Autoantibodies to Phosphatidylethanolamine (PE) Recognize a Kininogen-PE Complex

By Toshitaka Sugi and John A. McIntyre

Demonstration of autoimmune antiphospholipid antibodies (aPA) to negatively charged phospholipids (PL) in an enzyme-linked immunosorbent assay (ELISA) requires the presence of certain phospholipid-binding plasma proteins, eg, β2-glycoprotein I. We found a requirement for plasma proteins to detect certain autoantibodies in ELISA directed against the electrically neutral or zwitterionic phospholipid, phosphatidylethanolamine (PE). Two of these PE-binding plasma proteins were identified as high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK). We studied anti-PE antibody (aPE) seropositive plasma from 13 patients with SLE and/or recurrent spontaneous abortions by using partially purified kininogens and kininogen binding proteins from adult bovine serum isolated by carboxymethyl (CM)-papain affinity chromatography. Eleven of 13 sera recognized a kininogen-PE complex and/or a kininogen-binding protein-kininogen-PE complex. Some aPE-positive sera were shown to recognize highly purified HMWK and LMWK by ELISA only when the kininogens were presented on a PE substrate. These aPE sera did not recognize PE, HMWK, or LMWK when they were presented independently as the sole antigens on the ELISA plates. Other aPE-positive sera that did not react with PE-bound HMWK or LMWK reacted with the CM-papain column eluate when it was bound to PE, which suggests that these aPE recognize factor XI or prekallikrein, which normally bind to HMWK. The aPE ELISA reactivity of two patient sera were inhibited by preincubation of the CM-papain column eluate in the ELISA plate. These data show that most aPE are not specific for PE but require the presence of certain PL-binding plasma proteins that are kininogens or proteins in complex with kininogens. Our studies indicate that aPE bind to different plasma proteins than those implicated in anionic PL, aPA ELISA reactivity.

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AUTOANIBODIES KNOWN as anticardiolipin antibodies (aCL) and lupus anticoagulants (LA) have generated interest because of their association with vascular thrombosis, thrombocytopenia, and recurrent spontaneous abortion.1,4 It initially was assumed that antiphospholipid antibodies (aPA) were directed against simple phospholipid (PL) molecules, but it has been shown that the antigens detected by many aPA involve several PL binding proteins.5,6 Most LA and aPA literature is limited to descriptions of anionic PL5-10; however, antiphosphatidylethanolamine antibodies (aPE) also have been described as the sole aPA in patients with severe thrombotic diseases.11,12 Many laboratories do not screen for aPE routinely. A requirement for an aPA enzyme-linked immunosorbent assay (ELISA) is the addition of 10% adult bovine serum (ABS) to replenish native PL-binding proteins upon dilution of patient plasma. The need for serum in the ELISA was first attributed to a cofactor.6 Later, the cofactor was shown to be the target antigen for anionic aPA.8,9 The presence of negatively charged PL induced antigenic conformational changes in the PL-binding protein.13,14 We also found a serum requirement for detection of aPE in the ELISA. We chose to use ABS because it was the source of cofactor for detecting antibodies to anionic PL and was readily available in our laboratory. Two PL-binding proteins, β2-glycoprotein I (β2GPI) and prothrombin described as cofactor for aPA ELISA reactivity with anionic PL were unable to sustain aPE reactivity. We thus conducted a series of experiments designed to characterize the substance in ABS responsible for aPE cofactor activity. Our data show that the plasma proteins in ABS required for aPE ELISA activity are high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK). The finding that certain aPA detect kininogens bound to PE provides new insight into mechanisms that may predispose patients to vasculopathy, thrombocytopenia, and recurrent pregnancy loss.

