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Arginine Residues Are Important in Determining the Binding of Human Monoclonal Antiphospholipid Antibodies to Clinically Relevant Antigens

Ian Giles,†‡ Nancy Lambrianides,† Nisha Pattni,† David Faulkes,‡ David Latchman,‡ Pojen Chen,§ Silvia Pierangeli,§ David Isenberg,* and Anisur Rahman* †

In the antiphospholipid syndrome (APS), antiphospholipid Abs (aPL) bind to anionic phospholipids (PL) and various associated proteins, especially β₂-glycoprotein I (β2GPI) and prothrombin. In the present study, we show that altering specific Arg residues in the H chain of a human pathogenic β2GPI-dependent aPL, IS4, has major effects on its ability to bind these clinically important Ags. We expressed whole human IgG in vitro by stable transfection of Chinese hamster ovary cells with expression plasmids containing different VH and VL sequences. VH sequences were derived from IS4 by altering the number of Arg residues in CDR3. VH sequences were those of IS4, B3 (anti-nucleosome Ab), and UK4 (β2GPI-independent aPL). Binding of the expressed H/L chain combinations to a range of anionic, neutral, and zwitterionic PL, as well as prothrombin, β2GPI, dsDNA, and chicken OVA, was determined by ELISA. Of four Arg residues in IS4VH CDR3 substituted to Ser, two at positions 96 and 97 reduced binding to β2GPI but increased or decreased binding to different PL. Eleven of 14 H/L chain combinations displayed weak binding to OVA with Arg to Ser replacements of all four Arg residues enhancing binding to this Ag. Only one H/L chain combination bound neutral PL and none bound dsDNA; hence, these effects are particularly relevant to Ags important in antiphospholipid syndrome. We hypothesize that these four Arg residues have developed as a result of somatic mutations driven by an Ag containing both PL and β2GPI. The Journal of Immunology, 2006, 177: 1729–1736.

Antiphospholipid Abs (aPL)† comprise a heterogeneous group of Ab directed against a variety of phospholipids (PL) and protein cofactors. The antiphospholipid syndrome (APS) is diagnosed where aPL are present in association with vascular thromboses and/or pregnancy morbidity (1). However, aPL can also occur in healthy adults and patients with infectious, malignant, or drug-related disorders, without causing vascular thromboses or pregnancy morbidity. These nonpathogenic aPL are generally IgG in vitro by stable transfection of Chinese hamster ovary cells with expression plasmids containing different VH and VL sequences. VH sequences were derived from IS4 by altering the number of Arg residues in CDR3. VH sequences were those of IS4, B3 (anti-nucleosome Ab), and UK4 (β2GPI-independent aPL). Binding of the expressed H/L chain combinations to a range of anionic, neutral, and zwitterionic PL, as well as prothrombin, β2GPI, dsDNA, and chicken OVA, was determined by ELISA. Of four Arg residues in IS4VH CDR3 substituted to Ser, two at positions 96 and 97 reduced binding to β2GPI but increased or decreased binding to different PL. Eleven of 14 H/L chain combinations displayed weak binding to OVA with Arg to Ser replacements of all four Arg residues enhancing binding to this Ag. Only one H/L chain combination bound neutral PL and none bound dsDNA; hence, these effects are particularly relevant to Ags important in antiphospholipid syndrome. We hypothesize that these four Arg residues have developed as a result of somatic mutations driven by an Ag containing both PL and β2GPI. The Journal of Immunology, 2006, 177: 1729–1736.

APS is the commonest cause of acquired hypercoagulability (13), and the most important treatable cause of recurrent miscarriage (14). Despite the currently best available treatments of aspirin and/or anticoagulation, morbidity and mortality remain high in patients with APS (15–17). Furthermore, anticoagulation can have severe side effects, and the optimum dosing regimen for long-term anticoagulation remains controversial (18). Hence, it is vital to develop a greater understanding of how aPL interact with their target Ags. This process will help in defining pathogenic mechanisms and hence new—more effective and accurately targeted—treatments for APS may be developed.

Sequence analysis of human monoclonal aPL shows that certain sequence features are consistently found more commonly in IgG aPL isolated from patients with APS than in polyreactive IgM aPL isolated from healthy individuals (19). Major distinguishing features of IgG aPL are somatic mutations leading to accumulation of Arg, Asn, and Lys residues in the CDRs of VH and VL.

