE ANTIPHOSPHOLIPID SYNDROME

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ABSTRACT

Annexin A5 (A5) forms 2-dimensional crystals over phospholipid bilayers, blocking their availability for coagulation reactions. Recently, human antiphospholipid (aPL) mAbs have been demonstrated by atomic force microscopy (AFM) to disrupt this crystallization and accelerate coagulation. We therefore performed a study with small, well-defined groups of patients to investigate whether these effects on A5 binding and activity are also detectable in plasmas from patients with the aPL syndrome.

A5 binding to phospholipid was significantly reduced by plasmas of patients with aPL syndrome and thromboembolism compared to normal healthy controls (mean±SD: 26.7±4.3 ng/well, n=25 vs. 30.5±3.1 ng/well, n=20, p<0.01,) and the non-aPL thromboembolism group (29.9±3.2 ng/well, n=15, p<0.05). A5 anticoagulant activity was reduced by plasmas of patients with aPL syndrome and thromboembolism compared to aPL antibodies without thrombosis (182±31 %, n=25 vs. 210±35 %, n=26, p<0.01), non-aPL thromboembolism (229±16 %, n=15, p<0.001), and normal healthy controls (231±14 %, n=30, p<0.001).

In conclusion, in accordance with recent AFM data with monoclonal human aPL antibodies, plasmas from patients with aPL antibodies with thromboembolism reduce both A5 binding to phospholipid and A5 anticoagulant activity. This “annexin A5 resistance” identifies a novel mechanism for thrombosis in the aPL syndrome.
INTRODUCTION

The antiphospholipid (aPL) syndrome is a disorder in which thrombosis and recurrent pregnancy losses are associated with the presence of antibodies directed against anionic phospholipids and putative cofactors.\textsuperscript{1,2} The pathophysiologic mechanism(s) for thrombosis in this disorder has (have) not yet been established (for a recent review see \textsuperscript{3}).

Annexin A5 (previously known as annexin-V) is a potent anticoagulant protein whose anticoagulant properties are a consequence of its high affinity for anionic phospholipid.\textsuperscript{4,5} The protein forms 2-dimensional crystals on phospholipid surfaces\textsuperscript{6-8} that shield the phospholipids from availability for the phospholipid-dependent coagulation reactions.\textsuperscript{9} Annexin A5 is highly expressed by human endothelial cells\textsuperscript{10} and trophoblasts\textsuperscript{11} and is present on the surfaces of these cells.\textsuperscript{11,12} Annexin A5 is required for the maintenance of placental integrity in mice.\textsuperscript{13} Infusion of anti-annexin A5 IgG antibodies into pregnant animals decreased the availability of annexin A5 to bind to the trophoblast surfaces and caused placental thrombosis, necrosis, and fetal loss. However, it is interesting to note that a transgenic annexin A5-deficient mouse was found to be fertile.\textsuperscript{14}

We recently showed, through atomic force microscopic imaging, that aPL antibodies can disrupt the crystallization of annexin A5 over phospholipid membranes, expose underlying phospholipid membranes for availability for coagulation reactions, and thereby reduce the anticoagulant effects of annexin A5.\textsuperscript{15} These findings led us to investigate whether disruption of annexin A5 binding and reduction of its anticoagulant effect could be observed in plasmas from patients with aPL antibodies.
We therefore performed a small study with well-defined groups of patients to determine the effects of their plasmas on these parameters. Plasma-based assays were devised for this purpose and were validated with control plasmas spiked with purified aPL IgG and with plasmas from patients with the aPL syndrome. We then applied these assays to investigate plasmas from 3 well-defined groups – 1) patients with the aPL syndrome with histories for thromboembolic events, 2) patients with the aPL antibodies without previous histories of thrombosis or embolism, and 3) patients with thromboembolism without any evidence for aPL antibodies - compared to normal healthy control donors.

MATERIALS AND METHODS

Proteins and Antibodies

Annexin A5 was purified from human placentas according to the method of Funakoshi et al.4 Oregon green-conjugated annexin A5 (OG-annexin A5) (Molecular Probes, Inc., Eugene, Oregon, USA) was used for annexin A5 binding assays. Human \( \beta \)2GPI was purified as previously described.16 Human factor Xa, bovine factor Va and human prothrombin were generous gifts from Dr. Yale Nemerson (Division of Thrombosis, Mount Sinai School of Medicine, New York, NY). Recombinant human tissue factor (TF) (amino acids 1-243) was a gift from Genentech, Inc (San Francisco, CA, USA ). IgG antibodies were isolated from sera of three patients with severe antiphospholipid syndrome and three normal healthy subjects with protein G-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden), as previously described.17 A previously characterized monoclonal aPL antibody IS4, was derived from a patient with the primary aPL syndrome.18 The antibody is an IgG3 that recognized human \( \beta \)2GPI-cardiolipin complexes, human \( \beta \)2GPI alone and, to a small degree, cardiolipin alone.18 A
monoclonal IgG3 from a patient with a monoclonal gammopathy (Sigma, St. Louis, MO, USA) was used as its control.

**Plasma Specimens**

After Institutional Review Board approval, blood specimens were collected under informed consent from patients who were recruited through the Rheumatology and the High-Risk Thrombosis Clinics at Duke University Medical Center. The samples were prepared by double centrifugation. Lupus anticoagulants were identified using the criteria proposed by the Subcommittee on Lupus Anticoagulants/Antiphospholipid Antibodies of the International Society of Thrombosis and Haemostasis, as previously described. Anticardiolipin antibody IgG and IgM levels were determined by enzyme-linked immunosorbant assay using an in-house test developed in the Clinical Immunology lab in Duke University Medical Center, as described by Loizou and colleagues. Antiphospholipid antibody testing was performed on one or more occasions at a time when the patient was clinically stable. Repeat anticardiolipin antibody analysis was performed on all patient samples. The DRVVT/Confirm test was done with a commercially available method (Diagnostica Stago, Parsippany, NJ, USA). The PT and aPTT tests were run using an MDA-180 instrument (Organon Teknika, Research Triangle Park, NC, USA) with their standard reagents (Simplastin L and Platelin L, respectively).

