The intrinsic cysteine and histidine residues of the anti-Salmonella antibody Se155-4: a model for the introduction of new functions into antibody-binding sites

Young, N. Martin; Watson, David C.; Cunningham, Anna M.; Mackenzie, C. Roger

Publisher's version / Version de l'éditeur:
https://doi.org/10.1093/protein/gzu018
Protein Engineering Design and Selection, 27, 10, pp. 383-390, 2014-05-14

The intrinsic cysteine and histidine residues of the anti-Salmonella antibody Se155-4: a model for the introduction of new functions into antibody binding-sites

N. Martin Young[1], David C. Watson, Anna M. Cunningham and C. Roger MacKenzie

National Research Council Canada, 100 Sussex Drive, Ottawa, Ontario, Canada K1A 0R6

[1] To whom correspondence should be addressed: Martin.Young@nrc-cnrc.gc.ca

Abstract

New functions can be incorporated into anti-hapten or anti-protein antibodies by mutating selected residues in the binding-site region either to Cys, to allow alkylation with reagents bearing the desired functional groups, or to His, to create metal-binding sites or to make antigen binding pH-sensitive. However, choosing suitable sites for these mutations has been hampered by the lack of antibodies with these features, to serve as models. Remarkably, the anti-carbohydrate antibody Se155-4, specific for the Salmonella group B lipopolysaccharide, already has a Cys and two pairs of His residues close to the antigen-binding pocket in its structure, and shows pH-dependent antigen binding. We therefore investigated modification of its Cys94L in an scFv version of the antibody with the aims of creating a "reagentless" fluorescent sensor and attaching a metal-binding group that might confer lyase activity. These groups were successfully introduced, as judged by mass spectrometry, and had only slightly reduced antigen binding in ELISA. The fluorescent product was sensitive to addition of antigen in a solution format, unlike a modification of a more distant Cys introduced into the VH CDR4 loop. Two other routes to modulate antigen binding were also explored, metal-binding by the His pair alongside the antigen-binding pocket and insertions into CDR4 to extend the antigen contact area. The His residues adjacent to the antigen-binding pocket bound copper, causing a five-fold decrease in antigen binding. In CDR4 of the VH domain, the preferred insert length was four residues, which gave stable antigen-binding products but did not improve overall antigen affinity.

Keywords: binding site modification / histidine and cysteine residues/ metal affinity/ CDR4 loops

Introduction

Mutating an antibody in its binding-site region to introduce Cys or His residues can enable the direct detection of antigen binding by the attachment of reporter groups, or modulation of binding by adding metal atoms or changing pH. This necessitates selecting positions for mutation that are adjacent to the site, while simultaneously avoiding undesired effects on affinity, either indirectly by causing structural perturbation of the CDRs or directly by altering key antigen-contact residues. The current view of antigen binding is that affinity is conferred by some major contact residues but fine-tuned by other residues introduced by somatic mutation (Persson and Ohlin, 2007; Raghanuthan *et al.*, 2011). This view was originally based on experiments that recreated the putative germ-line versions of antibodies of known crystal structure, directed to either a protein antigen (England *et al.*, 1999) or a hapten conjugate (Wedemeyer *et al.*, 1997; Yang and Schultz, 1999), and characterized the mutations which led to the final antibodies. These studies showed that the central contact residues were usually germ-line in origin, i.e. arose from combinatorial diversity among the V, D and J gene segments, while somatic mutations around the perimeter served to refine the binding site and to increase its affinity for antigen, but rarely produced significant new binding contacts. Subsequent analyses of the PDB database of antibody structures and the germline genes from which they were formed have (Raghunathan *et al.*, 2011), and studies of haptens on different carriers (Persson and Ohlin, 2007) have confirmed this general picture of a core modulated by a perimeter region. Residues in the perimeter should therefore be very suitable candidates for mutation in order to introduce new functionalities, or for the introduction of non-natural amino acids with novel chemical features, as pioneered by Schultz and associates (e.g. Mills *et al.*, 2009).

However, natural models to guide the design of suitable Cys or His mutations into

antibodies are almost non-existent. Antibodies with intrinsic metal-binding properties are rare, though some calcium-requiring antibodies have been reported (Ward and Ingham, 1992; Hopp *et al.*, 1996; Zhou *et al.*, 2005), and free cysteines seldom occur in CDRs. Hence designs for new functionalities that avoid undesired structural perturbations or reductions in affinity have had to be based on general structural data for antibodies. The first examples mutated selected residues to His in order to create metal-binding sites that could coordinate zinc ions, in the manner of enzymes such as carbonic anhydrase (reviewed by Roberts and Getzoff, 1995). These experiments were conducted with anti-hapten antibodies, either specific for fluorescein or for enzyme intermediates (abzymes), whose crystal structures were known. The mutations successfully produced metal-binding sites, without altering hapten binding by the antibodies (Iverson *et al.*, 1990; Roberts *et al.*, 1990). An N-terminal sequence containing histidines has also been used to introduce metal-binding sites adjacent to the antigen binding pocket (Trisler *et al.*, 2007). Recently, screening of antibody or VHH libraries with multiple introduced His residues led to species whose antigen binding could be controlled by metals (Fanning *et al.*, 2011) or by pH (Igawa et al., 2010; Murtaugh *et al.*, 2011). Mutating residues to Cys has allowed subsequent alkylation with reagents incorporating the desired functionality, or using its disulfide form as a target for a nucleophile bearing an affinity label (Miao *et al.*, 2008). A method for identifying suitable sites for mutation to Cys has been reported (Junutula *et al.*, 2008), but modification of a naturally occurring Cys in CDRH3 of a monoclonal antibody reduced its stability and antigen binding (Banks *et al.*, 2008). However, fluorescent labels for reagentless sensors (Renard and Bedouelle, 2004; Renard *et al.*, 2002) and a chelating group for Yt (Corneillie *et al.*, 2004) have been successfully introduced by Cys labelling.

