Thrombin Binding Predicts the Effects of Sequence Changes in a Human Monoclonal Antiphospholipid Antibody on Its In Vivo Biologic Actions

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The mechanisms by which antiphospholipid Abs (aPL) cause thrombosis are not fully understood. It is clear that binding to a number of phospholipid-associated Ags is important but it is difficult to identify which Ag-binding properties are most closely linked to the ability to cause biologic effects such as promotion of thrombosis and activation of endothelial cells. We have previously used an in vitro expression system to produce a panel of human monoclonal IgG molecules between which we engineered small differences in sequence leading to significant well-defined changes in binding properties. In this study, we assess the properties of five of these IgG molecules in assays of biologic function in vitro and in vivo. The i.p. injection of these IgG into mice subjected to a femoral vein pinch stimulus showed that only those IgG that showed strong binding to thrombin promoted in vivo venous thrombosis and leukocyte adherence. However, this finding did not hold true for the effects of these IgG on activation of cultured endothelial cells in vitro, where there was a less clear relationship between binding properties and biologic effects. The Journal of Immunology, 2009, 182: 4836–4843.

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3 Abbreviations used in this paper: APS, antiphospholipid syndrome; aPL, antiphospholipid Ab; β2GPI, β2-glycoprotein I; CL, cardiolipin; EC, endothelial cell; EMP, endothelial microparticle; GPLU, IgG phospholipid unit; PC, phosphatidylcholine; PS, phosphatidylyserine.

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Abs with well-defined differences in sequence and binding properties. We have addressed this problem by using site-directed mutagenesis, L chain exchange, and in vitro expression to create a panel of monoclonal human IgG aPL that differ from each other only at points in the sequence engineered by us and have corresponding differences in binding properties. In this study, we have selected five members of this panel on the basis of their divergent binding properties and tested their biologic properties in vitro and in vivo.

All five IgG used were based on the human monoclonal IgG aPL IS4 (originally derived from a patient with APS), which binds β2-gp1 (25) and thrombin (10) and is known to be pathogenic in the femoral vein thrombosis model (17). Previously, we showed that mutations of one or more surface-exposed arginine residues in the CDR3 of IS4 H chain variable region (IS4VH) to serine dramatically altered binding to a range of phospholipids Ags and β2-gp1. In most cases, these changes led to reduced binding because the H chain is dominant in determining the binding properties of IS4 (26, 27). The variable region of the IS4 L chain (IS4VLL), however, played a more ambivalent role, and by altering the sequence of this chain by less than 7% either markedly increased or reduced binding to phospholipids Ags. Therefore, by selecting a panel of five of these IS4 variants, with different VH and VL sequence combinations, we have now studied the biologic effects of Abs with a range of clearly established sequence and binding properties (28). In particular we correlated the effects of the mAbs upon thrombosis and leukocyte adherence to EC in vivo with EC activation in vitro and binding to phospholipids, β2-gp1 and thrombin.

Materials and Methods

Human IgG mAbs

The production of the Abs IS4VH/IS4VLL, IS4VH/B3VLL, IS4VH/UK4VLL, IS4VHi&ii/IS4VLL, and IS4VHi&ii/B3VLL by stable expression in Chinese hamster ovary cells has been previously described (26, 28–30). The IS4VHi&ii differs from IS4VH in two arginine to serine mutations at positions 96 and 97. The large scale production and purification of IgG was outsourced to Chemicon International and Harlan.

Purified human IgG was passed through a endotoxin removal gel (Pierce Endogen) and determined to be free of endotoxin contamination (≤0.125 endotoxin U/ml) by Limulus amoebocyte assay (Sigma-Aldrich). The concentration of IgG was checked by ELISA (26) and spectrophotometry. The αCL activity, in IgG phospholipid units (GPLU), was measured as previously described (28). ELISA to detect thrombin binding was conducted as described by Hwang et al. (10). In brief, high binding ELISA plates (Costar) were coated with 10 μg/ml human α-thrombin (Hematologic Technologies) diluted in TBS on the test half of the plate and TBS alone was placed on the control half. Plates were incubated overnight at 4°C and then blocked with TBS/0.3% gelatin for 1 h at room temperature. Then monoclonal IgG (at 100 μg/ml) in TBS/0.1% gelatin were loaded in triplicate and incubated for 1.5 h followed by the addition of goat anti-human IgG Fc-specific alkaline phosphatase conjugate in TBS/0.1% gelatin for 1 h followed by substrate and absorbance read at 405 nm. Lupus anticongulant activity of each IgG was measured by a modified kaolin clotting time test (17) and considered positive if the ratio of the clotting time of a test IgG to that of normal IgG exceeded 1.2.

