Arginine mutation alters binding of a human monoclonal antibody to antigens linked to systemic lupus erythematosus and the antiphospholipid syndrome

Anastasia Lambrianides PhD [1], Ian Giles PhD MRCP [1,2], Yiannis Ioannou BMedSci, MRCP [1,2], Lesley Mason PhD [2], David S Latchman DSc [1], Jessica J Manson Bsc MRCP [2], David A Isenberg MD FRCP [2], Anisur Rahman PhD FRCP [1,2*]

[1] Medical Molecular Biology Unit, Institute of Child Health, University College London, 30 Guilford Street, London WC1N 1EH, United Kingdom.

[2] Centre for Rheumatology Research, Division of Medicine, University College London, Room 331, Windeyer Building, 46 Cleveland Street, London W1T 4JF, United Kingdom.

* To whom correspondence should be addressed

T: +44 (0) 207905 2621; F: +44 (0) 207905 2301

e-mail T: +44 (0) 207679 9270; F: +44 (0) 207679 9143

e-mail anisur.rahman@ucl.ac.uk

Grant Support: Dr Lambrianides was supported by the Rosetrees Trust. Dr Giles, Dr Ioannou, Dr Mason and Dr Manson were supported by the Arthritis Research Campaign.

Abstract

Objective: Previous studies have demonstrated the importance of somatic mutations and arginine residues in the complementarity determining regions (CDRs) of pathogenic anti-dsDNA antibodies in human and murine lupus. Studies of murine antibodies suggested that mutations at position 53 in V_HCDR2 are important. Previously, we have demonstrated *in-vitro* expression and mutagenesis of the human monoclonal IgG antibody B3. Here we use this expression system to investigate the importance of the arginine residue at position 53 (R53) in B3V_H.

Methods: R53 was altered by site-directed mutagenesis to serine, asparagine or lysine to create three expressed variants of V_H. In addition the germline sequence of the V_H3-23 gene (from which B3V_H is derived) was expressed either with or without arginine at position 53. These five new heavy chains, as well as wild-type B3V_H were expressed with four different light chains and the resulting antibodies assessed for binding to nucleosomes, alpha-actinin, cardiolipin, ovalbumin, beta-2-glycoprotein I (β_2GPI), and the N-terminal domain of β_2GPI (Domain I) using direct binding assays.

Results: The presence of R53 was essential but not sufficient for binding to dsDNA and nucleosomes. Conversely, the presence of R53 reduced binding to alpha-actinin, ovalbumin, β_2GPI and Domain I of β_2GPI. The combination B3(R53S)V_H/B3V_L binds human but not bovine β_2GPI.

Conclusion: The fact that the R53S substitution significantly alters binding of B3 to different clinically relevant antigens, but in opposite directions implies that this arginine residue plays a critical role in the affinity maturation of the antibody B3.

Systemic lupus erythematosus (SLE) is characterised by the presence of autoantibodies directed against a wide variety of different antigens. Antibodies to double-stranded DNA (anti-dsDNA) are especially important (1). Serum levels of these antibodies frequently reflect disease activity (2) and their presence correlates with the occurrence of renal disease (3, 4). In both clinical studies and animal models, pathogenicity is associated with IgG isotype and with high affinity binding to dsDNA rather than ssDNA (1, 2). However, only some anti-dsDNA antibodies are pathogenic in mice and this pathogenicity does not always correlate with affinity for dsDNA (5-8). This finding implies that dsDNA is not the only clinically relevant antigen in the pathogenesis of lupus nephritis. In particular, nucleosomes and alpha-actinin have been suggested as pathologically relevant antigens by different groups (9-12).

Sequence analysis of murine and human monoclonal anti-dsDNA antibodies (13, 14) has shown multiple somatic mutations in their heavy chain variable region (V_H) and light chain variable region (V_L) sequences. These somatic mutations are not distributed randomly but are clustered in the complementarity determining regions (CDRs), which form the antigen-binding site, suggesting that they are antigen-driven. Antigen-driven somatic mutation in many of these high affinity monoclonal anti-dsDNA antibodies results in accumulation of arginine (R), asparagine (N) and lysine (K) in the CDRs. (13, 15, 16) *In-vitro* expression experiments have shown that altering R residues in the CDRs of these antibodies can lead to significant alterations in binding to dsDNA (17-21) but few studies have looked at the influence of particular somatic mutations on binding to nucleosomes or alpha-actinin.

It is increasingly accepted that the generation and affinity maturation of autoantibodies in SLE is driven by the presence of autoantigens present on cell fragments released by apoptosis. Clearance of these fragments is retarded in patients with SLE (22). These autoantigens include nucleosomes, anionic phospholipids (PL) and beta-2-glycoprotein I (β_2GPI). Anti-PL and anti-β_2GPI antibodies are important in the pathogenesis of the antiphospholipid syndrome (APS), which often co-exists with SLE. Within β_2GPI, binding to epitopes on the N-terminal domain – Domain I - is particularly important in APS (23).

It is important to distinguish the somatic mutations in CDRs that enhance binding to multiple antigens on apoptotic cell debris from the mutations that focus binding onto a particular antigen involved in pathogenesis (e.g nucleosomes). In murine and human antibodies some arginine residues have been shown to enhance binding to both dsDNA and PL (21, 24) and others enhance binding to one of these antigens but have no effect on the other (25).

Amino acids at certain positions within CDRs seem likely to play a critical role in determining the binding properties of autoantibodies. Position 53 in V_HCDR2 is an important site of antibody-antigen contact in a number of murine anti-DNA antibodies (13) particularly the murine antibody 3H9 where R53 is important in binding to ssDNA, dsDNA, PL and β_2GPI (24). An arginine at position 53 of V_HCDR2 of the human monoclonal IgG1 antibody B3 appears to make a critical interaction with dsDNA in a computer model of the B3-DNA complex (26). B3 binds more strongly to nucleosomes than to dsDNA (27) and also binds cardiolipin (PL) (25). There is evidence that B3 is pathogenic in severe combined immunodeficiency (SCID) mice

(5, 27). In previous experiments we showed that the prediction of the computer model that R27a in $B3V_L$ was important in binding dsDNA/nucleosomes was confirmed by a reduction in binding when R27a was altered to serine (S) (20). In the current experiment we used similar techniques to investigate the importance of V_H R53 on binding to a range of antigens, to test the hypothesis that the introduction of this R played a critical role in making B3 a high-affinity anti-nucleosome antibody. $B3V_H$ is derived from germline gene V_H 3-23, but differs from it at a number of sites other than R53. To test the effect of R53 in isolation we expressed germline V_H 3-23 with and without introduction of R at position 53.

