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The critical role of arginine residues in the binding of human monoclonal antibodies to cardiolipinIan Giles^{1,2}, Nancy Lambrianides^{1,2}, David Latchman², Pojen Chen³, Reginald Chukwuocha³, David Isenberg¹ and Anisur Rahman^{1,2}¹Centre for Rheumatology, Department of Medicine, University College London, UK²Medical Molecular Biology Unit, Institute of Child Health, University College London, UK³Department of Medicine, Division of Rheumatology, University of California, Los Angeles, USACorresponding author: Ian Giles, i.giles@ich.ucl.ac.uk

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Arthritis Res Ther 2005, **7**:R47-R56 (DOI 10.1186/ar1449)© 2004 Giles *et al.*, licensee BioMed Central Ltd.This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is cited.**Abstract**

Previously we reported that the variable heavy chain region (V_H) of a human β_2 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 (V_L) determined the strength of CL binding. In the present study, we examine the importance of specific arginine residues in IS4 V_H and paired V_L in CL binding. The distribution of arginine residues in complementarity determining regions (CDRs) of V_H and V_L sequences was altered by site-directed mutagenesis or by CDR exchange. Ten different 2a2 germline gene-derived V_L sequences were expressed with IS4 V_H and the V_H of an anti-dsDNA antibody, B3. Six variants of IS4 V_H , containing different patterns of arginine residues in CDR3, were paired with B3 V_L and IS4 V_L . The ability

of the 32 expressed heavy chain/light chain combinations to bind CL was determined by ELISA. Of four arginine residues in IS4 V_H CDR3 substituted to serines, two residues at positions 100 and 100 g 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, V_L containing B3 V_L 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 V_L CDR2 or V_L 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; β_2 GPI = β_2 glycoprotein I; CDR = complementarity determining region; CL = cardiolipin; dsDNA = double-stranded DNA; ELISA = enzyme-linked immunosorbent assay; Fab = antigen-binding fragment; V_H = variable heavy chain region; V_L = 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 beta₂ glycoprotein I (β₂GPI) [10-13]. β₂GPI 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/β₂GPI 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 β₂GPI, can bind to β₂GPI 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 cardiolipin (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 β₂GPI, and to human β₂GPI 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 human 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 β₂GPI 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 UK4V_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 V_L expression vectors were digested with *Acc65 I* and *Pvu I* (Promega, Southampton, UK). *Acc65 I* cuts IS4, B3 or UK4 V_L sequences at a position in FR2 that is 106 base pairs from the beginning of V_L , but does not cut the expression vector outside the insert. *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 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 V_L expression vector was ligated with the 1.5 kb fragment derived from the other. The resulting plasmid would contain CDR1 of one V_L sequence and CDR2 and CDR3 of another V_L sequence.

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

Site-directed mutagenesis of IS4V_H

We generated six mutant forms of IS4V_H in which particular arginine residues were mutated to serine, using the Quik-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 D_H gene of IS4V_H CDR3 is unknown. Four mutants, named IS4V_Hi, IS4V_Hii, IS4V_Hiii and IS4V_Hiv, contained single mutations of arginine residues at positions 96, 97, 100 and 100 g, respectively. The remaining two forms contained two arginine to serine mutations, at positions 96 and 97 in the IS4V_Hi&ii mutant and at all four sites in mutant IS4V_Hx.

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 IS4V_L, UK4V_L, B3V_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 B3aV_L, shown in Fig. 1a, was derived from B3V_L by site-directed mutagenesis of Arg27a to serine [37].

The V_L sequences of IS4, B3 and UK4 are all encoded by the germline V_λ gene 2a2, but differ in their patterns of somatic mutation. B3V_λ contains two adjacent arginine residues in CDR1, both produced by somatic mutations. UK4V_λ has a single somatic mutation to arginine in CDR3 at position 94. A serine residue in CDR3 of IS4V_L is replaced by asparagine.