Fifty-one patients with aPL antibodies were enrolled into the studies and were subclassified into two groups: 1) aPL syndrome with thromboembolism and 2) aPL antibodies without thrombosis history.
The aPL syndrome with thromboembolism group contained 25 patients. All of the patients satisfied the Sapporo investigational criteria for the diagnosis of the aPL syndrome. These patients (13 male, 12 female) had sustained one or more thromboembolic events three or more months prior to analysis. Eighteen patients sustained a venous thromboembolic event, four of whom also sustained an arterial thromboembolic event. Seven patients in this group sustained isolated thromboembolic cerebrovascular events (mean age, 37 yr; range, 18-49 yrs). With one exception, all of these patients were on oral anticoagulant therapy at the time of testing.

The aPL antibodies without thrombosis history group contained 26 patients with aPL antibodies who had not sustained a thromboembolic event (7 male, 19 female), although 4 of these patients had sustained one or more miscarriages. One of these 26 patients was on oral anticoagulant therapy for a prosthetic mitral valve.

Fifteen additional patients (7 male, 8 female) who sustained a thromboembolic event (14 venous, one arterial) but who had no evidence for an aPL antibody composed the non-aPL thromboembolism group. Of these 15 patients, 4 were heterozygous for factor V Leiden, 1 was heterozygous for the prothrombin G20210A polymorphism, and 1 was homozygous for the thermolabile variant of methylene tetrahydrofolate reductase. Fourteen of these patients were on oral anticoagulant therapy at the time of enrollment into the studies. In addition, thirty plasma specimens were obtained from normal blood bank donors at Mount Sinai Medical Center.

Annexin A5 Binding Studies

The annexin A5 binding and coagulation methods are outlined in Table 1. We first performed
quantitative assessment of effects of aPL IgG on the binding of annexin A5 to phospholipid by exposing the phospholipid to test plasma and then to labeled annexin A5 as previously described, with minor modifications. aPTT reagent-phospholipid (50 µl) (Actin FS) (Dade Behring Inc, Newark, DE, USA) was incubated with 50 µl of citrated plasma that was spiked with aPL or normal IgG (7.5 mg/ml) for 10 min at 25°C. The mixture of aPTT reagent-phospholipid and IgG-spiked plasma was sedimented with a microcentrifuge (Eppendorf Centrifuge 5417R, Brinkmann Instrument, Westbury, NY) for 15 min at 20,800g at 25°C. The IgG-spiked plasma-treated phospholipid pellets were washed 3x with HEPES buffer saline (HBS) (0.01M HEPES, 0.14M NaCl, pH 7.5) and resuspended in 50 µl of HBS containing 5 mM CaCl₂. OG-annexin A5 (32.5 ng) in the calcium-containing buffer (50 µl) was then added into the phospholipid suspension and incubated for 5 min at 25°C. After centrifugation as described above, the phospholipid pellets were resuspended in 100 µl of HBS containing 10 mM EDTA and sedimented with the microcentrifuge. The supernatant was removed to a microtiter plate (FluoroNunc, Fisher Scientific, Pittsburgh, PA) filled with 100 µl/well of HBS containing 10 mM EDTA and 0.05% BSA. The quantity of phospholipid-associated annexin A5 in the supernatant was then detected by measuring wavelengths of excitation at 485 nM and emission at 535 nm using a microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). The results of the assay were expressed as ng of labeled annexin A5 per aliquot (50 µl) of aPTT reagent-phospholipid (ng/aliquot of PL), as previously described.

We then determined whether depleting plasmas of IgG would alter the observed effect on annexin A5 binding. 120 µl of plasma from aPL patients or normal controls was incubated with 200 µl of protein G-Sepharose or Sepharose 2B (Amersham Pharmacia Biotech) overnight at
4°C. The beads were sedimented with the microcentrifuge for 10 min at 20,800g at 25°C and removed. The treatment with protein G-Sepharose does not leave any detectable IgG in the supernatants. aPTT reagent-phospholipid (50 µl) was incubated with 50 µl of protein G-Sepharose-pretreated plasma or Sepharose 2B-pretreated plasma and incubated for 10 min at 25°C. The mixture of aPTT reagent-phospholipid and Sepharose-pretreated plasma was sedimented with the microcentrifuge for 15 min at 20,800g at 25°C. The pellets were washed 3x with HBS and resuspended in 50 µl of 5 mM calcium-containing HBS. OG-annexin A5 (32.5 ng) in the calcium-containing buffer (50 µl) was then added to the suspension and incubated for 5 min at 25°C. After centrifugation for 15 min at 20,800g, the phospholipid pellets were resuspended in 100 µl of 10 mM EDTA containing HBS and then sedimented with the microcentrifuge. The phospholipid-associated annexin A5 that was released into the supernatant was then determined using the spectrofluorometer as described above.