Materials and Methods

Human monoclonal antibodies from which the expressed V_H and V_L sequences were derived

B3 (28), 33H11 (16) and UK4 (29) are all human IgG monoclonal antibodies produced from lymphocytes of three different patients. 33H11 was a kind gift from Dr. Thomas Winkler (Erlangen, Germany). B3 and UK4 were produced in the laboratories of the authors in London. The V_L regions of all three antibodies are encoded by the gene 2a2 and therefore show >90% homology. They differ only at sites of somatic mutation as shown in Figure 1. The chimeric light chains B33 and BU were constructed such $B33V_L$ is exactly the same as $B3V_L$ up to a *Kpn* I site in FR2 and exactly the same as $33H11V_L$ after it. BUV_L is exactly the same as $B3V_L$ up to the *Kpn* I site and exactly the same as $UK4V_L$ after it.

Assembly of constructs for expression

The wild type heavy and light chain constructs, the chimeric light chain constructs and the point mutant B3(R27aS)V_L were prepared as detailed fully in previous papers (30, 31).

A plasmid (neSLE122H45.4) that encodes a human V_H3-23, D6-25, J_H5 IgH chain was a kind gift from Dr Hedda Wardemann (The Rockefeller University, New York, USA). This VDJ sequence was amplified by PCR to incorporate a *HindIII* restriction site at the 5' end and a *XhoI* site at the 3' end of the J region. The insert was then ligated into a final expression vector that contains Cγ1 constant region cDNA.

Site-directed mutagenesis of B3V_H

The aim of mutagenesis was to produce variant forms of B3V_H in which the positively charged R residue at position 53 had been altered to S which is uncharged and which is present at that position in the unmutated sequence of V_H 3-23 (germline reversion), N which is also uncharged, or K which is positively charged. We also made a variant of the germline V_H3-23 sequence in which S53 was mutagenized to R**.** The Quikchange site-directed mutagenesis kit (Stratagene,UK) was used to create all mutations. The primers used are shown in Table 1.

Transient expression of whole IgG molecules

The whole IgG molecules were expressed in COS-7 cells as described previously (30). Equal quantities (10μg) of recombinant heavy chain and recombinant light chain vector were transfected into 10^7 COS-7 cells in 700μl phosphate buffered saline (PBS) by electroporation (1.9 kV, 25μF). In each transfection experiment, a negative

control sample was prepared by subjecting an aliquot of cells to the same treatment but in the absence of plasmid DNA. The transfected COS-7 cell supernatants were harvested after 72 hours and were treated with *DNase* I at 37^{o}C for one hour. The *DNase* I was inactivated by addition of EDTA (final concentration of 15mM).

Due to the relatively low concentrations of IgG produced by the transient expression system, the cell supernatants were concentrated prior to the ELISA analysis using Centricon-YM30 centrifugal concentrators (Millipore,UK).

Stable expression of whole IgG molecules

Eight different IgG secreting lines were made. The lines were designated 1) SVBL [expressed the heavy/light combination $B3V_H/B3V_L$]. 2) SVBLX [expressed $B3V_H$ /$B3(R27aS)V_L$], 3) SVB33 [expressed $B3V_H/B33V_L$], 4) SVBU [expressed $B3V_H/BUV_L$], 5) SVBL(R53S) [expressed $B3(R53S)V_H/B3V_L$], 6) SVB33(R53S) [expressed $B3(R53S)V_H/B33V_L$], 7) SVBL(R53K) [expressed $B3(R53K)V_H/B3V_L$], 8) SVBL(R53N) [expressed $B3(R53N)V_H/B3V_L$]. The method used for the production of the stable cell lines is described in (27). A negative control CHO line was prepared by electroporation of the CHO*dhfr*$^{-}$ cells in the absence of plasmid DNA.

Affinity purification of antibody from CHO cells

The cell lines were transferred to Chemicon Europe Ltd Southampton, UK and expanded in larger quantities. Human IgG was purified from the supernatant using a Protein A column and the product was analysed for purity by SDS PAGE and

quantified by spectrophotometry. Purified human IgG was sent back to our unit where the amount of whole IgG antibody was quantified by ELISA.

Detection and quantification of whole IgG molecules by ELISA

Whole IgG molecules were detected and quantified using a direct ELISA, as described in previous papers (20, 25, 30).

Detection of binding to DNA and nucleosomes by ELISA

The dsDNA was prepared and used in an anti-DNA ELISA, as described previously (31). Binding to ssDNA was tested using exactly the same protocol except that the dsDNA was denatured by boiling at 100^{o}C for 30 minutes before being used to coat the plates. Nucleosomes were prepared and used in an anti-nucleosome ELISA as described previously (27).

Detection of binding to alpha-actinin by ELISA

Binding of IgG molecules to alpha-actinin was measured by direct ELISA using the method described previously (32).

Detection of binding to cardiolipin by ELISA

The binding of IgG molecules to CL in the presence of fetal calf serum (FCS), containing β_2GPI, was measured by direct ELISA as described previously (25). In some cases, the CL ELISA was modified by replacing the FCS with human serum to test whether the presence of human β_2GPI was important.

Detection of binding to β_2GPI by ELISA

The binding of IgG molecules to human β_2GPI was measured by direct ELISA as described previously (25). The human β_2GPI ELISA was modified by replacing the human β_2GPI with bovine β_2GPI (SCIPAC, UK) for the purpose of testing species-specificity of the binding of purified IgG antibodies.

Detection of binding to purified recombinant hexahistidine(his_6)-tagged Domain I of β_2GPI by ELISA

Recombinant his_6-tagged Domain I was produced by expression in *Escherichia coli*, as described elsewhere (33). The direct binding ELISA to purified recombinant his_6-tagged DI was performed as described previously (33).

Detection of binding to ovalbumin by ELISA

Monoclonal antibodies were also analysed by ELISA for binding to ovalbumin as previously described (34) using maxisorp (Nunc, UK) microtitre plates.

Results

Transient expression in COS-7 cells shows that R53 is necessary but not sufficient for binding to nucleosomes and dsDNA

The amino acid sequence of B3V_H, B3(R53S)V_H, B3(R53N)V_H, B3(R53K)V_H, the corresponding germline gene, V_H3-23 and its point mutant V_H3-23(S53R) are displayed in Figure 1a. When compared to V3-23, there are 11 replacement mutations in B3V_H, of which 6 are in the CDRs and only one (S53R) creates an extra R. Amino

acid sequences of $B3V_L$, $B3(R27aS)V_L$, $33H11V_L$, $UK4V_L$ and the corresponding germline gene, 2a2 are shown in Figure 1b.

Each of these four different light chains - $B3V_L$, $B3(R27aS)V_L$, $33H11V_L$ and $UK4V_L$ - was expressed transiently in COS-7 cells with each of the six different heavy chains - $B3V_H$, $B3(R53S)V_H$, $B3(R53N)V_H$, $B3(R53K)V_H$, V_H3-23, V_H3-23(S53R). Three expression experiments were carried out for each of these 24 combinations. In every expression experiment, a negative control sample supernatant, from COS-7 cells electroporated without plasmid DNA, contained no detectable IgG.

