Research article

The critical role of arginine residues in the binding of human monoclonal antibodies to cardiolipin

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Abstract

Previously we reported that the variable heavy chain region (VH) of a human beta2 glycoprotein I-dependent monoclonal antiphospholipid antibody (IS4) was dominant in conferring the ability to bind cardiolipin (CL). In contrast, the identity of the paired variable light chain region (VL) determined the strength of CL binding. In the present study, we examine the importance of specific arginine residues in IS4VH and paired VL in CL binding. The distribution of arginine residues in complementarity determining regions (CDRs) of VH and VL sequences was altered by site-directed mutagenesis or by CDR exchange. Ten different 2a2 germline gene-derived VL sequences were expressed with IS4VH and the VH of an anti-dsDNA antibody, B3. Six variants of IS4VH, containing different patterns of arginine residues in CDR3, were paired with B3VL and IS4VL. The ability of the 32 expressed heavy chain/light chain combinations to bind CL was determined by ELISA. Of four arginine residues in IS4VH CDR3 substituted to serines, two residues at positions 100 and 100g had a major influence on the strength of CL binding while the two residues at positions 96 and 97 had no effect. In CDR exchange studies, VL containing B3VL CDR1 were associated with elevated CL binding, which was reduced significantly by substitution of a CDR1 arginine residue at position 27a with serine. In contrast, arginine residues in VL CDR2 or VL CDR3 did not enhance CL binding, and in one case may have contributed to inhibition of this binding. Subsets of arginine residues at specific locations in the CDRs of heavy chains and light chains of pathogenic antiphospholipid antibodies are important in determining their ability to bind CL.

Keywords: antiphospholipid antibodies, arginine, binding, cardiolipin

Introduction

The identification of antiphospholipid antibodies (aPL) is a key laboratory feature in the diagnosis of patients with antiphospholipid antibody syndrome (APS). The cardinal manifestations of this syndrome are vascular thrombosis, recurrent pregnancy loss, livedo reticularis and thrombocytopenia [1,2]. APS may affect any organ of the body, leading to a broad spectrum of manifestations [3]. It is the commonest cause of acquired hypercoagulability in the general population [4] and a major cause of pregnancy morbidity.

APS may occur as a ‘freestanding’ syndrome (primary APS) [5] or in association with other autoimmune rheumatic diseases (secondary APS) [6]. In both primary APS and secondary APS, recurrence rates of up to 29% for thrombosis and a mortality of up to 10% over a 10-year follow-up period have been reported [7]. The only treatment that reduces the risk of thrombosis in APS is long-term anticoagulation [8]. This treatment may have severe side effects, notably bleeding. It is therefore important to develop a greater understanding of how aPL interact with their target antigens so that new treatments for APS, which are both more effective and more accurately targeted to the causes of the disease process, may be developed.

aPL = antiphospholipid antibodies; APS = antiphospholipid syndrome; β2GPI = beta2 glycoprotein I; CDR = complementarity determining region; CL = cardiolipin; dsDNA = double-stranded DNA; ELISA = enzyme-linked immunosorbent assay; Fab = antigen-binding fragment; VH = variable heavy chain region; VL = variable light chain region.
aPL occur in 1.5–5% of healthy people and may also occur in various medical conditions without causing clinical features of APS [9]. The aPL that are found in patients with APS differ from those found in healthy people in that they target predominantly negatively charged phospholipid antibodies and are in fact directed against a variety of phospholipid binding serum proteins. These proteins include protein C, protein S, prothrombin and beta_2_ glycoprotein I (beta_2GPI) [10-13]. beta_2GPI is the most extensively studied of these proteins and appears to be the most relevant clinically [14-16]. Furthermore, high levels of IgG aPL, rather than IgM aPL, are closely related to the occurrence of thrombosis in APS [17,18].

Sequence analysis of human monoclonal aPL has shown that IgG aPL, but not IgM aPL, often contain large numbers of somatic mutations in their variable heavy chain region (V_H) and variable light chain region (V_L) sequences [19]. The distribution of these somatic mutations suggests that they have accumulated under an antigen-driven influence [20]. These monoclonal aPL tend to have accumulations of arginine residues, asparagine residues and lysine residues in their complementarity determining region (CDRs). Arginine residues have also been noted to play an important role in the CDRs of some murine monoclonal aPL [21,22].