MATERIALS AND METHODS

We characterized by ELISA 13 systemic lupus erythematosus (SLE) and/or recurrent spontaneous abortion patients seropositive for aPE IgG. To find the PE-binding protein(s) for aPA to PE in the ELISA we chose to study one patient whose plasma had a high titered ABS-dependent aPA IgG.15 To determine the aPE titers, we screened 80 normal individuals for aPE reactivity by ELISA. From these data we calculated the number of multiples of the median (MoM) that accounted for 95% of our normal values.15 For aPA, this value was 4 MoM. The patient sera chosen for isolating IgG aPE was 49 MoM, which places it 45 MoM greater than the control group threshold. IgG was purified from this plasma by diethyl amineethyl (DEAE)-Sepharose (Pharmacia LKB Biotechnology AB, Upsala, Sweden) followed by protein G-Sepharose (Pharmacia) chromatography. Plasma (1 mL) previously dialyzed in 20 mmol/L Tris/HCl, pH 7.4, was applied to the DEAE-Sepharose column (1 × 10 cm) eluted with 20 mmol/L Tris/HCl, pH 7.4, and washed with the equilibration buffer at a flow rate of 20 mL/h. The effluent from the DEAE-Sepharose column was applied to a protein G-Sepharose column (5 mL) eluted with 20 mmol/L Tris/HCl, pH 7.4. After washing with buffer, protein G-bound IgG was eluted with a 0.2 mol/L glycine/HCl buffer, pH 3.0, and immediately neutralized with saturated Tris and dialyzed against Tris-buffered saline (TBS; 0.02 mol/L Tris, 0.15 mol/L NaCl, pH 7.3).

Monoclonal antibodies (MoAbs) used in these studies were to kininogen heavy chain (11-2), provided by Dr S. Reddigiari (State University of New York at Stony Brook, NY); to bovine IgG (DAS2), provided by Dr S.S. Kumaran (University of Nebraska, Lincoln, NE); to HMWK, light chain (anti-HMWK #1) and to PKK.
Kaolin adsorption of kininogens was performed according to the method of Jiang et al.,9 with slight modification. Briefly, 10% ABS was mixed with increasing concentrations of kaolin (Sigma, St Louis, MO; 0.5% to 6% [w/v]) at 37°C for 15 minutes. After centrifugation at 3,000g for 5 minutes, the supernatants were collected and visualized in immunoblots by using an MoAb (11-2) that detects both LMWK and HMWK heavy chain.

Bovine HMWK and LMWK were isolated from ABS by carboxymethyl (CM)-papain Sepharose 4B affinity column according to published methods,10 with slight modification. Briefly, ABS (20 mL) containing 2 mol/L NaCl was applied to a column of CM-papain-Sepharose 4B (20 mL) equilibrated with 50 mmol/L Tris/HCl containing 2 mol/L NaCl, pH 7.4, at a flow rate of 40 mL/h. The column was washed with 100 mL of equilibration buffer followed by 50 mmol/L Tris/HCl, pH 7.4, until the A280 decreased to less than 0.01. Bound proteins were eluted in a single peak with 50 mmol/L Tris/NaOH, pH 11.5. Fractions were collected into tubes, rapidly neutralized with 1 N HCl, and dialyzed against TBS. The HMWK prepared from ABS was bradykinin (BK) free because plasma kallikrein generated during the clotting process liberates the BK. The LMWK, in contrast, was isolated intact from the ABS. Our Western blot data showed that LMWK retained its BK until it was digested with tissue kallikrein.

The kininogen-containing eluate from the CM-papain-Sepharose affinity column was further purified by using a Resource Q column (Pharmacia; 1 mL) on a Pharmacia FPLC apparatus (flow rate, 2 mL/min). After the column was washed with 50 mmol/L Tris/HCl, pH 8.0, a chromatogram was produced by using a 30-mL linear gradient from 0.1 to 0.4 mol/L NaCl in 50 mmol/L Tris/HCl, pH 8.0, at a flow rate of 1 mL/min. Two-milliliter fractions were collected at 2-minute intervals. Fractions comprising the peaks were combined and concentrated (Centriprep 10; Amicon, Beverly, MA). HMWK and LMWK were separated by chromatography over Sephacryl S-200 HR (2.6 × 70 cm; Pharmacia) equilibrated with TBS at a flow rate of 20 mL/h. Kininogen concentrations were measured in terms of absorbance at 280 nm by using a Beckman Spectrophotometer (Beckman Instruments, Inc, Fullerton, CA). The extinction coefficients ε 1% (1% cm) of purified HMWK and LMWK at 280 nm were 7.4 and 6.7, respectively,11 and these values were used to prepare purified kininogen solutions of definite concentration.