Analysis of thrombus dynamics and adhesion of leukocytes to endothelium in vivo

The effects of these monoclonal IgG upon thrombus formation and endothelial activation in vivo were studied as previously described (17, 24). All animals were housed in the animal care facilities of the Morehouse School of Medicine (Atlanta, GA) and handled by trained personnel according to Institutional Animal Care and Use Committee guidelines. CD1 mice (weight 30–43 g, 4–5 animals per group; Charles River Breeding Laboratories) were injected i.p. with 100 μg/ml test monoclonal IgG or a control monoclonal IgG (Calbiochem), which lacked aPL reactivity (0 GPLU) at time 0 and at 48 h later. After 72 h, mice were anesthetized, the left femoral vein exposed, and a standardized mechanical pinch (1500 g/mm²) applied to induce a thrombus. The size (in micrometers squared) and duration of each thrombus was visualized and its digital image measured 1 min after each pinch. Immediately after this surgical procedure, the cremaster muscle of each mouse was exposed and visualized under the microscope. After a stabilization period of 30 min, the number of adhering (stationary) leukocytes within five different postcapillary venules for 30 s was then determined and the average expressed for each animal. Data on thrombus size and leukocyte adherence are shown for all animals tested apart from one animal in the control group, which died during surgery and one animal in the native IS4 treatment group that lacked cremaster circulation.

Measurement of aPL-induced EC activation in vitro by FACS analysis

Fresh HUVECs obtained by informed consent and approved by the local ethics committees (University College, London, U.K.) were extracted from healthy donors and grown to near confluence (at passage 2 or less) in 0.1% gelatin-coated 12-well plates with complete MCDB 131 medium/10% FCS at 37°C. The night before each experiment, the medium was replaced with MCDB 131 containing 1% FCS. On the day of the experiment, wells were treated for 4 h at 37°C with 500 μg/ml test monoclonal IgG or a control monoclonal IgG (a recombinant mAb with no binding to a number of Ags, previously described in Ref. 31), which lacked aPL reactivity (0 GPLU), or treated with 3 μg/ml LPS (Sigma-Aldrich). Cells were then harvested with trypsin-EDTA and centrifuged at 100 × g for 5 min, then incubated for 30 min at 4°C with mouse anti-human mAbs against E-selectin, VCAM-1, ICAM-1, and tissue factor (BD Biosciences). All Abs were PE-conjugated except anti-ICAM-1, which was Cy5-conjugated and had been titrated for optimal dilution. Positivity was defined using appropriate manufacturer’s isotype controls. Cells were washed with PBS/5% FCS/0.02% sodium azide and then fixed (CellFix; BD Biosciences). Cell fluorescence were measured on a BD Biosciences FACSscan flow cytometer, and data were analyzed with CellQuest Pro software.

The effect of aPL upon EMP production from these cultured HUVECs was measured as previously described (32). In brief, supernatant from these experiments was removed and labeled with fluorochrome-conjugated Annexin V. Monoclonal Abs against EC markers E-selectin, P-selectin, VCAM-1, ICAM-1, VE-cadherin (CD144), endoglin (CD105), and tissue factor were also used to stain EMP. The EMP phenotype and quantification were assessed using two-color flow cytometry, as described. EMPs were defined as particles with FSC less than that of 3-μm latex beads that stained positively for Annexin V and a second endothelial marker.

Statistical analysis

Nonparametric statistical analyses were performed on the results of in vivo and in vitro biologic assays. The Mann-Whitney-Wilcoxon test was conducted to compare two groups of unpaired data using GraphPad Prism software.