Figure 1a also shows the amino acid sequences of the V_λ 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

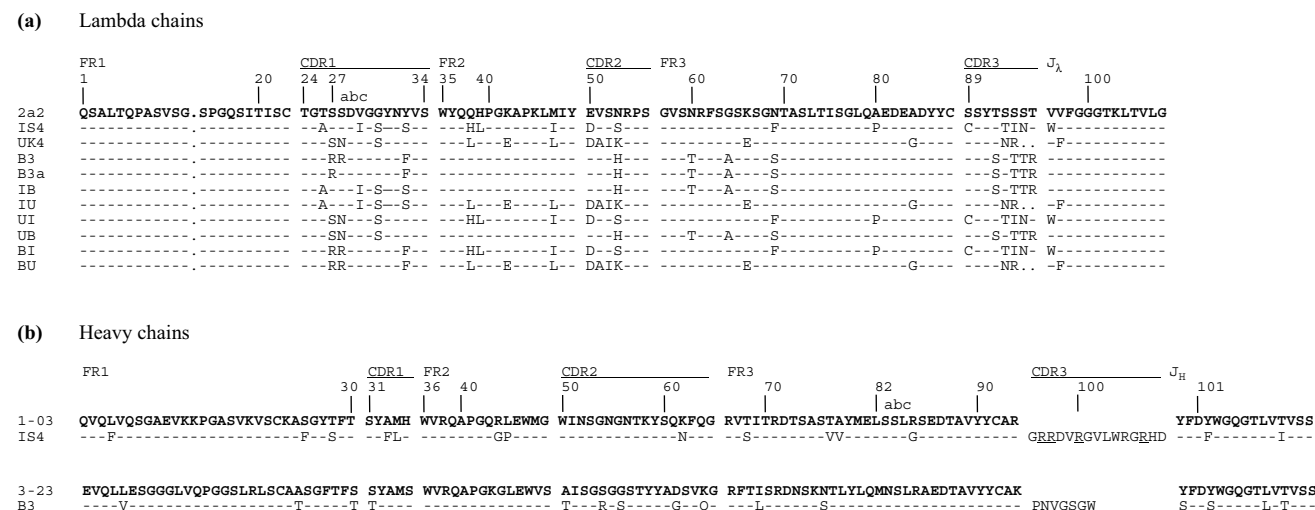
The amino acid sequences of IS4V_H and B3V_H chain and the corresponding germline genes are displayed in Fig. 1b. B3V_H has a single somatic mutation to arginine in CDR2. The CDR2 of IS4V_H contains an asparagine residue created by somatic mutation and in CDR3 there are multiple arginine residues, which 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 IS4V_H are underlined in Fig. 1b.

Expression of whole IgG

Each of the 10 light chains shown in Fig. 1a was paired with B3V_H and IS4V_H. Each of the six mutant forms of IS4V_H was paired with IS4V_L and B3V_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

Figure 1



Sequence alignment of the expressed variable light chainregion (V_L) and variable heavy chainregion (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_H regions could not be matched to germline genes. Arginine residues altered by site-directed mutagenesis to serine residues in IS4 V_H complementarity determining region (CDR) 3 are underlined. Amino acids are numbered according to Kabat. Dots inserted to facilitate the alignment. Dashes indicate homology with the corresponding germline sequence. FR, framework region.

