Autoantibodies to Heparin From Patients With Antiphospholipid Antibody Syndrome Inhibit Formation of Antithrombin III-Thrombin Complexes

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The antiphospholipid antibody syndrome (APS) is characterized by thrombosis, fetal loss, and is associated with a set of autoantibodies to phosphatidylserine (aPLs) that have been sequenced that binds antithrombin III and plays a physiologic role in coagulation. Antibodies to the native molecule on the endothelial cell surface that is present in heparin, a related glycosaminoglycan. We hypothesized that a subset of antiphospholipid antibodies with high affinity for heparan sulfate/heparin epitopes may inhibit the function of HS, promoting a procoagulant state. Purified IgG from all seven patients with APS studied were reactive with heparin by enzyme-linked immunosorbent assay, whereas none of five controls had antiheparin reactivity. IgG antiheparin antibodies were purified from two APS patients by affinity chromatography on heparin-Sepharose. Specificity studies showed that low-affinity electrostatic interactions clearly did not account for the observed reactivity with heparin, and that APS IgG antiheparin antibodies were specifically reactive with a disaccharide present in the heparin pentasaccharide that binds antithrombin III. Moreover, APS IgG antiheparin antibodies inhibited heparin-accelerated formation of antithrombin III-thrombin complexes. We conclude that antiheparan sulfate/heparin antibodies may be a cause of autoimmune vascular thrombosis in the antiphospholipid antibody syndrome.

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fied serum IgG (30 µg/mL) obtained from patients with APS (n = 7) and from normal controls (n = 5) to (A) cardiolipin, (B) heparan sulfate (HS), and (C) heparin. The results are expressed as the mean ODmax of triplicate assays.

Fig 1. Direct binding of purified serum IgG (30 µg/mL) obtained from patients with APS (n = 7) and from normal controls (n = 5) to (A) cardiolipin, (B) heparan sulfate (HS), and (C) heparin. The results are expressed as the mean ODmax of triplicate assays.

III (TAT) complexes, suggesting a noninflammatory immune-mediated mechanism of vascular injury induced by autoimmunity to HS. In the current report, we show the presence of antiheparin antibodies in patients with APS. APS affinity-purified IgG antiheparin antibodies reacted with a disaccharide found in the specific heparin pentasaccharide that binds antithrombin III, and inhibited the heparin-accelerated formation of antithrombin III-thrombin complexes. These results indicate that antiheparin antibodies from patients with APS may promote a procoagulant state and thrombosis via inhibition of physiologic heparin-dependent mechanisms of anticoagulation.

MATERIALS AND METHODS

Reagents. Heparin, heparin disaccharides, heparinase (I and III), HS, cardiolipin, heparin-sulfate, and goat-antihuman IgG-coupled Sepharose 4B were purchased from Sigma Chemical Co (St. Louis, MO). Biotinylated goat-antihuman IgG and streptavidine-alkaline phosphatase were purchased from Jackson ImmunoResearch (West Grove, PA). Cardiolipin cofactor (β2-microglobulin) was purified by previously described methods.

Human subjects. Sera from patients and controls were obtained from the Division of Rheumatology, Department of Medicine, Hospital for Special Surgery (Dr. Gharavi) and from the Coagulation Laboratory, Division of Hematology, Mount Sinai Hospital, New York, NY. All patients with APS fulfilled the major criteria for the disease.