Results

Previously known sequence and binding properties of the five Abs tested

The five mAbs tested in this experiment have been previously described (26–28, 33) and their binding properties are summarized in Table I. The index mAb IS4 shows strong but selective binding to the anionic phospholipids, cardiolipin (CL) and phosphatidylinerine (PS), and does not convincingly bind other phospholipids. Replacing IS4VL with VL from mAb B3 or UK4 has dramatic effects on binding properties. IS4VHi&ii/IS4VL, and IS4VHi&ii/B3VL by stable expression in Chinese hamster ovary cells has been previously described (26, 28–30). The IS4VHi&ii differs from IS4VH in two arginine to serine mutations at positions 96 and 97. The large scale production and purification of IgG was outsourced to Chemicon International and Harlan.

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Table I. Overall binding characteristics of the five H chain/L chain combinations

<table>
<thead>
<tr>
<th>H Chain</th>
<th>L Chain</th>
<th>β₂GPI</th>
<th>CL</th>
<th>PS</th>
<th>PI</th>
<th>PC</th>
<th>PA</th>
<th>PE</th>
<th>dsDNA</th>
<th>OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS4VH</td>
<td>IS4VL</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IS4VHii&amp;ii</td>
<td>IS4VL</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IS4VHii&amp;ii</td>
<td>B3VL</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>IS4VH</td>
<td>B3VL</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>IS4VH</td>
<td>UK4VL</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

*a Summary of binding of purified IgG to cardiolipin (CL), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), β₂GPI, prothrombin (PT), dsDNA, and OVA. The identity of native H and L chains is clearly indicated. IS4VHii&ii contains two arginine to serine replacements at positions 96 and 97. Each V_H/V_L combination was tested at 1 μg/ml in triplicate and the degree of binding was defined from the mean absorbance as follows: – = OD < 0.1; + = OD 0.1-0.4; ++ = OD 0.4-0.8; +++ = OD 0.8-1.2; and ++++ = OD > 1.2. These findings have been fully described previously (28) and are summarized to aid interpretation of the new data in this study.

Binding studies

Previous experiments have shown IS4, produced from hybridoma supernatant, to be thrombogenic in the in vivo model of thrombosis and microcirculation (17) at a concentration of 100 μg/ml. When we tested our five mAb at 100 μg/ml for ability to bind CL, we found that CL binding values decreased in the same order previously demonstrated at 1 μg/ml, with the highest binding obtained for IS4VH/B3VL (158 GPLU) and IS4VHii&ii/B3VL (157 GPLU) followed by IS4VH/IS4VL (98 GPLU) then IS4VH/UK4VL (10 GPLU), and finally IS4VHii&ii/IS4VL (1 GPLU). In contrast, none of the IgG displayed any lupus anticoagulant activity. None of the control monoclonal IgG used in these experiments bound CL (0 GPLU) when tested in this assay.

Of the five mAb tested at 100 μg/ml for antithrombin binding, only two that had strong and more selective binding to CL and PS (IS4VH/IS4VL and IS4VHii&ii/B3VL) displayed strong binding to thrombin (Fig. 1). The other three IS4 variants with poor selectivity or weak/no phospholipid binding had negligible antithrombin binding similar to the control IgG. Interestingly, (IS4VH/B3VL), which binds prothrombin and every phospholipid tested, did not bind thrombin.

Thrombin binding predicts the in vivo thrombogenic properties of these mAbs

All mice had measurable levels of circulating human IgG on the day of surgery (Fig. 2 and Table II). There was no statistically significant difference between the median IgG concentrations found in the mice injected with control IgG and those injected with IS4VH/IS4VL (p = 0.1). The mice treated with IS4VH/IS4VL had significantly higher levels of human IgG than those treated with the other V_H/V_L combinations: p < 0.004 for mice treated with IS4VHii&ii/IS4VL or IS4VHii&ii/B3VL; and p < 0.008 for mice treated with IS4VH/B3VL or IS4VH/UK4VL (Fig. 2).