Table 1. Identical concentrations were obtained for the combination IS4 V_{Hii} /B3 V_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 B3 V_H /BIV L , B3 V_H /UIV L and IS4 V_H /UIV L combinations compared with the other antibody combinations. The phenomenon of variable expression with different V_H and V_L constructs is well documented both in this antibody expression system and in other systems [35,37], although the reason for the occurrence 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 IS4 V_H

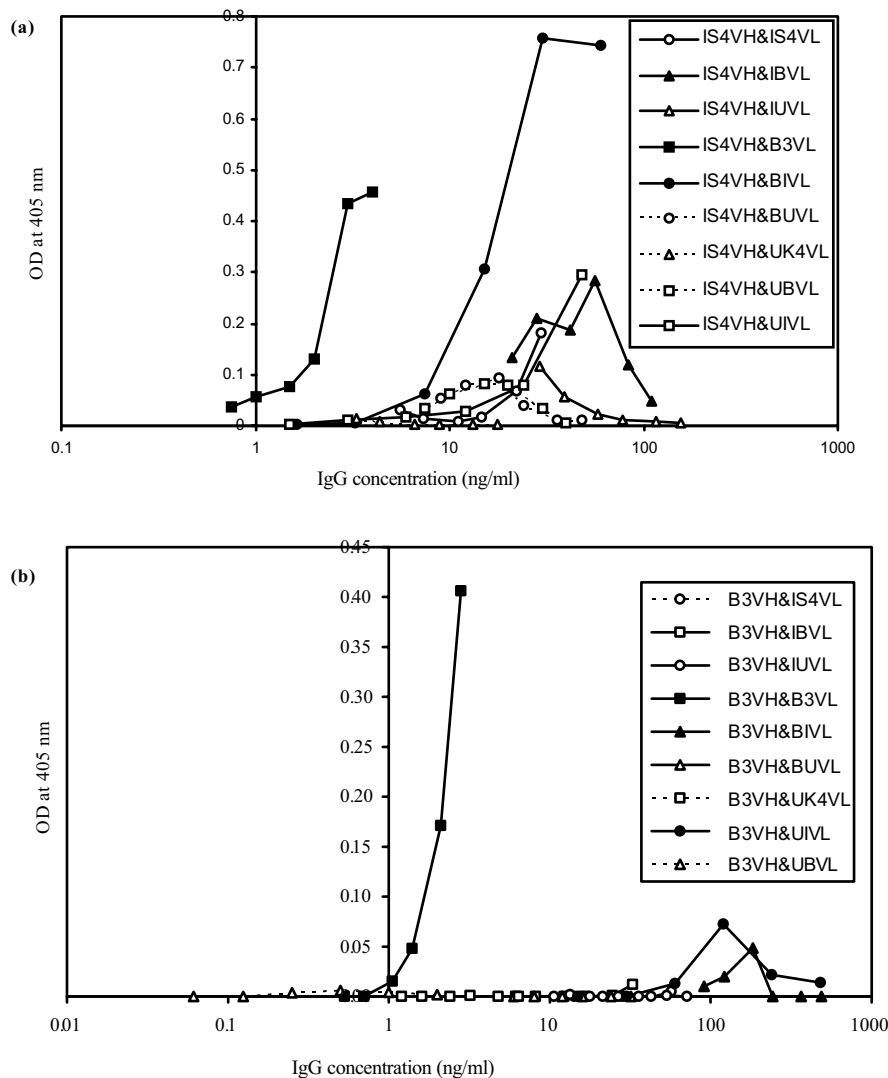
As reported previously, the presence of the heavy chain of IS4 plays a dominant role in binding to CL [29]. IS4 V_H binds CL in combination with six of the 10 light chains tested (see Figs 2a and 3): B3 V_L , B3a V_L , BIV L , IS4 V_L , IB V_L and UIV L . Only one of these light chains (B3 V_L) binds CL in combination with B3 V_H (Fig. 2b).

To identify the features of IS4 V_H that enhance binding to CL, we focused on the combination IS4 V_H /B3 V_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 IS4 V_H CDR3 to serine, as shown in Fig. 4. Substitution of all four arginine residues with serine residues (IS4 V_{Hx}) 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 (IS4 V_{Hiii} and IS4 V_{Hiv}) displayed approximately 50% weaker binding to CL in combination with B3 V_L than did the wild-type IS4 V_H /B3 V_L combination. In contrast, there were no reductions in CL binding for the heavy chains containing arginine to serine mutations at position 96 (IS4 V_{Hi}), at position 97 (IS4 V_{Hii}) or at both positions (IS4 $V_{Hi&ii}$).