Binding of each of the heavy/light chain combinations to nucleosomes is shown in Figure 2a and to dsDNA in Figure 2b. In each case, similar results were seen in each of the expression experiments and the figures show results of a single representative experiment. Only three of the 24 combinations bound these antigens. These were $B3V_H/B3V_L$, $B3V_H/33H11V_L$ and $B3V_H/B3(R27aS)V_L$. The strongest binding to both dsDNA and nucleosomes was seen with the combinations $B3V_H/B3V_L$ and $B3V_H/33H11V_L$. Both of these heavy/light chain combinations bound to both these antigens at very low IgG concentrations. The results of the dsDNA ELISA were consistent with those obtained in previous studies of these combinations in COS-7 cells (20, 25, 31). The three mutagenised versions of $B3V_H$ in which R53 had been changed to S, N or K did not bind either dsDNA or nucleosomes in combination with $B3V_L$, $33H11V_L$ or $B3(R27aS)V_L$ at any concentration tested. Thus the presence of R53 in $B3V_H$ was essential for binding to these antigens.

The germline V_H3-23 (with D6-25 and J_H5) heavy chain did not bind either dsDNA or nucleosomes in combination with any of the four light chains tested and this finding did not change when S at position 53 of the germline sequence was changed to R (V_H3-23(S53R)). Thus introducing the S53R mutation alone into this germline gene is not sufficient to engender binding to these antigens and the other somatic mutations in $B3V_H$ or sequence features in $B3V_H$ CDR3 must also play a role.

We went on to test the combinations of V_H3-23 and V_H3-23(S53R) with all four light chains in assays of binding to CL, alpha-actinin, β_2GPI and Domain I. There was no binding to any of these antigens (data not shown).

Stable expression of whole IgG molecules in CHO cells

Four stably transfected lines - SVBL (secreting the combination $B3V_H/B3V_L$), SVB33 (secreting $B3V_H/B33V_L$), SVBLX (secreting $B3V_H/B3(R27aS)V_L$) and SVBU (secreting $B3V_H/BUV_L$) had previously been produced and submitted to methotrexate amplification (27). We produced four more lines SVBL(R53S), SVBL(R53N), SVBL(R53K) and SVB33(R53S) in this study. These lines secrete $B3(R53S)V_H/B3V_L$, $B3(R53N)V_H/B3V_L$, $B3(R53K)V_H/B3V_L$ and $B3(R53S)V_H/B33V_L$ respectively. The last combination was included because previous experiments had shown that the chimeric light chain $B33V_L$ confers stronger anti-dsDNA and anti-nucleosome activity than the wild type light chain $B3V_L$, and we wanted to see if that effect would overcome the negative effect of the R53S change in the heavy chain. Yields of whole IgG were different for each cell line. The maximum yield of IgG produced by these stable cell lines was in the range of 130ng/10^6 cells/day to 6700ng/10^6 cells per day. Thus the maximum concentration of purified

antibody available for testing was up to 10μg/ml for some antibodies but 1μg/ml for others. As expected, the control CHO*dhfr*$^-$ cell line, transfected with empty expression vector (i.e. contains no heavy or light chain variable region cDNA) produced no detectable IgG.

Testing affinity-purified antibodies confirms that the R53S mutation abolishes binding to nucleosomes and shows that none of the antibodies binds ssDNA or dsDNA

Figure 3 shows binding of the eight affinity-purified antibodies to nucleosomes. The combinations $B3V_H/B3V_L$, $B3V_H/B33V_L$, and $B3V_H/B3(R27aS)V_L$) bind nucleosomes. Strength of binding to nucleosomes was similar for these three combinations. There was a tendency to increased strength of binding in the order $B3V_H/B3(R27aS)V_L < B3V_H/B3V_L < B3V_H/B33V_L$. The other five combinations do not bind nucleosomes. Thus mutation of R53 to either S, N or K abolishes binding of B3 to nucleosomes even when the antibodies are purified and tested at high concentrations. Similar patterns of binding were obtained with each antibody from repeated experiments hence representative results from a single experiment are shown in Figure 3a. We also tested the antibodies for binding to ssDNA and dsDNA. There was no binding to ssDNA or dsDNA for any of the eight purified antibodies despite good binding of the positive control in these ELISAs. The lack of binding to dsDNA, even for heavy/light chain combinations that bound well to nucleosomes, is consistent with our previous finding that purified recombinant $B3V_H/B3V_L$ does not bind dsDNA, but that binding can be re-constituted by the addition of supernatant from COS-7 cells (27).

The R53S mutation, but not R53N or R53K, enhances binding to alpha-actinin

Figure 3b shows binding of the eight affinity-purified antibodies to alpha-actinin. In complete contrast to the results of the anti-nucleosome ELISA, the presence of the R53S mutation enhances binding to alpha-actinin. The wild-type combination $B3V_H/B3V_L$ did not bind alpha-actinin in this experiment. However, $B3(R53S)V_H/B3V_L$ did bind alpha-actinin. Both $B3V_H/B33V_L$ and $B3(R53S)V_H/B33V_L$ bind alpha-actinin but the latter binds more strongly, again showing that the R53S mutation enhances binding to this antigen. The B33 light chain appears to confer stronger binding to alpha-actinin than $B3V_L$, just as was previously seen for binding to dsDNA and nucleosomes. None of the other combinations binds alpha-actinin. In particular, the other mutations at position R53 (R53N and R53K) did not enhance binding.

The R53S mutation enhances binding to whole β_2GPI and Domain I of β_2GPI

Figure 3c shows binding of the eight affinity-purified antibodies to β_2GPI. Binding was only seen at concentrations 10 times higher than those that gave binding of the wild-type $B3V_H/B3V_L$ to nucleosomes. As with alpha-actinin, however, the introduction of the R53S mutation enhances binding to β_2GPI. $B3(R53S)V_H/B3V_L$ binds β_2GPI whereas $B3V_H/B3V_L$ does not. Similarly, $B3(R53S)V_H/B33V_L$ binds β_2GPI better than $B3V_H/B33V_L$.

Interestingly, on this occasion the R27aS mutation in $B3V_L$ also enhances binding because $B3V_H/B3(R27aS)V_L$ binds β_2GPI whereas $B3V_H/B3V_L$ does not. The loss of this light chain R has not previously been found to enhance binding to any antigen (20, 31).