We examined the effect of each mAb upon thrombus size in the mice (Fig. 3 and Table II). Treatment with recombinant IS4VH/IS4VL and IS4VHii&ii/B3VL both significantly (p < 0.05) enhanced thrombus size in mice compared with those treated with the control monoclonal IgG. These findings confirm previous observations with hybridoma-derived IS4 (17) in this model. The two Abs (IS4VH/UK4VL and IS4VHii&ii/IS4VL) with little or no ability to bind phospholipids or thrombin both had little ability to promote thrombosis. Thrombi in these mice were much smaller than thrombi in the IS4VH/IS4VL-treated mice (p = 0.056 for IS4VH/UK4VL and p = 0.016 for IS4VHii&ii/IS4VL). More surprisingly three of four mice treated with IS4VH/B3VL, the strong but nonselective phospholipid binder, which lacks thrombin binding, also produced thrombi no larger than those seen in animals treated with control IgG. Excluding the one outlier from this IS4VH/B3VL-treated group, there was a significant reduction in thrombus size compared with mice treated with IS4VH/IS4VL (p = 0.018). IS4VHii&ii/B3VL, which has strong thrombin binding gives thrombi as large as those seen with native IS4VH/IS4VL (p = 0.155) and far larger than those seen with IS4VH/B3VL,
from that of control IgG-treated mice. The median thrombus size in each group is clearly marked in micrometers squared. For the identity of mutant H chains, see Table I. The median thrombus size in 48 h later and the effects upon thrombus size were measured at 72 h. Individual results from each animal are shown and the median thrombus size in each group is marked by horizontal bar. For the identity of microvascular leukocytes compared with the control IgG. When comparing the different test Abs with each other, however, we see the same pattern observed in the experiments on size of thrombus.

Effects of the Abs on leukocyte adherence in vivo mirror effects on thrombus formation
To determine the effects of each mAb upon in vivo endothelial activation we measured leukocyte adherence to endothelium of the cremaster muscle, in mice treated with the five test mAb and the control IgG (Fig. 4 and Table II). All five of the IS4 variants produced a statistically significant increase in the number of adhering leukocytes compared with the control IgG. When comparing the different test Abs with each other, however, we see the same pattern observed in the experiments on size of thrombus.

Effects on activation of cultured HUVECs do not show a clear differentiation between mAb with different binding and biologic properties
We next tested the effects of the mAb on up-regulation of several cell adhesion molecules and tissue factor on cultured HUVECs in vitro. The overall expression of E-selectin was low in all cells treated with control and test IgG, although a significant increase in thrombus size in each group was observed in the experiments on size of thrombus.

Table II. Binding properties and biologic effects of monoclonal IgG aPLa

<table>
<thead>
<tr>
<th>H Chain</th>
<th>L Chain</th>
<th>Cardiolipin Binding, GPLU</th>
<th>Thrombin Binding</th>
<th>IgG Median, μg/ml (range)</th>
<th>Thrombus Sizeb</th>
<th>Leukocyte Adherencec</th>
<th>E-selectin Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS4VH</td>
<td>IS4VL</td>
<td>98</td>
<td>Strong</td>
<td>130 (50–130)</td>
<td>16c</td>
<td>8c</td>
<td>1.8c</td>
</tr>
<tr>
<td>IS4VHi&amp;ii</td>
<td>IS4VL</td>
<td>1</td>
<td>None</td>
<td>20 (10–35)</td>
<td>2.6</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>IS4VHi&amp;ii</td>
<td>B3VL</td>
<td>157</td>
<td>Strong</td>
<td>12 (9–12)</td>
<td>22c</td>
<td>6c</td>
<td>1.1</td>
</tr>
<tr>
<td>IS4VH</td>
<td>B3VL</td>
<td>158</td>
<td>None</td>
<td>7 (5–10)</td>
<td>2.7</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>IS4VH</td>
<td>UK4VL</td>
<td>10</td>
<td>None</td>
<td>12 (6–17)</td>
<td>6.5</td>
<td>3.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

a Binding of each VH/VL combination (at 100 μg/ml) is shown to cardiolipin and thrombin. The effects in fold increase of each VH/VL combination on thrombus size and leukocyte adherence in vivo (at 100 μg/ml) and E-selectin expression in vitro (at 500 μg/ml) are shown relative to control IgG. For the identity of the mutant H chain, see Table I.

b Fold increase was calculated by dividing the median value of each group of animals/cells treated with monoclonal aPL by the median value of corresponding animals/cells treated with monoclonal control IgG, which lack cardiolipin binding.

c Statistically significant differences were measured.