The importance of arginine residues in the light chain CDRs

Six light chains bound CL in conjunction with IS4 V_H (Figs 2a and 3). The strongest binding was seen with light chains containing B3 V_L CDR1, namely B3 V_L , B3a V_L and BI V_L , in combination with IS4 V_H . In contrast, light chains IB V_L and UB V_L , containing CDR2 and CDR3 from B3, showed weak binding and no binding to CL, respectively, in combination with IS4 V_H .

To test the hypothesis that the arginine at position 27a in B3 V_L CDR1 is responsible for the favourable effect of this CDR on binding to CL, we expressed combinations of IS4 V_H and B3 V_H with B3a V_L , in which Arg27a has been

Figure 2

Effect of complementarity determining region exchange in the light chains. Cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type heavy chains expressed with wild-type or hybrid light chain constructs. **(a)** Light chains expressed with IS4 variable heavy chain region (V_H). **(b)** Light chains expressed with B3V_H. Presented as concentration of IgG in the supernatant versus optical density (OD) at 405 nm in the anti-cardiolipin ELISA.

mutated to serine. As shown in Fig. 3, there was a significant decrease in CL binding of B3V_H/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.

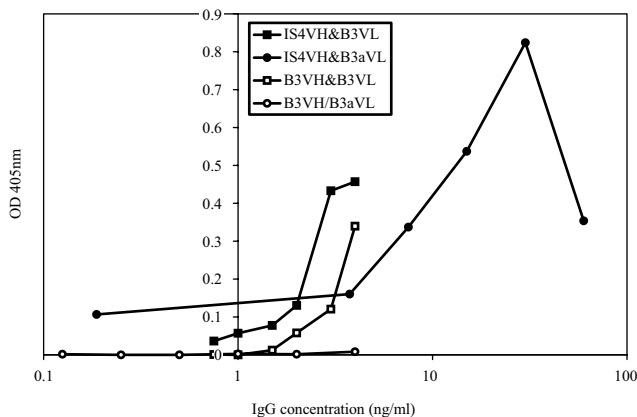
Table 1**The range of IgG concentrations (ng/ml) produced by expression of the 32 heavy chain/light chain combinations**

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	BI	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	BI	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
IS4V _H i	IS4	IS4	IS4	50–56
IS4V _H ii	IS4	IS4	IS4	65–70
IS4V _H iii	IS4	IS4	IS4	48–90
IS4V _H iv	IS4	IS4	IS4	48–90
IS4V _H x	IS4	IS4	IS4	78–94
IS4V _H i&ii	IS4	IS4	IS4	74–80
IS4V _H i	B3	B3	B3	24–54
IS4V _H ii	B3	B3	B3	30
IS4V _H iii	B3	B3	B3	30–34
IS4V _H iv	B3	B3	B3	28–30
IS4V _H x	B3	B3	B3	32–34
IS4V _H i&ii	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 IS4V_Hii/B3V_L from two different expression experiments.

The importance of arginine residues at specific positions in the V_H and V_L 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 V_H CDR3 often play a particularly important role in binding to this antigen [27,37,41-43].

Figure 3

Effect of point mutation Arg27a to serine in B3 variable light chain-region (V_L) complementarity determining region 1. Comparison of cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type heavy chains expressed with B3V_L or B3V_La. Presented as concentration of IgG in the supernatant versus optical density (OD) at 405 nm in the anti-cardiolipin ELISA.

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 Cocco

and colleagues that arginines at particular positions can enhance binding to both DNA and CL [21].