$B3V_H/BUV_L$ does not bind β_2GPI even at an IgG concentration of 10μg/ml but it is difficult to reach any firm conclusions about the other combinations because we could not test them at concentrations above 1μg/ml (at which only $B3(R53S)V_H/B33V_L$ showed binding). In particular, it is impossible to tell from Figure 3c whether mutation of R53 to N or K would have the same effect as R53S. If we consider Figure 3d, however, it becomes clear that the mutation R53S in $B3V_H$ has an effect on binding to Domain I of β_2GPI which is not shared by R53N or R53K. This figure shows binding of all eight affinity-purified antibodies to Domain I over the same range of concentrations up to a peak of 1μg/ml. Only $B3(R53S)V_H/B3V_L$ shows any significant binding to Domain I at these concentrations. Here $B3(R53S)V_H/B33V_L$ binds much less strongly than $B3(R53S)V_H/B3V_L$ showing that the B33 light chain does not enhance binding to Domain I, contrary to its effects on binding to nucleosomes and alpha-actinin.

Binding of $B3(R53S)V_H/B3V_L$ to β_2GPI is species-specific.

Figure 3e shows binding of the eight affinity-purified antibodies to CL in the presence of FCS, containing bovine β_2GPI. With the exceptions of combinations $B3V_H/B3V_L$ and $B3(R53S)V_H/B33V_L$, binding is either absent or weak and only present above 1μg/ml. Surprisingly, the combination $B3(R53S)V_H/B3V_L$ did not bind CL despite binding well to β_2GPI and Domain I at the same concentrations of IgG. In many cases human anti-β_2GPI antibodies bind CL, using β_2GPI as a co-factor. One possible explanation was that the strong binding of $B3(R53S)V_H/B3V_L$ to human β_2GPI and Domain I is species-specific. This hypothesis would predict that this combination

would bind CL in the presence of human but not bovine β_2GPI. Therefore, we replaced the FCS in the cardiolipin ELISA with human serum to test this hypothesis. Figure 3f shows the binding of the purified antibody B3(R53S)V_H/B3V_L to CL in the presence of human serum in comparison to a human monoclonal anti-PL antibody run as a positive control. Binding to CL is seen. To confirm the species-specificity still further, B3(R53S)V_H/B3V_L was tested for binding to pure bovine β_2GPI in a direct ELISA and did not bind (data not shown).

Binding to ovalbumin is enhanced by the mutation of R53 to N or K

In a recent paper (34) we used similar techniques to study the importance of R residues in V_HCDR3 of the human monoclonal IgG anti-PL antibody IS4. We showed that R to S changes at a number of positions in this CDR reduced binding to PL antigens but increased binding to ovalbumin. Figure 3g shows that the mutations R53N and R53K enhance binding of B3V_H/B3V_L to ovalbumin whereas these mutations had not enhanced binding to any other antigen. R53S increased binding to ovalbumin slightly in combination with B33V_L but not with B3V_L.

Reproducibility of results

Figures 2 and 3a to f all show representative ELISA curves. To confirm reproducibility of the findings we tested each antibody against each antigen in triplicate at a fixed IgG concentration of 1μg/ml. These results confirmed the different patterns of binding seen against different antigens in figures 2 and 3 (data not shown).

Discussion

Our results show that a single change from R to S in V_HCDR2 causes a dramatic shift in the binding properties of the human monoclonal IgG antibody B3. R at position 53 is critical for binding to dsDNA and nucleosomes. Changing this R to S, in the absence of any other change in sequence in either the heavy or light chain, abolishes binding to those two antigens but creates binding to alpha-actinin, whole β_2GPI and Domain I of whole β_2GPI. Other groups have previously shown simultaneous decreases in binding of human anti-dsDNA antbodies to dsDNA and nucleosomes (19) or to dsDNA and PL (24) after loss of particular R residues by mutagenesis. No group, however, has shown dramatic shifts of binding in opposite directions to different clinically relevant antigens caused by a single R to S mutation.

We chose to study binding to nucleosomes and alpha-actinin because of the evidence that antibodies that react with these antigens are important in pathogenesis of lupus nephritis. Two groups have shown that binding to alpha-actinin is an important factor determining the pathogenicity of anti-dsDNA antibodies in mouse models (9, 10, 35). These results were supported in a clinical study (32). No group has previously published an analysis of effects of somatic mutations in human anti-dsDNA antibodies on ability to bind alpha-actinin. Sequence analysis of pathogenic murine anti-alpha-actinin antibodies did not suggest that any particular sequence features favour binding to alpha-actinin (9).

Anti-nucleosome antibodies are found in patients with SLE (36) and develop before anti-dsDNA antibodies in murine models of lupus (37). Some antibodies known to be pathogenic in mice were initially thought to bind naked dsDNA but have

subsequently been shown to bind nucleosomes or chromatin instead (11, 27, 38). Anti-nucleosome antibodies are thought to cause glomerulonephritis by formation of nucleosome/anti-nucleosome complexes, which interact with heparan sulphate in the glomerular basement membrane (11, 12). Wellmann et al (19) recently showed that two somatic mutations in the light chain and one in the heavy chain of the human monoclonal anti-dsDNA antibody 33.C9 were essential for binding to either dsDNA or nucleosomes.

Cocca et al (24) showed that reversion of an R at position 53 in the heavy chain of the murine anti-dsDNA antibody 3H9 to serine reduces binding to DNA, PL, β_2GPI and apoptotic cells. They suggested that some autoantibodies are produced in response to autoantigens derived from apoptotic cell debris and that some somatic mutations could therefore enhance binding to several such antigens. Accumulation of further mutations might then be expected to increase the affinity of the antibody for one autoantigen while reducing its affinity for others. Our experiments showed that $B3V_H/B3V_L$ binds nucleosomes at much lower antibody concentrations than the concentrations of $B3(R53S)V_H/B3V_L$ required to bind alpha-actinin and β_2GPI. The computer model of the B3/dsDNA complex showing an interaction between dsDNA and R53 suggests a mechanism by which R53 could be important in affinity maturation of this antinucleosome antibody (26). An alternative hypothesis is that the R53 residue does not contact dsDNA directly, but stabilizes a protein structure that supports the antibody combining site. Since the germline sequence of V_H3-23 did not confer intrinsic binding to any of the antigens tested, our results could suggest that a precursor of B3 accumulated some somatic mutations to attain relatively broad but low affinity antigen-reactivity including alpha-actinin and β_2GPI, but that the

introduction of the heavy chain S53R somatic mutation created an antibody with specificity and high affinity for nucleosomes. It should be noted however, that unmutated V_H3-23 in combination with other D and J sequences than the one we tested is known to encode a number of polyreactive antibodies (reviewed in 14 and 24).