The aPE ELISA followed a previously described procedure,12 with slight modifications. Immulon 3 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 30 μL of 50 μg/mL PE (Avanti Polar Lipids, Birmingham, AL) diluted in chloroform-methanol (1:3) per well and dried under nitrogen. Each well was blocked (Avanti Polar Lipids, Birmingham, AL) diluted in chloroform-methanol (1:3) per well and dried under nitrogen. Each well was blocked with tissue kallikrein.

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8% homogeneous resolving gel and 3% stacking gel. Samples were boiled for 2 minutes in 2% SDS containing 0.1 mol/L Tris/HCl, pH 6.8, 20% glycerol, and 0.2% bromophenol blue. Gels were stained with Coomassie brilliant blue. Transfer to nitrocellulose membranes was performed overnight at 0.1 amps. Membranes were blocked for 1 hour with 1% BSA in phosphate-buffered saline (PBS), pH 7.3. Incubation with MoAb to kininogen heavy chain (11-2) and HMWK light chain (anti-HMWK #1) and prekallikrein (anti-PKK #4) was performed for 1 hour followed by three washes with 0.03% Tween 20/PBS. The membrane was exposed to peroxidase-conjugated rabbit antimouse IgG (1:1,000; Dako, Glostrup, Denmark) for 1 hour, followed by washing as described above. The immunoblots were developed by using 3, 3'-diaminobenzidine tetrahydrochloride (Polysciences, Warington, PA) containing 0.03% hydrogen peroxide.

**RESULTS**

**Discovery of kininogens as the aPE ELISA cofactor.** The requirement for ABS to show aPE in a dose-dependent man-
The reactivity of the purified IgG, aPE, was similar to the
DEAE column was used to remove other plasma pro-
teins such
from an aPE-positive patient plasma by DEAE column chro-
matography followed by protein G affinity chromatography. The DEAE column was used to remove other plasma proteins such as HMWK and LMWK that bind to protein G. The reactivity of the purified IgG, aPE, was similar to the native plasma or serum reactivity (Table 1). Sephacryl S-300 HR gel filtration showed the aPE cofactor in ABS to be less than 300 kD. Absorptions with solid-phase lectins, Concanavalin A (Con A), Helix Pomatia, and Lentil showed that Con A was capable of retaining cofactor activity, thus limiting our search to a glycoprotein. Attempts to elute cofactor activity of ABS from polyacrylamide columns consisting of 83% cholesterol and 17% PE were successful; however, the purity we desired was not observed on SDS gels. Substantial progress was realized when we observed that the ABS eluate from a solid-phase protein G Sepharose column contained cofactor activity with dose-response kinetics (data not shown). This finding was taken in context with the report by Sjobring et al who showed that protein G bound four plasma proteins: (1) IgG, (2) albumin, (3) α-2 macroglobulin, and (4) the kininogens. We focused immediately on the kininogens because previous experiments had indicated that purified bovine IgG did not have cofactor activity, bovine albumin was used as our negative control in the ELISA, and α-2 macroglobulin with a molecular weight (mol. wt.) of approximately 800 kDa was too large to be considered.

Kaolin absorptions to remove kininogens from ABS. Removal of HMWK and LMWK was performed by adsorption of 10% ABS with kaolin. A shown in Fig 2A, immunoblots using MoAb to kininogen heavy chain recognized bovine HMWK and LMWK at 90 and 60 kD, respectively, under nonreducing conditions. The MoAb to kininogen heavy chain (HC) designated (11-2) was produced to purified re-
duced and alkylated human HC. We chose this particular MoAb because of its crossreactivity with bovine kininogen HC. Other MoAbs to human HC tested did not recognize bovine HC. Both HMWK and LMWK decreased after adsorption with 3% concentrations of kaolin. The aPE ELISA reactivity decreased concomitantly with loss of the kininogen immunobands (Fig 2B). Theoretically, kaolin should not be effective in removing LMWK from serum. Nevertheless, Jiang et al reported that kaolin could consume LMWK at concentrations of 6% or higher and we confirmed their data. The faint bands in lane 5 and 6 (Fig 2A) testify to the sensitivity of the Western blot procedure; however, the small amount of kininogens detected was of insufficient concentra-
tion to serve as cofactor in the aPE ELISA.