The anti-carbohydrate antibody Se155-4 is remarkable in containing in its native state all of the desirable His and Cys features mentioned above. An IgG1/λ1 mouse hybridoma antibody,

Se155-4 is specific for the Salmonella type B lipopolysaccharide (Bundle *et al.*, 1994), whose O-chain repeating unit is the tetrasaccharide -3Galα1-2-{Abeα1-3}-Manα1-4-Rhaα1-. Its primary binding site for the trisaccharide epitope -Galα1-2-{Abeα1-3}-Manα1-4 is a central pocket formed by the CDRs (Cygler *et al.*, 1991; Cygler *et al.*, 1993; Zdanov *et al.*, 1994). Se155-4 has two pairs of adjacent histidines, one of which is on the perimeter of the antigen-binding pocket (Cygler *et al.*, 1991). Furthermore, it shows pH dependence of antigen binding (Bundle *et al.*, 1994), attributable to a fifth His situated in the antigen binding pocket, and it has a free cysteine in CDR L3 at residue 94L (Bundle *et al.*, 1994). The occurrence of a Cys and two His pairs so close to the pocket (Fig. 1) is remarkable, particularly since four of them have arisen by somatic mutation, i.e. three of the His and the Cys (Bundle *et al.*, 1994), yet they appear to have no significant roles in the overall affinity of the antibody. Protein engineering studies of the Fab and scFv showed it has a remarkable tolerance for mutations close to the pocket, including ones in the CDR H3 and the salt-bridge at the base of its loop structure (Brummell *et al.*, 1993; Deng *et al.*, 1994; 1995).

Se155-4 therefore provides a natural guide for the introduction of new functions into antibody binding sites. We report experiments to characterize the His pairs and to modify the free Cys residue in Se155-4. The external loops of the two V-domains were also investigated as sites for modification. These β-turns, sometimes termed CDR4, are on the periphery of the binding-site region alongside the CDRs, and are comprised of residues within the framework 3 segment, around residue 74 in the Kabat nomenclature. In Se155-4, extrapolation of the bound heptasaccharide (Fig. 1) suggested these loops would come close to a bound polysaccharide antigen. We explored extending this loop in the VH domain and introducing a Cys into it.

Materials and methods

DNA procedures

All DNA manipulations were carried out using standard methods. Helper phage M13K07 was purchased from Life Technologies, Inc. Restriction enzymes and modifying enzymes were purchased from New England Biolabs and from Life Technologies, Inc. Randomized oligonucleotides were prepared using an Applied Biosystems Inc DNA synthesizer model 394.

Antibody fragments and ELISA

Previously described procedures were used to produce native Fab from Se155-4 IgG (Bundle *et al.*, 1994), recombinant Fab (Brummell *et al.,* 1994) and scFv (Deng *et al.,* 1994), and to analyse binding to LPS antigen by ELISA (Deng *et al.,* 1994).

Cloning, expression and purification of scFv

A version of the scFv with a C-terminal 6xHis tag was obtained from the previous 3B1 construct (Deng *et al.,* 1994) by PCR amplification with Phusion DNA polymerase (New England Biolabs Inc.). The primers used incorporated either *EcoR*I or *Bgl*II cloning sites and a 6xHis tag in the 3P PCR primer. The primer pair used was 5'-GGGAAACCCGAATTCATGAAAAAAACCGCTATCGCGATCGCAGTTGCACTG-3' (forward primer) and 5'-GTCAGTCAGTCAAGATCTTTATTAGTGATGGTGATGGTGATGGGAGGACACGGTCAGGCTC GCGCCTTGGCC-3' (reverse primer). The PCR product was gel purified and cloning sites generated by double digestion with *EcoR*I and *Bgl*II restriction enzymes (New England Biolabs Inc.). The gene was cloned into the phagemid pSK4 and the construct maintained in *E.coli* TG1. Cells from positive clones, as judged by sequence analysis, were grown in minimal media, induced, subjected to periplasmic extraction and the scFv was purified by Ni^{2+} immobilized metal

ion affinity chromatography.

Site-directed mutagenesis of scFv

To introduce Cys residues, the 3B1-His_6 scFv was mutagenized using a two-stage mutagenesis protocol. 5' and 3' PCR primers both containing the desired mutation were used in two separate PCR reactions to generate two overlapping gene fragments. Both PCR products were then used as template with the primer pair described above in a third PCR reaction to generate full length mutagenized scFv. Full length PCR products were subsequently cloned, verified by sequence analysis, expressed and purified as described above. Three single mutations to Cys at Asp71L, Ser94L, and Thr74H were obtained in this way, and expressed and purified as above.

Labelling of cysteine residues

The reagents IANDB ester (*N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole; Life Technologies/ Molecular Probes) and FeBABE (iron (*S*)-1-(*p*-bromoacetamido-benzyl)ethylenediaminetetraacetate; Thermo-Fisher Scientific/ Pierce Biotechnology) were used to modify the various cysteines introduced into the scFv, according to the manufacturers' suggested protocols. As a control, a third sample was labelled with a 10% excess of iodoacetamide in Tris-HCl buffer, 0.1 M pH 8.0, at room temperature for 1h. The incorporation of the derivatives was checked by electrospray ionization mass spectrometry.

The IANDB derivative was assayed with Salmonella LPS using black EIA plates previously blocked with 300µl of 1% BSA per well for I hr. The final volume in each well was 200 µl of PBS containing 15µg of labelled scFv and 0-5µg of LPS. After 30 min at room temperature, the wells' fluorescence was determined with a Spectromax M2 plate reader (Molecular Devices LLC) with excitation at 472nm and emission at 536nm.

The FeBABE derivative was tested for its ability to cleave the Salmonella antigen by

activating it with ascorbate and hydrogen peroxide and incubating it with antigen. A non-cross reactive LPS from *Actinobacillus pleuropneumoniae* served as a control for non-specific cleavage. Deoxycholate-PAGE with silver staining was used to analyse the reaction products (Tsai and Frasch, 1982).

Fluorescence quenching by copper

The instrument used was an SLM 8000C spectrofluorometer, with 4 nm excitation and emission band pass. For the titrations, solutions of the Se155-4 Fab, scFv or mutants in 0.05 M ammonium acetate pH 6.5, 0.15 M NaCl, with A_{280nm} values of approximately 0.1 were titrated at 20° with solutions of copper sulphate, with continuous stirring. The excitation wavelength was 295nm and fluorescence emission was recorded at 340nm.

CDR4 insertions

These insertions were made in the synthetic scFv gene construct 3B1, which was designed with unique restriction sites distributed throughout its sequence (Anand *et al.,* 1991). The nucleotide segments carrying the CDR4L or CDR4H regions were excised with the appropriate restriction enzymes and replaced with oligonucleotide duplexes containing the inserts YPY, WRY, SYPYD or SWRYS as previously described (Brummell et al, 1993; Deng et al., 1994). The scFvs were expressed and purified by affinity chromatography on the Salmonella antigen column (Bundle *et al.,* 1994).