Several authors have noted a preponderance of R, N and K residues at the antigen-binding sites of human and murine anti-dsDNA antibodies (13, 14, 16). Using a database of 1875 antibody sequences Collis et al (39) showed that both R and N, but not K, are significantly over-represented in the combining sites of antibodies that bind nucleotide antigens, in comparison to antibodies binding other types of antigen. K, like R, is positively charged and could potentially interact with negatively charged antigens. R, N and K can all form hydrogen bonds with dsDNA. One might therefore hypothesise that the R53N and R53K mutations would have less adverse effect on binding to dsDNA and nucleosomes than R53S but we did not find this to be the case. It seems likely that a property of R apart from charge or hydrogen bond formation is crucial to its interaction with dsDNA in B3. Perhaps the length and shape of the arginine side chain are important. In contrast R53S, R53N and R53K had very different effects on interaction with alpha-actinin and Domain I of whole β_2GPI. Only the germline reversion R53S enhanced binding to those antigens. Heavy/light combinations containing R53N and R53K did not bind any antigen except ovalbumin. In our previous paper (34) we found that alteration of arginines in IS4V_H CDR3 to serines led to an increase in binding to ovalbumin and hypothesized that this might be due to a reversal of serine to arginine somatic mutations that took place during affinity maturation. However, we could not be sure of this since the germline

sequence corresponding to IS4V_H CDR3 is unknown. In the current paper, however, the germline reversion R53S did not increase binding to ovalbumin even though it increased binding to other antigens. We conclude that the identity of the residue at position 53 is very important in determining antigen-specificity of the antibodies that we tested, but the effects of particular changes in this residue are difficult to predict.

The production of an antibody, B3(R53S)V_H/B3V_L which was found to bind human but not bovine β_2GPI was unexpected and has not previously been described. Zhu et al (40) reported two human IgG monoclonal antibodies IS1 and IS2 that bound bovine but not human β_2GPI. Sequence analysis later showed that these antibodies were derived from the same B cell clone (41). There are 11 amino acid differences between the sequence of Domain I of human and bovine β_2GPI, therefore it is likely one of these differences plays a role in the specificity of B3(R53S)V_H/B3V_L for human β_2GPI. Our group has expressed two variant forms of Domain I of human β_2GPI that specifically target these residues of interest, therefore it will be possible in future experiments to determine the binding affinity of B3(R53S)V_H/B3V_L to these variant forms.

Acknowledgements

The authors would like to thank Mrs Alison Levy and Drs C.A. Kettleborough & T. Jones (AERES Biomedical, London, U.K.) for their kind gifts of the modified Chinese Hamster Ovary cells (CHO*dhfr*$^-$) and the expression vectors. We thank Professor Jo Berden and Dr Johan van der Vlag (Nijmegen, Netherlands) for their help in setting up the anti-nucleosome assay. We are grateful to Dr Hedda Wardemann and Professor Michel Nussenzweig (The Rockefeller University, New

York) for sending us the V_H3-23 construct. We would also like to thank the Rosetrees Trust and the Arthritis Research Campaign for funding this work. The research was funded by arc grants 17045, 16555 and G0585.

References

1. Isenberg DA, Ravirajan CT, Rahman A, Kalsi J. The role of antibodies to DNA in systemic lupus erythematosus--a review and introduction to an international workshop on DNA antibodies held in London, May 1996. Lupus 1997;6(3):290-304.
2. Okamura M, Kanayama Y, Amastu K, Negoro N, Kohda S, Takeda T, et al. Significance of enzyme linked immunosorbent assay (ELISA) for antibodies to double stranded and single stranded DNA in patients with lupus nephritis: correlation with severity of renal histology. Ann Rheum Dis 1993;52(1):14-20.
3. Koffler D, Schur PH, Kunkel HG. Immunological studies concerning the nephritis of systemic lupus erythematosus. J Exp Med 1967;126(4):607-24.
4. Winfield JB, Faiferman I, Koffler D. Avidity of anti-DNA antibodies in serum and IgG glomerular eluates from patients with systemic lupus erythematosus. Association of high avidity antinative DNA antibody with glomerulonephritis. J Clin Invest 1977;59(1):90-6.
5. Ehrenstein MR, Katz DR, Griffiths MH, Papadaki L, Winkler TH, Kalden JR, et al. Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice. Kidney Int 1995;48(3):705-11.
6. Madaio MP, Carlson J, Cataldo J, Ucci A, Migliorini P, Pankewycz O. Murine monoclonal anti-DNA antibodies bind directly to glomerular antigens and form immune deposits. J Immunol 1987;138(9):2883-9.
7. Ravirajan CT, Rahman MA, Papadaki L, Griffiths MH, Kalsi J, Martin AC, et al. Genetic, structural and functional properties of an IgG DNA-binding monoclonal antibody from a lupus patient with nephritis. Eur J Immunol 1998;28(1):339-50.
8. Raz E, Brezis M, Rosenmann E, Eilat D. Anti-DNA antibodies bind directly to renal antigens and induce kidney dysfunction in the isolated perfused rat kidney. J Immunol 1989;142(9):3076-82.
9. Mostoslavsky G, Fischel R, Yachimovich N, Yarkoni Y, Rosenmann E, Monestier M, et al. Lupus anti-DNA autoantibodies cross-react with a glomerular structural protein: a case for tissue injury by molecular mimicry. Eur J Immunol 2001;31(4):1221-7.
10. Deocharan B, Qing X, Lichauco J, Putterman C. Alpha-actinin is a cross-reactive renal target for pathogenic anti-DNA antibodies. J Immunol 2002;168(6):3072-8.
11. Kramers C, Hylkema MN, van Bruggen MC, van de Lagemaat R, Dijkman HB, Assmann KJ, et al. Anti-nucleosome antibodies complexed to nucleosomal antigens show anti-DNA reactivity and bind to rat glomerular basement membrane in vivo. J Clin Invest 1994;94(2):568-77.