Two cases of APS were studied in depth. Patient 1 was a 58-year-old white man who was admitted with increased lethargy and right-flank pain. The past medical history was significant for a progressive dementia of 1 year’s duration, peripheral neuropathy, and retinal artery occlusion 4 months before admission, at which time the patient was found to have elevated IgG anticardiolipin antibodies (greater than 143 ACA GPL units; normal is less than 23). There were no IgM anticardiolipin antibodies. He was treated with warfarin. On admission, a chest x-ray showed a right lower lobe infiltrate with congestive heart failure. An electrocardiogram showed a new right bundle branch block pattern with a left-anterior hemiblock and an old inferior wall myocardial infarction. A head computed tomograph showed marked cerebral atrophy for the patient’s age and an old right temporal-occipital infarct. Laboratory studies showed a negative antinuclear antibody, anti-ds DNA antibody, Venereal Disease Research Laboratory (VDRL), and cryoglobulins. The platelet count was 95,000/mm3; bone marrow showed normal megakaryocytes. The activated partial thromboplastin time (APTT) was 64.6 seconds (control = 34.1 seconds; reference range = 25.0 to 33.0 seconds) and prothrombin time was 21.6 seconds (control = 10.9 seconds; reference ranges = 11.0 to 13.2 seconds). Mixing studies showed the presence of an inhibitor. The bleeding time was 8.5 minutes. Anticardiolipin IgG titers were above 1043 ACA GPL units (normal range is less than 23); IgM ACA was within normal limits. Two days after admission the patient became increasingly dyspneic and hypertensive while on anticoagulant therapy. Ventilation/perfusion lung scanning showed a pulmonary embolism. He was placed on intravenous heparin, but despite large doses of heparin (up to 2,000 U/hr), the APTT remained just at or below therapeutic range, and he required a Greenfield filter. The patient then developed two subendocardial myocardial infarctions despite continued anticoagulation. His mental status deteriorated and he went into a coma. He required steroids because of bilateral adrenal hemorrhages and adrenal insufficiency. Because of his deteriorated condition despite therapy, the patient was treated with a course of plasmapheresis. After a long and complicated course, he recovered completely with continued plasmapheresis and intravenous heparin. He was discharged 4 months after admission and given Coumadin and aspirin.

Patient 2 was a 41-year-old woman of Peruvian origin who presented to clinic for evaluation of spontaneous bruising that had developed over the preceding 6 months. Her history was significant for a right-lower extremity deep vein thrombosis that had been treated with anticoagulant therapy 15 years before. The obstetrical history was significant for two normal pregnancies, followed by four sequential miscarriages. The patient was known to have a biologic false positive VDRL. On examination the patient had multiple, scat-
tered 1-cm ecchymoses in her upper and lower extremities with some of the ecchymoses having central raised tender nodules. Her laboratory studies were significant for a hematocrit 32.1%, platelets 124,600, positive Coombs test that showed IgG, IgA, IgM, C3D, and C4 on her red blood cells, along with a positive HAM test with 10.5% hemolysis; antiplatelet antibodies were negative. Coagulation screening studies showed prothrombin time of 14.9/11.7 seconds, APTT of 104.0/52 seconds. Mixing studies were positive for a circulating lupus anticoagulant. The patient was diagnosed as having SLE with the antiphospholipid syndrome.

The concentration of dialyzed IgG solution was determined by capture ELISA using commercially available human IgG (Sigma) as a standard sample. Figure 2. Direct binding of purified serum IgG and affinity-purified antiheparin IgG obtained from two APS patients and two normal controls to (A) cardiolipin (CL), (B) heparan sulfate (HS), and (C) heparin. The results are expressed as mean OD of triplicate assays. [Symbols: (○), patient 1 total IgG; (□), patient 2 total IgG; (△), NL1 total IgG; (●), NL2 total IgG; (□), patient 1 antiheparin IgG; (■), patient 2 antiheparin IgG.

**Preparation of IgG from human sera.** Igs from human sera were initially purified on protein G. To further purify the IgG fraction, the IgG was extensively dialyzed against 0.01 mol/L phosphate buffer saline (PBS), pH 7.4, and applied to a goat-antihuman IgG-coupled Sepharose 4B column (Sigma). Unbound proteins were washed off with PBS and the bound IgG was eluted with 0.1 mol/L glycine-HCl pH 2.7. The eluate was immediately neutralized with 1.0 mol/L TRIS-HCl pH 9.0 and extensively dialyzed against PBS. The concentration of dialyzed IgG solution was determined by capture enzyme-linked immunosorbent assay (ELISA) using commercially available human IgG (Sigma) as a standard sample.