Three randomized libraries of inserts into CDR4H were made in a similar way. The phagemid expressing Se155-4 single mutant 3B1-geneIII (249-406) fusion (Deng *et al.,* 1994) was digested with the restriction enzymes *Spe*I and *Mlu*I to remove the fragment from position 74 to 97 in FRH3. Synthetic oligonucleotide duplexes containing randomized $(NNK)_4$, $(NNK)_5$, $(NNK)_6$ and $(NNK)_7$ at the desired insertion point were ligated in separate reaction mixtures in a final

volume of 200µl using approximately 1µg of vector and 300ng of insert. After cleaning the ligation reactions using a Qiagen PCR Kit and eluting in a final volume of 50µl, a 3-5µl aliquot was used to test the size of the library by tranforming into electrocompetent *E.coli* TG1 and plating onto LB-carbenicillin. The remainder of the ligation mixture was stored until the library size was determined, which should ideally be $1x10^6$. Following this determination, the remaining ligation reaction was transformed, using no more than 5µl/transformation, plated out on large 2YT-carbenicillin plates (1ml/plate) and incubated overnight at 37°C. The next day the transformants were scraped off the plates, using either 2YT-ampicillin or LB-ampicillin, in as small a volume as possible and stored at -80°C in 15% glycerol.

Once the libraries from each construct were obtained and their sizes determined, phage was prepared following the protocol outlined in Harrison *et al.* (1996) and their titers determined. Each library was panned separately against serotype-B lipopolysaccharide, which was coated onto a microtiter plate at a concentration of 10µl/ml. Microtiter plates were washed with phosphate-buffered saline to remove non-binders and the binders were eluted in 200µl of 0.2 M glycine-HCl, pH 2.2 + 1mg/ml BSA and neutralized with 30µl of 2 M Tris.HCl, pH 9.5. This eluate was then used for the next round of panning by amplification in *E.coli* TG1.

Screening of clones

After three rounds of panning, 60 clones were randomly selected from each library, inoculated into microtiter plates containing 150µl 2YT-ampicillin-2% glucose and incubated at 37°C with gentle shaking until they reached an A_{600nm} of 0.2-04. Each well was then infected with 25µl of helper phage containing 10^9 pfu and incubated at 37°C for 30 min without shaking. The microtiter plate was then centrifuged, the supernatant was removed, and the pellet was re-suspended in 150µl of 2YT-ampicillin-kanamycin and allowed to grow at 30°C overnight. In the meantime a flat-bottomed microtiter plate was coated with 10µg/ml of LPS diluted in sodium carbonate buffer, pH 9.7. Phage

ELISA assays were then carried out as previously described (Deng *et al.,* 1994). Clones showing the highest activity were selected for sequencing.

Expression and isolation of soluble mutant scFvs

For expression purposes, the amber codon TAG was mutated to CAG (Gln) in the mutants selected for further studies. Specifically designed primers and the universal forward primer were used to obtain PCR products for each mutant. The resulting PCR products were digested with *SpeI* and *HindIII* and ligated into the expression vector which had also been digested with the same restriction enzymes. The construct was transformed into electrocompetent *E.coli* TG1 and plated onto LB-carbenicillin plates. Colony PCRs were performed to screen transformants for sequencing purposes. The mutants of interest were then grown up as previously described (Deng *et al.,* 1994). Periplasmic extracts of the cells were checked for expression using Western blots and the scFvs were purified by affinity chromatography on the Salmonella antigen column (Bundle *et al.,* 1994). The eluate fractions were examined for monomer and dimer content by size-exclusion chromatography, using a Superdex™ 75 column. The monomer and dimer fractions were collected for SPR analysis.

Histidine mutants

Changes of His residues to Gln were carried out in a similar manner to the construction of the CDR4 inserts, i.e. by cleavage of the synthetic scFv gene at appropriate restriction sites and insertion of replacement oligonucleotides bearing a codon for Gln instead of His. Expression and purification of the mutant scFvs was performed as above.

Surface plasmon resonance analysis

Association rate and dissociation rate constants for the interaction of purified scFv fragments with

BSA-antigen conjugates were determined by SPR on a Biacore™ biosensor system (GE Healthcare), as previously described (MacKenzie *et al.,* 1996). Immobilizations were carried out using the amine coupling kit supplied by the manufacturer. BSA-trisaccharide and BSA-O chain were immobilized in 10 mM sodium acetate, pH 4.5, at concentrations and constant times that yielded about 200 resonance units of immobilized material. One resonance unit corresponds to an immobilized protein concentration of ~1 pg/mm^2. All measurements were performed at ambient temperature in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.44 mM EDTA at a flow rate of 5µl/min. Surfaces were regenerated with 10 mM HCl. Binding constants were calculated from the association rate and dissociation rate constants with the Biacore™ software (GE Healthcare).

Results and Discussion

Modification of Cys 94L to introduce new functionalities

Residue 94L of Se155-4 is Cys in the parental hybridoma and Tyr in the germline gene. In the synthetic gene constructed for the scFv form (Anand *et al.*, 1991), a Ser was introduced instead of the Cys in order to enhance the yield of soluble protein in *E. coli*. To enable the modification experiments, 94L was changed back to Cys, and a His_6 tag was added to replace the previous antigen-based affinity purification with an IMAC procedure. Before the reactions described below, the scFv samples were reduced with dithiothreitol to remove small thiols that had become attached to the Cys94L during expression.

Cysteine modifications were carried out with two commercially-available reagents ie IANBD to introduce a fluorescent tag, and Fe-BABE to introduce a metal-binding functional group which has been used to cleave neighbouring chains of DNA or the protein itself, by a free radical process. The SH-reactive moieties of these reagents are respectively an iodoacetyl and a bromoacetyl group.

Samples of scFv modified with these reagents or with iodoacetamide were examined by mass spectrometry, and their antigen binding was compared to Se155-4 IgG and the Ser94L form of the scFv by ELISA. Electrospray mass spectrometry (Table 1) showed excellent labelling with iodoacetamide and the fluorescent reagent, and approximately 50% modification with Fe-BABE, which had been used at sub-optimal levels. In ELISA assays with the Salmonella LPS antigen (Fig. 2), the antigen binding by the derivatives was only slightly weaker than that of the Ser 94L scFv. Hence modification with bulky groups at this Cys position can be carried out with little loss of activity, despite it being in a CDR loop very close to the antigen binding pocket.