Arginine residues, lysine residues and asparagine residues also occur very commonly in the CDRs of human and murine antibodies to dsDNA (anti-dsDNA) [23-25], particularly arginine residues in V_H CDR3 [25-27]. It has been suggested that the structure of these amino acids allows them to form charge interactions and hydrogen bonds with the negatively charged DNA phosphodiester backbone [25,28]. We hypothesise that the same types of interaction may occur between negatively charged epitopes upon phospholipid antibodies/beta_2GPI and arginine residues, asparagine residues and lysine residues at the binding sites of high-affinity pathogenic IgG aPL.

We have previously described a system for the in vitro expression of whole IgG molecules from cloned V_H and V_L sequences of human monoclonal aPL antibodies [29]. This system was used to test the binding properties of combinations of heavy chains and light chains derived from a range of human antibodies. One of these antibodies, IS4, is an IgG antibody derived from a primary APS patient. IS4 binds to anionic phospholipid antibodies only in the presence of beta_2GPI, can bind to beta_2GPI alone and is pathogenic in a murine model [30]. It is therefore likely to be relevant in the pathogenesis of APS.

We found that the sequence of IS4V_H was dominant in conferring the ability to bind cardioliopin (CL) while the identity of the V_L paired with this heavy chain was important in determining the strength of CL binding [29].

Modelling studies have shown that multiple surface-exposed arginine residues were prominent features of the heavy chains and light chains that conferred the highest ability to bind CL. The CDR3 region of IS4V_H contains five arginine residues, of which four are predicted by the model to be surface-exposed, and therefore is potentially important in binding to CL [29].

The purpose of the study reported in this paper was to define the contribution of different CDRs, and of individual arginine residues within those CDRs, in binding to CL. Patterns of CDR arginine residues in the cloned V_H and V_L sequences were altered by site-directed mutagenesis or by CDR exchange. The altered heavy chains and light chains were expressed transiently in COS-7 cells. Binding of the different heavy chain/light chain combinations to CL was tested by direct ELISA.

**Materials and methods**

**Human monoclonal antibodies**

IS4, B3 and UK4 are all human IgG monoclonal antibodies produced from lymphocytes of three different patients. IS4 was derived from a primary APS patient by the Epstein–Barr virus transformation of peripheral blood mononuclear cells and fusion with the human-mouse heterohybridoma K6H6/B5 cell line [31]. IS4 binds to CL in the presence of bovine and human beta_2GPI, and to human beta_2GPI alone [31]. B3 [32] and UK4 [33] were isolated by fusion of peripheral B lymphocytes from systemic lupus erythematosus patients with cells of the mouse heteromyeloma line CB-F7. B3 binds single-stranded DNA, dsDNA, CL and histones [32,34]. UK4 binds negatively charged (but not neutral) phospholipid antibodies in the absence of beta_2GPI and does not bind DNA [33].

**Assembly of constructs for expression**

**Wild-type heavy chain and light chain constructs**

Constructs containing the wild-type heavy chain and light chain were prepared as detailed fully in previous articles [29,35]. UK4V_H could not be cloned into the appropriate plasmid, hence only UK4_L was available for analysis. The expression vectors (pLN10, pLN100 and pG1D210) were all kind gifts from Dr Katy Kettleborough and Dr Tarran Jones (Aeres Biomedical, London, UK).

**Hybrid V_L chain constructs**

Each hybrid V_L chain construct contained the CDR1 of one of the human monoclonal IgG antibodies IS4, B3 or UK4 and the CDR2 and CDR3 of a different one of these antibodies. Two hybrid V_L chains (BU and UB) had previously been made by Dr Haley and colleagues [36], and a further
four chains (IB, IU, BI and UI) were made by a similar method, as follows.