The effect of the aPL plasma concentration on annexin A5 binding was also determined. Citrated plasmas from 2 different patients with the aPL syndrome were serially diluted in normal plasma at 1, 2, 5, 10, 20, 50 and 100 % ratios. 50 µl of aPTT reagent-phospholipid was incubated with 50 µl of the plasma for 10 min at 25°C. The mixture of aPTT reagent-phospholipid and the plasma was sedimented, washed and resuspended in 50 µl of the calcium containing HBS, followed by addition of 32.5 ng of OG-annexin A5 in the buffer (50 µl) as described above. After incubation and centrifugation, the phospholipid pellets were resuspended in 100 µl of 10 mM EDTA containing HBS and then sedimented, the phospholipid pellets were resuspended in 100 µl of the EDTA buffer and sedimantated, the phospholipid-associated annexin A5 was then detected using the spectrofluorometer as described above.
Annexin A5 Binding Assays for Patient Plasma Groups

Annexin A5 binding assays were performed for plasmas from the 3 groups of well-defined patients and normal healthy controls using phospholipid-coated plates. Microtiter plates (Nunc-Immuno Plate, MaxiSorp Surface) (Fisher Scientific, Pittsburgh, PA, USA) were coated with phospholipids composed of 50 µM of 30% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (PS) and 70% 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) (Avanti Polar-Lipids, Inc., Alabaster, AL, USA) as previously described. The composition of phospholipid that was used was identical to that used for the atomic force imaging studies of aPL antibodies and annexin A5. Citrated plasma (50 µl/well) was added and incubated for 10 min at 37°C. The plate was washed 3x with HBS. OG-annexin A5 (50 ng) in HBS containing 5 mM CaCl2 (50 µl) was then added and incubated for 90 min at 37°C. The plate was washed 3x with HBS containing 5 mM CaCl2 and 0.1% BSA, followed by 100 µl of 10 mM EDTA in HBS with 0.1% BSA. The quantity of annexin A5 dissociated from the phospholipid-coated wells was determined by the microplate spectrofluorometer as described above. The results were reported as ng/well. To determine the precision and accuracy of the annexin A5 binding assay, we tested a plasma from a normal control 15 times in the same test-run and 3 times over a period of 3 weeks. The coefficient of variation (C.V.) for the assay was between 2.6% and 4.5%, and the inter-assay C.V. was 3.7%.

Annexin A5 Resistance Studies

The effects of a previously characterized monoclonal aPL antibody IS4 on annexin A5 anticoagulant activity were measured using the prothrombinase reaction and then confirmed in plasma. Experiments were performed at room temperature. Microtiter plates were coated with 30% PS/70% PC as previously described. This phospholipid composition is identical to that
which had been used for the atomic force imaging studies\textsuperscript{15} and was validated in pilot studies performed with microtiter plates coated with variable proportions of PS/PC. HBS containing 0.1 % BSA (350 µl) was added to the wells and incubated for 60 min. The wells were then filled with 300 µl of HBS containing 1.25 mM CaCl\textsubscript{2} (a concentration that is in the physiologic range of free calcium ion concentration in plasma) and 0.1% BSA. aPL mAb (IS4) and an isotype-matched control mAb IgG3 (80 µg/ml) in triplicate, together with or without human β2GPI (15 µg/ml) and incubated for 30 min. The wells were washed 4x with the buffer, followed by addition of annexin A5 (7 µg/ml) in the buffer, and incubated for 60 min. After wash 4x with the buffer, factor Xa (final concentration 1.5 nM) and factor Va (1.5 nM) were added and incubated for 10 min. Prothrombin (34 nM) was then added to reach a total volume of 350 µl. The enzyme mixture in the well was serially aliquotted (50 µl/well) into another microtiter plate (DYNEX Technologies, Chantilly, VA) filled with 100 µl/well of bicine buffer (50 mM bicine, 0.1M NaCl, 0.1% BSA, 25 mM EDTA, pH 8.5). Chromogenic substrate S-2238 (Chromogenix, Biogenic France) was then added (0.57 mM). The thrombin generation rate (TGR) was measured using a SPECTRAmax Plus 384 microplate reader (Molecular Devices, Menlo Park, CA) at 405 nM and 490 nM dual beam at 35°C.

The effects of the aPL mAb on plasma coagulation and annexin A5 anticoagulant activity were studied as follows. Phospholipid vesicles, consisting of 30% PS and 70% PC, were incorporated with 1-243 TF using octyl glucoside as previously described.\textsuperscript{23,24} The aPL IS4 or control IgG3 mAb (50 µg/ml) were incubated with the relipidated TF (TF/PL, 3.05 nM tissue factor and 13.4 µM phospholipids) (a total of 100 µl) for 15 min at room temperature. HBS (150 µl) was added to increase the volume of the mixture to 250 µl. The mixture (50 µl) was then incubated with 50 µl of pooled normal plasma for 20 sec at 37°C in a ST4 Coagulation Instrument (American
Bioproducts Co. Parsippany, NJ), after which, 0.02 M CaCl₂ or 0.02 M CaCl₂ (50 µl) containing amnexin A5 (30 µg/ml) was added. Duplicate tests, with and without annexin A5 were done. The coagulation times were measured and the mean times of the duplicate tests were recorded. The anticoagulant activity of annexin A5 was expressed as the “annexin A5 anticoagulant ratio” as follows:

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\text{annexin A5 anticoagulant ratio} = \frac{\text{coagulation time in the presence of annexin A5}}{\text{coagulation time in the absence of annexin A5}} \times 100\%
\]

