Competition of Annexin V and Anticardiolipin Antibodies for Binding to Phosphatidylserine Containing Membranes

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ABSTRACT: Annexin V, an intracellular protein with a calcium-dependent high affinity for anionic phospholipid membranes, acts as an inhibitor of lipid-dependent reactions of the blood coagulation. Antiphospholipid antibodies found in the plasma of patients with antiphospholipid syndrome generally do not interact with phospholipid membranes directly, but recognize (plasma) proteins associated with lipid membranes, mostly prothrombin or β_2-glycoprotein I (β_2GPI). Previously, it has been proposed that antiphospholipid antibodies may cause thrombosis by displacing annexin V from procoagulant cell surfaces. We used ellipsometry to study the binding of annexin V and of complexes of β_2GPI with patient-derived IgG antibodies to β_2GPI, commonly referred to as anticardiolipin antibodies (ACA), to phospholipid bilayers composed of phosphatidylcholine (PC) and 20% phosphatidylserine (PS). More specifically, we investigated the competition of these proteins for the binding sites at these bilayers. We show that ACA−β_2GPI complexes, adsorbed to PSPC bilayers, are displaced for more than 70% by annexin V and that annexin V binding is unaffected by the presence of ACA−β_2GPI complexes. Conversely, annexin V preadsorbed to these bilayers completely prevents adsorption of ACA−β_2GPI complexes, and none of the preadsorbed annexin V is displaced by ACA−β_2GPI complexes. Using ellipsometry, we also studied the effect of ACA−β_2GPI complexes on the interaction of annexin V with the membranes of ionophore-activated blood platelets as a more physiological relevant model of cell membranes. The experiments with blood platelets confirm the high-affinity binding of annexin V to these membranes and unequivocally show that annexin V binding is unaffected by the presence of ACA−β_2GPI. In conclusion, our data unambiguously show that ACA−β_2GPI complexes are unable to displace annexin V from procoagulant membranes to any significant extent, whereas annexin V does displace the majority of preadsorbed ACA−β_2GPI complexes from these membranes.

The antiphospholipid syndrome (APS)[1] is associated with an increased risk of thrombosis and a substantially increased risk of fetal loss (reviewed in refs *1*−*4*), commonly thought to be related to placental thrombosis. The antiphospholipid antibodies present in the plasma of these patients are generally thought to be linked to these thrombotic conditions and are detected in the patient's plasmas either by solid-phase immuno assays or by their ability to inhibit phospholipid-dependent blood coagulation tests (*5, 6*).

An increasing number of studies have shown that these antibodies are directed against lipid-binding plasma proteins rather than against lipid membranes per se. Although other plasma proteins have been implicated (*7*−*11*), it appears that prothrombin and β_2GPI are the most frequently observed antigens (*12*−*15*). Anticardiolipin antibodies (ACA) isolated from APS patients by liposomal adsorption, representing an IgG fraction highly enriched in anti-β_2GPI antibodies, have been shown to display low affinity (K_d in the micromolar range) for β_2GPI in solution (*16*). The high affinity found for binding to lipid-associated β_2GPI was demonstrated to be caused by divalent interaction (*16, 17*).

Annexin V is an intracellular protein, abundant in endothelial cells, with a calcium-dependent, high affinity for negatively charged phospholipid membranes (*18*−*21*). As anticipated from its high-affinity binding, annexin V has the potency to interfere with the binding of blood coagulation factors to procoagulant membranes (*22*), which explains the strong anticoagulant activity of this protein in several lipid-dependent coagulation assays (*18, 19, 23*−*25*). The physiological role of annexin V remains as yet unclear, although a role as anticoagulant (*19, 23, 24*), as modulator of phospholipase activity (*26*), and in membrane-dependent inflammation reactions (*27*) has been proposed.

The thrombotic tendency observed in patients with antiphospholipid antibodies at first sight seems paradoxical in view of the anticoagulant effects of these antibodies reported for in vitro tests. However, both procoagulant and antico-

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[1] Abbreviations: ACA, anticardiolipin antibody; APS, antiphospholipid syndrome; β_2GPI, β_2-glycoprotein I; PS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid.

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agulant reactions of the blood coagulation system depend on membranes containing negatively charged phospholipids, and antiphospholipid antibodies may equally well interfere with the anticoagulant reactions (*7*, *28*−*31*). In addition, alternative procoagulant effects of antiphospholipid antibodies have been proposed, such as enhanced Factor Xa generation (*32*, *33*), and induction of tissue factor expression and procoagulant activity in endothelium, monocytes, and blood platelets (*34*−*37*).

Recently, a new mechanism for the thrombotic action of antiphospholipid antibodies was proposed. It was argued that annexin V binding to membranes is essential to shield the procoagulant sites present at cell membranes and that these antibodies, by virtue of their high affinity for phospholipids, are able to displace membrane-bound annexin V. Implicitly, it was assumed that the antiphospholipid antibodies, despite their high affinity, leave enough free sites at the membrane for interaction with blood coagulation factors (*38*−*42*).

In the present study, we compared the binding of anticardiolipin antibodies purified from patients with APS, and annexin V to PS containing planar bilayers composed of 20% DOPS and 80% DOPC, using ellipsometry to measure the protein binding. In addition, we used ellipsometry to assess the effect of ACA−β_2GPI complexes on the binding of annexin V to membranes of activated blood platelets. Our findings are at variance with the reports of Rand et al. and Vogt et al. (*39*, *41*, *42*) and show that annexin V cannot be displaced by ACA−β_2GPI complexes. In contrast, we show that preadsorbed annexin V prevents any adsorption of ACA−β_2GPI complexes.

