



Research paper

Isolation of human anti-idiotypic antibodies by phage display for clinical immune response assays

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Abstract

The clinical development of therapeutic proteins requires assays that measure the pharmacokinetic (PK) profile of, and the potential immune response (IR) to, the protein agent. Each assay requires reagents that are highly specific for the therapeutic protein. For therapeutic monoclonal antibodies, anti-CDR-specific, or anti-idiotypic (anti-id), antibodies are an ideal class of reagents suitable for these assays because of their high specificity and affinity to the drug antibody. We generated anti-ids to two human antibodies by antibody phage display using the MorphoSys HuCAL GOLD[®] Fab library. To selectively target the CDR regions, serum and a framework-matched mAb were included as competitors during the phage selection process. Panels of CDR-specific Fabs, with low to sub-nM affinities, were isolated against both targets. The CDR specificity of these Fabs was shown by their lack of binding to a framework-matched control mAb and by competition of this binding with the soluble antigens of the respective therapeutic mAb targets. The candidate anti-id Fabs were able to detect both immobilized and soluble target Ab without being affected by serum, a requirement for both PK assay and the IR bridging assay format. Combinations of the Fabs for PK detection assays were identified by pairwise binding studies, although the pair for one target mAb lacks the desired sensitivity for PK assays. To evaluate their potential as anti-drug antibodies (ADAs), the best Fabs for one of the targets were converted and produced as the required bivalent human mAbs. In comparison to rodent mAbs and primate polyclonal serum, the phage display derived human mAbs were equally effective as reference standards. Our results demonstrate that competition-based phage selection can be an effective method for the isolation of anti-idiotypic antibodies for PK and IR assay development, and in this latter case, overcome limitations of current methods using rodent derived anti-ids.

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Abbreviations: pK, pharmacokinetics; IR, immune response; Ab, antibody; mAb, monoclonal antibody; ADA, anti-drug antibody; IgG, immunoglobulin; λ , lambda light chain of an antibody; κ , kappa light chain of an antibody; V_H , variable region of the heavy chain of an antibody; V_L , variable region of the light chain of an antibody; CDR, complementarity determining regions in antibody V_H or V_L ; Fab, antigen binding fragment of an antibody; CAM35, 35 μ g/ml chloroamphenicol; Glu, glucose; NHS, normal human serum; FM-Ab, framework-matched antibody; TBS, Tris buffer saline; CFU, colony forming unit; IPTG, Isopropyl β -D-1-thiogalactopyranoside.

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1. Introduction

Monoclonal antibodies are proven therapeutic agents with eighteen now approved for treatment of a variety of disease indications (Carter, 2006). Pre-clinical and clinical development of these antibodies requires highly specific detection reagents and assays to measure their pharmacodynamics, bio-distribution and immune responses in human subjects (Mire-Sluis et al., 2004; Mahmood and Green, 2005). This becomes particularly challenging for human-antibody therapeutics due to the high background of human antibody in serum and the expected low immune response.

Pharmacokinetic studies require highly specific and high-affinity reagents to detect the presence of the therapeutic drug in patient serum. For therapeutic antibody development, anti-idiotypic antibodies (anti-ids) and sometimes antigen are good reagents for the assays. As ELISA is the commonly used assay format for PK studies, a pair of non-competing anti-ids, in addition to high specificity and affinity, are highly desirable.

Similar to PK assays, critical to the evaluation of an immune response is a highly specific reference antibody against which to measure any anti-drug response (Geng et al., 2005). Unlike PK assays, the species of the assay antibody is an important consideration for an IR reference standard. Ideally, during the clinical phases of drug development, this reference should be a human antibody so as to reflect the natural immune response. With the technological progression from murine, to chimeric, to humanized and human therapeutic antibodies, the specificity of ADAs has become increasingly localized to the CDR regions, which in turn requires robust and highly selective assays (Schantz et al., 2001). A further consequence of humanization has been a lower incidence of ADA, trending down to the point where a human reference sample cannot always be obtained from treated subjects (Pendley et al., 2003). Moreover, pre-clinical generation of reference antibodies would permit timely development of the clinical assays. None of the methods currently in use satisfy all of these desired parameters (Mire-Sluis et al., 2004). Anti-ids, which target the CDR regions of the therapeutic antibody, provide exquisite specificity for the target antibody in the background of serum antibodies. However, anti-ids are typically murine mAbs derived by classic hybridoma technology. These anti-ids can be converted to a pseudo-human reference by molecular cloning and chimerization but this is rather laborious. In addition, they tend to be of high affinity and thus may be poor models of clinical responses, which are likely to be of more modest affinity. An alternative approach is the immunization of

primates to generate a polyclonal reference standard; however, successful immunization to human antibody can be sporadic and is a potentially controversial use of these animals. Lastly, human sera from early clinical trials of the therapeutic antibody can serve as a reference standard for later stage development, if ADAs are generated. This latter material is difficult to obtain in sufficient quantity and can be obtained only after initiation of clinical trials. Moreover, changing the ADA reference sample midway through clinical development necessitates assay revalidation.