The effects of aPL IgGs on annexin A5 anticoagulant activity was then determined using a two stage method (Table 1), which exposed phospholipid to test plasma followed by an exposure to normal pooled plasma, as previously described, with modifications. One vial of lyophilized recombinant human TF (Innovin) (Dade Behring Inc) was reconstituted with 10 ml suspension of aPTT reagent (Actin FSL) (Dade Behring Inc). The mixture of TF and aPTT reagent-phospholipid was washed 2x with HBS by sedimentation and resuspension with the microcentrifuge for 15 min at 20,800g at 25°C. The pellet was resuspended in a total of 10 ml of HBS. 100 µl of this washed phospholipid of TF-aPTT reagent was incubated with 50 µl of normal plasma that was spiked with aPL or normal IgG to a final concentration of 7.5 mg/ml for 5 min at room temperature. The mixture of TF-aPTT phospholipid and IgG-spiked-plasma was sedimented with the microcentrifuge as described above. The pellets were washed 1x with HBS and resuspended in 220 µl of the buffer. The pretreated-phospholipid (50 µl) was then incubated with 50 µl of pooled normal plasma for 20 sec at 37°C in the ST4 Coagulation Instrument, which was then recalcified with 50 µl of 0.02 M CaCl₂ with and without annexin A5 (30 µg/ml), the annexin A5 anticoagulant ratios of the duplicate tests were determined as described above.
The effects of depleting aPL IgG from plasmas on annexin A5 anticoagulant activity were then investigated. Plasmas from aPL patients or normal controls were pretreated with protein G-Sepharose or Sepharose 2B as for the annexin A5 binding studies described above. 100 µl of the washed phospholipid of TF-aPTT reagent, prepared as described above, was incubated with 50 µl of protein G-Sepharose pretreated-plasma or Sepharose 2B-pretreated plasma for 5 min at room temperature. The mixtures were pelleted with the microcentrifuge, washed 1x in HBS and resuspended in 220 µl of the buffer. The phospholipid (50 µl) was then incubated with 50 µl of pooled normal plasma for 20 sec at 37°C in the ST4 Coagulation Instrument, which was then recalcified with 50 µl of 0.02 M CaCl₂, with and without added annexin A5 (30 µg/ml), the annexin A5 anticoagulant ratios were determined as described above.

To observe the effects of varying concentration of aPL plasma on annexin A5 anticoagulant activity, aPL plasma was serially diluted in a normal control plasma. 100 µl of the washed TF-aPTT reagent-phospholipid, described above, was incubated with 50 µl of the plasma for 5 min and the annexin A5 resistance was measured as described above.

**Annexin A5 Resistance Assays for Patient Plasma Groups**

Annexin A5 resistance assay for the plasmas from the 4 groups described above was tested using prothrombin time (PT) reagent (tissue factor-phospholipid complex) (Thromboplastin-C Plus, Baxter Diagnostics Inc, Deerfield, IL, USA). A vial of PT reagent was washed 2x with HBS by sedimentation and resuspension with the microcentrifuge as for TF-aPTT reagent-phospholipid described above. The pellet of the washed PT reagent-phospholipid was resuspended in 10 ml of
HBS; aliquots (50 µl) were then incubated with 50 µl of test plasma for 5 min at room temperature, washed and resuspended in 220 µl of HBS as described above. A portion of this (50 µl) was incubated with 50 µl of pooled normal plasma in the ST4 Coagulation Instrument, which was then recalcified with 50 µl of 0.02 M CaCl₂, with and without added annexin A5 (30 µg/ml), the annexin A5 anticoagulant ratio was determined as described above. The precision and accuracy of the annexin A5 resistance assay was determined in the same manner as described above for the annexin A5 binding assay. The intra-assay C.V. was between 2.0 % and 3.8 %, and the inter-assay C.V. was 2.7 %.

**Statistical Analysis**

Differences between aPL and control IgGs were assessed using unpaired two-tailed t test. Because of concern that the patient samples for the annexin A5 binding and resistance studies might have been drawn from non-normal distributions, pairwise comparisons of patient groups were assessed by Kruskal-Wallis test. Correlation coefficient (r) and 95% coefficient intervals between annexin A5 binding assay and annexin A5 resistance assay were evaluated by Spearman rank correlation within all groups. 95 % prediction intervals was generated by SigmaPlot software (SPSS Inc. Chicago, Illinois).

**RESULTS**

**Annexin A5 Binding Studies**

Quantitative assessment of annexin A5 binding to aPTT reagent-phospholipid showed that phospholipid preincubated with aPL IgG-spiked plasma bound significantly less labeled annexin A5 (mean±SD: 12.1±2.6 ng/aliquot of PL) than phospholipid preincubated with control IgG-
spiked plasma (16.7±0.7 ng/aliquot of PL, p<0.05) (n=3 pairs of different aPL and control IgGs) (Fig. 1A). In separate experiments, depletion of aPL IgG antibodies from plasmas by protein G-Sepharose significantly increased the binding of annexin A5 to phospholipid (8.4±2.5 ng/aliquot of PL) as compared to aPL plasmas pretreated with Sepharose 2B (5.8±2.4 ng/aliquot of PL, p<0.05) (Fig 1B); in control plasmas, there was no significant difference on the ability to bind annexin A5 between plasmas pretreated with protein G-Sepharose (9.0±1.3 ng) and plasmas pretreated with Sepharose 2B (10.1±1.9 ng) (n=10 different plasmas for each group) (Fig 1B). Dose-response studies on the effects of varying concentration of aPL plasmas on annexin A5 binding showed that the phospholipid-bound annexin A5 progressively decreased as the concentration of aPL plasma was increased in a normal plasma (Fig 1C).
**Figure 1B**

Bar chart showing the PL-Bound Annexin A5 levels in aPL plasma and control plasma after Sepharose 2B and Protein G pretreatment. The y-axis represents PL-Bound Annexin A5 (ng/ aliquot of PL), and the x-axis represents different plasma samples. The chart indicates significant differences with p-values of p<0.001 and p<0.05.