12. van Bruggen MC, Walgreen B, Rijke TP, Tamboer W, Kramers K, Smeenk RJ, et al. Antigen specificity of anti-nuclear antibodies complexed to nucleosomes determines glomerular basement membrane binding in vivo. Eur J Immunol 1997;27(6):1564-9.
13. Radic MZ, Weigert M. Genetic and structural evidence for antigen selection of anti-DNA antibodies. Annu Rev Immunol 1994;12:487-520.
14. 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(12):807-23.
15. Katz JB, Limpanasithikul W, Diamond B. Mutational analysis of an autoantibody: differential binding and pathogenicity. J Exp Med 1994;180(3):925-32.
16. Winkler TH, Fehr H, Kalden JR. Analysis of immunoglobulin variable region genes from human IgG anti-DNA hybridomas. Eur J Immunol 1992;22(7):1719-28.
17. Radic MZ, Mackle J, Erikson J, Mol C, Anderson WF, Weigert M. Residues that mediate DNA binding of autoimmune antibodies. J Immunol 1993;150(11):4966-77.
18. 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(7):2015-26.
19. Wellmann U, Letz M, Herrmann M, Angermuller S, Kalden JR, Winkler TH. The evolution of human anti-double-stranded DNA autoantibodies. Proc Natl Acad Sci U S A 2005;102(26):9258-63.
20. Rahman A, Haley J, Radway-Bright E, Nagl S, Low DG, Latchman DS, et al. The importance of somatic mutations in the V(lambda) gene 2a2 in human monoclonal anti-DNA antibodies. J Mol Biol 2001;307(1):149-60.
21. Giles I, Lambrianides N, Latchman D, Chen P, Chukwuocha R, Isenberg D, et al. The critical role of arginine residues in the binding of human monoclonal antibodies to cardiolipin. Arthritis Res Ther 2005;7(1):R47-56.
22. Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Rheum 1998;41(7):1241-50.
23. Giles IP, Isenberg DA, Latchman DS, Rahman A. How do antiphospholipid antibodies bind beta2-glycoprotein I? Arthritis Rheum 2003;48(8):2111-21.
24. Cocca BA, Seal SN, D'Agnillo P, Mueller YM, Katsikis PD, Rauch J, et al. Structural basis for autoantibody recognition of phosphatidylserine-beta 2 glycoprotein I and apoptotic cells. Proc Natl Acad Sci U S A 2001;98(24):13826-31.
25. Giles IP, Haley J, Nagl S, Latchman DS, Chen PP, Chukwuocha RU, et al. Relative importance of different human aPL derived heavy and light chains in the binding of aPL to cardiolipin. Mol Immunol 2003;40(1):49-60.
26. Kalsi JK, Martin AC, Hirabayashi Y, Ehrenstein M, Longhurst CM, Ravirajan C, et al. Functional and modelling studies of the binding of human monoclonal anti-DNA antibodies to DNA. Mol Immunol 1996;33(4-5):471-83.
27. Mason LJ, Lambrianides A, Haley JD, Manson JJ, Latchman DS, Isenberg DA, et al. Stable expression of a recombinant human antinucleosome antibody to investigate relationships between antibody sequence, binding properties, and pathogenicity. Arthritis Res Ther 2005;7(5):R971-83.

28. 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(4):1787-97.
29. Menon S, Rahman MA, Ravirajan CT, Kandiah D, Longhurst CM, McNally T, et al. The production, binding characteristics and sequence analysis of four human IgG monoclonal antiphospholipid antibodies. J Autoimmun 1997;10(1):43-57.
30. Rahman MA, 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(6):661-9.
31. Haley J, Mason LJ, Nagl S, Giles I, Latchman DS, Isenberg DA, et al. Somatic mutations to arginine residues affect the binding of human monoclonal antibodies to DNA, histones, SmD and Ro antigen. Mol Immunol 2004;40(11):745-58.
32. Mason LJ, Ravirajan CT, Rahman A, Putterman C, Isenberg DA. Is alpha-actinin a target for pathogenic anti-DNA antibodies in lupus nephritis? Arthritis Rheum 2004;50(3):866-70.
33. Ioannou Y, Giles I, Lambrianides A, Richardson C, Pearl LH, Latchman DS, et al. A novel expression system of domain I of human beta2 glycoprotein I in Escherichia coli. BMC Biotechnol 2006;6:8.
34. Giles I, Lambrianides N, Pattni N, Faulkes D, Latchman D, Chen P, et al. Arginine residues are important in determining the binding of human monoclonal antiphospholipid antibodies to clinically relevant antigens. J Immunol 2006;177(3):1729-36.
35. Zhao Z, Weinstein E, Tuzova M, Davidson A, Mundel P, Marambio P, et al. Cross-reactivity of human lupus anti-DNA antibodies with alpha-actinin and nephritogenic potential. Arthritis Rheum 2005;52(2):522-30.
36. Bruns A, Blass S, Hausdorf G, Burmester GR, Hiepe F. Nucleosomes are major T and B cell autoantigens in systemic lupus erythematosus. Arthritis Rheum 2000;43(10):2307-15.
37. Amoura Z, Piette JC, Bach JF, Koutouzov S. The key role of nucleosomes in lupus. Arthritis Rheum 1999;42(5):833-43.
38. Guth AM, Zhang X, Smith D, Detanico T, Wysocki LJ. Chromatin specificity of anti-double-stranded DNA antibodies and a role for Arg residues in the third complementarity-determining region of the heavy chain. J Immunol 2003;171(11):6260-6.
39. Collis AV, Brouwer AP, Martin AC. Analysis of the antigen combining site: correlations between length and sequence composition of the hypervariable loops and the nature of the antigen. J Mol Biol 2003;325(2):337-54.
40. Zhu M, Olee T, Le DT, Roubey RA, Hahn BH, Woods VL, Jr., et al. 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(1):102-9.
41. Chukwuocha RU, Zhu M, Cho CS, Visvanathan S, Hwang KK, Rahman A, et al. Molecular and genetic characterizations of five pathogenic and two non-pathogenic monoclonal antiphospholipid antibodies. Mol Immunol 2002;39(5-6):299-311.

	PRIMER SEQUENCE		
	$B3V_H$ wild-type		GGGTCTCAACTATTAGTGGTCGTGGTAGTAGCACATAC
1.	$B3(R53S)V_H$	Forward	5`GGGTCTCAACTATTAGTGGT**A**GTGGTAGTAGCACATAC 3`
2.		Back	5`GTATGTGCTACTACCAC**T**ACCACTAATAGTTGAGACCC 3`
3.	$B3(R53N)V_H$	Forward	5`GGGTCTCAACTATTAGTGGT**AA**TGGTAGTAGCACATAC 3`
4.		Back	5`GTATGTGCTACTACCA**TT**ACCACTAATAGTTGAGACCC 3`
5.	$B3(R53K)V_H$	Forward	5`GGGTCTCAACTATTAGTGGT**AAG**GGTAGTAGCACATAC 3`
6.		Back	5`GTATGTGCTACTACC**CTT**ACCACTAATAGTTGAGACCC 3`
	V_H3-23		CTCAGCTATTAGTGGTAGTGGTGGTAGCACATAC
7.	V_H3-23(53)	Forward	5`CTCAGCTATTAGTGGT**C**GTGGTGGTAGCACATAC 3`
8.		Back	5`GTATGTGCTACCACCAC**G**ACCACTAATAGCTGAG 3`

Table 1. Sequences of primers used in site directed mutagenesis. Mutagenic primers with sites of point mutation shown in bold. Primers 1+2 were used to mutate the Arg to Ser residue of $B3V_H$ at position 53. Primers 3+4 were used to mutate the Arg to Asn in $B3V_H$ at position 53. Primers 5+6 were used to mutate the Arg to Lys residue of $B3V_H$ at position 53. Primers 7+8 were used to mutate the Ser to Arg residue of V_H3-23 at position 53.