Similarly, Arg, Lys, and Asn residues occur commonly in the CDRs of human and murine Abs to dsDNA (20, 21), frequently as a result of somatic mutation (22). The anti-dsDNA affinity of F(ab′)₂ Abs and Abs isolated from patients with lupus has been shown to correlate with the presence of Arg residues in CDRs of both H (23) and L chains (24). In some of these Abs, high-affinity binding to dsDNA and nucleosomes has been shown to be acquired by stepwise accumulation of somatic mutations (24). Arg, Asn, and Lys are believed to enhance intermolecular Ab-Ag interactions of human IgG anti-dsDNA Abs, probably by virtue of their charge and/or ability to form hydrogen bonds.

Previously, we used in vitro transient expression of whole IgG molecules from cloned VH and VL sequences of human monoclonal aPL (25, 26) to investigate the importance of Arg, Asn, and Lys residues in the aPL-Ag interaction. These aPL included IS4—an
IgG aPL derived from a patient with APS—which binds to anionic PL only in the presence of β2GPI, binds to β2GPI alone, is pathogenic in a murine model (27), and is therefore likely to be relevant to the pathogenesis of APS. The sequence of IS4VH was dominant in conferring the ability to bind cardioliopin (CL), while the identity of the paired V_L was important in determining the strength of CL binding (25). Subsequently, we altered the patterns of CDR Arg necessary genetic material to express a whole IgG molecule. By creating 14 different stably transfected Chinese hamster ovary (CHO) cell lines, which continually secrete the H/L chain combinations of interest. We purified whole IgG from large volumes of CHO supernatants and tested each purified H/L combination in ELISA for binding to a range of Ags that have all been suggested as important targets in APS. These included anionic and zwitterionic PL, as well as β2GPI and PT. We also tested binding to a neutral PL, phosphatidylcholine (PC), to dsDNA, which shares some structural similarities with anionic PL but is not involved in APS, and to OVA, which shares neither structural features nor clinical relevance with PL. These three Ags were included to determine whether the relationships between Ab sequence and binding that we observed were specific for clinically important Ag/Ab interactions.

Materials and Methods

Human mAbs

IS4, B3, and UK4 are all human IgG mAbs previously produced from lymphocytes of three different patients. IS4 was derived from a primary antiphospholipid syndrome patient and binds to CL in the presence of bovine and human β2GPI and to human β2GPI alone (28), B3 (29) and UK4 (30) were isolated from patients with SLE, and B3 binds nucleosomes, CL, and histones (29, 31, 32). UK4 binds negatively charged (but not neutral) PL in the absence of β2GPI and does not bind DNA (30).

Preparation of Ab expression constructs

The expression vectors containing both H and L chain cDNA were adapted from the single chain expression vectors previously used in our transient expression experiments (25, 33). The original vectors (pLN10, pLN100, pG1D1, and pG1D210) were all gifts from Drs. C. A. Kettleborough and T. Jones (Aices Biomedical, Mill Hill, London).

Construction of a vector containing both H and L chain cDNA has been described previously in detail for the B3 Ab (31). In the same way, three additional vectors were made from four different recombinant expression vectors: pG1D210 containing IS4VH, pLN100 containing IS4VH; and pLN10 containing B3VL or UK4VL. An EcoRI fragment from each V_L containing the promoter, the thumb constant region gene, and the λ variable region gene (of the chosen Ab) was ligated into the EcoRI-linearized vector pG1D210 containing IS4VH in-frame with a promoter and Cγ1 constant region gene. Thus, each new recombinant vector contains the necessary genetic material to express a whole IgG molecule.

Site-directed mutagenesis of recombinant IS4VH

Three new recombinant vectors were made, all of which contained cloned IS4VH, but differed in the identity of their L chain sequence. The first contained the IS4VL sequence and was designated IS4VH/IS4VL, the second contained B3VL, designated IS4VH/B3VL, whereas the third contained UK4VL, designated IS4VH/UK4VL.

In IS4VH/IS4VL and IS4VH/B3VL, particular Arg residues in IS4VHCDR3 were mutated to Ser by site-directed mutagenesis or CDR exchange and reported that specific Arg residues in IS4VHCDR3 were especially important in conferring the ability to bind CL (26).