Two different wild-type $\mathrm{V}_L$ expression vectors were digested with Acc65 I and $\mathrm{Pvu}$ I (Promega, Southampton, UK). Acc65 I cuts IS4, B3 or UK4 $\mathrm{V}_L$ sequences at a position in FR2 that is 106 base pairs from the beginning of $\mathrm{V}_L$, but does not cut the expression vector outside the insert. $\mathrm{Pvu}$ I cuts the vectors at a single site approximately 1 kb downstream of the insert. Each vector was therefore digested into two linear bands; one of approximately 1.5 kb and the other of approximately 6 kb. The 1.5 kb fragment contained CDR2 and CDR3 of the IgG $\mathrm{V}_L$ region and also part of the downstream expression vector containing the lambda constant region cDNA, while the 6 kb fragment contained CDR1 and the rest of the vector. The 6 kb fragment derived from one $\mathrm{V}_L$ expression vector was ligated with the 1.5 kb fragment derived from the other. The resulting plasmid would contain CDR1 of one $\mathrm{V}_L$ sequence and CDR2 and CDR3 of another $\mathrm{V}_L$ sequence.

Since IS4, B3 and UK4 $\mathrm{V}_L$ sequences differ in their content of the restriction sites $\mathrm{Aat}$ II and $\mathrm{Ava}$ I, we checked that the desired parts of each sequence were present in the new hybrid sequences by carrying out $\mathrm{Aat}$ II, $\mathrm{Hind}$ III/$\mathrm{Ava}$ I and $\mathrm{Aat}$ II/$\mathrm{Bam}$ HI digests.

Site-directed mutagenesis of IS4$\mathrm{V}_{\mathrm{HL}}$
We generated six mutant forms of IS4$\mathrm{V}_{\mathrm{HL}}$ in which particular arginine residues were mutated to serine, using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s protocol. Serine was chosen because it is nonpolar. Germline reversion could not be performed because the exact germline $\mathrm{D}_{\mathrm{HL}}$ gene of IS4$\mathrm{V}_{\mathrm{HL}}$ CDR3 is unknown. Four mutants, named IS4$\mathrm{V}_{\mathrm{HL}i}$, IS4$\mathrm{V}_{\mathrm{HL}ii}$, IS4$\mathrm{V}_{\mathrm{HL}iii}$ and IS4$\mathrm{V}_{\mathrm{HL}iv}$, contained single mutations of arginine residues at positions 96, 97, 100 and 101 g, respectively. The remaining two forms contained two arginine to serine mutations, at positions 96 and 97 in the IS4$\mathrm{V}_{\mathrm{HL}i}$&ii mutant and at all four sites in mutant IS4$\mathrm{V}_{\mathrm{HL}x}$.

Expression of whole IgG molecules
The whole IgG molecules were expressed in COS-7 cells as described previously [29,37].

Detection and quantitation of whole IgG molecules in COS-7 supernatant by ELISA
Whole IgG molecules were detected and quantitated in the COS-7 cell supernatants using a direct ELISA, as described in previous papers [29,35,37].

Detection of binding to CL by ELISA
The binding of IgG molecules to CL was measured by direct ELISA as described previously [29].

Results
Sequences of light chains expressed
Amino acid sequences of IS4$\mathrm{V}_{\mathrm{L}}$, UK4$\mathrm{V}_{\mathrm{L}}$, B3$\mathrm{V}_{\mathrm{L}}$ and germline gene 2a2 are shown in Fig. 1a. All of these light chains contain numerous somatic mutations. Previous statistical analysis has shown that the observed pattern of replacement mutations in the CDRs of these sequences is consistent with antigen-driven selection [32,33,35,38-40]. The light chain B3a$\mathrm{V}_{\mathrm{L}}$, shown in Fig. 1a, was derived from B3$\mathrm{V}_{\mathrm{L}}$ by site-directed mutagenesis of Arg27a to serine [37].

The $\mathrm{V}_L$ sequences of IS4, B3 and UK4 are all encoded by the germline $\mathrm{V}_L$ gene 2a2, but differ in their patterns of somatic mutation. B3$\mathrm{V}_{\mathrm{L}}$ contains two adjacent arginine residues in CDR1, both produced by somatic mutations. UK4$\mathrm{V}_{\mathrm{L}}$ has a single somatic mutation to arginine in CDR3 at position 94. A serine residue in CDR3 of IS4$\mathrm{V}_{\mathrm{L}}$ is replaced by asparagine.