**Affinity purification of human IgG with heparin-agarose.** A 5-mL column of heparin-agarose was equilibrated with PBS. Purified serum IgG in PBS was loaded on the column, the fall through collected, and the column was washed with 20 mL PBS. Two mL fractions were collected. Bound IgG was eluted stepwise with 20 mL each of 0.3 mol/L, 0.6 mol/L, 1 mol/L, 3 mol/L NaCl and 4 mol/L guanidine HCl/2 mol/L NaCl, pH 7.4. The column was monitored by spectrophotometry at 280 nm. Unbound material in the flow-through and the wash were pooled and rechromatographed. Peak fractions from the first chromatography were pooled and dialyzed against PBS, and the concentration of IgG in the dialyzed material was determined by capture ELISA. This peak fraction IgG was used for further immunologic studies.

**Conjugation of HS to bovine serum albumin (BSA).** Intact HS was conjugated to BSA (Cohn fraction V) to assure adsorption of HS to the radioimmunoassay plates via protein portion of the conjugate. Twenty milligrams of HS were placed in 10 mL PBS and briefly sonicated until solubilized. Two and a half milliliters of 250 mmol/L benzoquinone in absolute ethanol were added to the HS solution and rotated in the dark at room temperature for 1 hour. Of 95% ethanol/0.1 mol/L sodium acetate (100 mL) was added, and the mixture was centrifuged at 12,100g for 20 minutes. The pellet was retained and washed twice with ethanol acetate. Then 10 mL of PBS was added to the pellet. The solution was briefly sonicated and dialyzed overnight against 4 L of PBS. 16 mg of BSA was added to the solution and rotated overnight at 4°C. The HS-BSA conjugate was then precipitated by the addition of 90 mL of saturated ammonium sulfate at 4°C. The mixture was then centrifuged at 12,100g for 20 minutes, and the pellet was resuspended in PBS. The solution was again dialyzed against PBS. The final solution, referred to as HS-BSA, contained 15 mg/mL of BSA and 16 mg/mL of HS, as estimated by protein BCA assay (Pierce, Rockford, IL) and glucuronate analysis respectively.

**Digestion of heparin with heparinase I and III.** Heparinase digests (3 μIU/mg heparin) were performed in 0.1 mol/L TRIS-HCl, pH 7.2 containing 5 mmol/L calcium acetate in the presence of protease inhibitors (10 mmol/L N-ethylmaleimide, 1 mmol/L...
phenylmethanesulfonyl fluoride, and 0.035 mmol/L peptatin A) for 1 hour with rotation. Subsequently, the mixture was heated at 90°C for 30 minutes to denature the enzyme. Then the sample was centrifuged and the supernatant was recovered for use in inhibition experiments. The relative completeness of the heparinase digestions was evaluated by gel chromatography on Biogel P6 in PBS by modifications of standard methods. The elutions from the column were monitored at 230 nm for digestion products and compared with the chromatographic profile of undigested heparin and the standard heparin disaccharides also used for inhibition studies. The results showed that heparinase I digested the majority of the material to small oligosaccharides (with a degree of polymerization ≤6 monosaccharides). Heparinase III resulted in slightly larger oligosaccharides (degree of polymerization = 10 monosaccharides). To calculate molar compositions of the intact heparin and the heparinase digested materials used for immunoinhibition experiments, an estimated mean molecular weight of the intact heparin preparation was used (30 kD).

ELISA

Detection of antiheparin and anti-HS antibodies: Direct binding assay. For detection of anti-HS antibodies, plates were coated with HS-BSA (10 μg HS/mL in 0.1 mol/L sodium carbonate, pH 9.8) overnight at 4°C. For detection of anti-heparin antibodies, microtiter plates (Nunc, Roskilde, Denmark) were coated with 10 μg/mL of protamine sulfate in 0.1 mol/L sodium carbonate, pH 9.8, for 1 hour at room temperature. After coating, protamine sulfate-coated plates were washed in PBS-0.1% Brij (PBSB) and then incubated with 10 μg/mL of human IgG from patients or controls diluted 1:1 in 50 μL 3% BSA-PBS for 2 hours at room temperature. After extensive washing, biotinylated goat-antihuman IgG in PBSB was added to the plates for further 60 minutes. Plates were then incubated with streptavidin-alkaline phosphatase in PBSB for 60 minutes, and p-nitrophenyl phosphate was added. After 30 minutes optical density (OD) at 405 nm was measured in an ELISA microplate reader to determine the amount of bound enzyme.