These previous studies were limited because the transient expression system did not allow production of enough Ab to purify it from the supernatant or to test high concentrations in ELISA. In the experiments reported here, all these problems were overcome by creating 14 different stably transfected Chinese hamster ovary (CHO) cell lines, which continually secrete the H/L chain combinations of interest. We purified whole IgG from large volumes of CHO supernatants and tested each purified H/L combination in ELISA for binding to a range of Ags that have all been suggested as important targets in APS. These included anionic and zwitterionic PL, as well as β2GPI and PT. We also tested binding to a neutral PL, phosphatidylcholine (PC), to dsDNA, which shares some structural similarities with anionic PL but is not involved in APS, and to OVA, which shares neither structural features nor clinical relevance with PL. These three Ags were included to determine whether the relationships between Ab sequence and binding that we observed were specific for clinically important Ag/Ab interactions.

Stable expression of whole IgG molecules

A total of 13 different IgG-secreting cell lines was made, which all contained the ISVH sequence or variants of IS4VH. Stable expression of whole IgG molecules was achieved as previously described for the B3V3/B3VL cell line, which was also the 14th line used in these experiments (31). In brief, these plasmids, containing the dihydrofolate reductase gene (dhfr), were transfected into CHO/dhfr cells (35) by electroporation. To select for genomic incorporation of the dhfr gene, transfected cells were grown in medium lacking ribonucleosides and deoxyribonucleosides. Individual colonies of cells were isolated, and, once confluent, the cell supernatants were tested for the presence of whole IgG by ELISA (see below). The specific Ab productivity (q Ab) of each clone was calculated and expressed as ng/10^6 cells/day. Thus, the highest producing clones from each transfection were identified for further selection by exposure to 10^-8 to 10^-10 M methotrexate (MTX), a competitive inhibitor of the intracellular activity of the dhfr enzyme. MTX resistance was proportional to the amount of dhfr activity and the degree of amplification of the dhfr gene, hence the Ig gene.

Affinity purification of mAbs

Cells were transferred to Chemicon Europe and grown in larger quantities as described previously (31). Purified human IgG was sent back to our unit, and the concentration of IgG was checked by ELISA, as described previously (25).

Western blot analysis for the detection of whole IgG and β2GPI

Purified Ab samples were resolved by SDS-PAGE, using a 10% gel under nonreducing conditions. The proteins were transferred to Hybond-C membranes (Amersham Biosciences) for 1 h. Membranes were blocked in 5% skimmed milk/PBS/0.1% Tween 20. The membranes were incubated with a 1/2000 dilution of anti-IgG HRP-conjugated secondary Ab for 1 h. Bound HRP was then detected using chemiluminescence (Amersham Biosciences).

In addition, the purified Ab samples were examined for the presence of β2GPI as described above, except that proteins were resolved by SDS-PAGE under reducing conditions, and the primary Ab was a murine monoclonal anti-β2GPI Ab (provided by Dr. M. Iverson, La Jolla Pharmaceutical, La Jolla, CA) diluted 1/2000, followed by incubation with a HRP-conjugated murine IgG diluted 1/2000. For identification and quantification of β2GPI in each sample, 125–750 ng of human β2GPI (ScilPac) was also loaded upon each gel.

Table I. Patterns of Arg to Ser replacements in IS4VHCDR3

<table>
<thead>
<tr>
<th>H chain</th>
<th>Position 96</th>
<th>Position 97</th>
<th>Position 100</th>
<th>Position 100g</th>
</tr>
</thead>
<tbody>
<tr>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>IS4VHiii</td>
<td>R</td>
<td>R</td>
<td>R100S</td>
<td>R</td>
</tr>
<tr>
<td>IS4VHiv</td>
<td>R</td>
<td>R100S</td>
<td>R100S</td>
<td>R</td>
</tr>
<tr>
<td>IS4VHii &amp; iii</td>
<td>R96S</td>
<td>R97S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>IS4VHii &amp; iv</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R100S</td>
</tr>
<tr>
<td>IS4VHiii &amp; iv</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R100S</td>
</tr>
<tr>
<td>IS4VHx</td>
<td>R96S</td>
<td>R97S</td>
<td>R100S</td>
<td>R100S</td>
</tr>
</tbody>
</table>