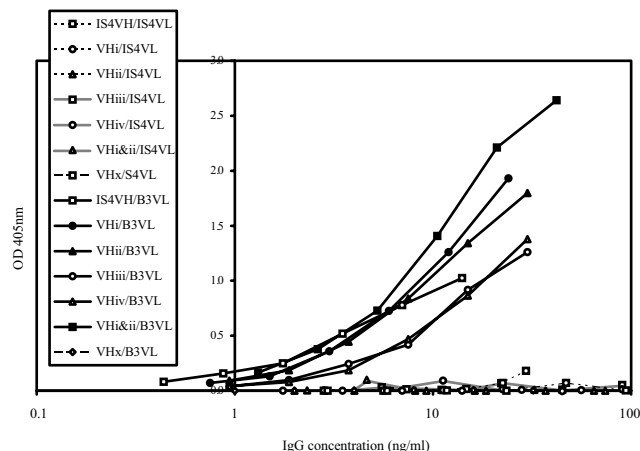
It is important, however, not to overlook the possible contribution of other amino acids in B3V_L to CL binding. For example, substitution of histidine at position 53 with lysine and substitution of serine at position 29 with glycine could significantly influence the stability of the antigen binding 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 4



Effect of arginine to serine point mutations in IS4 variable heavy chain-region (V_H) complementarity determining region 3. Cardiolipin binding of IgG in COS-7 cell supernatants containing wild-type or mutant forms of IS4 heavy chain expressed with wild-type B3 or IS4 light chains. The IS4 V_H mutants VHi, VHiI, VHiII and VHiV contain single arginine to serine point mutations at positions 96, 97, 100 and 100 g, respectively; VHi&II contains arginine to serine point mutations at positions 96 and 97; and VHX has an arginine to serine point mutation at all 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 V_H 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 V_H 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 V_H 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 V_H 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 participated in its design and coordination. All authors read and approved the final manuscript.

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References

- Hughes GR: **Thrombosis, abortion, cerebral disease, and the lupus anticoagulant.** *Br Med J (Clin Res Ed)* 1983, **287**:1088-1089.
- Hughes GR, Harris NN, Gharavi AE: **The anticardiolipin syndrome.** *J Rheumatol* 1986, **13**:486-489.
- Cervera R, Piette JC, Font J, Khamashta MA, Shoenfeld Y, Camps MT, Jacobsen S, Lakos G, Tincani A, Kontopoulou-Griva I, et al.: **Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients.** *Arthritis Rheum* 2002, **46**:1019-1027.
- Petri M: **Classification and epidemiology of the antiphospholipid syndrome.** In *The Antiphospholipid Syndrome II: Autoimmune Thrombosis* second edition. Edited by: Asherson RA, Cervera R, Piette J-C, Shoenfeld Y. Amsterdam: Elsevier Science BV; 2002:11-20.
- Asherson RA, Khamashta MA, Ordi-Ros J, Derksen RH, Machin SJ, Barquinero J, Outt HH, Harris EN, Vilardell-Torres M, Hughes GR: **The 'primary' antiphospholipid syndrome: major clinical and serological features.** *Medicine (Baltimore)* 1989, **68**:366-374.
- Morrow WJW, Nelson L, Watts R, Isenberg DA: *Autoimmune Rheumatic Disease* 2nd edition. Oxford: Oxford University Press; 1999.
- Shah NM, Khamashta MA, Atsumi T, Hughes GR: **Outcome of patients with anticardiolipin antibodies: a 10 year follow-up of 52 patients.** *Lupus* 1998, **7**:3-6.
- Khamashta MA, Cuadrado MJ, Mujic F, Taub NA, Hunt BJ, Hughes GR: **The management of thrombosis in the antiphospholipid-antibody syndrome.** *N Engl J Med* 1995, **332**:993-997.
- Greaves M, Cohen H, MacHin SJ, Mackie I: **Guidelines on the investigation and management of the antiphospholipid syndrome.** *Br J Haematol* 2000, **109**:704-715.
- Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ, Barbui T, Zwaal RF, Bevers EM: **Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor.** *Lancet* 1990, **335**:1544-1547.
- McNeil HP, Simpson RJ, Chesterman CN, Krilis SA: **Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H).** *Proc Natl Acad Sci USA* 1990, **87**:4120-4124.

12. Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T: **Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease.** *Lancet* 1990, **336**:177-178.
13. Ordi J, Selva A, Monegal F, Porcel JM, Martinez-Costa X, Vilardell M: **Anticardiolipin antibodies and dependence of a serum cofactor. A mechanism of thrombosis.** *J Rheumatol* 1993, **20**:1321-1324.
14. Tsutsumi A, Matsuura E, Ichikawa K, Fujisaku A, Mukai M, Kobayashi S, Koike T: **Antibodies to beta 2-glycoprotein I and clinical manifestations in patients with systemic lupus erythematosus.** *Arthritis Rheum* 1996, **39**:1466-1474.
15. McNally T, Mackie IJ, Machin SJ, Isenberg DA: **Increased levels of beta 2 glycoprotein-I antigen and beta 2 glycoprotein-I binding antibodies are associated with a history of thromboembolic complications in patients with SLE and primary antiphospholipid syndrome.** *Br J Rheumatol* 1995, **34**:1031-1036.
16. Kandiah DA, Sali A, Sheng Y, Victoria EJ, Marquis DM, Coutts SM, Krilis SA: **Current insights into the 'antiphospholipid' syndrome: clinical, immunological, and molecular aspects.** *Adv Immunol* 1998, **70**:507-563.
17. Alarcon-Segovia D, Deleze M, Oria CV, Sanchez-Guerrero J, Gomez-Pacheco L, Cabiedes J, Fernandez L, Ponce de Leon S: **Antiphospholipid antibodies and the antiphospholipid syndrome in systemic lupus erythematosus. A prospective analysis of 500 consecutive patients.** *Medicine (Baltimore)* 1989, **68**:353-365.
18. Lynch A, Marlar R, Murphy J, Davila G, Santos M, Rutledge J, Emlen W: **Antiphospholipid antibodies in predicting adverse pregnancy outcome. A prospective study.** *Ann Intern Med* 1994, **120**:470-475.
19. Giles IP, Haley JD, Nagl S, Isenberg DA, Latchman DS, Rahman A: **A systematic analysis of sequences of human antiphospholipid and anti-beta2-glycoprotein I antibodies: the importance of somatic mutations and certain sequence motifs.** *Semin Arthritis Rheum* 2003, **32**:246-265.
20. Shlomchik MJ, Marshak-Rothstein A, Wolfowicz CB, Rothstein TL, Weigert MG: **The role of clonal selection and somatic mutation in autoimmunity.** *Nature* 1987, **328**:805-811.
21. Cocco BA, Seal SN, D'Agnillo P, Mueller YM, Katsikis PD, Rauch J, Weigert M, Radic MZ: **Structural basis for autoantibody recognition of phosphatidylserine-beta 2 glycoprotein I and apoptotic cells.** *Proc Natl Acad Sci USA* 2001, **98**:13826-13831.
22. Kita Y, Sumida T, Ichikawa K, Maeda T, Yonaha F, Iwamoto I, Yoshida S, Koike T: **V gene analysis of anticardiolipin antibodies from MRL-lpr/lpr mice.** *J Immunol* 1993, **151**:849-856.
23. Rahman A, Latchman DS, Isenberg DA: **Immunoglobulin variable region sequences of human monoclonal anti-DNA antibodies.** *Semin Arthritis Rheum* 1998, **28**:141-154.
24. Ehrenstein MR, Katz DR, Griffiths MH, Papadaki L, Winkler TH, Kalden JR, Isenberg DA: **Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice.** *Kidney Int* 1995, **48**:705-711.
25. Radic MZ, Weigert M: **Genetic and structural evidence for antigen selection of anti-DNA antibodies.** *Annu Rev Immunol* 1994, **12**:487-520.
26. Radic MZ, Mackle J, Erikson J, Mol C, Anderson WF, Weigert M: **Residues that mediate DNA binding of autoimmune antibodies.** *J Immunol* 1993, **150**:4966-4977.