The fluorescently-labelled scFv was tested in a solution format designed to detect direct antigen binding through its effect on the fluorescence of the label. It showed an increase in fluorescence in the presence of antigen, in solution experiments in ELISA plates (Fig. 3). The

linear range for antigen detection was up to 1.5µg per well with 15µg of labelled scFv. Scatchard analysis of the titration data gave an apparent K_D of ~10 µM, calculated on the basis of the repeating unit. This value is comparable to the K_D value determined by SPR of 7.5 µM (MacKenzie *et al.,* 1996). Hence the advantages of direct measurement of a carbohydrate antigen in a solution format, compared to the multiple steps of an ELISA method, could be achieved with scFv fluorescently-modified at Cys94L. Notably, a Cys at this position also has been utilised in a similar format for reagentless assay of a protein antigen (Renaud *et al.,* 2002).

The derivative made with Fe-BABE which has an EDTA-like structure, was intended to cleave the polysaccharide antigen through a free radical process. This type of protein modification has been studied as a means of turning DNA regulatory proteins into pseudo-restriction enzymes and to map neighbouring chains in proteins (Datwyler and Meares, 2000). Since the lyase family of glycosidases cleave carbohydrates in a similar type of reaction, e.g. the glycosaminoglycan lyases (Garron and Cygler, 2010), it was hoped that the modified scFv would cut a bound polysaccharide antigen. To detect possible cleavage of LPS by the Fe-BABE derivative of the scFv, when activated with ascorbate and hydrogen peroxide, a deoxycholate-PAGE gel method (Tsai and Frasch, 1982) was used to examine the molecular weight distribution of the LPS. However, there was no obvious change in the antigen mass profile, i.e. no transfer of higher molecular weight components to the lower regions of the ladder in the gel (data not shown). Thus a metal-binding function was successfully introduced into Se155-4 with FeBABE, but it did not cleave the antigen to a detectable extent. It is relevant that the previous uses of this modification to cleave DNA or proteins required only a single cleavage to take place, whereas a considerable turnover would be necessary for polysaccharide cleavage to become apparent.

In order to compare the Cys94L derivatives with ones at Cys more distant from the central antigen-binding pocket, two site-directed mutations were made to introduce Cys residues into the extended VH and VL CDR4s of the scFv (see below) at Thr74H and Asp71L respectively. These

loops were predicted to be close to the position of a bound polysaccharide antigen, judging by the orientation of the heptasaccharide ligand in the binding site (Fig. 1). The VH version gave more soluble scFv expression than the VL one, so it was chosen for further characterization and comparison with the Cys94L scFv. The IANBD derivative of Cys74H in CDR4 showed a small change in fluorescence on addition of antigen (Fig. 3), considerably less than that of the Cys94L derivative. Nevertheless, this change is consistent with the bound antigen being near the CDR H4, as predicted from the structure of the heptasaccharide-Se155-4 complex (Fig. 1).

The His pairs of Se155-4 and its copper-binding

Remarkably, Se155-4 has four histidines around the binding-site region in the Fv, brought together by the folding of the molecule into two pairs spanning the VH/VL interface, 101H/34L and 62H/97L (Fig. 1). There are two other histidine residues in the scFv, His35H in the binding site pocket, which may be responsible for its antigen binding being sensitive to pH (Bundle *et al.*, 1994), and His44H at the base of the Fv, also near the VL/VH interface. The 101H/34L pair is close to the antigen binding site, and both residues may form H-bonds to the antigen (Cygler *et al.,* 1993), but mutagenesis of His101H to Glu, Gln or other residues did not much impair antigen binding (Brummell *et al.*, 1993). The His residues in each pair are close enough together that copper binding would be expected. Furthermore, each pair has a neighbouring residue that could also assist in metal binding. In the vicinity of the 101H/34L pair there is a negatively-charged residue, Asp52H. However it is not as symmetrically placed as in the metal-binding sites of Zn enzymes. The other pair of histidines, 62H/97L, is near to the position of the N-terminal Glu residue of the light chain (pyroGlu in the Fab). The sequence analysis of Se155-4 showed that three of the histidines (34L, 62H and 101H) and Asp 52H appear to have arisen by somatic mutation (Bundle *et al.*, 1994).

Fluorescence titrations with copper sulphate solutions were used to characterise metal

binding by Se155-4 recombinant Fab. Approximately 85% quenching of the protein's intrinsic tryptophan fluorescence was observed (Fig. 4). A single copper binding site was indicated with a K_D of 3.3 μM, which is similar to the affinity of the antibody for the trisaccharide epitope, 4.8 μM (Bundle *et al.*, 1994). This copper affinity is the same as that reported for an engineered His site in a VHH species (Fanning *et al.,* 2011), but below that of a site introduced into an anti-fluorescein antibody, 0.6 μM (Roberts *et al.,* 1990). Since the native and recombinant forms behaved similarly, it was probable that the site was not the one in the region of the N-terminus of the L chain. Addition of the trisaccharide, which does not cause any change in fluorescence, did not alter the copper binding significantly. Addition of copper to an antigen-antibody titration by ELISA led to a five-fold reduction in antigen binding (Fig. 5), similar to the 8-fold reduction seen with the VHH (Fanning *et al.,* 2011). The reduction is compatible with the loss of an H-bond between the antigen and the antibody, and shows that the copper binding site involves His101H. However, mutation of His101H to Gln did not affect copper quenching but mutation of His34L did. Compared to the K_D of the wildtype, 7.5 μM as measured by SPR (MacKenzie *et al.,* 1996), the 101H Gln mutant showed a reduction in antigen binding (K_D 26 μM); a smaller change was found with His34L Gln (K_D 12 μM) and mutations of the 97L and 62H His pair did not alter antigen binding significantly (K_D 9 μM).