Treatment with IS4VH/IS4VL gives the highest leukocyte adherence values. IS4VHi&ii/B3VL is the next highest, with no significant reduction in leukocyte adherence compared with IS4VH/IS4VL (p = 0.095). The strong but nonselective binder IS4VH/B3VL and the weak binders IS4VH/UK4VL and IS4VHi&ii/IS4VL gave similar results to each other in this assay. All gave significantly reduced leukocyte adherence values compared with IS4VH/IS4VL (p = 0.029 for IS4VH/B3VL, p = 0.029 for IS4VH/UK4VL, p = 0.008 for IS4VHi&ii/IS4VL). Similar to our findings with in vivo thrombosis, the pattern of mAb binding to thrombin was most closely associated with the ability of the mAb to promote leukocyte adherence in vivo.

Effects on activation of cultured HUVECs do not show a clear differentiation between mAb with different binding and biologic properties
We next tested the effects of the mAb on up-regulation of several cell adhesion molecules and tissue factor on cultured HUVECs in vitro. The overall expression of E-selectin was low in all cells treated with control and test IgG, although a significant increase...
was observed with native IS4 compared with all other test and
control IgG (Fig. 5A). VCAM-1 expression was also very low
in all cells treated with IgG but none of the IS4 variants caused
a significant increase compared with control IgG (Fig. 5B).

Constitutive ICAM-1 expression (Fig. 5C) was seen even in
cells treated with control IgG that does not bind phospholipids
and did not differ significantly when any of the other Abs were
added instead of control IgG. Tissue factor expression (Fig. 5D)
was not seen in cells treated with any of the mAb or the
control IgG.

We also examined EC activation by measuring EMP production
from HUVECs exposed to the different mAb (Fig. 6). EMP were
identified by the presence of Annexin V staining and positivity for
VE-cadherin, endoglin, E-selectin and/or ICAM-1; and the ab-
sence of staining for P-selectin, VCAM-1 or tissue factor. Com-
pared with control IgG none of the IS4 variants appreciably in-
creased the production of EMP after 4 h of exposure.

Discussion
This study has emphasized that sequence changes in both the VH
and VL of the recombinant human aPL IS4 are important in de-
termining its functional and pathogenic properties in vivo. Impor-
tantly, these functional effects are not always predicted by CL
binding or effects of the aPL on EMP production or surface ex-
pression of E-Selectin, ICAM-1, VCAM-1, or tissue factor on ECs
in vitro. Our findings of a statistically significant increase in throm-
bus size and leukocyte adherence with recombinant IS4 compared
with control monoclonal IgG (Figs. 3 and 4) corroborate previous
findings with native IS4 in this same animal model (17). This result
underlines the pathogenic potential of this monoclonal aPL and the
relevance of the study of our panel of Abs derived from IS4 to the
pathogenesis of APS. It is important to note, however, that many
structurally different forms of aPL exist so our panel of recombinant monoclonal aPL Abs may not be entirely representative of all pathogenic aPL. Therefore, future experiments using different aPL, such as those which cross-react with DNA, may lead to refinement of our conclusions.

These in vivo findings extend those of our previous studies in which we found that somatic mutations to arginine determine the ability of these V_H/V_L combinations to bind Ags relevant in the pathogenesis of the APS (26–28). In particular, these new data confirm the striking effects of making just two arginine to serine changes caused loss of binding to all phospholipids tested and ability to stimulate thrombosis and leukocyte adherence in vivo. In IS4VHii/B3VL however, these changes increased both selectivity of binding to anionic phospholipids as well as ability to stimulate thrombosis and leukocyte adherence in vivo. Furthermore, the opposing effects of these V_H mutations on IS4VH/IS4VL and IS4VH/B3VL show that the L chain too has a profound influence on both the binding and biologic properties of these Abs (Figs. 3 and 4).