* Variants of IS4VH are named using roman numerals to represent positions at which Arg residues in IS4VHCDR3 have been replaced by Ser, indicated in the appropriate column. R, Arg residue; S, Ser residue; VH, variable H chain sequence.
Immunological characterization of Abs

The binding of IgG molecules to CL, PC, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylethanolamine (Pe) were measured by direct ELISA, following the same consensus criteria protocols (36) at both laboratories in University College London and Morehouse School of Medicine. For example, polysorp (Nunc Polysorp) microtiter plates were coated with 50 μg/ml CL (Sigma-Aldrich) in ethanol or with ethanol alone overnight at 4°C. After washing with PBS plates were blocked with 10% FCS/PBS for 1 h at room temperature. Abs were then diluted in 10% FCS/PBS and incubated for 90 min at room temperature, followed by the addition of alkaline phosphatase-conjugated goat anti-human IgG and substrate and absorbance read at 405 nm. Background binding to control wells lacking PL was subtracted from binding to PL-coated wells in each ELISA. A modified CL ELISA was performed by replacing 10% FCS/PBS with 1% BSA/PBS in each relevant step of the consensus criteria protocol (36). Binding to β2GPI in the absence of PL was measured by direct ELISA as described previously (25). In each ELISA, samples were tested at serial dilutions and then at a fixed dose in triplicate by ELISA.

To detect anti-PT activity, high-binding (Costar) microtiter plates were coated with human PT at 10 μg/ml (American Diagnostica) in TBS—0.05 M Tris-HCl and 0.15 M NaCl (pH 7.5)—or with TBS alone overnight at 4°C. After washing with TBS, plates were blocked with 0.3% gelatin/TBS for 1 h at room temperature. Abs were then diluted in 0.1% gelatin/TBS and incubated for 90 min at room temperature, followed by the addition of alkaline phosphatase-conjugated goat anti-human IgG and substrate as above.

mAbs were also analyzed by ELISA for binding to dsDNA and OVA. The dsDNA ELISA was performed as described previously (31). For the OVA assay, nonirradiated microtiter plates (Titertek) were coated with 2 μg/ml OVA (Sigma-Aldrich) in PBS or PBS alone overnight at 4°C. Plates were then washed with PBS and blocked with 0.25% gelatin/PBS at room temperature for 1 h. Abs were diluted in 0.1% gelatin/PBS and incubated for an additional hour at room temperature, followed by the addition of alkaline phosphatase-conjugated goat anti-human IgG and substrate as above.

Results
Sequences of expressed Ab variable regions

The sequences of IS4, B3, and UK4 have all been described in detail previously (25, 29). All three Abs contain L chains encoded by the germline Vλ gene 2a2. These L chains differ in nucleotide sequence (although only by ≤7%) as a result of their different patterns of somatic mutation (25). Statistical analysis has shown that the observed patterns of replacement mutations in the CDRs of these sequences are consistent with Ag-driven selection (33, 37). There are five Arg residues in IS4VHCDR3. These are also highly likely to have arisen as a result of Ag-driven somatic mutation since this CDR does not match any known human D4H gene. Four of these Arg residues are surface exposed (25) and were mutated to Ser to create the mutant forms of IS4VH tested in the current study. In previous expression experiments in COS-7 cells, we had found that these surface-exposed Arg residues were especially important in conferring the ability to bind CL (26).

Stable expression of whole IgG in CHO cells

A total of 14 H/L chain combinations was expressed in CHO cells. All contained wild-type or mutagenized IS4VH, except the combination B3VH/B3VL. Western blot analyses of the purified Abs confirmed the presence of a strong band consistent with whole IgG on nonreducing gels (data not shown) with no free H chains. Different amounts of IgG were obtained from each cell line even after successive rounds of MTX amplification. The lowest dAbs was obtained from IS4VHii/IS4VL (88 ng/106 cells/day) and the highest from IS4VHii/IS4VL (5152 ng/106 cells/day). MTX amplification increased the yield of IgG by up to 75-fold in the case of IS4VH/IS4VL. The variable expression from CHO cells is well documented in both our hands (31) and others (38). This phenomenon occurs during subcloning in successive amplification steps when cells can increase the copy number of the dhfr gene without increasing their production rate (38). Thus, cells that do not produce an exogenous protein will usually have a higher growth rate and take over the culture, thereby reducing the chance of selecting a high producer cell. This variation in yield is not relevant to our results obtained using purified Abs, which were all tested at the same concentration (1 μg/ml in Figs. 1–4).