Figure Legends

Figure 1. Sequence alignment of expressed V_H and V_L regions. (a) Sequences of expressed V_H regions compared to V3-23. (b) Sequences of expressed V_L regions compared to 2a2. The amino acids altered by site-directed mutagenesis are shown in bold. The amino acids are numbered according to Kabat (39). A dash indicates homology with the corresponding germline sequence. Abbreviations: FR – Framework region; CDR – Complementarity determining region; B3 [1] – B3(R53S)V_H ; B3 [2] – B3(R53N)V_H; B3 [3] – B3(R53K)V_H; B3 [4] – B3(R27aS)V_L; 3-23 * – V_H3-23(S53R) .

Figure 2. Effect of point mutation in B3VH and VH3-23. The graphs show binding of IgG in COS-7 cell supernatants containing each heavy/light chain combination to (a) nucleosomes and (b) dsDNA. Only 3 combinations bind these antigens and these lines are labelled on the graph. All combinations containing B3(R53S)VH, B3(R53N)VH, B3(R53K)VH, VH3-23, VH3-23(53) as well as combination B3VH/UK4VL do not bind dsDNA or nucleosomes. These are the unlabelled lines on the graph. A fully labelled version of this figure is available on the UCL Eprints website at http://eprints.ucl.ac.uk/. Diluted serum from a patient with SLE was run on every plate as a positive control. The standard deviation of the positive control OD value between plates was <0.025. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained no IgG and no anti-nucleosome or anti-dsDNA activity. This also applies to Figure 3.

Figure 3. Figures 3a to 3e show binding of the eight purified antibodies in direct ELISA assays to 3a) nucleosomes, 3b) alpha-actinin, 3c) β_2GPI, 3d) Domain I of β_2GPI and 3e) cardiolipin in the presence of fetal calf serum. In each case diluted

serum from a patient with SLE or APS was run as a positive control. Figure 3f shows that antibody B3(R53S)V_H/B3V_L binds cardiolipin as well as a human monoclonal antiphospholipid antibody in the presence of human serum. Figure 3g shows binding of the eight purified antibodies (all at 1μg/ml) to OVA.

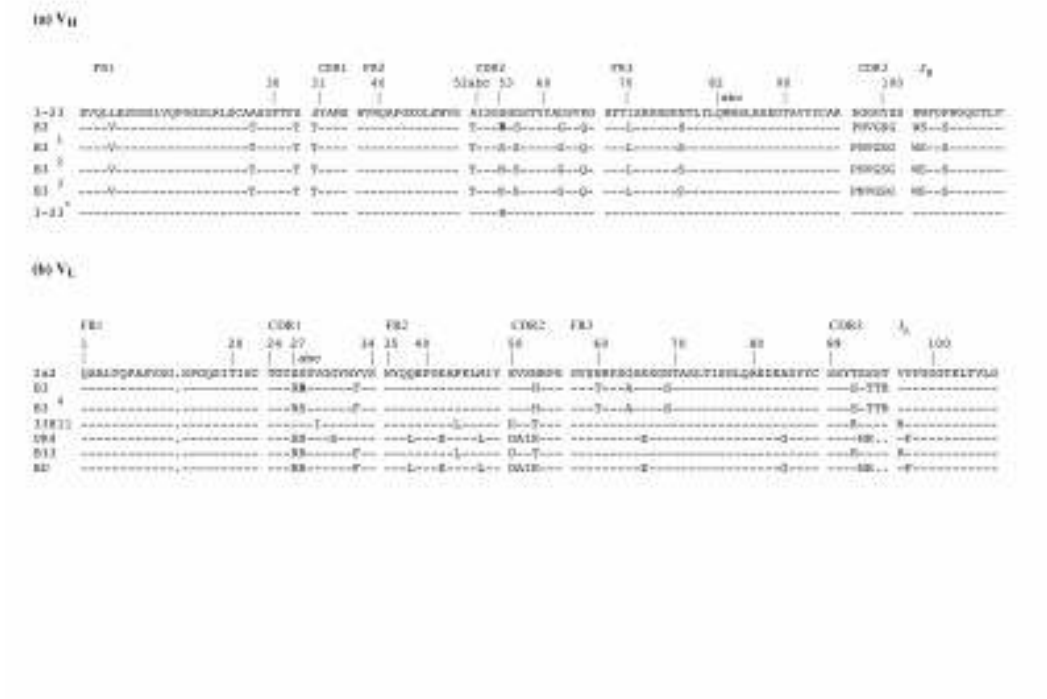
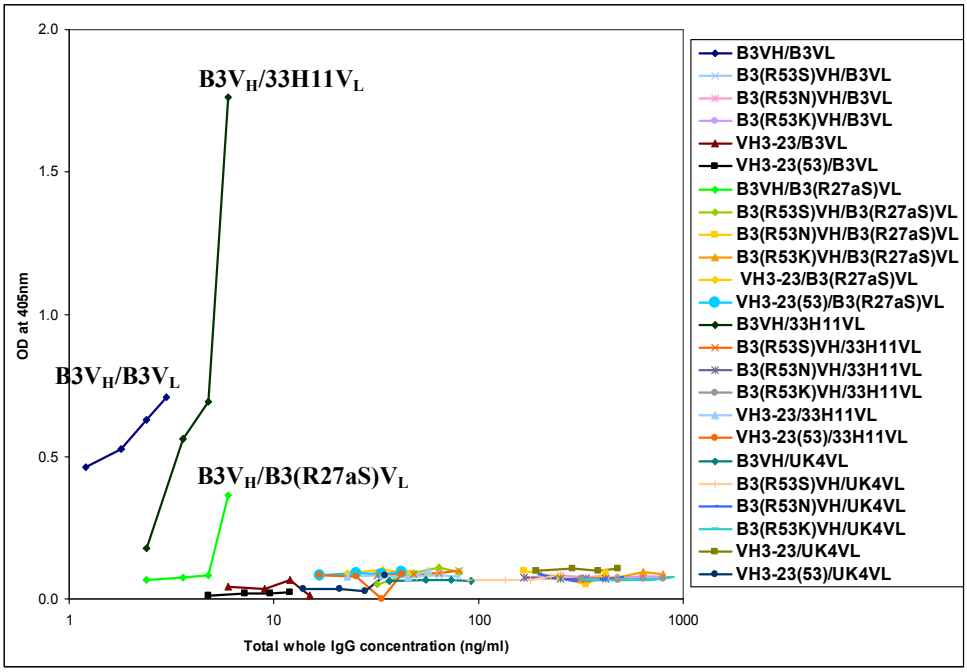
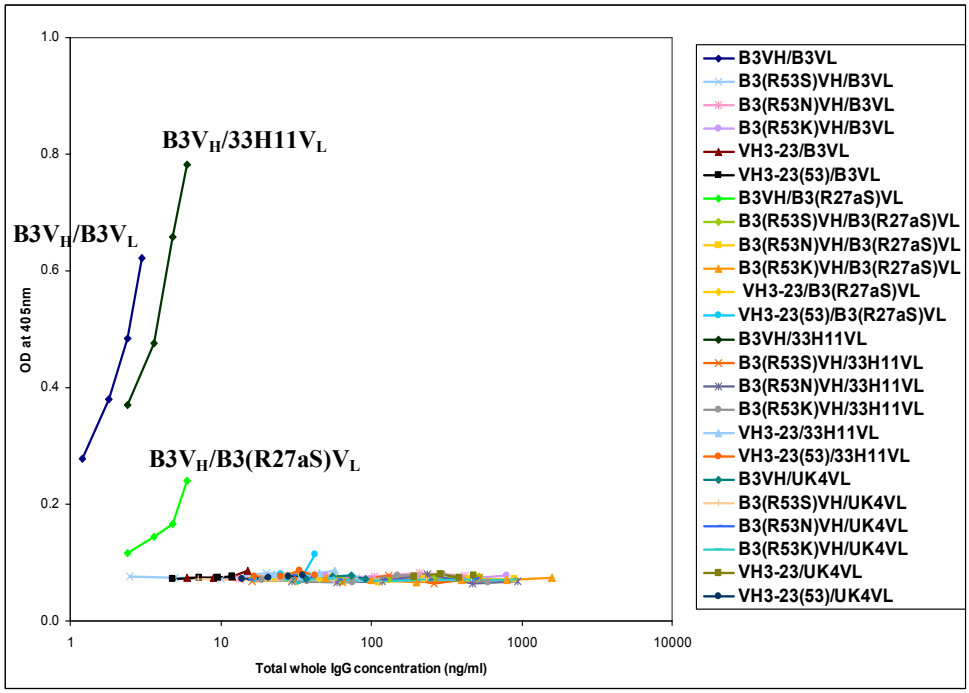