Detection of anticardiolipin antibodies. These studies were performed essentially as previously described. Microtiter plates were coated with 50 μg/mL of cardiolipin in absolute ethanol and dried up overnight at 4°C. Plates were blocked with 10% heat inactivated fetal bovine serum (FBS)-PBS and incubated with human IgG diluted with 10% PBS-PBS for 2 hours at 4°C. Then the binding of IgG was determined as above.

ELISA for detection of human IgG inhibition of thrombin-antithrombin III complex formation. Human antithrombin III (ATIII) was a gift from Dr D. Menache-Aronson (American National Red Cross Laboratories, Bethesda, MD). The ATIII was biotinylated using D-biotinyloaminocaproic acid N-hydroxysuccinimide ester according to the manufacturer’s directions (Boehringer-Mannheim, Indianapolis, IN). Human α-thrombin was generously provided by Dr Jody Fenton (NY State Department of Health, Albany, NY). Rabbits were immunized intradermally with human thrombin to obtain antiserum. The IgG fraction was isolated from the rabbit sera and affinity purified using immobilized thrombin.

The assay for detecting TAT complexes accelerated by heparin was performed with modifications of the method detailed for ELISA of thrombin-antithrombin complexes and our previously described methods. Microtiter plates (Nunc) were coated with 4 μg/mL of the IgG fraction of rabbit antithrombin thrombin overnight at 4°C and blocked with 3% BSA-PBS. Biotinylated ATIII, 0.5 μg/mL at final concentration, was preincubated with various amounts (0.01 to 0.5 μg/mL) of heparin for 15 minutes at 37°C. Purified human thrombin, 0.25 μg/mL at final concentration, was added to the mixture and incubated for 15 minutes at 37°C, and the reaction was terminated by the addition of 1 μg/mL D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (Calbiochem Inc, San Diego, CA). The mixtures were transferred to rabbit antithrombin thrombin precoated wells and incubated for 1 hour at room temperature. The plates were then incubated with streptavidin-alkaline phosphatase and then with the substrate p-nitrophenyl phosphate. After 30 minutes, OD was measured in an ELISA microplate reader to determine the amount of TAT complex formed in the assay. The amount of TAT complex increased proportionally to the dose of heparin preincubated with ATIII.
To examine whether human IgG antibody affected TAT complex formation by inhibiting the effect of heparin, 50 μL of various concentrations of APS and control IgG diluted in 3% BSA-PBS were preincubated with 50 μL of the optimum amount of heparin (0.1 μg/mL) for 30 minutes at 37°C and then for 30 minutes at 4°C. Finally, the amount of TAT complex was determined as above.

Data analysis. Statistical analysis was performed using the chi-square test.

RESULTS

Direct binding studies of human IgG to cardiolipin, HS, and heparin. Human IgG (30 μg/mL) purified from the sera of five normal controls and seven patients with APS were investigated by ELISA for direct binding to cardiolipin, HS, and heparin. The binding of normal serum IgG to cardiolipin, HS, and heparin was 0.201 ± 0.117, 0.264 ± 0.037, and 0.226 ± 0.078, respectively (mean ± SD of OD405nm). The data were then analyzed by chi-square analysis, using cut-off values that were two standard deviations beyond the mean for control samples. By this method, 6/7, 5/7, and 7/7 APS IgG were positive for anticardiolipin, anti-HS, and antiheparin antibody activity, respectively. The IgG antibody activity to cardiolipin (P = .019), HS (P = .06) and heparin (P = .004) were significantly higher in APS patients than in normal controls (Fig 1).

Purified IgG from two APS patients with high binding to heparin and cardiolipin and two normal controls were chosen for further investigation. Direct binding of purified serum IgG from these two APS patients to cardiolipin, HS, and heparin showed the dose-dependent nature of the reactivity to these molecules (Fig 2).

Affinity purification of human IgG with heparin-agarose.