His 35H and pH sensitivity

The Se155-4 binding site pocket contains a critical His35H that positions a water molecule to contact the abequose moiety of the antigen (Cygler *et al.,* 1991). As a consequence, antigen binding is lost below pH 5 and this sensitivity has been exploited for affinity purification of the IgG and its fragments (Bundle *et al.,*1994). When His35H was mutated to Gln, the scFv expressed less abundantly and did not bind to the affinity medium. Consequently this residue appears to be more responsible for the pH sensitivity than His101H or His34L. His35H is germ-line encoded, ie it

has not arisen by somatic mutation (Bundle *et al.,* 1994). It is remarkable that histidine residues occur commonly at this position in several human and murine VH classes, suggesting pH sensitivity should be encountered more often. The mutations found to confer pH sensitivity to a humanized antibody by Igawa *et al.* (2010) also included a Trp35H to His mutation. Hence this residue should be considered a prime candidate when trying to make an antibody pH sensitive.

Designed insertions into the CDR4 loop

The CDRs of antibody V domains form three β-turns, while an additional β-turn, often termed CDR4, lies on the outside of the binding-site region (Fig. 1). It is comprised of residues within the framework 3 segment (FR3), around residue 74 in the Kabat nomenclature. This region shows some limited variability in antibodies, mainly from germ-line gene differences, but it is more strongly variable in the V domains of T-cell receptor molecules where it has a role in MHC binding. However, inserts have been found in the CDR4 loops of two antibodies that were essential for antigen binding. The first case is of a VH domain, with a four residue insertion (Simon and Rajewsky, 1992), and the second is of a VL domain with an eight residue insertion and a corresponding deletion of a conserved leucine in the FR2 (Cook and Barber, 1995). Insertions at the CDR4 loop could increase the protein surface available for interaction with antigen, and hence improve affinity, or be useful for the introduction of new functions without affecting antigen binding. We therefore investigated whether Se155-4 could generally tolerate insertions at this point.

In a preliminary experiment, 3 or 5 residue segments containing pairs of aromatic amino acids were chosen for insertion into CDR4s after residue Asp71L or Ser75H; the sequences chosen were YPY, SYPYD, WRY and SWRYS. These constructs expressed satisfactorily, showing the Se155-4 loops could tolerate insertions well, and bound to the antigen affinity column, showing they retained activity. We therefore created phage-display libraries of randomised inserts

of four to seven amino acids in the VH CDR4 loop. Four individual libraries were constructed by inserting randomised oligonucleotide fragments $(NNK)_{4-7}$ after residue Ser75H, using nearby *SpeI* and *MluI* restriction sites. The monovalent phagemid/helper system was employed to generate phage display libraries. After three rounds of panning of each library against Salmonella serotype B LPS, several clones were selected and they were found to bind to the antigen affinity column. Their insert sequences (Table 2) showed no particular preferences for any type of amino acid; aromatic ones were not favored though they frequently occur in carbohydrate-binding sites. Remarkably the clones from the hexa- and hepta-peptide libraries also had only tetrapeptide inserts, i.e. they were deletion mutants. Hence a tetrapeptide appears to be the size of insert best tolerated in the CDR4 loop, despite the above results with the four tripeptide and pentapeptide inserts. Deletions could occur during the replication process due to the presence of a large population of random direct and indirect DNA repeats in the library constructs. The selection of deletion mutants could arise from selection pressure being imposed on the stability and the proper folding of the proteins displayed on the surface of the phage.

Each of the mutants was subcloned into an expression vector SK-1 and expressed in *E. coli*. Affinity purifications of the periplasmic fractions on an oligosaccharide antigen column showed all the mutants were able to bind to the antigen, and thus had retained their ability to fold into active proteins with antigen-binding activity. One product with the insert sequence QVTA was chosen for SPR analysis. Its K_D of 11.5 ± 0.8 μM was only slightly below that of the wildtype, 7.5 μM (MacKenzie *et al.,* 1996). Therefore this insert was well tolerated by the scFv, but did not enhance its binding affinity. The tetrapeptide insert is the same size as the natural insert reported by Simon and Rajewsky (1992), and a small, even number of inserted residues may best preserve the type I β-turn of the CDR H4.

Conclusions

The intrinsic Cys and His sites of the Se155-4 antibody have proved to be instructive about the tolerance of antibodies for the introduction of new functions. In particular, the Cys94L while very close to the antigen binding pocket, could be modified with bulky groups by alkylation reactions with little change in antigen affinity. Even residues that can form H-bonds to the antigen, such as His 101H, can be altered. More distant sites can be introduced in the CDR4H β-turn, which could be modified by Cys mutation and alkylation or by inserting residues to extend it. Though the β-turn loop could accept insertions of various lengths, a four-residue insert seems to be optimal for minimal disruption of the binding site region. These results and those of other studies show that antibodies may tolerate considerable redesign of regions either directly alongside or close to their binding sites, including changes in residues originally introduced by somatic mutation, without significant loss of antigen affinity. While these findings were made with an anti-carbohydrate antibody, they are encouraging for conferring new functions on antibodies generally.

Acknowledgements

We dedicate this paper to the memory of our late colleague, Dr Saran A. Narang. The authors thank Dr A.G. Szabo and Don Krajarski for assistance with the fluorescence measurements, Dr John Pezacki for access to ELISA equipment, Joe Michniewicz and Sonia Leclerc for DNA sequence analyses, Dr Jyothi Kumaran and Tom Devecseri for assistance with the figures, Ginette Dubuc, Tomoko Hirama, Anne M. Moffatt, Rebecca To and Jacek Stupek for excellent technical assistance.

References

Al-Lazikani B, Lesk AM, Chothia C. 1997. Standard conformations for the canonical structures of immunoglobulins. *J. Mol. Biol.* 273:927-948.

Almagro JC, Fransson J. 2008. Humanization of antibodies. *Frontiers Biosci.* 13:1619-1633.

Anand NN, Mandal S, MacKenzie CR, Sadowska J, Sigurskjold B, Young NM, Bundle DR, Narang SA. 1991. Bacterial expression and secretion of various single-chain Fv genes encoding proteins specific for a *Salmonella* serotype B O-antigen *J. Biol. Chem.* 266:21874-21879.

Banks DD, Gadgil HS, Pipes GD, Bondarenko PV, Hobbs V, Scavezze JL, Kim J, Jiang XR, Mukku V, Dillon TM. 2008. Removal of cysteinylation from an unpaired sulfhydryl in the variable region of a recombinant monoclonal IgG1 antibody improves homogeneity, stability, and biological activity. *J. Pharm. Sci.* 97:764-779.

Brummell DA, Sharma VP, Anand NN, Bilous D, Dubuc G, Michniewicz J, MacKenzie CR, Sadowska J, Sigurskjold BW, Sinott B, Young NM, Bundle DR, Narang SA. 1993. Probing the combining site of an anti-carbohydrate antibody by saturation-mutagenesis: role of the heavy-chain CDR3 residues. *Biochemistry* 32:1180-1187.