Immunological characterization of monoclonal aPL by ELISA

For each H/L chain combination that bound PL, the linear portion of the binding curve for absorbance against Ab concentration was determined empirically by dilution of Ab over a wide range of concentrations. We then tested each combination in triplicate against each Ag, using a fixed Ab concentration of 1 μg/ml, which was found to be optimal.

The H chain of IS4 is more important than the L chain in binding to CL, PS, and Pe

Previous transient expression experiments showed that the L chain, but not the H chain, of IS4 could be replaced without reducing binding to CL. Thus, COS-7 supernatant containing IS4VH/B3VL

![Figure 1](image-url)
showed increased binding to CL compared with the wild-type combination IS4VH/IS4VL, whereas IS4VH/UK4VL did not bind CL at all at the maximum concentration tested of 675 ng/ml (25).

The use of a stable expression system has allowed us to expand upon these earlier studies by testing Ab binding to five additional PL. We have found that this difference between the importance of IS4VH and IS4VL in binding to CL also holds true when purified Abs are tested at higher doses than was previously possible, shown in Fig. 1. In addition, we have demonstrated that this difference also holds true for binding to other PL, PS, and Pe. The wild-type combination IS4VH/IS4VL bound CL, PS, and Pe, but any reduction in the number of CDR3 Arg abolished binding to all three Ags. In contrast, replacement of the L chain of IS4 by B3VL (in IS4VH/B3VL) not only increased binding to CL, PS, and Pe but also created the ability to bind PI, PA, and PC. When the H chain of IS4 was then replaced by B3VH (in B3VH/B3VL), binding to all six PL Ags was abolished. As shown previously for CL, the L chain UK4VL conferred weaker binding to both PS and Pe than either IS4VL or B3VL.

Arg at 100 and 100g are important for binding to all PL tested, but the effects of Arg at positions 96 and 97 depend on the sequence at 100 and 100g

In both these and previous experiments, we found that binding of the combination IS4VH/IS4VL to PL Ags was abolished by substitution of any one of the four VHCDR3 Arg residues so that mutagenesis of IS4VH/IS4VL was not useful in determining the relative importance of these Arg. A major strength of this study—conferred by the stable expression system—was that we could study the effects of VHCDR3 Arg to Ser replacements on binding of the combination IS4VH/B3VL to six different PL Ags. In this case, binding was reduced but not always abolished by different patterns of Arg to Ser replacements so that useful comparisons between the degrees of reduction caused by substitutions at different positions could be made, shown in Fig. 1.

Substitution of all four Arg with Ser residues (IS4VHx/B3VL) abolished binding to all PL completely. It seems likely that all four of these residues contribute to PI and PC binding since double mutations of either Arg 96 and 97 (IS4VHi&ii/B3VL) or Arg 100 and 100g (IS4VHiii&iv/B3VL) also caused a complete loss of binding to these two Ags.

Binding to CL, PS, PA, and Pe, however, appears to be largely conferred by the Arg residues at positions 100 and 100g. Compared with the wild-type IS4VH/B3VL combination, the H chain containing Arg to Ser replacements at positions 100 and 100g (IS4VHiii&iv/B3VL) displayed ~50% weaker binding to CL, PS, and Pe in combination with B3VL and did not bind at all to PA. In contrast, IS4VHii&iii/B3VL showed increased binding to CL, PS, PA, and Pe in comparison to IS4VH/B3VL. However, Arg to Ser mutations at either position 96 or position 97 reduced binding to these Ags if the Arg at 100 and 100g had already been changed to Ser. In the triple mutants (IS4VHi,iii&iv/B3VL and IS4VHii,iii&iv/B3VL), binding to all PL Ags was very low and clearly lower than in the double mutant IS4VHiii&iv/B3VL.
The Ags can be divided into two groups according to their binding properties. PC, PI, and PA show very restricted binding to only IS4VH/B3VL (and IS4VHi&ii/B3VL in the case of PA alone). CL, PS, and Pe all bind 5 of the 14 aPL (IS4VH/IS4VL, IS4VH/B3VL, IS4VHi&ii/B3VL, IS4VHi&ii/B3VL, and IS4VH/UK4VL). CL and PS also show weak binding to the triple mutant IS4VHi,iii&iv/B3VL. Interestingly, these groupings do not depend on charge of the PL. CL, PS, and PA, and PI are all anionic but belong to different groups. CL and PS fall in a group with the zwitterionic Pe, whereas PA and PI behave like the neutral PC in these experiments.