Elution profiles of patient and control IgG samples were monitored by OD at 280 nm. Increasing concentrations (up to 3 N) of NaCl, and 0.1 mol/L glycine, pH 2.3 failed to elute the APS antiheparin antibody, essentially eliminating the possibility that low-affinity electrostatic interactions alone account for the binding of APS IgG to the heparin column. Only 4 mol/L guanidine HCl/2 mol/L NaCl eluted the affinity bound IgG, indicating that the binding affinity of APS IgG to heparin was very high. The peak fractions of the initial guanidine buffer elutions from the two APS patient IgG samples were pooled and dialyzed against PBS, and the amount of IgG eluted from the heparin agarose determined to be 20 μg/mL and 6 μg/mL, respectively. These samples were used below for further immunologic studies. No peak was seen in the guanidine elution profiles of the normal controls.

To estimate the percent of the loaded total IgG that bound the heparin agarose, all fractions in each of the stepwise elutions (except the peak fractions of the guanidine elutions described above) were pooled, dialyzed against PBS, and tested for IgG concentration. For the APS patients, these data were combined with the data obtained for the guanidine elution peak fractions. When 10 mg of purified APS IgG from two patients was applied to a 5-mL heparin agarose column, 0.19 mg (1.9%) and 0.17 mg (1.7%) of IgG was eluted with 4 mol/L guanidine/2 mol/L NaCl (Fig 3). Relative to the APS patients, only small quantities of IgG (0.05 mg or 0.5%) from normal controls were found in the pooled guanidine elutions from the heparin column.

APS affinity-purified IgG antiheparin antibody reacted with cardiolipin, HS, and heparin in a dose-dependent fash-
ion (Fig 2). The reactivity of the affinity-purified antiheparin IgG to heparin was significantly enriched compared with that of total IgG. For example, the binding to heparin of patient 1 and patient 2 antiheparin IgG (1 μg/mL) was 1.001 ± 0.069 and 1.069 ± 0.171, respectively (mean ± SD of OD_{405 nm}), whereas the binding of total IgG fractions from the same patients at the same concentration was 0.276 ± 0.014 and 0.225 ± 0.014, respectively. Although the degree of enrichment was clearly not as great, binding enrichment of APS antiheparin IgG compared with the total IgG fraction was also noted in reactivity to cardiolipin and HS. This result would be expected because heparin is the homologous antigen used in the affinity purification procedure. These results show the presence of anticardiolipin reactivity and anti-HS reactivity in the APS affinity-purified IgG antiheparin antibody fraction.

Fine immunospecificity of affinity-purified antiheparin antibodies obtained from patients with APS. To investigate the immunologic specificities of the affinity-purified APS antiheparin antibodies, liquid-phase competitive inhibition studies were performed using heparin as antigen (Fig 4). Affinity-purified APS antiheparin antibody was inhibited by HS, cardiolipin with cofactor, and heparin. Cardiolipin alone had little effect. To further characterize the immunodominant epitope recognized by these antibodies, heparinase I or III was used to digest sulfated and nonsulfated regions of heparin, respectively (Fig 5). Digestion of nonsulfated regions of heparin by heparinase III increased the antigenicity of the heparin preparations, whereas digestion of the highly sulfated regions of heparin by heparinase I reduced the antigenicity of heparin as recognized by APS affinity-purified antiheparin IgG.

Use of heparin disaccharides confirmed that specific sulfate residues were necessary for APS IgG antiheparin antibody recognition. The disaccharide α-ΔUA-2S-[1→4]-GlcNS-6S was the most effective inhibitor, almost equivalent to the intact heparin chain. Of interest, the same disaccharide is present in residues IV and V of the specific heparin pentasaccharide sequence that binds ATIII with high affinity. The disaccharides α-ΔUA-[1→4]-GlcNS-6S and α-ΔUA-[1→4]-GlcNS were poor inhibitors. The structure of these disaccharides is shown in Fig 6. Other data (not shown) showed that disaccharides containing N-acetylated disaccharides, α-ΔUA-[1→4]-GlcNAc and α-ΔUA-[1→4]-GlcNAc-6S, were completely ineffective inhibitors of APS IgG antiheparin antibody, further showing the specificity of APS IgG antiheparin antibody for the α-ΔUA-2S-[1→4]-GlcNS-6S disaccharide.
Inhibition of heparin-accelerated thrombin-antithrombin complex formation by APS IgG. We used a modification of our previously described methods to investigate whether APS IgG antiheparin antibody can inhibit the heparin accelerated formation of TAT complex formation, and therefore promote a procoagulant state. The addition of APS total IgG resulted in a dose-dependent inhibition of heparin-accelerated TAT complex formation (Fig 7). This inhibition was increased approximately 100-fold by APS affinity-purified antiheparin IgG. Essentially no inhibition of TAT complex formation was caused by normal IgG (up to 2 mg/mL). Approximately 5 μg/mL of APS affinity-purified antiheparin IgG was necessary to inhibit 50% of TAT complex formation, which is similar to the concentration of procoagulant antibody activity found in SLE sera containing lupus anticoagulants.