Only two previous studies have altered the sequences of monoclonal aPL to examine the complex relationship between sequence, structure, binding, and pathogenic effects. The findings of Lieby et al. (34) were straightforward in that the simultaneous germline reversion of three somatically mutated VLCDR1 asparagine residues caused a pathogenic aPL to lose its Annexin A5 dependency for CL binding and its ability to induce fetal loss in BALB/c mice. In contrast, sequence analysis of a range of aPL, including hybridoma-derived IS4, tested in the in vivo model of microcirculation in our laboratory (17) revealed no simple relationship between pathogenicity and strength of binding to CL. Two of the Abs tested, IS1 and IS2, contained identical V_H and their L chains differed from each other by only five amino acids (35). IS1VL contained more mutations than IS2VL, which improved its reactivity for Ag but abrogated its thrombogenic activity (17, 35).

In this current study, with variants of recombinant IS4, we have found a similarly complex relationship between aPL binding and biologic effects. In particular, IS4VH/B3VL binds CL and other Ags believed to be important in the pathogenesis of APS such as PS and β2GPI (28), but this V_H/V_L combination did not increase thrombus size or leukocyte adherence in three of four treated mice, compared with a control IgG, (Figs. 3 and 4). One possible explanation for this result could have been that the levels of circulating human IgG in mice injected with IS4VH/B3VL were lower than the levels of human IgG in the mice injected with IS4VH/IS4VL (Fig. 2). We do not believe, however, that differences in circulating human IgG levels can explain all the differences between groups because mice injected with IS4VHii/B3VL had similar circulating levels to those injected with IS4VH/B3VL but developed much larger thrombi (Table II). It is not clear why human IgG levels were higher in mice injected with IS4VH/IS4VL than in those injected with the other IS4 variants. Because the route of administration and suspension buffer were identical for all IgG preparations, one would expect the kinetics of their i.p. absorption into surrounding tissue and the bloodstream to be equal (36, 37). It is unlikely that the high binding V_H/V_L combinations (IS4VH/B3VL and IS4VHii/B3VL) may have fallen in concentration through binding to other structures during their equilibration phase within the peritoneum because the concentration of these IgG in serum was similar to the low/no CL binding IgG such as IS4VHiiii/IS4VL. Furthermore, technical problems such as accidental injection of IgG into the gastrointestinal tract are unlikely to have occurred because all injections were conducted by the same trained individual and (with the exception of the IS4VH/IS4VL group) all animals injected with the same Ab had very similar circulating IgG levels (Fig. 2 and Table II).

A major difference between IS4VH/IS4VL and IS4VH/B3VL is that the latter binds to a wide range of different phospholipids (Table I). Of the thirteen variants of IS4 we previously tested for binding to a panel of six different anionic and neutral phospholipids only IS4VH/B3VL bound phosphatidylcholine (PC) a neutral phospholipids (28). Generally, PC binding is recognized as a feature of nonpathogenic aPL (3), and murine IgM anti-PC Abs have been shown to be anti-atherogenic in mice (38). Given the small number of animals treated with IS4VH/B3VL it is difficult to draw firm conclusions, but we speculate that the anti-PC binding of this V_H/V_L combination may indicate it has a protective role despite its strong CL binding. This theory is supported by the fact that only the two mAb that bind CL and PS but not PC (IS4VHiiii/B3VL and IS4VH/IS4VL) enhance thrombosis in these mice. A complicating factor is that some or all of these mAb were probably binding phospholipids via the cofactor β2GPI, which is present in the mice. One possibility is that IS4VH/B3VL binds strongly to a range of phospholipids without the need for a cofactor, whereas IS4VHiiii/B3VL and IS4VH/IS4VL only bind anionic phospholipids in the presence of β2GPI. We cannot test this theory directly because we are unable to exclude β2GPI from our in vitro binding assays, as Western blotting has previously shown that it is present in the purified IgG (28).