Purified human kininogen as aPE ELISA cofactor. We also asked if kininogens prepared from human plasma had aPE cofactor activity. Commercially prepared human LMWK (Calbiochem, San Diego, CA) was substituted for ABS. As shown in Fig 3, the PE cofactor activity was pres-

See text for IgG purification scheme. aPE-positive patient plasma (1:200) or purified IgG from the same patient was diluted in 10% ABS and ELISA reactivity was assessed. Plasma IgG concentration of this patient was 15 mg/mL; thus, the patient’s plasma at 1:200 dilution was similar in ELISA reactivity to the purified IgG at 75 μg/mL.

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD₆₃₀</th>
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<tbody>
<tr>
<td>10% ABS + patient plasma 1/200</td>
<td>1.020</td>
</tr>
<tr>
<td>10% ABS + patient IgG (150 μg/mL)</td>
<td>1.899</td>
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<tr>
<td>10% ABS + patient IgG (75 μg/mL)</td>
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<tr>
<td>10% ABS + patient IgG (37.5 μg/mL)</td>
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Table 1. Isolated IgG aPE in ELISA

![Graph](image)

Fig 2. Immunoblot analysis and aPE ELISA reactivity of 10% ABS adsorbed with increasing concentrations of kaolin. Ten percent ABS was mixed with increasing concentrations of kaolin (0.5 to 6% [w/vol]) at 37°C for 15 minutes. After centrifugation at 3,000g for 5 minutes, the supernatants were collected and immunoblots and an aPE ELISA was performed. (A) Kininogens in kaolin-adsorbed ABS (10 μL/ lane) were visualized in immunoblots by using an MoAb (11-2) that detects both LMWK HC and HMWK HC. HMWK and LMWK immunobands were decreased with greater than 3% kaolin. (B) APE reactivity by ELISA adding kaolin-adsorbed ABS and aPE-positive patient plasma (1:200). (♦) PE; (□) blank. Loss of aPE ELISA reactivity corresponded to loss of kininogens.

![Graph](image)

Fig 3. A representative experiment that substituted purified human LMWK for ABS as the source of aPE cofactor. (■) PE; (□) blank.
Fig 4. The void volume peak from a Sephacryl S-200 HR column. After SDS-PAGE electrophoresis, proteins were transferred to nitrocellulose paper. Lane 1 shows MoAb to human PKK (anti-PKK #4) that recognizes bovine PKK and bovine FXI. Lane 2 is a normal mouse IgG control. A small amount of background IgG was present in this fraction.

ent. However, commercially prepared human HMWK (Calbiochem) did not have cofactor activity (data not shown). We attributed this difference to certain reagents commonly used in the purification of kininogens and other contact factors, eg, polybrene, EDTA, soybean trypsin inhibitor (SBTI), and benzamidine. When we added these reagents independently to ABS (40 μg/mL polybrene; 2 mmol/L EDTA; 0.2 mg/mL SBTI; 1 to 10 mmol/L benzamidine) they had variable, but inhibitory, effects on the ability of ABS to serve as cofactor in the aPE ELISA. Dialysis of the ABS subsequent to exposure to these reagents did not restore its cofactor activity. We intentionally avoided using human plasma and serum as the kininogen source because it contained an IgG contaminant that caused an unacceptably high background in the aPE ELISA. This was not a problem with bovine kininogen preparations because our MoAb antihuman IgG conjugate did not recognize bovine IgG, despite our observation that bovine IgG also contaminated the bovine kininogen preparations. Such residual IgG could be removed, but required additional FPLC chromatography.