Affinity-purified Abs contain β2GPI

Purified native IS4 bound equally well to CL in both the modified and standard ELISA (data not shown). Since this finding, suggesting a lack of serum dependency of native IS4 was at odds with those of Zhu et al. (28), we examined the purified samples for the presence of contaminating β2GPI by Western blot analysis. A faint band was visible on each blot consistent with the presence of bovine β2GPI—derived from the CHO cell culture medium—in each sample tested (Fig. 2).

Arg residues at all four positions have similar effects on binding to β2GPI

In our previous experiments using transient expression, we had not been able to demonstrate binding to β2GPI, a finding that was unsurprising in view of the low β2GPI affinity of the original hybridoma-derived IS4 (28). The development of the stable expression system allowed us to test concentrations of 1 μg/ml purified Ab. We found that IS4VH/IS4VL and IS4VH/B3VL bound β2GPI but IS4VH/UK4VL did not (Fig. 3). Although this order of binding was similar to the results obtained in the PL ELISA, the results obtained with the Arg to Ser substitutions were different. First, the binding of IS4VH/IS4VL to β2GPI was not abolished by all of these mutations. Single Arg to Ser mutations at 100 or 100g did not abolish binding, although double mutations at 96 and 97 or 100 and 100g did abolish it. Second, there was no major difference between the roles played by arginines at 96 and 97 and those at 100 and 100g when we tested these heavy chains in combination with B3VL. Both the double mutants (IS4VHi&ii/B3VL and IS4VHi&ii&iv/B3VL) showed similar reductions in binding to β2GPI compared with IS4VH/B3VL, and this binding was abolished altogether when any three or all four Arg were mutated.

All patterns of Arg to Ser replacements dramatically reduce binding to PT

The increased yield of IgG from the stable expression system also allowed us to examine binding to PT. IS4VH/B3VL displayed the strongest binding to PT, shown in Fig. 4. In fact, this Vιg/Vιc combination was the only Ab to convincingly bind to PT. Of the remaining Abs, it is clear that any pattern of Arg to Ser replacement in IS4VH dramatically reduces—by at least 7-fold—binding to PT.

Arg to Ser replacements enhance binding to OVA but not dsDNA

None of the purified Abs convincingly bound dsDNA, shown in Fig. 5. The results for binding to OVA were interesting in that 11 of the 14 Vιg/Vιc combinations bound this Ag (shown in Fig. 5). Most Arg to Ser replacements either had little effect or actually enhanced binding to OVA. Intriguingly, IS4VHi&ii/IS4VL displayed the strongest binding to OVA despite not binding to any other of the Ags tested in these experiments. Native IS4 displayed very weak binding to OVA, and double (IS4VHi&ii/IS4VL) as well as single (IS4VHi&ii/IS4VL and IS4VHi&iv/IS4VL) Arg to Ser replacements actually enhanced OVA binding. Multiple replacements that included both Arg residues at position 100 and 100g (IS4VHi&ii&iv/IS4VL and IS4VHi&iv/IS4VL) led to a complete loss of OVA binding. In the B3VL combinations, double replacement of the Arg 96 and 97 residues (IS4VHi&ii/B3VL) or 100 and 100g residues (IS4VHi&ii&iv/B3VL) increased binding to OVA slightly compared with IS4VH/B3VL. Even the replacement of all four Arg residues to Ser (IS4VHx/B3VL) did not lead to complete loss of binding. In fact, the weakest binding occurred with the triple replacement of Arg residues at positions 96, 100, and 100g, shown in Fig. 5.