**Figure 1C**

Line graph showing the change in PL-Bound Annexin A5 levels as the percentage of aPL plasma in control plasma increases. The x-axis represents the percentage of aPL plasma in control plasma, and the y-axis represents PL-Bound Annexin A5 (ng/ aliquot of PL). The graph indicates a significant decrease in PL-Bound Annexin A5 levels as the percentage of aPL plasma increases.
Annexin A5 Binding Assays for Patient Plasma Groups

Annexin A5 binding assays for plasmas from the well-defined 3 groups of patients and normal healthy controls showed that phospholipid-coated wells preincubated with plasmas from the aPL syndrome with thromboembolism group bound significantly less annexin A5 (mean±SD: 26.7±4.3 ng/well, n=25, range 17.2-34.3 ng/well, median 27.3 ng/well) than wells preincubated with plasmas from normal control group (30.5±3.1 ng/well, n=20, range 26.1-35.9 ng/well, median 31.1 ng/well, p<0.01.), non-aPL thromboembolism group (29.9±3.2 ng/well, n=15, range 24.8-34.9 ng/well, median 29.3 ng/well, p<0.05) (Fig 2). Four patients with aPL antibodies without thrombosis had miscarriages; two of these showed annexin A5 binding levels that were near the mean and median of the controls, with values of 31.3 and 33.3 ng/well respectively, while other two had levels that were 24.7 and 25.8 ng/well, respectively. The wells pretreated with plasmas from the group of patients with aPL antibodies without thrombosis also bound significantly less annexin A5 (28.2±3.7 ng/well, n=26, range 20.7-33.8 ng/well, median 27.5 ng/well) than the normal healthy controls (p<0.05). There were no significant differences in the binding of annexin A5 between the wells pretreated with plasmas from the aPL syndrome with thromboembolism and the aPL antibodies without thrombosis groups, and plasmas from the non-aPL thromboembolism and the normal healthy controls (Fig 2).
Annexin A5 Resistance Studies

We examined the effects of a previously characterized monoclonal aPL antibody, IS4,\textsuperscript{18} on the anticoagulant activity of annexin A5 using the prothrombinase reaction. We then determined whether the aPL mAb-mediated blunting of annexin A5 anticoagulant activity also occurs in plasma. The addition of factor Xa, factor Va and prothrombin to PS/PC coated wells yielded a baseline thrombin generation rate (TGR) of 2,402±210 pM/min (mean±SD). Addition of 7 µg/ml (final concentration) of annexin A5 significantly reduced the TGR to 358±18 pM/min (p<0.0001) (Fig 3A). In the absence of annexin A5, the addition of aPL or control mAb alone to
the system showed no significant difference on the TGR (1,605±78 pM/min vs 1,431±236 pM/min); the addition of the aPL mAb together with β2GPI (15 µg/ml) showed a non-statistically significant reduction of the TGR compared to the control mAb (1,302±96 pM/min vs 1,900±384 pM/min). In contrast, in the presence of annexin A5, the aPL mAb significantly increased the TGR (713±87 pM/min) as compared to the control mAb (347±28 pM/min, p<0.01). The TGR was further increased by the addition of β2GPI (859±77 pM/min, compared to 421±64 pM/min for the control mAb, p<0.01). When β2GPI alone was added to the prothrombinase system, the TGR was 1,296±11 pM/min and was 478±18 pM/min for β2GPI added together with annexin A5 (p<0.0001) (Fig 3A).

The effects of the aPL mAb on blunting annexin A5 anticoagulant activity in the prothrombin system were confirmed in plasma. Addition of TF/PL which had been incubated with the aPL
mAb to plasma resulted in a coagulation time of 39.3±4.0 sec vs 28.0±1.2 sec (mean±SD) for TF/PL treated with control mAb (n=4, p<0.01). When the plasma was recalcified together with annexin A5, the coagulation time with the aPL mAb treated TF/PL was significantly less prolonged, to 62.9±5.6 sec, as compared to prolongation to 73.0±5.1 sec for the control IgG3 (n=4, p<0.05). Thus, compared to the control mAb, the aPL mAb significantly prolonged the coagulation time in the absence of annexin A5 but accelerated the coagulation time in the presence of annexin A5, thereby reducing the annexin A5 anticoagulant ratio to 156±12% vs 278±2.1% for the control mAb (n=4, p<0.0001).

We investigated whether aPL IgGs isolated from aPL patient sera might have an effect on annexin A5 anticoagulant activity by monitoring the coagulation times. We found that TF-aPTT reagent-phospholipid that had been preincubated with aPL IgG-spiked plasma significantly reduced annexin A5 anticoagulant activity, expressed as “annexin A5 anticoagulant ratios” (mean±SD: 143±11 %), as compared to the phospholipids preincubated with control IgG-spiked plasma (180±8 %, p=0.01) (n=3 pairs of different IgGs) (Fig 3B). In separate experiments, aPL plasmas that were depleted of IgG by pretreatment with protein G-Sepharose significantly increased annexin A5 anticoagulant activity (235±28 %) as compared to aPL plasmas that were pretreated with Sepharose 2B without protein G (193±48 %, p<0.05); in control plasmas, there was no significant difference on annexin A5 anticoagulant activity between plasmas pretreated with protein G-Sepharose (239±15 %) and plasmas pretreated with Sepharose 2B alone (250±12 %) (n=10 pairs of different aPL and control plasmas) (Fig 3C). Dose-response studies on the effects of varying concentration of aPL plasmas on annexin A5 anticoagulant activity showed
that the annexin A5 anticoagulant ratio progressively decreased as the concentration of aPL plasmas was increased in a normal plasma (Fig 3D).
Figure 3C

Sepharose 2B pretreatment
Protein G-Sepharose pretreatment
n=10 for each group

Annexin A5 Anticoagulant Ratio (%)

p<0.01
p=NS
p<0.05
p=NS

Figure 3D

% aPL Plasma in Control Plasma
Annexin A5 Resistance Assays for Patient Plasma groups