Figure 1a also shows the amino acid sequences of the $\mathrm{V}_\lambda$ CDR hybrids in which each newly formed chain construct contains CDR1 of one antibody with CDR2 and CDR3 of a different antibody. These hybrid sequences were named by combining the names of the two parent antibodies such that the first letter represented the antibody from which CDR1 was derived and the last letter represented the antibody from which both CDR2 and CDR3 were derived. Hybrid IB thus contains CDR1 from IS4, and CDR2 and CDR3 from B3, whereas hybrid BI contains the reverse combination (CDR1 from B3, and CDR2 and CDR3 from IS4).

Sequences of heavy chains expressed
The amino acid sequences of IS4$\mathrm{V}_{\mathrm{H}}$ and B3$\mathrm{V}_{\mathrm{H}}$ chain and the corresponding germline genes are displayed in Fig. 1b. B3$\mathrm{V}_{\mathrm{H}}$ has a single somatic mutation to arginine in CDR2. The CDR2 of IS4$\mathrm{V}_{\mathrm{H}}$ contains an asparagine residue created by somatic mutation and in CDR3 there are multiple arginine residues, which are highly likely to have arisen as a result of antigen-driven influence. The four surface-exposed arginine residues that were mutated to serine to create the six mutant forms of IS4$\mathrm{V}_{\mathrm{H}}$ are underlined in Fig. 1b.

Expression of whole IgG
Each of the 10 light chains shown in Fig. 1a was paired with B3$\mathrm{V}_{\mathrm{H}}$ and IS4$\mathrm{V}_{\mathrm{H}}$. Each of the six mutant forms of IS4$\mathrm{V}_{\mathrm{L}}$ was paired with IS4$\mathrm{V}_{\mathrm{L}}$ and B3$\mathrm{V}_{\mathrm{L}}$. A total of 32 heavy chain/light chain combinations were expressed in COS-7 cells. At least two expression experiments were carried out for each combination. IgG was obtained in the supernatant for all of the combinations.

The range of concentrations of IgG obtained in COS-7 cell supernatants, determined by ELISA, from each of the 32 heavy chain/light chain combinations are presented in

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Fig. 1
(a) Lambda chains

(b) Heavy chains

Sequence alignment of the expressed variable light chain region (V\(_L\)) and variable heavy chain region (V\(_H\)), using DNAplot software in VBASE. (a) Sequences of expressed V\(_L\) regions compared with gene 2a2. (b) Sequences of expressed V\(_H\) regions compared with genes 1-03 (IS4) and 3-23 (B3). The D\(_\alpha\) regions could not be matched to germline genes. Arginine residues altered by site-directed mutagenesis to serine residues in IS4V\(_H\) (b). The DH regions could not be matched to germline genes. Arginine residues altered by site-directed mutagenesis to serine residues in IS4V\(_H\) (b).

Table 1. Identical concentrations were obtained for the combination IS4V\(_{H}\)ii/B3V\(_L\) from two different expression experiments. In each case the negative control sample, in which COS-7 cells were electroporated without any plasmid DNA, contained no detectable IgG. Consistently high yields were obtained with the B3V\(_H\)/BIVL, B3V\(_H\)/UIVL and IS4V\(_H\)/UIVL combinations compared with the other antibody combinations. The phenomenon of variable expression is not clear.

Results of anti-CL ELISA
For each heavy chain/light chain combination that bound CL, the linear portion of the binding curve for absorbance against antibody concentration was determined empirically, by dilution of antibody over a wide range of concentrations. Similar patterns of binding were obtained for each combination from repeated expression experiments, hence representative results from a single experiment only are shown in Figs 2,3,4.

The importance of arginine residues in IS4V\(_{H}\)
As reported previously, the presence of the heavy chain of IS4 plays a dominant role in binding to CL [29]. IS4V\(_{H}\) binds CL in combination with six of the 10 light chains tested (see Figs 2a and 3): B3V\(_{L}\), B3aV\(_{L}\), BIVL, IS4V\(_{L}\), IBV\(_{L}\) and UVL. Only one of these light chains (B3V\(_{L}\)) binds CL in combination with B3V\(_{H}\) (Fig. 2b).