DISCUSSION

In this study, we have shown the presence of IgG antiheparin antibodies in patients with APS. APS IgG antiheparin antibodies specifically reacted with a disaccharide present in the unique heparin pentasaccharide that binds antithrombin III. The autoimmune nature of the APS antiheparin antibodies was further shown by their reactivity with the cardiolipin/cofactor complex associated with APS. Previous studies have shown that aPL reactivity with cardiolipin alone is not associated with APS, that aPL do not react with β2-glycoprotein, and that aPL reactive with cardiolipin/cofactor complexes are associated with disease manifestations in APS. Although the exact relationship between aPL and antiheparin antibodies requires further study, it is likely that assays for aPL and antiheparin autoantibodies detect overlapping, but not identical, autoantibody populations. This was particularly illustrated by purification studies of APS IgG on heparin-Sepharose, which resulted in a significant enhancement of heparin-binding activity, but much less enhancement of cardiolipin reactivity (Fig 2). We may speculate that subpopulations of aPL with high affinity for heparin, or antiheparin antibodies, may be particularly effective in promoting a pathologic procoagulant state. Further population studies using larger sample sizes are necessary to investigate the hypothesis that antiheparin antibodies are associated with clinical episodes of thrombosis in patients with APS.

As noted previously, serum assays for the detection of antiheparin antibodies have generally proven unreliable. We have also found that assays for antiheparin antibodies using sera are unreliable (Shibata et al, unpublished data, 1993). There are numerous molecules in serum that may bind heparin and form macromolecular complexes that may interfere with the detection of specific antiheparin IgG by increasing nonspecific interactions. In addition, the presence of endogenous circulating heparin in serum and HS epitopes that may be exposed along the entire vasculature would also confound assays for circulating antiheparin antibodies. Indeed, considering that antiheparin antibodies, like other circulating autoantibodies, most likely exist in serum in a state of antigen excess, it is remarkable that they can be detected in serum at all. By using highly purified IgG, we were able to detect antiheparin antibodies, and to discriminate between patients and controls in our small sample. Presumably, removal of interfering substances by the purification of IgG in our studies enabled us to identify APS sera containing high-affinity antiheparin antibodies and to perform immunospecificity studies of APS antiheparin antibodies.

Several mechanisms to explain vascular injury and throm-
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Fig 7. Inhibition of heparin-accelerated TAT complex formation by APS IgG. Various amounts of APS total IgG and affinity-purified IgG antiheparin antibodies and normal control total IgG were preincubated with heparin (0.1 µg/mL). Antibody inhibition of heparin-accelerated formation of TAT complex was determined by ELISA. The results are expressed as percent inhibition of TAT complex formed in the absence of human IgG.

bosis associated with aPLs have been proposed, primarily based on their phospholipid reactivity. In this study, we showed the presence of IgG autoantibodies to HS/heparin in patients with the antiphospholipid syndrome that inhibits the heparin-accelerated formation of antithrombin III-thrombin complexes. Antiheparin antibodies may also inhibit other physiologic HS-dependent anticoagulation mechanisms, such as that involving heparin cofactor II. Considering the known physiologic importance of HS/heparin in normal anticoagulation, aPLs with high affinity for heparin or antiheparin antibodies may be an important cause of autoimmune vascular thrombosis in the aPL syndrome.

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Autoantibodies to heparin from patients with antiphospholipid antibody syndrome inhibit formation of antithrombin III-thrombin complexes [see comments]

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