Annexin A5 resistance assays were performed for plasmas from the 3 groups of well-defined patients and normal healthy controls using PT reagent-phospholipid. Pilot studies to examine the influence of oral anticoagulant therapy on annexin A5 anticoagulant activity showed that there was no significant difference of annexin A5 anticoagulant ratio between phospholipid pretreated with plasmas from non-aPL patients who were on oral anticoagulant for prosthetic heart valves (mean±SD: 230±13 %, n=10) and plasmas from normal healthy controls (228±12 %, n=10). The assays for plasmas from the 4 groups described above showed that plasmas from the aPL syndrome with thromboembolism group had a significant reduced annexin A5 anticoagulant ratio (182±31 %, n=25, range 120-226 % median 187 %) compared to plasmas from the aPL antibodies without thrombosis group (210±35 %, n=26, range 112-250 %, median 220 %, p<0.01), non-aPL thromboembolism group (229±16 %, n=15, range 205-275 %, median 229 %, p<0.001), and the normal healthy control group (231±14 %, n=30, range 208-268 %, median 234 %, p<0.001) (Fig 4). Plasmas from the aPL antibodies without thrombosis group also showed a significantly reduced annexin A5 anticoagulant ratio as compared to the normal healthy control group (p<0.05) (Fig 4). Of the four patients with miscarriages in this group, the two who had the lower levels of annexin A5 binding also showed low annexin A5 anticoagulant ratios (137 and 178 % respectively), while the other two patients had levels (226 and 235 % respectively) that were near the mean and median values of the normal healthy controls. There was no significant difference between the non-aPL thromboembolism and normal healthy control groups (Fig 4).
Correlations

There was a weak, but statistically significant correlation between the quantity of phospholipid-bound annexin A5 and the annexin A5 anticoagulant ratio ($r=0.42$, $95\%$ confidence intervals: $0.23 – 0.59$, $p<0.0001$, $n=86$) (Fig 5).
DISCUSSION

There is significant evidence to support the hypothesis that aPL antibodies may promote thrombosis in the aPL syndrome by disruption of the anticoagulant annexin A5 shield. IgG fractions from patients with the aPL syndrome reduce the quantity of phospholipid-bound annexin A5 on the apical surfaces of cultured trophoblasts and endothelial cells, and accelerate coagulation of plasma added to these cells.\textsuperscript{12} IgG fractions from aPL patients reduce annexin A5 on non-cellular phospholipid surfaces\textsuperscript{17,25} and accelerate plasma coagulation and thrombin
generation.\textsuperscript{17,26} Sera from aPL patients reduce the quantity of annexin A5 on platelets.\textsuperscript{27} We recently provided morphologic evidence with atomic force microscopy that human monoclonal aPL antibodies disrupt the ordered crystallization of annexin A5 and expose phospholipid for availability for coagulation reactions, and showed that the antibodies accelerate thrombin generation.\textsuperscript{15}

In the present study, using fluorescence-labeled annexin A5, we demonstrated that plasmas which had been spiked with purified IgG fractions from patients with the aPL syndrome reduce annexin A5 binding to phospholipid; the reduction of annexin A5 binding is mediated by aPL IgG as evidenced by the effects of depleting IgG from plasmas with protein G-Sepharose. This reduction occurs in aPL plasmas in a concentration-dependent manner. Measurement of the effects upon the annexin A5 binding for the patient groups showed a statistically significant reduction of annexin A5 using plasmas from patients with a clinical history for the aPL syndrome with thrombosis compared to normal healthy controls and the non-aPL thromboembolism patients. We also showed that an aPL mAb blunted the anticoagulant effect of annexin A5 on a purified prothrombinase reaction, and that this effect could also be demonstrated in plasma by measuring coagulation times with tissue factor-phospholipid complex that had been exposed to the aPL mAb. It is interesting that this mAb reduced the anticoagulant effect of annexin A5 even in the absence of β2GPI - an effect that had been described for some other aPL mAbs.\textsuperscript{15} It is also interesting to note that although this aPL mAb was not previously found to have lupus anticoagulant activity in a dilute Russell’s viper venom time (DRVVT) assay,\textsuperscript{18} it did prolong tissue factor-mediated coagulation in this system.
In order to assay patient samples for resistance to annexin A5 anticoagulant activity, we designed a two-stage coagulation assay that uses pooled normal plasma for the coagulation phase of the test. This was done in order to address the variable baseline coagulation times of individual patients. Plasmas spiked with aPL IgGs reduce the anticoagulant activity of annexin A5 on the coagulation time. The reduction of annexin A5 anticoagulation activity is mediated by aPL IgG as evidenced by loss of this effect after plasmas were depleted of IgG by protein G-Sepharose. This effect occurs in a manner that is dependent upon the concentration of aPL plasma. Measurements of the effects of plasmas upon the anticoagulant activity of annexin A5 showed a statistically significant reduction with plasmas from patients with a clinical history for the aPL syndrome with thrombosis. A remarkable aspect of this study, that stands in contrast to the paradoxical “lupus anticoagulant” effect, is the aPL antibody-mediated acceleration of coagulation that occurs in this system containing annexin A5.

We observed that the differences between the aPL syndrome group and the other groups were more significant with the annexin A5 resistance assay than the binding assay. Remarkably, 18 out of the 25 aPL syndrome patients had annexin A5 anticoagulant ratios that were lower than the lowest measurements obtained from the normal healthy controls and the non-aPL thromboembolism group and also below 2 standard deviation of the mean of the normal healthy controls. As to the difference in annexin A5 results between the symptomatic and asymptomatic groups of aPL patients, we hypothesize that reduced annexin A5 binding and anticoagulant activity reflect increased antibody avidity. However, future studies will be needed to investigate this. Interestingly, 6 of the 26 patients with aPL antibodies, but without a thrombosis history also had similar low levels of annexin A5 anticoagulant ratios. We speculate that these asymptomatic
individuals may be at high risk for thrombosis. Future studies would be needed to address this possibility. We also speculate that the coagulation assay reports the quantity of unshielded phospholipid available for coagulation reactions more precisely than does the annexin A5 binding assay.