Biochemical purification and analyses of kininogens. Bovine HMWK and LMWK were purified by using a CM-papain Sepharose affinity column, FPLC Resource Q column followed by Sephacryl S-200 HR chromatography. The S-200 HR column yielded 3 protein peaks. All 3 peaks supported aPE activity in the ELISA. The first peak off this column (void volume peak) contained the high mol. wt. material (mol. wt. >200 kD). From our Western blot data, the MoAb to HMWK light chain (LC; anti-HMWK #1) recognized this high mol. wt. material in the void volume fraction. Other investigators also have reported this high mol. wt. material as aggregates of HMWK.20-22 The first or void volume peak also contained HMWK complexed to either factor XI (FXI) or prekallikrein (PKK). Figure 4 shows a Western blot of the void volume fraction with an MoAb (anti-PKK #4) produced to human PKK. This particular MoAb recognizes bovine PKK as well as bovine FXI due to the high degree of homology between PKK and FXI.

The purified HMWK and LMWK that eluted from the S-200 HR column at the expected mol. wts. retained cofactor activity. Figure 5A shows an SDS-PAGE of the second and third peaks. The third peak contained 3 approximately 60-kD bands, all of which were identified in immunoblots using MoAb (11-2) as LMWK. The second peak shows an approximate 90-kD band under nonreducing conditions and a 60-kD band HC under reducing conditions, indicating that this peak contained two-chain HMWK. This finding was confirmed by using an MoAb to the HMWK LC (anti-HMWK #1) that recognized a 90-kDa band under nonreducing conditions and a 30-kDa band (LC) under reducing condition (data not shown). Figure 5B shows the dose-dependent aPE binding activity supported by the addition of highly purified LMWK to a 1/200 dilution of aPE-positive patient plasma. (○) PE; (●) blank.
Kininogen-PE complexes. We showed directly using ELISA the formation of a complexes between purified kininogens and PL. Purified two-chain HMWK and LMWK were incubated in ELISA wells previously coated with either anionic or zwitterionic PL. Binding of HMWK was detected by MoAb to the HMWK LC (anti-HMWK #1). As shown in Fig 6A, HMWK bound to all PL tested. Purified LMWK-PL binding was detected by an affinity-purified polyclonal antibody (PoAb) to BK. Our Western blot data had shown that this PoAb to BK did not recognize purified HMWK under reducing or nonreducing conditions. In contrast, this PoAb recognized purified LMWK under reducing and nonreducing conditions. This finding indicated that our purified LMWK was not digested by plasma kallikrein. Subsequent to digestion of LMWK with tissue kallikrein, this PoAb did not recognize LMWK. LMWK bound best to PE and phosphatidylcholine (PC; Fig 6B) and did not bind significantly to phosphatidylserine (PS) or cardiolipin (CL). We do not know why LMWK does not bind to CL. Binding of HMWK to CL may implicate binding by HMWK LC, which is different from the LC of LMWK. Of primary interest was the observation that the patient aPE recognized only the HMWK and LMWK bound to PE. No patient aPE binding was observed in wells in which the HMWK bound PS, CL, or PC (manuscript in preparation). In further support of the requirement for a PE kininogen complex to detect aPE were the observations that purified HMWK and LMWK were not recognized by aPE-positive patient plasma in Western blots (data not shown). In summary, when bound to PE, both intact and BK free kininogens can serve as cofactor for aPE.

Effect of purified kininogens on the aPE ELISA. Sero-positive aPE IgG plasma from 13 patients with SLE and/or recurrent spontaneous abortions were investigated for kininogen dependence in an ELISA. All patients' plasmas were diluted (1:100) in either the partially purified kininogen preparation eluted from the CM-papain-Sepharose affinity column (500 μg/mL) or 1% BSA as control. Figure 7 shows
that 11 of 13 patients’ aPE reactivities were optimized by the addition of exogenous kininogens or HMWK-binding proteins found in the CM-papain column eluate. Two patient plasmas did not produce an increased aPE ELISA signal as a result of their dilution in the CM-papain column eluate. To the contrary, addition of the CM-papain column eluate markedly inhibited aPE binding by these two plasmas. Thus, one can occasionally find aPE without having ABS in the assay. This situation is similar to the inhibition reported between aCL and β3GPI in patients with false-positive Vener- Real Disease Research Laboratory tests.23