To identify the features of IS4V\(_{H}\) that enhance binding to CL, we focused on the combination IS4V\(_{H}/B3V_{L}\). This combination shows high binding to CL. This binding could be altered by the replacement of some or all of the four surface-exposed arginine residues in IS4V\(_{H}\) CDR3 to serine, as shown in Fig. 4. Substitution of all four arginine residues with serine residues (IS4V\(_{H}\)x) abolished CL binding completely. This effect seems probably due entirely to the changes at positions 100 and 100 g. This is supported by the fact that heavy chain combinations containing arginine to serine mutations at these positions (IS4V\(_{H}\)iii and IS4V\(_{H}\)iv) displayed approximately 50% weaker binding to CL in combination with B3V\(_{L}\) than did the wild-type IS4V\(_{H}/B3V_{L}\) combination. In contrast, there were no reductions in CL binding for the heavy chains containing arginine to serine mutations at position 96 (IS4V\(_{H}\)ii), at position 97 (IS4V\(_{H}\)ii) or at both positions (IS4V\(_{H}\)ii).
mutated to serine. As shown in Fig. 3, there was a significant decrease in CL binding of B3V_L/B3aV_L compared with B3V_H/B3V_L. Although the combination IS4V_H/B3aV_L binds CL less strongly than does IS4V_H/B3V_L, reduction in binding is not as great as that seen when these light chains are combined with B3V_H. This observation is consistent with the idea that IS4V_H plays a dominant role in binding to CL.

Despite being tested at a range of concentrations up to 75 times higher than those that gave maximal CL binding for the other combinations containing IS4V_H, none of the light chains containing CDR2 and CDR3 derived from UK4V_L, including UK4 wild-type, IU and BU, showed any binding to CL.

**Discussion**

Previously we have shown the important roles played in antigen binding by IS4V_H and B3V_L, which both contain multiple nongermline-encoded arginine residues in their CDRs, supporting the idea that this amino acid is important in creating a CL binding site [29]. The results described in the present study demonstrate that it is not just the presence of, but the precise location of arginine residues in the CDRs that is important in determining the ability to bind CL.
The importance of arginine residues at specific positions in the VH and VL sequences of anti-DNA antibodies has been examined by many groups, by expressing the antibodies in vitro and then altering the sequence of the expressed immunoglobulins by chain swapping or mutagenesis [27,37,41-43]. In general, these studies have shown that altering the numbers of arginine residues in the CDRs of these antibodies can lead to significant alterations in binding to DNA. Arginines in VH CDR3 often play a particularly important role in binding to this antigen [27,37,41-43].