METHODS

Materials. Bovine serum albumin (BSA, essentially fatty acid free) was from Sigma (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-dioleoyl-*sn*-glycero-3-L-phosphoserine (PS) were from Avanti Polar Lipids (Alabaster, AL). Silicon slides were obtained from Aurel GmbH (Landsberg, Germany). Human β_2GPI was purified according to the procedure of ref *43*, as described earlier (*16*). Polyclonal antibodies against human β_2GPI were raised in rabbits: 0.5 mg of purified human β_2GPI in complete Freund's adjuvant was injected intradermally. At 2-week intervals, boosts of 0.25 mg were given in incomplete Freund's adjuvant, and the rabbits were bled 2 weeks after the last injection. IgG was purified from the immune serum using affinity chromatography over protein A−Sepharose. Recombinant annexin V, prepared as described (*44*), was a kind gift of Dr. Reutelingsperger.

Patients. This study included antiphospholipid IgG preparations isolated from the plasma of six APS patients. All patient plasmas were positive for both ACA and lupus anticoagulant (LA). Five out of six patients had at least one thromboembolic event; one patient had rheumatoid arthritis and thrombocytopenia. None of the patients met the revised criteria of the American Rheumatism Association for the diagnosis of Systemic Lupus Erythematosus (*45*).

Isolation of IgG and of ACA. Total IgG was isolated from patient plasma or rabbit serum by affinity chromatography over protein A−Sepharose. Anticardiolipin antibodies (ACA) were purified by adsorption of patient plasma to cardiolipin-containing liposomes and subsequent affinity chromatography over protein A−Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in the presence of *n*-octyl β-D-glucopyranoside to disrupt the liposomes, as described earlier (*12*). The final preparation was run over an anti-β_2GPI affinity column, to ensure that ACA was free of β_2GPI contamination. Purity was checked by SDS−PAGE, performed in 10−15% gradient gels using a Phast system from Pharmacia (Upsala, Sweden).

Isolation and Ionophore Treatment of Platelets. Blood from healthy volunteers was collected on ACD, and washed platelets were isolated by differential centrifugation as described before (*46*). Platelets were resuspended in Hepes buffer, composed of 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose, 1 mg/mL human serum albumin, adjusted to pH 7.4. Platelets, at a concentration of 3×10^8 mL^{-1}, were activated with ionomycin (2.5 μM) in the presence of 3 mM $CaCl_2$ for 15 min at 37 °C.

Ellipsometric Determination of Protein Adsorption to Lipid Bilayers. Protein adsorption to planar bilayers was measured by ellipsometry (*16*). Briefly, planar bilayers deposited on silicon slides were prepared as described previously (*47*) by immersion of the hydrophilic silicon slide for 5 min in a stirred suspension of small unilamellar lipid vesicles (30 μM) in Tris buffer (50 mM Tris, 120 mM NaCl, 3 mM $CaCl_2$, pH 7.5). The instrument and data analysis have been described earlier (*48*, *49*). Measurements were performed using silicon slides at an angle of incidence of the light beam (HeNe laser) of 68° (*50*). Experiments were performed at ambient temperature (20−22 °C) under continuous stirring in a trapezoidal cuvette in Tris buffer (50 mM Tris, 120 mM NaCl, 0.5 mg·mL^{-1} bovine serum albumin, pH 7.5) containing 3 mM $CaCl_2$ unless indicated otherwise.

Measurement of Annexin V Binding to Blood Platelets. Ionomycin-treated blood platelets, $(0-2) \times 10^7$ platelets·mL^{-1}, were incubated with 75 nM annexin V in 1.1 mL of Tris-HCl buffer (50 mM Tris, 120 mM NaCl, 3 mM $CaCl_2$, 0.5 mg·mL^{-1} BSA, pH 7.5) for 20 min. Subsequently, the platelets were sedimented at 20000*g* for 25 min. To estimate the concentration of unbound annexin V, 1 mL of supernatant was added to the ellipsometer cuvette, containing 4 mL of buffer, and the adsorption to a planar lipid bilayer composed of 20% PS and 80% PC was measured. The effect of ACA and β_2GPI upon the annexin V adsorption to activated platelets was assessed in similar experiments, in which $(1.6-2) \times 10^7$ blood platelets·mL^{-1} were incubated with 75 nM annexin V for 20 min followed by a 45 min incubation in the presence of ACA (50 μg·mL^{-1}) and β_2GPI (1 μM). Unbound annexin V in the supernatant was again estimated by ellipsometry from the adsorption to a planar PSPC bilayer.

RESULTS

Figure 1 shows the adsorption of annexin V to PSPC phospholipid bilayers containing 20% PS. The left panel, Figure 1A, shows the time-dependent protein adsorption for several annexin V concentrations (3, 7.5, 30, and 150 nM, lower to upper curve). The initial rate of adsorption is proportional to the concentration of annexin V added to the cuvette. The final equilibrium adsorption, however, appears to depend only weakly on the annexin V concentration, as the adsorption amounts from $\Gamma = 0.165$ to 0.190 μg·cm^{-2}