**DISCUSSION**

Autoantibodies reactive to anionic PL have been detected frequently in patients with collagen-vascular diseases such as SLE. Recent evidence shows that many of these aPA are not specific for anionic PL per se, but are directed to PL-binding proteins.5-9 Because the initial nature and specificity of the PL antigenic epitopes were not understood, the PL-binding proteins were designated as cofactors. Most investigators now agree that the cofactors are the antigen and that the role of the PL is to induce conformational changes within these plasma proteins subsequent to PL-binding.15,14

A few investigators have looked for and found aPE.11,12,24,25 Although serum may be required in the plasma diluent to show optimal aPE reactivity in the ELISA, the specificity of aPE regarding PL-binding proteins was unknown previously. Our data clearly show that some aPE recognized both purified LMWK and HMWK in the ELISA, but only when kininogens were presented on a PE-coated surface. HMWK, LMWK, and PE were not recognized when they served as the sole ELISA target antigens. These data indicate that binding of kininogens to PE induces novel antigenic epitopes. The requirement for ABS in the aPE-positive plasma diluent is necessary because of the low concentrations of native kininogens at the dilutions (1/100) used in the aPE ELISA. As shown in Fig 1B, reduction of ABS to less than 5% resulted in the loss of aPE ELISA reactivity. Indeed, in many patients there was no positive ELISA signal obtained until 5% or greater concentrations of ABS were present. The option of using native kininogens in patient samples diluted 1/10 or 1/20 is untenable because of increased IgG background readings found with such high serum concentrations.

Ten of 12 other patients positive for aPE also recognized kininogen preparations from CM-papain-Sepharose in the aPE ELISA; however, only some patient sera recognized purified HMWK and LMWK bound to PE. Because virtually all circulating FXI and approximately 75% of circulating PKK27,28 exist as complexes with HMWK, the eluate from the CM-papain column also contains FXI and PKK. Our preliminary data indicate that other aPE-positive patient sera recognize FXI or PKK or their activated intermediates bound to HMWK because they were not positive in the ELISA when purified HMWK or LMWK was bound to PE. Whether the antigenic epitope(s) recognized by these patient sera exists on conformationally altered HMWK, FXI, or PKK induced by the complex or its binding to PE is still under investigation. However, what is clear is that the majority of aPE detected by ELISA recognize either a PE-kininogen complex and/or a PE-kininogen-FXI, PKK complex and not PE. We have not measured these aPE-positive plasmas for HMWK or LMWK levels.

In the ELISA, aCL associated with syphilis and other infectious diseases do not require PL-binding proteins. The presence of β3GPI in this instance competes with the aCL and causes decreased ELISA reactivity.23 Similarly, for 2 patients with aPE, the addition of kininogens decreased their aPE ELISA reactivity. This finding suggests that some patients produce aPE that is cofactor independent. However, at this time, we cannot rule out the possibility that the aPE binding by these 2 patients’ sera may require another PE-binding protein that is sufficiently present in their diluted plasmas. Further studies are needed to clarify this possibility.

These findings offer new insights into the vascular pathology associated with aPE. Both HMWK and LMWK can bind to platelet membranes through their respective heavy chains (domain 3), and, when bound, they inhibit thrombin-induced platelet activation.29 A platelet kininogen receptor has not been described. The outer leaflet of the resting and activated platelet membrane reportedly contains 9.5% and 36% PE, respectively.30 We hypothesize that kininogens may bind to platelets by virtue of membrane PE. When targeted by aPE, this platelet-kininogen-aPE complex may no longer render the platelet refractory to thrombin activation, thus predisposing the patient with aPE to thrombosis. This hypothesis is currently under investigation by studying thrombin-induced platelet aggregation in the presence of putative aPE.

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Autoantibodies to phosphatidylethanolamine (PE) recognize a kininogen- PE complex

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