### Table 1

| Heavy chain | Light chain contributing CDR1 | Light chain contributing CDR2 and CDR3 | Light chain name | IgG concentration (ng/ml) |
|-------------|-------------------------------|--------------------------------------|-----------------|--------------------------|
| IS4         | IS4                           | IS4                                  | IS4             | 24–368                   |
| IS4         | IS4                           | B3                                   | IB              | 22–140                   |
| IS4         | IS4                           | UK4                                  | IU              | 70–194                   |
| IS4         | B3                            | B3                                   | B3              | 5–14                     |
| IS4         | B3                            | IS4                                  | B3              | 50–60                    |
| IS4         | B3                            | UK4                                  | BU              | 5–60                     |
| IS4         | UK4                           | UK4                                  | UK4             | 11–22                    |
| IS4         | UK4                           | IS4                                  | UI              | 50–480                   |
| IS4         | UK4                           | B3                                   | UB              | 9–50                     |
| B3          | IS4                           | IS4                                  | IS4             | 71–192                   |
| B3          | IS4                           | B3                                   | IB              | 41–96                    |
| B3          | IS4                           | UK4                                  | IU              | 89–376                   |
| B3          | B3                            | B3                                   | B3              | 3.5–6                    |
| B3          | B3                            | IS4                                  | B3              | 120–608                  |
| B3          | B3                            | UK4                                  | BU              | 40–68                    |
| B3          | UK4                           | UK4                                  | UK4             | 8–28                     |
| B3          | UK4                           | IS4                                  | UI              | 60–480                   |
| B3          | UK4                           | B3                                   | UB              | 2–20                     |
| IS4         | B3(Arg27aSer)                 | B3                                   | B3a             | 48–60                    |
| B3          | B3(Arg27aSer)                 | B3                                   | B3a             | 2.5–4                    |
| IS4\(V_{H1}\) | IS4                           | IS4                                  | IS4             | 50–56                    |
| IS4\(V_{H2}\) | IS4                           | IS4                                  | IS4             | 65–70                    |
| IS4\(V_{H3}\) | IS4                           | IS4                                  | IS4             | 48–90                    |
| IS4\(V_{H4}\) | IS4                           | IS4                                  | IS4             | 48–90                    |
| IS4\(V_{H5}\) | IS4                           | IS4                                  | IS4             | 78–94                    |
| IS4\(V_{H6}\) | IS4                           | IS4                                  | IS4             | 74–80                    |
| IS4\(V_{H7}\) | B3                            | B3                                   | B3              | 24–54                    |
| IS4\(V_{H8}\) | B3                            | B3                                   | B3              | 30                        |
| IS4\(V_{H9}\) | B3                            | B3                                   | B3              | 30–34                    |
| IS4\(V_{H10}\) | B3                            | B3                                   | B3              | 28–30                    |
| IS4\(V_{H11}\) | B3                            | B3                                   | B3              | 32–34                    |
| IS4\(V_{H12}\) | B3                            | B3                                   | B3              | 32–47                    |

IgG concentrations in COS-7 cell supernatants were determined by ELISA. The hybrid light chains were named by combining the names of the two parent antibodies such that the first letter represented the antibody from which the complementarity determining region (CDR) 1 was derived and the last letter represented the antibody from which both the CDR2 and CDR3 were derived. At least two expression experiments were carried out for each combination; identical concentrations were obtained for IS4\(V_{H1}/B3\) from two different expression experiments.
Behrendt and colleagues recently demonstrated that the affinity of human phage-derived anti-dsDNA Fabs from a lupus patient correlated with the presence of somatically mutated arginine residues in CDR1 and CDR2 of the heavy chain [44].

Previous studies of the contribution of aPL heavy chains or light chains to CL binding have yielded conflicting results. Different groups have reported important contributions from the heavy chain [21,45], from the light chain [46], or from both chains [43,47]. In one of these studies the role of arginine residues was examined in a murine antibody (3H9) with dual specificity for phospholipid antibodies and DNA [21]. The introduction of arginine residues into the V_{H} at positions known to mediate DNA binding enhanced binding to phosphatidylserine-β_{2}GPI complexes and to apoptotic cell debris, which may be an important physiological source of both these antigens [48].

Our data show that combinations of IS4V_{H} with light chains containing CDR1 of B3 (B3V_{L}, B3aV_{L} and BIV_{L}) produced the strongest binding to CL. The CDR1 of B3V_{L} and BIV_{L} contains two surface-exposed arginine residues at positions 27 and 27a, while B3aV_{L} contains only one arginine at position 27. Previous modelling studies have suggested that the binding of B3V_{H}/B3V_{L} to dsDNA is stabilised by the interaction of dsDNA with Arg27a in CDR1 and Arg54 in CDR2 of the light chain [34]. Expression and mutagenesis studies from our group confirmed that mutation of Arg27a to serine led to a reduction in binding to DNA [37]. In the present study the same change has been shown to reduce binding to CL, supporting the conclusion of Coca and colleagues that arginines at particular positions can enhance binding to both DNA and CL [21].

It is important, however, not to overlook the possible contribution of other amino acids in B3V_{L} to CL binding. For example, substitution of histidine at position 53 with lysine and substitution of serine at position 29 with glycine could significantly influence the stability of the antigen binding site. In fact, we have previously shown that introduction of the Ser29 to glycine mutation in addition to the Arg27a to serine mutation in the light chain of B3V_{L}/B3V_{H} leads to a further reduction in binding to dsDNA [37].