Bundle DR, Eichler E, Gidney MAJ, Meldal M, Ragauskas A, Sigurskjold B, Sinnott B, Watson DC, Yaguchi M, Young NM. 1994. Molecular recognition of a *Salmonella* epitope by monoclonal antibody Se155-4. *Biochemistry* 33:5172-5182.

Chothia C, Lesk AM. 1987. Canonical structures for the hypervariable regions of immunoglobulins.*J. Mol. Biol.* 196:901-917.

Cook J, Barber BH. 1995. Recombinant antibodies containing an engineered B-cell epitope capable of eliciting conformation-specific antibody responses. *Vaccine* 13:1770-1778.

Corneillie TM, Lee KC, Whetstone PA, Wong JP, Meares CF. 2004. Irreversible engineering of the multielement-binding antibody 2D12.5 and its complementary ligands. *Bioconj. Chem.*

15:1392-1402.

Cygler M, Shan W, Zdanov A, Bundle DR, Rose DR. 1993. Recognition of a carbohydrate antigenic determinant of *Salmonella* by an antibody. *Biochem. Soc. Trans.* 21:437-441.

Cygler M., Rose DR, Bundle DR. 1991. Recognition of a cell-surface oligosaccharide of pathogenic *Salmonella* by an antibody fragment. *Science* **253**, 442-445.

Datwyler SA, Meares CF. 2000. Protein-protein interactions mapped by artificial proteases: where σ factors bind to RNA polymerase. *Trends Biochem. Sci.* **25**, 498-414.

Deng,S.-J., MacKenzie,C.R., Hirama,T., Brousseau,R., Lowary,T.L., Young,N.M., Bundle,D.R. and Narang,S.A. 1995. *Proc. Natl. Acad. Sci. USA* **92**, 4992-4996.

Deng S-J, MacKenzie CR, Sadowska J, Michniewicz J, Young NM, Bundle DR, Narang SA. 1994. Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. *J. Biol. Chem.* 269:9533-9538.

England P, Nageotte R, Renard M, Page A-L, Bedouelle H. 1999. Functional characterization of the somatic hypermutation process leading to antibody D1.3, a high affinity antibody directed against lysozyme. *J. Immunol.* 162:2129-2136.

Fanning SW, Murtaugh ML, Horn JR. 2011. A combinatorial approach to engineering a dual-specific metal switch antibody. *Biochemistry* 50:5093-5095.

Garron ML, Cygler M. 2010. Structural and mechanistic classification of uronic acid-containing polysaccharide lyases. *Glycobiology* 20:1547-1573.

Harrison JL, Williams SC, Winter G, Nissim A. 1996. Screening of phage antibody libraries. *Methods Enzymol.* 267:83-109.

Hopp TP, Gallis, B, Prickett, KS. 1996. Metal-binding properties of a calcium-dependent monoclonal antibody. *Mol. Immunol.* 33:601-608.

Igawa T, Ishii, S, Tachibana, T, Maeda A, Higuchi, Y, Shimaoka S, Moriyama C, Watanabe, T, Takuba R, Doi, Y, Wakabayashi T, Hayasaka A, Kadono S, Miyazaki T, Haraya K, Sekimori Y,

Kojima T, Nabuchi, Y Aso Y, Kawabe Y, Hattori K. 2010. Antibody recycling by engineered pH-dependent antigen binding improves the duration of antigen neutralization. *Nature Biotech.* 28:1203-1207.

Iverson BL, Iverson SA, Roberts VA, Getzoff ED, Tainer JA, Benkovic SJ, Lerner RA. 1990. Metalloantibodies. *Science* **249**, 659-662.

Junutula JR, Bhakta S, Raab H, Ervin KE, Eigenbrot C, Vandlen R, Scheller RH, Lowman HB. 2008. Rapid identification of reactive cysteine residues for site-specific labeling of antibody-Fabs. *J. Immunol. Meth.* 332:41-52.

Mackenzie CR, Hirama T, Deng S-J, Bundle DR, Narang SA, Young NM. 1996. Analysis by surface plasmon resonance of the influence of valence on the ligand binding affinity and kinetics of an anti-carbohydrate antibody. *J. Biol. Chem.* 271:1527-1533.

Miao Z, McCoy MR, Singh DD, Barrios B, Hsu OL, Cheal SM, Meares CF. 2008. Cysteinylated protein as reactive disulphide: an alternative route to affinity labelling. *Bioconj. Chem.* 19:15-19.

Mills JH, Lee HS, Liu CC, Wang J, Schultz PG. 2009. A genetically encoded direct sensor of antibody-antigen interactions. *ChemBioChem* 10:2162-2164.

Murtaugh ML, Fanning SW, Sharma TM, Terry AM, Horn JR. 2011. A combinatorial histidine library scanning approach to engineer highly pH-dependent protein switches. *Protein Science* 20:1619-1631.

Persson H, Ohlin M. 2007. Exploring central and peripheral diversity in antibody evolution. *Mol. Immunol.* 44:2729-2736.

Raghunathan G, Smart J., Williams J, Almagro JC. 2012. Antigen-binding site anatomy and somatic mutations in antibodies that recognize different types of antigens. *J. Mol. Recognit.* 25:103-113.

Renard M, Bedouelle H. 2004. Improving the sensitivity and dynamic range of reagentless fluorescent immunosensors by knowledge-based design. *Biochemistry* 43:15453-15462.

Renard M, Belkadi L, Hugo N, England P, Altshuh D, Bedouelle H. 2002. Knowledge-based design of reagentless fluorescent biosensors from recombinant antibodies. *J. Mol. Biol.* 318:429-442.

Roberts VA, Getzoff ED. 1995. Metalloantibody design. *FASEB J.* 9:94-100.

Roberts VA, Iverson BL, Iverson SA, Benkovic SJ, Lerner RA, Getzoff ED, Tainer JA. 1990. Antibody remodelling: a general solution to the design of a metal-coordination site in an antibody binding pocket. *Proc. Natl. Acad. Sci. USA* 87:6654-6658.

Simon T, Rajewsky K. 1992. A functional antibody mutant with an insertion in the framework region 3 loop of the V_H domain: implications for antibody engineering. *Prot. Eng.* 3:229-234.