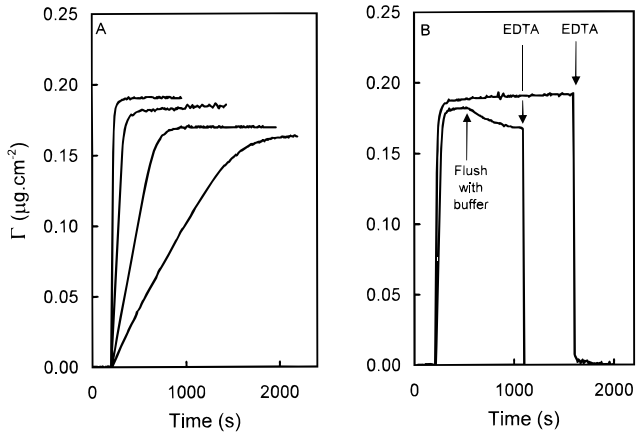

FIGURE 1: Annexin V binding to PSPC bilayers containing 20 mol % PS. The mass, Γ (μg·cm^{-2}), of adsorbed protein, measured by ellipsometry, is plotted as a function of time. (A) At $t = 200$ s, annexin V (3, 7.5, 30, or 150 nM, lower to upper curve) was added to the cuvette, and the adsorption was monitored by ellipsometry. (B) The upper curve shows the adsorption of annexin V (150 nM), added at 200 s, followed by addition of EDTA (4 mM) at 1600 s to deplete Ca^{2+} ions from the buffer. The lower curve shows the adsorption of 60 nM annexin V, added at 200 s. Only a minor desorption is observed when at $t = 500$ s the contents of the cuvette were flushed with 50 mL of buffer (in order to deplete annexin V from the cuvette solution). During desorption, the cuvette solution was refreshed with buffer at a rate of 1 mL·min^{-1} to prevent accumulation of desorbed protein. At 1100 s, EDTA (4 mM) was added to deplete calcium. Experiments were performed at ambient temperature (20−22 °C) in Tris-HCl buffer (pH 7.5, 120 mM NaCl, 50 mM Tris, 3 mM $CaCl_2$) containing 0.5 mg·mL^{-1} BSA.

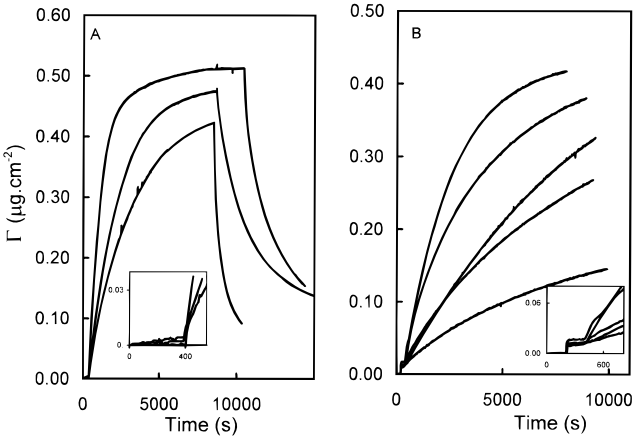

FIGURE 2: β_2GPI-dependent adsorption of anticardiolipin antibodies. (A) At $t = 200$ s, ACA (2.5, 5, or 10 μg·mL^{-1}, lower to upper curve) was added to the cuvette, followed by 100 nM β_2GPI at 400 s. After approximately 3 h, equilibrium adsorption was attained, and unbound ACA and β_2GPI were depleted from the cuvette buffer by rapidly (5−10 s) flushing with buffer (50 mL). The inset shows the initial phase of the adsorption. Accumulation of desorbed protein in the buffer was prevented by refreshing the cuvette buffer at a rate of 1 mL·min^{-1}. (B) First, β_2GPI (100 nM at $t = 200$ s) was added to the cuvette and adsorption was followed until equilibrium. Subsequently, at $t = 400$ s, 5 μg·mL^{-1} ACA was added: upper to lower curves correspond to preparations of 5 different patients. The inset shows the initial phase of the adsorption. The experimental conditions are as described for Figure 1.

for annexin V concentrations of 3 and 150 nM, respectively. Even an annexin V concentration as low as 1 nM ultimately results in binding of 0.156 μg·cm^{-2} (data not shown). This independence of the equilibrium adsorption of annexin V on the concentration of annexin V indicates an extremely high affinity of this protein for PSPC membranes containing 20% PS. Analysis of binding data using the Langmuir model resulted in estimations of the maximal binding, $\Gamma_{max} = 0.193$ μg·cm^{-2}, and of the dissociation constant, $K_d = 0.15$ nM, in agreement with earlier reports (*20*, *21*, *51*). The right panel, Figure 1B, shows that depletion of annexin V from the cuvette solution only results in a rather slow and limited desorption. At equilibrium, the desorption amounts to less than 10−15% of the original adsorption. This desorption pattern is consistent with the high-affinity binding of annexin V. Figure 1B also shows that the binding of annexin V to lipid membranes is instantaneously and completely reversed upon calcium depletion by addition of EDTA.

As the aim of our study is to compare the binding of annexin V and of ACA−β_2GPI complexes under physiological relevant conditions, we have chosen to perform all experiments at 120 nM NaCl and 3 mM $CaCl_2$. Earlier findings (*16*) have shown that the presence of calcium results in decreased affinity of β_2GPI for PSPC. Therefore, the experiments were carried out using sufficiently high β_2GPI concentrations (100 nM or higher) in order to approach saturation of the ACA−β_2GPI binding with respect to β_2GPI.