The presence of UK4V_{L} CDR2 and CDR3 in any light chain blocked binding to CL, even when combined with B3V_{L} CDR1 (light chain BU). UK4V_{L}CDR1, however, does not block binding. We have previously shown that the presence of UK4V_{L} CDR2 and CDR3 blocks binding to DNA and histones but not to the Ro antigen [36,37]. Modelling studies have shown that an arginine at position 94 in CDR3 of UK4V_{L} hinders DNA binding sterically. A similar effect may be occurring with regards to the binding of UK4V_{L} to CL.

The effect of point mutations of specific arginine residues in CDR3 of IS4V_{H} upon CL binding is shown in Fig. 4. The low binding of IS4V_{H}/IS4V_{L} was abolished by inclusion of any one of these mutations. This is not the case, however, when these mutants are expressed with B3V_{L}. In this case the arginine residues at 100 and 100 g confer a greater effect on CL binding compared with the arginine residues at positions 96 and 97. Substitutions of all four of these IS4V_{H} CDR3 arginine residues were sufficient to completely abolish all binding to CL.

An accumulation of arginine residues in V_{H} CDR3 has been noted in most, but not in all, sequences of pathogenic monoclonal aPL. From our detailed analysis of all published sequences of monoclonal aPL we found that of 13 monoclonal aPL that had been examined in various biological assays, eight monoclonal aPL had been shown to be pathogenic [49]. Three aPL derived from patients with primary APS and a healthy subject induced a significantly higher rate of foetal resorptions and a significant reduction in foetal and placental weight following intravenous injection into mated BALB/c mice [50,51]. Five other aPL derived from patients with primary APS and systemic lupus erythematosus/APS were found to be thrombogenic in an in vivo model of thrombosis [30]. We compared the sequences of these eight pathogenic antibodies with those of the other five antibodies, observing no evidence of pathogenicity in these bioassays. There was no evidence of preferential gene usage in either antibody group and somatic mutations were common in both groups. The presence of arginine residues in V_{H} CDR3, however, did differ between patho-

![Figure 3](http://arthritis-research.com/content/71/R47)
region (VH) complementarity determining region 3. Cardiolipin binding IS4VH mutants VHi, VHii, VHiii and VHiv contain single arginine to serine point mutations at positions 96, 97, 100 and 100 g, respectively; VHx contains an arginine to serine point mutation at four positions. Presented as concentration of IgG in the supernatant versus optical density (OD) at 405 nm in the anti-cardiolipin ELISA.

genic aPL and nonpathogenic aPL. Six of the eight pathogenic aPL, but only one of five nonpathogenic aPL, contain at least two arginine residues in VH CDR3 [49].

Our data confirm that the effect of arginine residues on binding to CL is highly dependent on the positions that they occupy in the sequence. The precise location of arginine residues has been shown to be important in the binding of both murine and human anti-dsDNA to DNA in numerous studies [25,26,37]. Interestingly, Krishnan and colleagues have demonstrated a strong correlation between specificity for dsDNA and the relative position of arginine residues in VH CDR3 [52,53]. They reported that the frequency of arginine expression among murine anti-dsDNA antibodies was highest at position 100, and they postulate that the importance of this residue in binding to dsDNA lies in its position at the centre of the VH CDR3 loop in the structure of the antigen combining site [52]. Assuming that this loop would be projected outward from the antigen combining site, an arginine residue at position 100 would be located at the apex of the VH CDR3 loop.

Conclusion
We have demonstrated the relative importance of certain surface-exposed arginine residues at critical positions within the light chain CDR1 and heavy chain CDR3 of different human monoclonal antibodies in conferring the ability to bind CL in a direct ELISA. It is now important to test the effects of sequence changes involving these amino acids on pathogenic functions of these aPL, by expressing the altered antibodies in larger quantities from stably transfected cells, and then testing them in bioassays.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
IG produced four hybrid light chains, participated in the production of the mutant heavy chains, antibody expression and study design, and drafted the manuscript. NL participated in the production of the mutant heavy chains and antibody expression. PC and RC produced the human monoclonal aPL IS4. DL and DI participated in study design and coordination. AR conceived of the study, and approved the final manuscript.

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