27. Li Z, Schettino EW, Padlan EA, Ikematsu H, Casali P: **Structure-function analysis of a lupus anti-DNA autoantibody: central role of the heavy chain complementarity-determining region 3 Arg in binding of double- and single-stranded DNA.** *Eur J Immunol* 2000, **30**:2015-2026.
28. Katz JB, Limpanasithikul W, Diamond B: **Mutational analysis of an autoantibody: differential binding and pathogenicity.** *J Exp Med* 1994, **180**:925-932.
29. Giles I, Haley J, Nagl S, Latchman D, Chen P, Chukwuocha R, Isenberg D, Rahman A: **Relative importance of different human aPL derived heavy and light chains in the binding of aPL to cardiolipin.** *Mol Immunol* 2003, **40**:49-60.
30. Pierangeli SS, Liu X, Espinola R, Olee T, Zhu M, Harris NE, Chen PP: **Functional analyses of patient-derived IgG monoclonal anticardiolipin antibodies using *in vivo* thrombosis and *in vivo* microcirculation models.** *Thromb Haemost* 2000, **84**:388-395.
31. Zhu M, Olee T, Le DT, Roubey RA, Hahn BH, Woods VL Jr, Chen PP: **Characterization of IgG monoclonal anti-cardiolipin/anti-beta2GP1 antibodies from two patients with antiphospholipid syndrome reveals three species of antibodies.** *Br J Haematol* 1999, **105**:102-109.
32. Ehrenstein MR, Longhurst CM, Latchman DS, Isenberg DA: **Serological and genetic characterization of a human monoclonal immunoglobulin G anti-DNA idiotype.** *J Clin Invest* 1994, **93**:1787-1797.
33. Menon S, Rahman MA, Ravirajan CT, Kandiah D, Longhurst CM, McNally T, Williams WM, Latchman DS, Isenberg DA: **The production, binding characteristics and sequence analysis of four human IgG monoclonal antiphospholipid antibodies.** *J Autoimmun* 1997, **10**:43-57.
34. Kalsi JK, Martin AC, Hirabayashi Y, Ehrenstein M, Longhurst CM, Ravirajan C, Zvelebil M, Stollar BD, Thornton JM, Isenberg DA: **Functional and modelling studies of the binding of human monoclonal anti-DNA antibodies to DNA.** *Mol Immunol* 1996, **33**:471-483.
35. Rahman MAA, Kettleborough CA, Latchman DS, Isenberg DA: **Properties of whole human IgG molecules produced by the expression of cloned anti-DNA antibody cDNA in mammalian cells.** *J Autoimmun* 1998, **11**:661-669.
36. Haley J, Mason L, Giles I, Nagl S, Latchman D, Isenberg D, Rahman A: **Somatic mutations to arginine residues affect the binding of human monoclonal antibodies to DNA, histones S_mD and R_{antigen}.** *Mol Immunol* 2004, **40**:745-758.
37. Rahman A, Haley J, Radway-Bright E, Nagl S, Low DG, Latchman DS, Isenberg DA: **The importance of somatic mutations in the V(lambda) gene 2a2 in human monoclonal anti-DNA antibodies.** *J Mol Biol* 2001, **307**:149-160.
38. Winkler TH, Fehr H, Kalden JR: **Analysis of immunoglobulin variable region genes from human IgG anti-DNA hybridomas.** *Eur J Immunol* 1992, **22**:1719-1728.
39. Chukwuocha R, Zhu M, Cho C, Visvanathan S, Hwang K, Rahman A, Chen P: **Molecular and genetic characterizations of five pathogenic and two non-pathogenic monoclonal antiphospholipid antibodies.** *Mol Immunol* 2002, **39**:299-311.