In fact, the in vivo thrombogenic effects of IS4VH/IS4VL and IS4VHiiii/B3VL were most closely mirrored by their pattern of binding to thrombin, a serine protease and key effector enzyme in hemostasis. Thrombin converts fibrinogen to fibrin allowing the formation of stable fibrin clots and provides positive feedback amplification of the coagulation cascade to enhance the proteolytic conversion of prothrombin to thrombin. Thrombin generation is negatively regulated by serine protease inhibitors (serpins), particularly antithrombin, heparin cofactor II, and protease nexin I, which bind thrombin and inactivate it irreversibly (39). Abs that bind to thrombin may mask these serpin epitopes and thus prevent inactivation of thrombin. Perhaps IS4VH/IS4VL and IS4VHiiii/B3VL enhance thrombosis by inhibiting serpin-mediated thrombin inactivation. However, it was previously shown that hybridoma-derived IS4 bound thrombin without reducing in vitro antithrombin-mediated inactivation (10). The ability of IS4 to inhibit heparin cofactor II and protease nexin I inactivation of thrombin has not previously been tested and will be the focus of future studies to try and determine the significance of the antithrombin binding found in these pathogenic mAbs.

Previously, we found that aPL with strong CL binding that enhanced thrombus size and leukocyte adherence in vivo also increased in vitro ICAM-1, VCAM-1, E-selectin, and tissue factor expression on HUVECs, though this finding was not true for every Ab tested (17, 24). In particular IS4 had very small effects, with only a 2-fold increase in E-selectin and VCAM-1 expression compared with control IgG (17). From our current panel of five mAbs we found that only native IS4 displayed any significant evidence of EC (E-selectin expression, Fig. 5A) activation in vitro. The dissonance between the properties of these mAb in the in vitro HUVEC assays and in vivo (shown in Table II) could arise for a number of different reasons. For instance, EC may express a different phenotype in cultured HUVECs compared with femoral vein EC in vivo and thus be an imperfect predictor of pathogenicity. Alternatively, the effects of the aPL on other cells (such as platelets or monocytes) or directly upon the clotting cascade itself may be largely responsible for differences in their ability to cause thrombosis in vivo.
These potential differences between the effects of aPL upon in vitro and in vivo EC may also explain our finding of a lack of correlation between EMP expression and aPL pathogenicity, which contrasts with the results of two recent clinical studies. Dig- nat-George et al. (22) found circulating EMP to be elevated in patients with APS (with vascular thromboses but no pregnancy morbidity) and systemic lupus erythematosus patients with aPL but not in systemic lupus erythematosus patients without aPL or in non-aPL-related venous thrombosis (22). Although Jy et al. (40) found EMP to be elevated in all patients with (thrombotic and nonthrombotic) APS. Thus, both studies found that the generation of EMP was specific to the presence of aPL per se rather than vascular thromboses. In fact, Jy et al. (40) found that it was platelet microparticles, which were significantly elevated in patients with thrombotic APS compared with nonthrombotic APS and controls.

It is surprising that none of the IS4 variants enhanced expression of tissue factor on the cultured HUVECs because numerous previous studies have demonstrated aPL mediated up-regulation of tissue factor from HUVECs (15, 16, 41). Furthermore, enhanced tissue factor expression is thought to be a major mechanism of thrombosis in patients with APS. Our findings, however, do not exclude a role for tissue factor in aPL-mediated thrombosis in vivo because aPLs also enhance tissue factor expression on monocytes (42), which will have been present in our in vivo but not our in vitro assays. Hence, tissue factor expression may more importantly in vivo due to aPL-mediated effects on monocytes rather than EC and future experiments will be conducted to address this issue.

In conclusion, we have used a stable expression system to investigate the relationship of aPL sequence, binding, and patho- genic properties in the human monoclonal IgG aPL IS4. We found that minor alterations in the pattern of arginine residues in VHV and changes in the pattern of somatic mutations in Vι of IS4 have profound effects upon its in vivo pathogenicity. The ability, how- ever, to promote thrombosis in vivo is related to both strength and selectivity of binding to several key Ags, particularly thrombin.

Acknowledgments

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Disclosures

S.P. is a co-owner and founder of Louisville APL Diagnostics, Inc., which supplied the calibrators used for detection of aCL Abs. The remaining authors have no financial conflict of interest.

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