Figure 2 shows the β_2GPI-dependent binding of ACA to PSPC bilayers containing 20% PS. Panel A demonstrates that ACA alone does not cause any measurable adsorption; upon addition of ACA (2.5, 5, or 10 μg·mL^{-1}) at $t = 200$ s, the protein adsorption remains below 0.003 μg·cm^{-2}. Only after addition of β_2GPI (100nM at $t = 400$ s) a significant adsorption of protein is detectable (see inset). Total IgG collected from control subjects did not cause any enhancement of the β_2GPI adsorption nor did it result in a measurable adsorption in the absence of β_2GPI (data not shown). The rate of adsorption increases with increasing concentrations of ACA. The final adsorption is nearly saturated (maximal) at ACA levels exceeding 2.5 μg·mL^{-1}. Protein depletion by flushing with buffer results in rapid desorption of the ACA−β_2GPI complexes. This desorption, however, appears to be incomplete. Exponential analysis of the desorption curves indicates that an amount of 0.06−0.11 μg·cm^{-2} of ACA−β_2GPI complexes remains virtually irreversibly bound to the phospholipid bilayer. The experiments shown in Figure 2A were performed with ACA isolated from patient Gh. Panel B of Figure 2 shows β_2GPI-dependent adsorption of ACA of five other patients. Adsorption was initiated by addition of β_2GPI (100 nM) at 200 s, which results in a very small but measurable adsorption of about 0.01 μg·cm^{-2}, as shown earlier (*16*). This minor β_2GPI adsorption is apparently sufficient to mediate a massive (up to 0.50 μg·cm^{-2}) binding of anti-β_2GPI−β_2GPI complexes upon addition of ACA (5 μg·mL^{-1} at $t = 400$ s). No significant adsorption was observed in the absence of β_2GPI (data not shown). It is obvious from Figure 2B that widely different adsorption rates are observed for the various preparations, most likely reflecting differences between the patient plasmas with respect to the concentration and/or affinity of the anti-β_2-GPI antibodies. In a previous study (*16*) we found that, although ACA preparations are enriched in anti-β_2GPI activity compared to plasma IgG, nevertheless only a fraction of the ACA−IgG preparation possesses anti-β_2GPI activity, supporting the notion that differences in anti-β_2GPI content

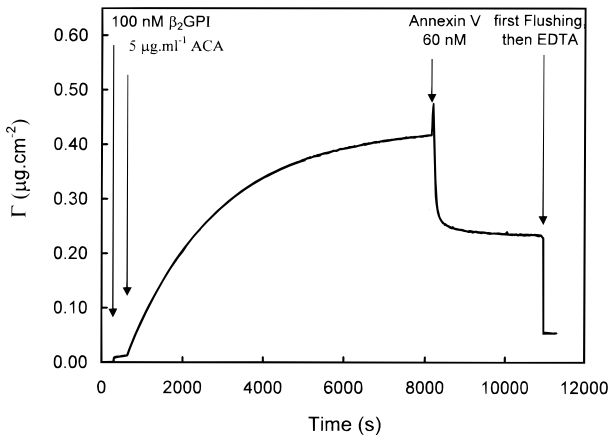

FIGURE 3: Partial displacement of ACA−β_2GPI complexes by annexin V. After addition of β_2GPI (100 nM at $t = 300$ s) and ACA (5 μg·mL^{-1} of patient Sa at $t = 600$ s), the adsorption of complexes was allowed to attain equilibrium. Then, annexin V (60 nM) was added to the cuvette buffer, and adsorption and desorption of protein was followed until equilibrium was reached. Finally, unbound protein was depleted from the cuvette buffer by flushing the cuvette with 50 mL of buffer, immediately followed by addition of EDTA in order to remove calcium ions from the solution. Other experimental conditions were as described for Figure 1.

between preparations may cause differences in adsorption rate.

Figure 3 shows that preadsorbed ACA−β_2GPI complexes do not prevent the binding of annexin V. Adsorption of ACA−β_2GPI complexes is initiated by addition of β_2GPI (100 nM at $t = 300$ s) and ACA (5 μg·mL^{-1} at $t = 600$ s; patient Sa). After about 2 h, adsorption equilibrium ($\Gamma = 0.42$ μg·cm^{-2}) is reached. Subsequent addition of annexin V (60 nM) causes a rapid but transient additional adsorption of annexin V to a maximum of about 0.48 μg·cm^{-2}, reached within 50 s after addition of annexin V. This is followed by an apparently spontaneous desorption representing ACA−β_2GPI complexes being replaced by annexin V. This replacement of ACA−β_2GPI by annexin V results, as anticipated, in a net desorption of protein because maximal surface coverage by annexin V (0.19 μg·cm^{-2}, see Figure 1) is substantially lower than maximal surface coverage by ACA-β_2GPI (∼0.4−0.5 μg·cm^{-2}). Finally, after completion of the desorption, the cuvette was rapidly flushed, within 50 s followed by addition of EDTA in order to provoke instantaneous release of annexin V from the phospholipid membrane. Desorption during the 50 s after flushing was less than 0.005 μg·cm^{-2}. The cuvette was flushed before EDTA addition to avoid the rapid readsorption of ACA−β_2GPI after EDTA addition (see also Figure 4). Considering that the affinity of β_2GPI and ACA−β_2GPI increases upon lowering the calcium concentration (*16*), the stepwise (within 10 s) release of protein observed in Figure 3 must reflect calcium depletion-induced release of annexin V (cf. Figure 1). This EDTA-induced desorption step is used to estimate the total amount of annexin V bound to the PSPC membrane just prior to addition of EDTA (0.17 μg·cm^{-2}), while the protein that remains adsorbed (0.06 μg·cm^{-2}) represents the ACA−β_2-GPI, that could not be displaced by annexin V.