Trisler K, Looger LL, Sharma V, Baker M, Benson DE, Trauger S, Schultz PG, Smider VV. 2007. A metalloantibody that irreversibly binds a protein antigen. *J. Biol. Chem.* 282:26334-26353.

Tsai CM, Frasch CE. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analyt. Biochem.* 119:115-119.

Ward SL, Ingham KC. 1991. A calcium-binding monoclonal antibody that recognizes a non-calcium-binding epitope in the short consensus repeat units (SCRs) of complement C1r. *Mol. Immunol.* 29:83-93.

Wedemayer GJ, Patten PA, Wang LH, Schultz PG, Stevens RC. 1997. Structural insights into the evolution of an antibody combining site. *Science* 276:1665-1669.

Yang PL, Schultz PG. 1999. Mutational analysis of the affinity maturation of antibody 48G7. *J. Mol. Biol.* 294:1191-1201.

Zdanov, A, Li Y, Bundle DR, Deng S-J, MacKenzie CR, Narang SA, Young NM, Cygler M. 1994. Structure of a single-chain antibody variable domain (Fv) fragment complexed with a carbohydrate antigen at 1.7 Å resolution. *Proc. Natl. Acad. Sci. USA* 91:6423-6427.

Zhou T, Hamer DH, Hendrickson WA, Sattentau QJ, Kwong PD. 2005. Interfacial metal and

antibody recognition. *Proc. Natl. Acad. Sci. USA* 102:14575-14580.

Figure Legends

Fig.1. Location of the Cys and His residues in the Se155-4 binding-site region, and the CDR H4. The image is based on the heptasaccharide-Fab complex, PDB entry 1MFB (Cygler *et al.,* 1993). The VH domain is shown in dark grey, and the VL in light gray. His101H (nitrogens shown in blue) is to the left of the ligand and below it is His34L; His35H is under the ligand; His62H and 97L are to the right; the sulphur atom of Cys94L is in yellow. Thr74H of CDR H4 is shown at the top of the structure in solid colour and Asp61L of CDR L4 is at the bottom. Residues are numbered here and in the text according to the VL and VH sequences (Bundle *et al.*, 1994); to obtain the numbering of the VH region given in the PDB file1MFB, add 250.

Fig.2. ELISA assays of wild-type and Cys94 modified forms of Se155-4. IgG (■); unmodified scFv S94C (□); S94C modified with iodoacetamide (▲); scFv S94C modified with IANDB (▼); scFv S94C modified with FeBABE (○).

Fig.3. Fluorescence titrations of the IANBD-labelled scFv with LPS. Upper panel, labelled Cys94L scFv (▲) and labelled Cys74H in CDR4H (■); lower panel, Scatchard plot of the Cys94L data.

Fig.4. Quenching of the intrinsic fluorescence of Se155-4 by copper. Scatchard plot of a copper titration, and the emission spectra before and after copper addition (inset).

Fig.5. ELISA assay of the effect of copper on antigen binding by Se155-4. IgG titration in the presence (♦) and absence of copper (△).

Table I. Mass spectrometry of Cys94L derivatives

Derivative	Mass (Da)	Mass change from thiol form (Da)	Expected change (Da)
Ser94L	27116		
Cys94L	27132		
Cys94L + IANDB	27411	279	279
Cys94L + FeBABE	27132, 27628	496	491
Cys94L + iodoacetamide	27191	59	59

Table 2. Representative clones recovered from panning of CDR H4 libraries

Clone	Planned size of CDR4 insertion (aa)	Insert sequence found
4B6	Four	QRTL
4B7	Four	LGGL
4E9*	Four	QVTA
4G6	Four	WSQL
6G11	Six	SQNY
7G2	Seven	QLAG
7G10	Seven	VTEE