Discussion

We have successfully established a stable expression system to produce recombinant human monoclonal aPL. Our current findings extend those of our previous studies, using a transient expression system in which we found that somatic mutations to Arg determine the ability of these H and L chains to bind CL (25, 26). A major limitation of the transient expression studies was the low yield of IgG, which only allowed us to investigate binding of nonpurified IgG to a single PL Ag (CL). The concentration of IgG in COS-7 cell supernatants transfected with different Vιg/Vιc combinations varied widely so that it was impossible to test all combinations at the same concentration. Using the stable expression system, we were able to test binding of purified IgG at concentrations of at least 1 μg/ml to multiple Ags.

Increasingly, it is recognized that a number of other clinically relevant Ags in addition to CL (39, 40) are important in the pathogenesis of the APS. A positive correlation has been reported between anti-β2GPI (10), as well as anti-PT (reviewed in Ref. 41), Ab and clinical manifestations of the APS in some but not all studies (reviewed in Ref. 42). Despite these conflicting, results...
better standardization of anti-β2GPI assays has led to their inclusion in the revised international classification criteria for APS (43). Other PL are also important since anti-PS Abs have been shown to have a statistically significant association with arterial thrombosis (11), and Ab directed against other anionic PL (PI and PA), as well as the zwitterionic PC (44), have also been implicated in the pathogenesis of the APS.

Binding to PS is of particular interest because the introduction of Arg residues into the V<sub>H</sub> of a murine Ab (39H) with dual specificity for PL and DNA (45) at positions known to mediate DNA binding enhanced binding to PS-β2GPI complexes and to apoptotic cells, an important physiological source of both these Abs (46). In addition, externalization of PS characterizes early placentation development during cytotrophoblast differentiation and occurs independently of apoptosis (47). Therefore, exposure of PS may occur by several mechanisms in patients with APS.

In this study, we were able to examine binding to a wide panel of Abs. We found that the strongest binding of the wild-type IS4VH/IS4VL Ab was to CL, PS, Pe, and β2GPI. This finding is consistent with the hypothesis that development of pathogenic aPL such as IS4 is driven by a complex of anionic PL and β2GPI. If this hypothesis is the case, one might be able to identify specific amino acids within the V<sub>H</sub> or V<sub>L</sub> sequences of IS4 that have been created by somatic mutations in the germline genes and that promote binding to anionic PL, β2GPI, or both. We believe that the Arg residues at positions 100 and 100g fit this description since all heavy/light combinations, in which either or both of these Arg were changed to Ser, showed reduced binding to CL, PS, and Pe, depending on whether positions 96 and 97 could enhance or reduce binding to CL, PS, and Pe, depending on whether positions 100 and 100g were occupied by Arg or Ser may be explained in a number of ways. The most likely explanation is that Arg<sub>96</sub> and Arg<sub>97</sub> were acquired before Arg<sub>100</sub> and Arg<sub>100g</sub> during clonal expansion of the B cell line that produced IS4 in the original patient. Thus, an early member of the clone produced a H chain identical to IS4VHiii&iv, and the affinity of that H chain for both PL and β2GPI was enhanced by the subsequent addition of Arg<sub>100</sub> and Arg<sub>100g</sub>. If Arg<sub>96</sub> and Arg<sub>97</sub> had been present first (i.e., in an H chain identical to IS4VHii&ii), then addition of Arg<sub>100</sub> and Arg<sub>100g</sub> would have reduced affinity for any driving PL Ag, which would have inhibited expansion of the clone. The idea that Arg<sub>96</sub> and Arg<sub>97</sub>, in particular, was a late addition to IS4VHCDR3 makes sense in that it is a distal residue in a very long CDR3 and may have been incorporated partially by N addition. Alternatively, Arg<sub>96</sub> and Arg<sub>97</sub> may be concerned primarily with binding to β2GPI or in forming an interaction with IS4VL such that the apparently adverse effect of these Arg residues on binding to PS, Pe, and CL was irrelevant to the expansion of the IS4 clone in vivo.

The process of Ag-driven somatic mutation in B cell clones tends to increase affinity for the driving Ag and reduce affinity for other Ags. The idea that the Arg in IS4VHCDR3 has been accumulated by this process is supported by our finding that, when any of them are changed to Ser, there is in many cases an increase in binding to the non-PL Ag OVA (Fig. 5). We do not know that Ser was the amino acid present at positions 96, 97, 100, and 100g before mutation to Arg in the original IS4 clone in vivo because we do not know the sequence of the original germline DNA gene in that B cell clone. However, because the AGT and AGC Ser codons are very easily mutated to AGA and AGG, which encode Arg, Ser-to-Arg somatic mutations occur frequently in Ig sequences. It is likely that one or more of the Arg to Ser replacements that we made represents a true germline reversion.