Experiments as shown in Figure 3 were performed with ACA from five patients (with ACA and β_2GPI concentrations adjusted to obtain comparable adsorption rates). Table 1 summarizes the results. The data show that annexin V adsorption in the presence of preadsorbed ACA−β_2GPI reaches values (mean ± SD) of 0.177 ± 0.006, close to the maximal surface coverage of annexin V, i.e., 0.186 μg·cm^{-2}, despite the large preadsorption, 0.38−0.47 μg·cm^{-2}, of ACA−β_2GPI complexes. It is noteworthy that after displacement by annexin V, a fraction of the ACA−β_2GPI complexes remains bound to the membrane (15−30% of initial binding). Apparently, the amount of irreversibly bound ACA−β_2GPI differs among different patient preparations. Despite the presence on the bilayer of these remnant ACA−β_2GPI complexes, the binding of annexin V is nearly equal to the maximal surface coverage as observed in the absence of ACA and β_2GPI.

Next we investigated whether the order of addition of annexin V and ACA−β_2GPI complexes may influence the final adsorption of these proteins. Figure 4 shows that preadsorption of annexin V prevents any additional adsorption of β_2GPI and ACA−β_2GPI complexes. The addition of EDTA (4 mM) again results in instantaneous (within 10 s) and virtually complete reversal of the adsorption from 0.185 to 0.018 μg·cm^{-2}. This indicates that attachment to the membrane of virtually all bound protein was calcium-dependent, i.e., consisted of annexin V only. Immediately following the EDTA-induced desorption, a calcium-independent adsorption is observed, which we interpret as binding of β_2GPI and ACA−β_2GPI complexes, resulting in a final adsorption of 0.54 μg·cm^{-2}. This amount was also observed for the adsorption of ACA−β_2GPI complexes in the absence of annexin V, when the experiment was performed in buffer without added $CaCl_2$.

To verify whether these findings are specific for ACA prepared from patient plasma by liposomal adsorption or whether they can be extrapolated to anti-β_2GPI antibodies in general, the experiments described in Figures 3 and 4 also were performed with total IgG preparations from patients as well as polyclonal rabbit anti-human β_2GPI IgG (total IgG). ACA is highly enriched in anti-β_2GPI IgG. Therefore, much more patient IgG than ACA was required to attain similar adsorptions. The rabbit anti-β_2GPI preparation represents the other end of the scale: the content of specific anti-β_2GPI antibodies is higher. Moreover, these antibodies appeared to possess a higher affinity for β_2GPI, requiring much less β_2GPI to saturate the adsorption (rate). The higher affinity was also apparent from the much slower desorption rate of lipid-bound anti-β_2GPI IgG−β_2GPI complexes. In the experiments, we correspondingly adapted the concentrations of the IgG preparations, 100−300 μg·mL^{-1} for total IgG from patients and 2.5−10 μg·mL^{-1} for the rabbit anti-β_2GPI IgG. Essentially identical results were obtained as found for ACA preparations (data not shown): preadsorption of annexin V completely prevents adsorption of anti-β_2GPI IgG−β_2GPI complexes, whereas preadsorbed anti-β_2GPI IgG−β_2GPI complexes not only are unable to prevent annexin V adsorption but also are largely dislodged by annexin V from the lipid bilayer.

These results thus show that annexin V is not displaced from PSPC bilayers by ACA−β_2GPI complexes. To test whether these findings can be extrapolated to biological membranes, we have studied annexin V binding to ionophore-activated blood platelets and the effect of ACA−β_2-GPI complexes thereupon. We used ellipsometry to estimate unbound annexin V in a similar fashion as described earlier

Table 1: Displacement of ACA−β_2GPI Complexes by Annexin V[a]

patient	[β_2GPI] (nM)	[ACA] (μg·mL^{-1})	$\Gamma_{ACA-\beta2GPI}$ (μg·cm^{-2})	$\Gamma_{ACA-\beta2GPI\&Annexin\ V}$ (μg·cm^{-2})	$\Gamma_{ACA-\beta2GPI-residual}$ (μg·cm^{-2})	$\Gamma_{annexin\ V}$ (μg·cm^{-2})
Ca	100	5	0.380	0.231	0.050	0.181
Gh	100	5	0.468	0.339	0.160	0.179
Lo	500	10	0.388	0.224	0.057	0.167
Sa	100	5	0.417	0.233	0.053	0.180
Ta	500	10	0.428	0.215	0.038	0.177

[a] Experiments as shown in Figure 3, were performed with ACA preparations of 5 patients. [β_2GPI] and [ACA] represent the total concentrations of the two proteins. $\Gamma_{ACA-\beta2GPI}$ represents the equilibrium adsorption of ACA−β_2GPI complexes. $\Gamma_{ACA-\beta2GPI\&AnnexinV}$ is the total amount of protein remaining bound after the transient annexin V adsorption. $\Gamma_{ACA-\beta2GPI-residual}$ is the residual amount of protein attached to the bilayer after the EDTA addition, representing the ACA−β_2GPI complexes that remain bound after (partial) displacement by annexin V. $\Gamma_{annexin\ V}$ represents the annexin V adsorption as estimated from the difference between $\Gamma_{ACA-\beta2GPI\&Annexin\ V}$ and $\Gamma_{ACA-\beta2GPI-residual}$. Experiments were performed in Tris-HCl buffer containing 50 mM Tris, 120 mM NaCl, 0.5 mg·mL^{-1} BSA, and 3 mM $CaCl_2$.