* Clone expressed and purified for SPR

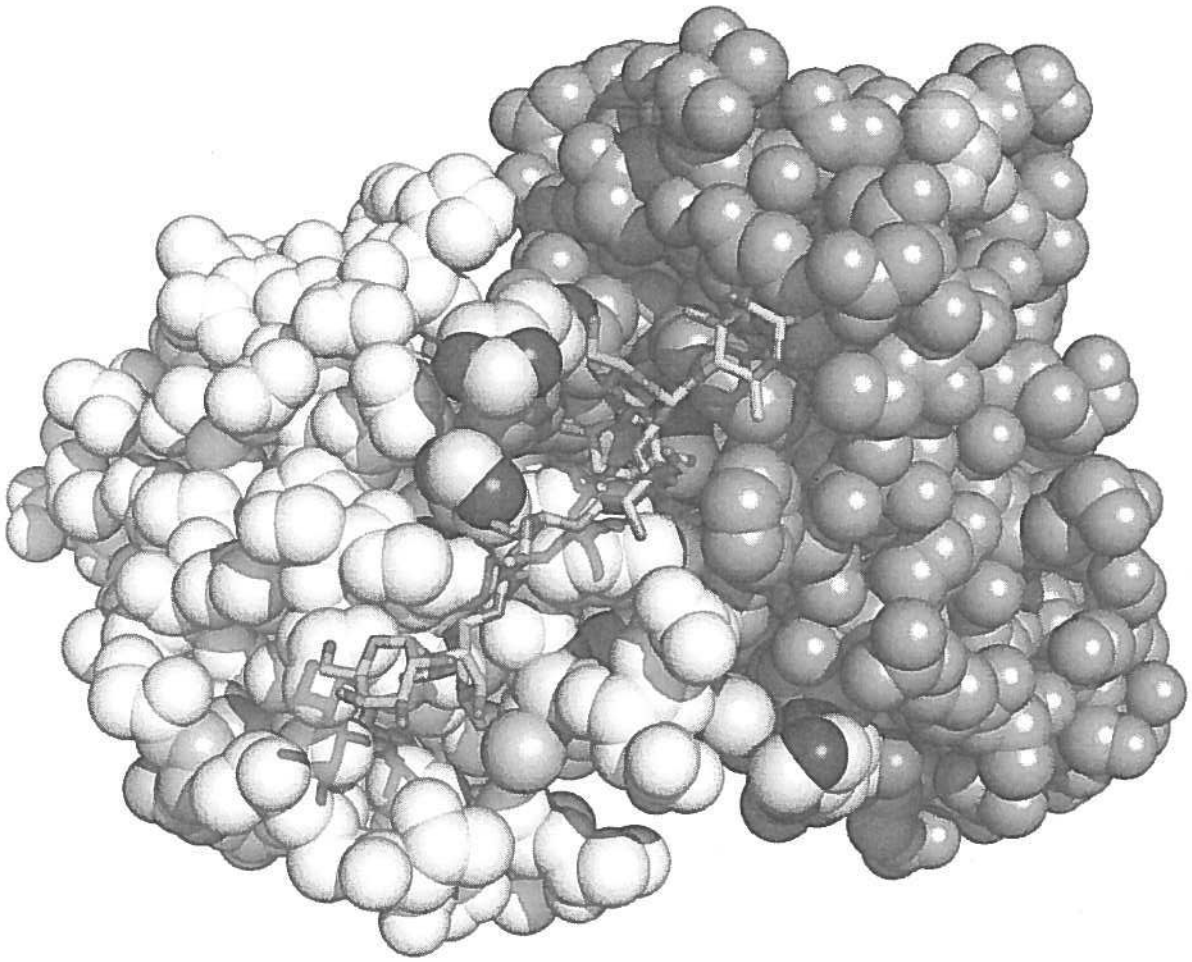

Fig. 1

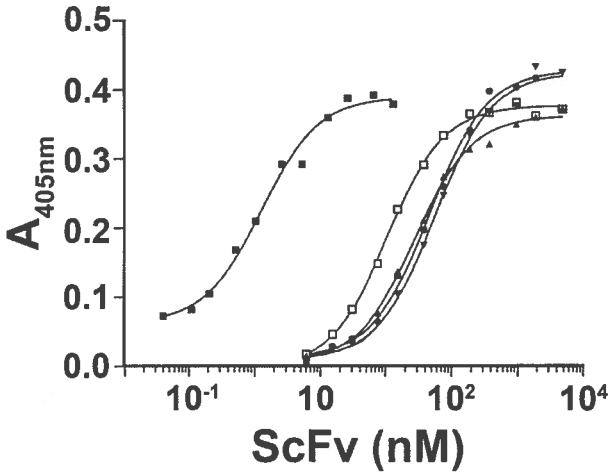
0.5
0.4
0.3
0.2
0.1
0.0
A_{405nm}
10^{-1}
10
10^{2}
10^{4}
ScFv (nM)

Fig.2

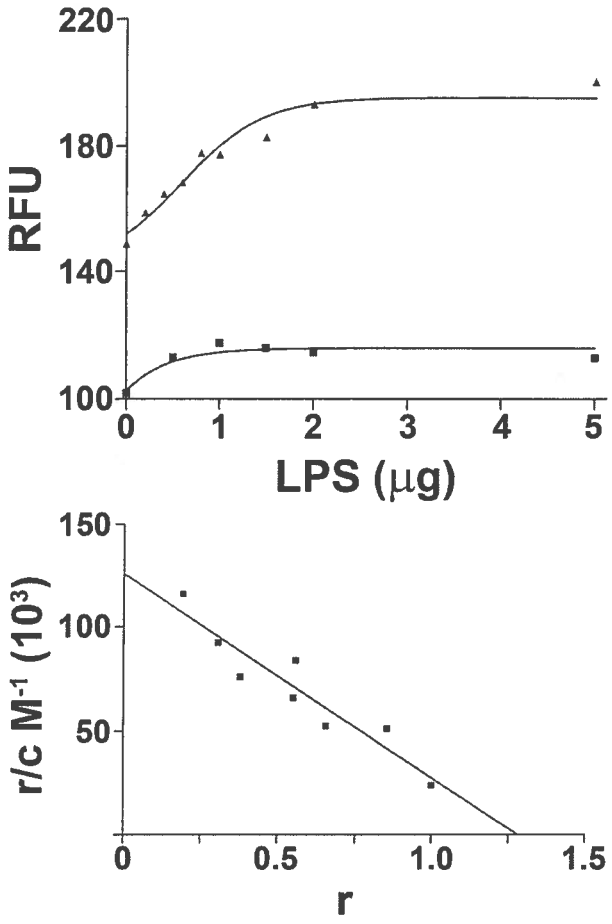
220
180
140
100
RFU
0
1
2
3
4
5
LPS (μg)
150
100
50
r/c M^{-1} (10^{3})
0
0.5
1.0
1.5
r

Fig. 3

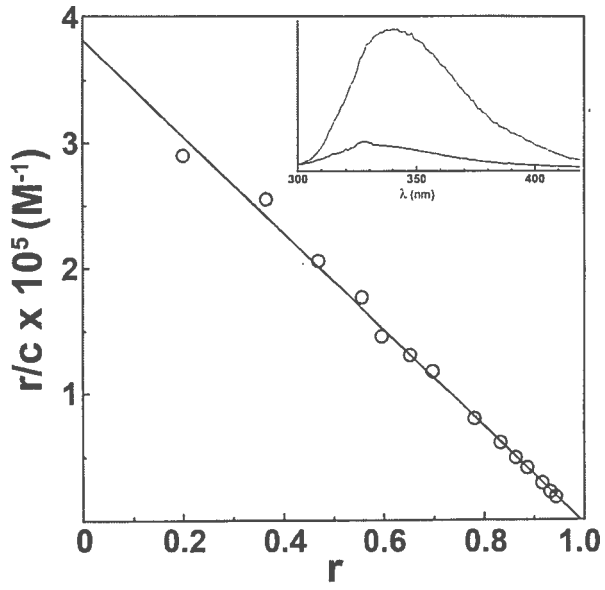

Fig.4

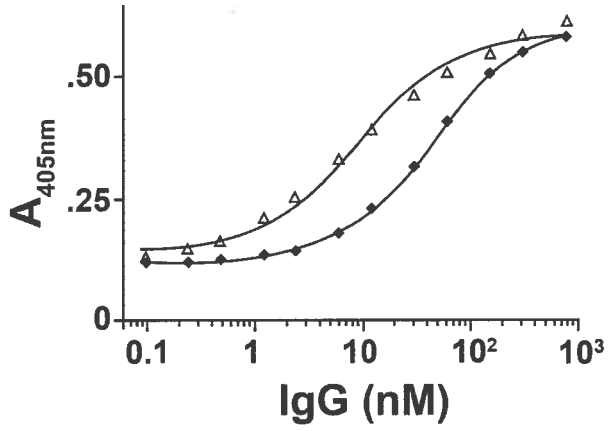

Fig.5