There have been very few other studies of the relative importance of H and L chain sequence motifs in the binding of whole human aPL to clinically relevant Ags. Several groups have studied either murine aPL or human F(ab'<sub>2</sub>)<sub>2</sub> primarily directed against dsDNA; hence, the direct relevance of these studies to APS in humans is limited (reviewed in Ref. 48). Only one other group has examined the effects of sequence alteration upon the ability of recombinant human aPL to bind relevant Abs. Lieby et al. (49) showed that simultaneous germline reversion of three somatically mutated VLCDR1 Asn residues in a pathogenic aPL caused the Ab to lose its annexin A5 dependency for CL binding. No group, however, has previously shown how the stepwise accumulation of somatic mutations contributes to binding to both protein and PL Ags in a monoclonal aPL.

Our results add to the previous data supporting the importance of arginine residues in binding to PL and β2GPI (19) and suggest that these residues are also important in binding to other PL. The exact structural interactions between these Abs and Ags, however, are difficult to define. Attraction of positively charged arginines on the Ab to negatively charged phosphate groups on PL may play a role but is probably not the major interaction. Binding to the PL Ags could not be distinguished on the basis of their charge since several aPL bound anionic, zwitterionic, and neutral PL. If charge was a dominant factor, one might expect the pattern of binding of our panel of Abs to negatively charged dsDNA to resemble that of binding to PL, but in fact none of the H/L chains that we tested bound dsDNA at all. The stark difference in binding to dsDNA and PL may be partially due to interactions between the Abs and the hydrophobic fatty acid chains of the PL molecules (which have no equivalent in the structure of dsDNA). In vivo, however, these fatty acid groups would usually be buried in membranes and might not be exposed to Ab molecules. The regular, repeating three-dimensional structure of dsDNA has enabled the generation by computers of docking models to predict the way in which Ab surfaces interact with dsDNA (32). Similar models have not been produced for binding to PL or β2GPI, which do not have regularly repeating structures so that modeling of surface interactions between Ab and Ag would be speculative unless the exact location of the major epitopes were known. Despite the three-dimensional structure of both β2GPI (50) and membrane-bound PT (51) having been identified, the dominant immunogenic epitope for aPL on both of these proteins remains unresolved. The fact that B3VH/B3VL does not bind dsDNA confirms our recent detailed assessment of this Ab where we found that it is actually specific for nucleosomes and does not bind dsDNA when purified (31).

One limitation of our study is that we could not remove β2GPI completely from the Ab preparations, as shown by Western blotting (Fig. 2). We were therefore unable to demonstrate a loss of CL binding when purified IS4VH/IS4VL was tested in the modified CL ELISA in the absence of FCS. In contrast, Zhu et al. (28) conducted more stringent purification of IS4 from hybridoma supernatant and were thus able to remove β2GPI from purified aPL. They were able to demonstrate that IS4 does not bind CL in the absence of β2GPI. Nevertheless, our results suggest that IS4VH probably makes contacts with PL that are separate from its interaction with β2GPI since effects of Arg to Ser substitutions on binding to β2GPI were markedly different to the effects on binding to the PL Ags tested (Figs. 1 and 3). In particular, the combination IS4VHii&ii/B3VL shows strong binding to CL, PS, and Pe but no binding to β2GPI.

Previously, native IS4 produced from hybridoma cells tested at 1 μg/ml had been shown to weakly bind PT with an OD of ~0.2 (52). In contrast, we found native IS4 at the same dose to bind PT
with an OD of just <0.1. One possible explanation for this discrepancy is that our recombinant IS4VH/IS4VL is an IgG1, whereas the hybridoma IS4 is an IgG3, which is more prone to form dimers (53) and thus may have higher avidity toward PT.

It is interesting that IS4VH/B3VL also binds PC. In view of its direct binding to B2GPI and PT, one might predict that this V_{Hl}/V_{Ll} combination would be pathogenic in vivo. Therefore, IS4VH/B3VL would not be expected to bind to the neutral PC because this combination would be pathogenic in vivo. Therefore, IS4VH/B3VL is an IgG1, with an OD of just