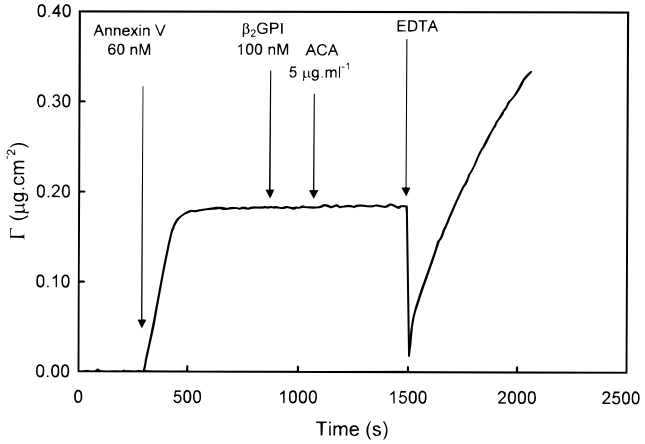

FIGURE 4: Preadsorption of annexin V prevents binding of β_2GPI and β_2GPI−ACA complexes. Annexin V (60 nM) was added to the cuvette buffer at $t = 300$ s. After completion of the annexin V adsorption, β_2GPI (100 nM at 800 s) and subsequently ACA (5 μg·mL^{-1} at $t = 1100$) were added to the cuvette. At 1500 s, EDTA (4 mM) was added to the cuvette buffer to deplete calcium.

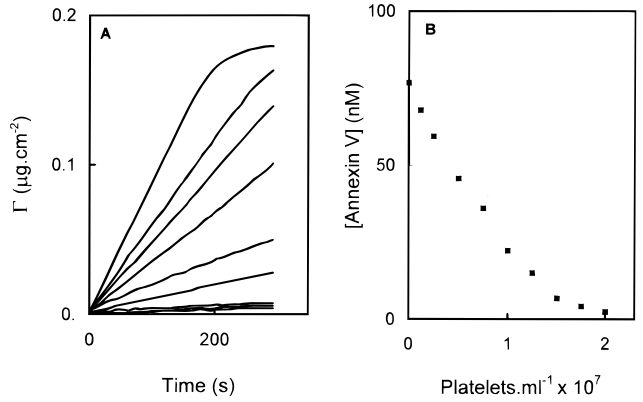

FIGURE 5: Ellipsometric determination of annexin V binding to activated blood platelets. (A) Ionophore-activated blood platelets were incubated with 75 nM annexin V for 20 min. After centrifugation (25 min, 22000*g*), 1 mL of supernatant was added ($t = 0$) to the ellipsometer cuvette (final volume 5 mL) for measurement of the adsorption of the remaining free annexin V to the PSPC bilayer. The adsorption from the supernatant of 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 × 10^7 platelets·mL^{-1} (upper to lower curve) is shown. (B) The initial rates of adsorption of the curves as shown in panel A were converted to concentrations of unbound annexin V present in the supernatant by using a reference curve constructed with known amounts of annexin V. Indicated is the free annexin V concentration (mean value of at least 3 experiments) as a function of the platelet concentration.

(*22*). Annexin V (75 nM) was incubated with various amounts of activated blood platelets (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 × 10^7 cells·mL^{-1}). Free and platelet-bound annexin V were separated by centrifugation, and the supernatant was assayed for annexin V by ellipsometric measurement of the adsorption to PSPC bilayers of the free (unbound) annexin V present in the supernatant. Figure 5A shows a decrease in adsorption rate of the residual unbound annexin V in the supernatant with increasing amounts of platelets in the incubation mixture. At platelet concentrations exceeding 1.5 × 10^7 cells·mL^{-1}, nearly all annexin V is bound. Control experiments showed that preincubation of annexin V with the supernatant of the platelet suspension (2 × 10^7 cells·mL^{-1}) has no appreciable effect on the rate of adsorption of annexin V to planar bilayers. Using a reference curve determined for annexin V concentrations ranging from 0.75 to 30 nM, the initial rates of experiments as shown in Figure 5A are plotted as the concentration of unbound annexin V as a function of the platelet concentration (Figure 5B). Figure 5B clearly shows that platelet concentrations above 1.5 × 10^7 cells·mL^{-1} result in binding of more than 95% annexin V (75 nM). From the initial slope of the decrease of the free annexin V concentration with increasing amounts of platelets in the incubation mixture, it is estimated that 1.5 × 10^7 platelets·mL^{-1} are required to bind 75 nM annexin V; i.e., maximal binding amounts to about 50 pmol of annexin V per 10^7 cells (i.e., 3 × 10^6 molecules of annexin V per platelet).

The effect of ACA and β_2GPI on annexin V binding to ionophore-activated blood platelets was analyzed by quantitating the amount of annexin V in the supernatant of the platelet incubation by ellipsometry. Since this supernatant also contains the unbound β_2GPI and ACA, it was essential to assess the annexin V binding in the presence of β_2GPI and ACA. For these experiments, we used the rabbit anti-β_2GPI IgG because of its higher affinity than ACA and patient total IgG. Rabbit anti-β_2GPI IgG (50 μg·mL^{-1}) and β_2GPI (1 μM) were preincubated for 20 min with various amounts of annexin V (2−15 nM) in Tris−BSA buffer containing 3 mM $CaCl_2$. This incubation mixture (1 mL) was added to 4 mL of buffer in the ellipsometer cuvette, and the combined adsorption of annexin V and anti-β_2GPI−β_2GPI complexes to a planar PSPC bilayer was measured by ellipsometry for 750 s (Figure 6A). Then adsorption was stopped by flushing the cuvette, and the ensuing desorption was followed for 300 s. Finally, EDTA (4 mM) was added in order to cause immediate release of bound annexin V. It is apparent from Figure 6A that this instantaneous desorption