40. Rahman A, Giles I, Haley J, Isenberg D: **Systematic analysis of sequences of anti-DNA antibodies - relevance to theories of origin and pathogenicity.** *Lupus* 2002, **11**:807-823.
41. Radic MZ, Mascelli MA, Erikson J, Shan H, Weigert M: **Ig H and L chain contributions to autoimmunity specificities.** *J Immunol* 1991, **146**:176-182.
42. Mockridge CI, Chapman CJ, Spellerberg MB, Isenberg DA, Stevenson FK: **Use of phage surface expression to analyze regions of human V4-34(VH4-21)-encoded IgG autoantibody required for recognition of DNA: no involvement of the 9G4 idiotope.** *J Immunol* 1996, **157**:2449-2454.
43. Pewzner-Jung Y, Simon T, Eilat D: **Structural elements controlling anti-DNA antibody affinity and their relationship to antiphosphorylcholine activity.** *J Immunol* 1996, **156**:3065-3073.
44. Behrendt M, Partridge LJ, Griffiths B, Goodfield M, Snaith M, Lindsey NJ: **The role of somatic mutation in determining the affinity of anti-DNA antibodies.** *Clin Exp Immunol* 2003, **131**:182-189.
45. Blank M, Waisman A, Mozes E, Koike T, Shoenfeld Y: **Characteristics and pathogenic role of anti-beta2-glycoprotein I single-chain Fv domains: induction of experimental antiphospholipid syndrome.** *Int Immunol* 1999, **11**:1917-1926.
46. Pereira B, Benedict CR, Le A, Shapiro SS, Thiagarajan P: **Cardiolipin binding a light chain from lupus-prone mice.** *Biochemistry* 1998, **37**:1430-1437.
47. Kumar S, Kalsi J, Ravirajan CT, Rahman A, Athwal D, Latchman DS, Isenberg DA, Pearl LH: **Molecular cloning and expression of the Fabs of human autoantibodies in *Escherichia coli*. Determination of the heavy or light chain contribution to the anti-DNA-cardiolipin activity of the Fab.** *J Biol Chem* 2000, **275**:35129-35136.
48. Cocco BA, Cline AM, Radic MZ: **Blebs and apoptotic bodies are B cell autoantigens.** *J Immunol* 2002, **169**:159-166.
49. Giles I, Isenberg D, Rahman A: **Lessons from sequence analysis of monoclonal antiphospholipid antibodies.** In *Hughes Syndrome: Antiphospholipid Syndrome* 2nd edition. Edited by: Khamashta MA. London: Springer-Verlag in press.
50. Ikematsu W, Luan FL, La Rosa L, Beltrami B, Nicoletti F, Buyon JP, Meroni PL, Balestrieri G, Casali P: **Human anticardiolipin monoclonal autoantibodies cause placental necrosis and fetal loss in BALB/c mice.** *Arthritis Rheum* 1998, **41**:1026-1039.

51. Lieby P, Poindron V, Roussi S, Klein C, Knapp AM, Garaud JC, Cerutti M, Martin T, Pasquali JL: **Pathogenic antiphospholipid antibody: an antigen selected needle in a haystack.** *Blood* 2004, **104**:1711-1715.
52. Krishnan MR, Jou NT, Marion TN: **Correlation between the amino acid position of arginine in VH-CDR3 and specificity for native DNA among autoimmune antibodies.** *J Immunol* 1996, **157**:2430-2439.
53. Krishnan MR, Marion TN: **Comparison of the frequencies of arginines in heavy chain CDR3 of antibodies expressed in the primary B-cell repertoires of autoimmune-prone and normal mice.** *Scand J Immunol* 1998, **48**:223-232.
54. Tomlinson IM, Williams SC, Corbett SJ, Cox JPL, Winter G: *VBASE: A Database of Human Immunoglobulin Variable Region Genes* Cambridge: MRC Centre for Protein Engineering; 1998.