Figure 1.



(a) Nucleosomes



(b) dsDNA

Figure 2.

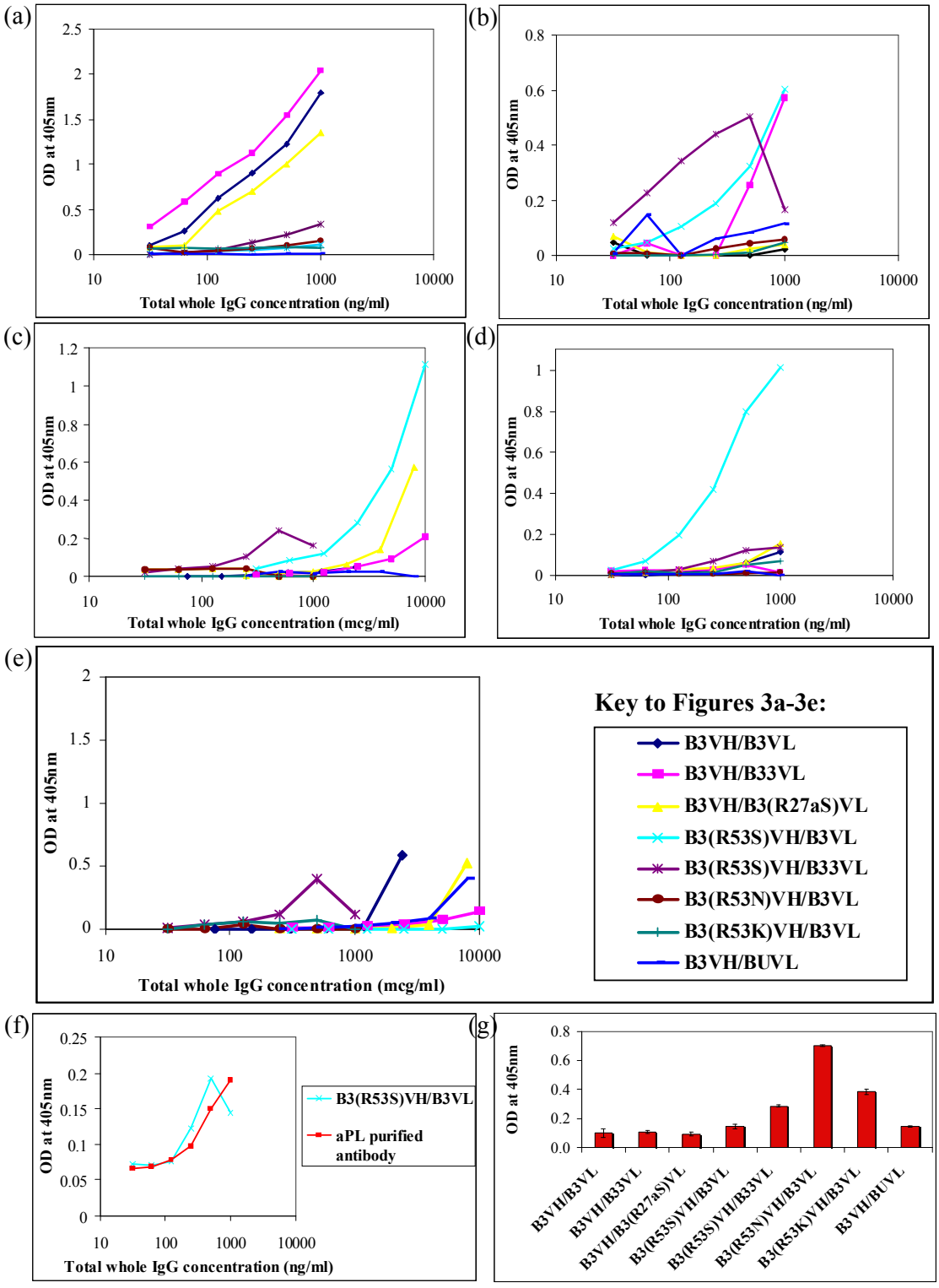

(a)
OD at 405nm
Total whole IgG concentration (ng/ml)
(b)
OD at 405nm
Total whole IgG concentration (ng/ml)
(c)
OD at 405nm
Total whole IgG concentration (mcg/ml)
(d)
OD at 405nm
Total whole IgG concentration (ng/ml)
(e)
OD at 405nm
Total whole IgG concentration (mcg/ml)
Key to Figures 3a-3e:
B3VH/B3VL
B3VH/B33VL
B3VH/B3(R27aS)VL
B3(R53S)VH/B3VL
B3(R53S)VH/B33VL
B3(R53N)VH/B3VL
B3(R53K)VH/B3VL
B3VH/BUVL
(f)
OD at 405nm
Total whole IgG concentration (ng/ml)
B3(R53S)VH/B3VL
aPL purified antibody
(g)
OD at 405nm
B3VH/B3VL
B3VH/B33VL
B3VH/B3(R27aS)VL
B3(R53S)VH/B3VL
B3(R53S)VH/B33VL
B3(R53N)VH/B3VL
B3(R53K)VH/B3VL
B3VH/BUVL

Figure 3.