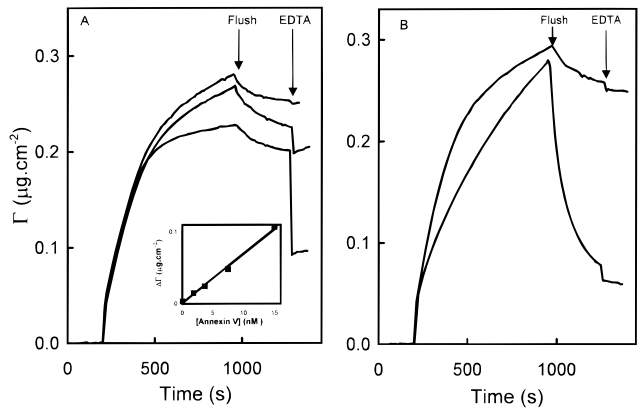

FIGURE 6: Annexin V bound to activated blood platelets is not displaced by β_2GPI−ACA complexes. (A) Measurement of annexin V concentration in the presence of anti-β_2GPI IgG and β_2GPI. Various concentrations of annexin V (0, 3.75, and 15 nM, upper to lower curve), rabbit anti-β_2GPI IgG (50 μg·mL^{-1}), and β_2GPI (1 μM) were incubated for 20 min before addition to the ellipsometer cuvette containing 4 mL of buffer. Adsorption of the protein mixture was followed for 750 s, after which adsorption was interrupted by flushing the cuvette with 50 mL of buffer, effectively depleting protein from the solution. The desorption of protein was monitored for 300 s, followed by addition of EDTA (4 mM) to induce instantaneous release of annexin V. The inset shows the EDTA-induced desorption as a function of the concentration of added annexin V. (B) Blood platelets (2×10^7 platelets·mL^{-1}) were incubated for 20 min with annexin V (75 nM); then β_2GPI (1 μM) and ACA of patient Gh (lower curve) or rabbit anti-β_2GPI IgG were added, and the mixture was incubated for another 45 min. Finally, free and bound proteins were separated by centrifugation (25 min, 22000*g*), and the supernatant was added to the ellipsometer cuvette to measure the annexin V content as described in panel A.

step increases with the annexin V concentration in the incubation mixture. The inset in Figure 6A clearly shows that the size of the instantaneous desorption step is a linear function of the annexin V concentration, with a slope marginally lower (15%) than predicted from the initial rates of the annexin V adsorption in the absence of anti-β_2GPI and β_2GPI. Thus, the procedure shown in Figure 6A can be used to quantitate the amount of annexin V in mixtures with ACA and β_2GPI, enabling the quantitation of the effect of ACA (or anti-β_2GPI IgG) and β_2GPI on annexin V binding to membranes of activated blood platelets.

To assess the ability of ACA−β_2GPI complexes to interfere with annexin V binding to membranes of activated blood platelets, we performed the experiments shown in Figure 6B. Annexin V (75 nM) was incubated with ionophore-activated blood platelets (2×10^7 platelets·mL^{-1}); after 20 min, ACA (50 μg·mL^{-1}) and β_2GPI (1 μM) were added, and the mixture was incubated for an additional 45 min followed by centrifugation (25 min, 22000*g*). The supernatant (1 mL) was added to the ellipsometer cuvette in order to determine the annexin V content following the procedure as shown in Figure 6A. It is apparent that the EDTA-induced desorption is minor (0.015 and 0.008 μg·cm^{-2} for the lower curve, ACA of patient Gh, and the upper curve, rabbit anti-β_2GPI IgG, respectively). Using the reference line shown in the inset of Figure 6A, these values correspond to a concentration of unbound annexin V of 2.1 and 1.1 nM, respectively, i.e., less than 3% of bound annexin V.

Similar experiments were performed several times, also using ACA from another patient (Ca). The displacement of annexin V was estimated as the difference between the concentrations of unbound annexin V in the presence of ACA (or anti-β_2GPI IgG) and β_2GPI, estimated as in Figure 6A, and in the absence of these proteins, estimated as in Figure 5A. The results of these experiments, with more than 70 nM annexin V bound to the platelets in the absence of ACA and β_2GPI, show a displacement of -0.3 ± 0.5 nM annexin V (mean ± SEM; $n = 12$) upon addition of ACA (or anti-β_2-GPI IgG) and β_2GPI. Thus, it is concluded that the displacement of annexin V from membranes of activated blood platelets by ACA−β_2GPI complexes is negligible.

DISCUSSION

The data presented in this study unequivocally show that annexin V preadsorbed at PSPC membranes is not displaced by ACA−β_2GPI complexes. Moreover, this preadsorbed annexin V appears to bind so tightly to the phospholipid membrane that it completely prevents adsorption of ACA−β_2GPI complexes. The notion that ACA−β_2GPI has insufficient capacity to displace annexin V from membranes is supported by the observation that the bulk of preadsorbed ACA−β_2GPI can in fact be displaced by annexin V.

Protein interaction with phospholipid bilayers was measured by ellipsometry, which affords accurate kinetic measurement of protein adsorption to planar lipid bilayers deposited on silicon (*47*). These lipid bilayers, formed by exposure of the hydrophilic surface to small PSPC vesicles, have been used extensively as model membranes in the study of protein adsorption (*16*, *22*, *47*, *52*, *53*), and in the study of lipid-dependent reactions of blood coagulation (*47*, *52*, *54*, *55*). These bilayers apparently cover the silicon surface completely as deposition of a bilayer of pure DOPC at the silicon prevents any adsorption of, e.g., HMWK and fibrinogen (*56*), whereas these proteins, in the absence of the lipid membrane, show sizable adsorptions at silicon of about 0.4 μg·cm^{-2}. This study adds a similar observation for IgG: neither IgG obtained from healthy control subjects nor IgG isolated from APS patients exhibited any significant interaction with PSPC deposited on the silicon. In contrast, for uncovered silicon we found an IgG adsorption of about 0.5 μg·cm^{-2} (*16*).