These are model systems that, like all model systems are subject to limitations. It may be argued that the sequence of addition, with plasma containing the antibodies added before the annexin A5, and the absence of calcium in the first plasma incubation favor the competition of the antibodies over the annexin A5. This sequence and was necessary on practical grounds since a first incubation with annexin A5 would require calcium concentrations that would already clot the patient plasma used for the first stage and not enable us to do a second stage with the pooled normal plasma. We previously addressed the question of order of addition in the atomic force imaging study, where we found that either order of addition (annexin A5 first or antibodies cofactors first) resulted in disruption of annexin A5 crystallization, and where the effect occurred in the presence of a physiologic concentration of calcium.\textsuperscript{15}

Analysis of the relationship between annexin A5 binding assays and annexin A5 resistances assays showed a weak, but statistically significant, positive correlation between these two assays. Larger studies are planned to further evaluate the relationships between these types of assays and clinical conditions. It was recently reported that annexin A5 levels are increased in the plasmas of patients with SLE and primary antiphospholipid syndrome compared to normal healthy controls but that this was not correlated with thrombosis,\textsuperscript{28} however, conclusions about the displacement of annexin A5 cannot be drawn from those data since neither annexin A5 binding
nor anticoagulant activity were measured in those patients.

The data presented here demonstrate that aPL antibody-mediated blunting of annexin A5 binding to phospholipid and of the anticoagulant activity of annexin A5 can be detected in plasmas from aPL patients. These data are consistent with the hypothesis that disruption of the annexin A5 antithrombotic shield is a mechanisms for thrombosis in the aPL syndrome. Also, our data indicate that the two types of assays – the annexin A5 binding assay and the annexin A5 resistance assay – may identify patients who are at risk for thrombosis with aPL syndrome. Although one group had previously concluded through ellipsometry that aPL antibodies “unambiguously” do not displace annexin A5,\textsuperscript{29} the current data with patient plasmas extend the atomic force microscopic imaging data with human aPL mAbs\textsuperscript{15} to support the conclusion that aPL antibodies can indeed interfere with annexin A5 crystallization and with its anticoagulant activity. This concept is also supported by the work of others using phospholipid-coated microtiter plates\textsuperscript{25} and freeze-thawed platelets.\textsuperscript{27}

Several other mechanisms have been described to explain thrombosis in the aPL syndrome (for recent review see \textsuperscript{2,30}. Among these are mechanisms in which the antibodies have been proposed to interfere with endogenous anticoagulant mechanisms, such as the protein C-protein S system \textsuperscript{31-33} and tissue factor pathway inhibitor, \textsuperscript{34} and also mechanisms in which the antibodies promote the expression of proinflammatory and pro-adhesive phenotypes such as stimulation of tissue factor expression and cell adhesion molecules.\textsuperscript{30,35}
While this study provides data that support a role for aPL antibodies in disrupting annexin A5 binding and anticoagulant activity, it does not establish whether this mechanism occurs in vivo. It remains possible that antibody-mediated interference with annexin A5 binding and anticoagulant activity correlate with thrombosis in the aPL syndrome because they are surrogate markers for high avidity antibodies that actually have other pathologic effects. At this time, one can only speculate about whether one, or several, of the proposed thrombogenic mechanisms acting in concert are involved in the pathophysiology of the aPL syndrome. In any event, this type of study that translates a basic science observation with monoclonal antibodies, purified proteins and phospholipids into clinical assays using blood from small groups of well-defined patients is a significant step in further defining the mechanism(s) of this syndrome in humans.

In conclusion, we have demonstrated that plasmas from patients with the aPL syndrome show inhibition of annexin A5 binding and resistance to annexin A5 anticoagulant activity. These results are in accordance with recent atomic force imaging results with human monoclonal aPL antibodies. The inhibition of annexin A5 binding and the resistance to its anticoagulant activity are attributable to the aPL antibodies in plasma and stand in contrast to the paradoxical “lupus anticoagulant” effect that has been extensively described. Plasma tests for reduction of annexin A5 binding and resistance to annexin A5 anticoagulant activity correlate with thrombosis in the aPL syndrome and appear to identify a pathophysiologically relevant mechanism for thrombosis in these patients.

ACKNOWLEDGMENTS
We thank Ms. Karen Moore for performing the anticardiolipin assays, and Ms. Elizabeth Thames
for patient identification and recruitment. This paper is dedicated to the memory of Dr. Peter C. Harpel whose support contributed to the development of this work.
Table 1. Schematic of Annexin A5 Binding and Resistance Assays

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<th>Annexin A5 binding assay</th>
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<tr>
<td>Incubate phospholipid with test sample</td>
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<tr>
<td>Wash phospholipid with HEPES buffered saline (HBS)</td>
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<tr>
<td>Incubate the phospholipid with labeled annexin A5 in HBS containing CaCl₂</td>
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<td>Wash the phospholipid with HBS containing CaCl₂</td>
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<td>Add HBS containing EDTA to the phospholipids to dissociate phospholipid-bound annexin A5</td>
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<td>Fluorimetry with excitation 485 nM and emission 535 nm</td>
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<td>Expressed results as ng/aliquot of PL or ng/well</td>
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<table>
<thead>
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<th>Annexin A5 Resistance assay</th>
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<tr>
<td>Incubate phospholipid with test sample</td>
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<tr>
<td>↓</td>
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<tr>
<td>Wash phospholipid with HBS</td>
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<tr>
<td>Incubate the phospholipids with pooled normal plasma at 37°C</td>
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<tr>
<td>Recalcify the plasma with 0.02 M CaCl₂ or 0.02 M CaCl₂ containing annexin A5</td>
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<tr>
<td>Measure the coagulation times in the presence and absence of annexin A5</td>
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<td>Express results as “annexin A5 anticoagulant ratio”</td>
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LEGENDS

Figure 1. Annexin A5 binding studies.

A) Effects of spiking plasmas with aPL IgG on annexin A5 binding to phospholipid.
aPL or control IgG was added to a normal plasma to a final concentration of 7.5 mg/ml and incubated with aPTT reagent-phospholipid. The phospholipid preincubated with aPL IgG-spiked plasma bound significantly less labeled annexin A5 (mean±SD: 12.1±2.6 ng/aliquot of PL) compared to the phospholipid preincubated with control IgG-spiked plasma (16.7±0.7 ng/aliquot of PL, p<0.05). (n=3 pairs of different IgG).

B) Effects of depleting aPL IgG from plasmas on annexin A5 binding.
aPL plasmas that were pretreated with protein G-Sepharose significantly increased the ability of annexin A5 binding to phospholipid (mean±SD: 8.4±2.5 ng/aliquot of PL) compared to aPL plasmas preincubated with Sepharose 2B (5.8±2.4 ng/aliquot of PL, p<0.05). In control plasmas, no significant difference on the binding of annexin A5 was observed between plasmas preincubated with protein G-Sepharose (9.0±1.3 ng) and plasmas pretreated with Sepharose 2B (10.1±1.9 ng) (n=10 plasmas for each group).

C) Effects of varying concentration of aPL plasmas on annexin A5 binding.
aPL plasmas were serially diluted in a normal plasma and incubated with aPTT reagent-phospholipid. The quantity of annexin A5 bound to phospholipid decreased progressively as the concentration of aPL plasma, diluted in a normal control plasma, was increased (each point shows the mean of 2 aPL patient plasmas)

Figure 2. Annexin A5 binding assays for patient plasma groups.
PS/PC coated wells that had been preincubated with plasmas from the aPL syndrome with
thromboembolism group (A) bound significantly less annexin A5 (mean±SD: 26.7±4.3 ng/well, n=25) than wells preincubated with plasmas from normal healthy control group (D) (30.5±3.1 ng/well, n=20, p<0.01) and plasmas from non-aPL thromboembolism group (C) (29.9±3.2 ng/well, n=15, p<0.05). Wells pretreated with plasmas from the aPL antibodies without thrombosis group (B) also bound significantly less annexin A5 (28.2±3.7 ng/well, n=26) than the normal healthy controls (D) (p<0.05). There were no significant differences in the binding of annexin A5 between the wells pretreated with plasmas from the aPL syndrome with thromboembolism (A) and the aPL antibodies without thrombosis (B) groups, and plasmas from the non-aPL thromboembolism (C) and the normal healthy controls (D). Error bars are shown for mean ±2SD of normal healthy controls (D).

Figure 3. Annexin A5 resistance studies.

A) Effect of aPL mAb on inhibition of prothrombinase reaction by annexin A5.
Factor Xa, factor Va and prothrombin added to the PL bilayer yielded a baseline thrombin generation rate (TGR) of 2,402±210 pM/min (mean±SD). Addition of annexin A5 significantly reduced the TGR to 358±18 pM/min (p<0.0001). Addition of the aPL mAb to the system containing the annexin A5, reversed the anticoagulant effect of annexin A5 and increased the TGR (713±87 pM/min as compared to 347±28 pM/min for the control mAb p<0.01), a reversal that was further enhanced by the addition of β2GPI. (859±77 pM/min compared to 421±64 pM/min for the control mAb, p<0.01).

B) Effects of spiking plasma with aPL IgG on annexin A5 anticoagulant activity.
aPL or control IgG was added to a normal plasma. The annexin A5 anticoagulant ratio for the
phospholipid preincubated with aPL IgG and β2GPI was significantly less (mean±SD: 143±11 %) than the phospholipid preincubated with control IgG and β2GPI (180±8 %, p=0.01). (n=3 pairs of different IgG).

C) Effects of depleting aPL IgG from aPL plasmas on annexin A5 anticoagulant activity.
aPL plasmas that were pretreated with protein G-Sepharose significantly increased annexin A5 anticoagulant ratios (mean±SD: 235±28 %) compared to plasmas pretreated with Sepharose 2B (193±48 %, p<0.05). In control plasmas, no significant difference on annexin A5 anticoagulant activity was observed between plasmas pretreated with protein G-Sepharose (239±15 %) and plasmas pretreated with Sepharose 2B (250±12 %). (n=10 pairs of aPL and control plasmas)

D) Effects of varying aPL plasma concentration on annexin A5 anticoagulant activity.
Varying dilutions of aPL plasma in control plasma were incubated with TF-aPTT reagent-phospholipid. The annexin A5 anticoagulant ratio progressively decreased as the concentration of aPL plasma was increased in control plasma. (Each point is the mean of 2 aPL patient plasmas)

Figure 4. Annexin A5 resistance assays for patient plasma groups.
The annexin A5 anticoagulant ratio for the aPL syndrome with thromboembolism group (A) was significantly decreased (mean±SD: 182±31 %, n=25) as compared to the aPL antibodies without thrombosis history group (B) (210±35 %, n=26, p<0.01), the non-aPL thromboembolism group (C) (229±16 %, n=15, p<0.001), and the normal healthy control group (D) (231±14%, n=30, p<0.001). The ratio for the plasmas from the aPL antibodies without thrombosis history group (B) also significantly reduced as compared to the normal healthy control group (D) (p<0.05). There were no significant differences in annexin A5 anticoagulant ratio for the non-aPL
thromboembolism group (C) as compared to the aPL antibodies without thrombosis history group (B) and the normal healthy control group (D). Error bars are shown for mean ±2SD of normal healthy controls (D).

**Figure 5. Correlation analysis for annexin A5 resistance and annexin A5 binding assays.**

There was a weak, but statistically significant correlation between the quantity of PL-bound annexin A5 and the annexin A5 anticoagulant ratio (r=0.42, p<0.0001, n=86).


Detection of Antibody-Mediated Reduction of Annexin A5 Anticoagulant Activity in Plasmas of Patients with the Antiphospholipid Syndrome

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