Ellipsometric measurement reports on the total amount (μg/cm^2) of proteins adsorbed and normally does not allow splitting of the total adsorption of protein mixtures into the separate adsorptions of its individual constituents. However, the different calcium requirements for binding to PSPC bilayers of annexin V (strictly dependent on the presence of calcium) and of ACA−β_2GPI complexes (no calcium required) allowed us to elucidate the competitive adsorptions of these proteins. Figure 1 shows that calcium depletion results, within 10 s, in an abrupt desorption of all bound annexin V, whereas Figure 2 shows that the amount of adsorbed ACA−β_2GPI complexes is virtually unchanged in this time span. Therefore, it can be safely assumed that the extent of the desorption step induced by EDTA (cf. Figures 4−6) represents an accurate estimate of the amount of annexin V present at the surface.

The experiments presented in Figure 1 confirm the high affinity of annexin V for PS containing membranes, that was reported earlier (*19*−*21*). Similarly, the experiments shown in Figure 2 confirm the high affinity of the ACA−β_2GPI

complexes for PSPC membranes. The dissociation constant, defined as the ACA concentration required to attain half-maximal surface coverage in the presence of 100 nM β_2GPI (saturating concentration), was estimated to be in the nanomolar range (*16*). In contrast to the minor desorption observed for annexin V after depletion of protein from solution, we found a major and rapid desorption of β_2GPI and ACA. It is noteworthy that desorption appears to be incomplete, with 10−30% of the ACA−β_2GPI complexes remaining bound. It is hypothesized that during prolonged adsorption, part of the ACA−(β_2GPI)$_2$ complexes become interconnected through multivalent binding, as could be expected from the polyclonal origin of the IgG.

The observation that coverage of the PSPC bilayer with annexin V completely prevents the adsorption of ACA−β_2-GPI is compatible with the notion that annexin V bound to the membrane forms very tight binding clusters (*22*). When annexin V was added to the cuvette after formation of ACA−β_2GPI complexes at the PSPC membrane, 70−90% of these preadsorbed complexes were displaced, thus leaving 10−30% of the ACA−β_2GPI complexes irreversibly bound. In the absence of annexin V, a similar amount of ACA−β_2-GPI complexes remains irreversibly bound after depletion of protein from the solution (Figure 2). This partial coverage of the lipid surface with irreversibly bound ACA−β_2GPI complexes, however, does not hamper annexin V binding to any significant extent (Table 1).

We used a modification of a procedure developed previously for the determination of annexin V binding to small and large unilamellar phospholipid vesicles (*22*) to quantitate annexin V binding to blood platelets (cf. Figures 5 and 6). Accurate determination of protein binding to membranes by this procedure requires the ability to deplete an appreciable fraction of protein from the solution by binding and is therefore only applicable for high-affinity interactions between protein and the membrane. Figure 5 shows that this condition was fulfilled for annexin V binding to blood platelets. In principle, this technique would also be suitable to measure high-affinity binding of ACA−β_2GPI complexes to platelet membranes. In exploratory experiments, however, we were unable to show a depletion of ACA and β_2GPI binding activity from the solution by addition of blood platelets or even PSPC vesicles to a similar extent as found for annexin V (cf. Figure 5). It remains to be investigated whether this inability was caused by limitations of our experimental setup or reflects a poorer binding to platelets of ACA−β_2GPI complexes compared to annexin V. Nevertheless, the experiments presented in Figures 5 and 6 unequivocally demonstrate that even an large excess of ACA−β_2GPI complexes does not cause an appreciable displacement of annexin V from the natural membrane of activated blood platelets.

The present findings are discordant with data obtained by Rand et al. (*42*). In a seemingly identical experimental setup, these investigators found that preadsorption of annexin V to PS/PC membranes containing 30% PS does not prevent adsorption of ACA−β_2GPI complexes. Moreover, the greater part (>70%) of preadsorbed annexin V was found to be displaced by ACA−β_2GPI complexes. Closer inspection of their data, however, reveals several other discrepancies: even after preadsorption of annexin V, they found a large adsorption of patient IgG (in the absence of β_2GPI). Also a large adsorption was found for control IgG (in the presence of β_2GPI). In our experiments, binding of patient IgG is strictly dependent on β_2GPI, and no binding whatsoever is found for IgG isolated from healthy controls. In contrast to the claim of these authors that lipid bilayers were prepared as described earlier by our laboratory (*22, 47*), it appears that they introduced a modification in the preparation of the lipid bilayers by adding BSA to the buffer from which the vesicle suspension was adsorbed to the silicon slide. Using their modified procedure (addition of BSA) for the preparation of PSPC "bilayers", we also found adsorption of control and patient IgG independently of β_2GPI as well as a partial displacement of preadsorbed annexin V. As a likely explanation we propose that addition of BSA during the lipid deposition interferes with the formation of a continuous lipid bilayer, resulting in adsorption of a binary mixture of protein (BSA) and phospholipid (patches).

The data presented here show that substitution of annexin V by antiphospholipid antibodies is an unlikely explanation for the observed displacement of annexin V from cell surfaces and subsequent exposure of a procoagulant lipid surface (*39*−*42*, *57*). Therefore, other actions of antiphospholipid antibodies, such as the interference with lipid-dependent feedback (anti-coagulant) reactions of the blood coagulation system, or cellular activation resulting in induction of the expression of tissue factor, should be considered as a possible cause of the increased risk for thrombosis. Alternatively, as was previously suggested (*15*), it cannot be excluded that the antiphospholipid antibodies arise as a consequence of a prothrombotic state rather than being a cause of thrombosis.

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