Here we describe the direct isolation of human anti-ids to the mAbs to human IL-6 and IL-13 by antibody phage display and their characterization as detection and reference standard reagents for PK and IR assays. Phage display was carried out with the MorphoSys HuCAL GOLD® library, a synthetic human Fab combinatorial library (Knappik et al., 2000; Krebs et al., 2001; Kretzschmar and von Ruden, 2002). The recovered Fabs bound to their target Abs with low to sub-nM affinities, and had the anti-idiotypic characteristics of specificity for the CDR regions and inhibition by the corresponding antigens. These antibodies were functional in pharmacokinetic detection assays developed in a sandwich format. Upon conversion to mAbs, the antibodies were suitable reference standards for immune response assays, conducted in a bridging format.

2. Materials and methods

2.1. Target antibodies

The first target (Ab1) is an IgG1/λ human antibody directed against human IL-13. The Vh and Vλ regions are synthetic derivatives of human germline genes IGHV3-30-3 (vb_3-30.3) (Vh3 family) and IGLV3-1 (vb_3r) (VλIII family), respectively; the gene names are according to IMGT®, the international ImMunoGeneTics information system® <http://imgt.cines.fr> with a prior locus naming convention given in parentheses (see V Base at <http://vbase.mrc-cpe.cam.ac.uk>). The second target (Ab2) is an IgG1/κ human engineered antibody directed against human IL-6. The Vh and Vκ framework regions are synthetic derivatives of the human germline genes IGHV3-21 (vb_3-21) (Vh3 family) and IGKV3-11 (vb_L6) (VκIII family), respectively. These antibodies were chemically biotinylated using EZ-Link NHS-LC-Biotin (Pierce, #21335). An EZ-Link-Biotin solution (1 mM in PBS pH 7.2) was incubated with the Ab at a 5:1 molar ratio in a 1 ml reaction volume for 2 h at room temperature (RT). Excess biotinylation reagent was removed by extensive dialysis against PBS at 4 °C.

Although not evaluated for these antibodies, other antibodies and proteins treated in this manner have shown 2–3 biotin moieties per molecule as measured by mass spectrometry.

2.2. Phage selection

The Human Combinatorial Antibody Library HuCAL GOLD® (MorphoSys AG, Germany) was panned in solution essentially as described by Krebs (2001). For the Ab1, six separate sub-libraries, $V_{H1}-V_{H6}$ at a titer of 10^{10} CFU in $0.5\times$ Chemiblocker (CB) (Chemicon, #2170) and $0.5\times$ TBS with 0.025% Tween-20 (TBST), were panned against biotinylated Ab1 in the absence (round 1) or presence (rounds 2 and 3) of 10% pooled human serum. The Ab specific phage were enriched by capture of the biotinylated Ab on streptavidin-coated beads (Dyna, Dynabead M280 Streptavidin) and washed with $1\times$ TBST to remove non-bound phage. The stringency of the selection was increased in successive rounds of panning by increasing the number and duration of the washing steps and by reducing the concentration of biotinylated Ab1 from 100 nM in the first round of panning to 50 nM in the second and the third rounds. Phage specific for the target were eluted with 150 μ L of 50–100 mM DTT in 10 mM Tris pH 8.0 for 10 min at 4 °C. For Ab2, panning was performed with three pools of the sub-libraries, V_{H1+3} , V_{H2+5} , and V_{H4+6} , each with a titer of 10^{10} CFU in $0.5\times$ TBST, $0.5\times$ CB, 10% pooled human serum, and 0.7 μ M of a framework-matched control monoclonal antibody. Other panning conditions were similar to those for Ab1, except Ab2 concentration was lowered to 10 nM in the third round of selection.

2.3. Fab screening and identification

After the third round of selection, colonies were scraped from agar plates and suspended in $2\times$ YT with 35 μ g/ml chloramphenicol, and suspended colonies were grown overnight at 37 °C. Plasmids were isolated from overnight cultures using a QIAprep spin miniprep kit (Qiagen, #27106), and Fab-encoded inserts were liberated with the restriction enzymes XbaI and EcoRI. The inserts were ligated into XbaI/EcoRI-digested pMORPHx7-MH vector, which expresses soluble Fab with c-myc and His₆ tags. The ligation reactions were transformed into electrocompetent TG1 cells (Stratagene, #200123). The transformed cells were allowed to recover, spread onto LB agar Q-trays containing 35 μ g/ml chloramphenicol and 10% glucose (Teknova, # L4807), and incubated overnight at 37 °C.

The Fab transformants were grown in 60 μ l of $2\times$ YT containing 35 μ g/ml chloramphenicol and 1% Glucose

($2\times$ YT/CAM35/Glu) within a master 384-well plate at 37 °C overnight. Duplicate cultures without glucose (40 μ L) were inoculated from cultures in this master plate and were incubated at 37 °C with shaking for 5–6 h. Fab expression was induced by the addition of 10 μ l per well of $2\times$ YT/CAM35/0.5 mM IPTG, and cultures were grown overnight with shaking at 30 °C. Induced cultures were lysed with the addition of 15 μ l/well of BEL buffer (200 mM Boric Acid, 150 mM NaCl, 2 mM EDTA, 2.5 mg/ml Lysozyme) and incubated for 1 h at RT. Cell lysates were blocked for 30 min at RT with 30 μ l/well CB.

To screen Fab-containing lysates, Maxisorp plates (Nunc, #43300-4548) were coated with the Abs at 7 nM in $1\times$ PBS at 4 °C overnight and blocked with Chemiblocker (CB). 10 μ l of Fab-expressing culture lysate was added to each well and incubated for 1 h at RT. Wells were washed 3 times with $1\times$ TBST. A 1:5000 dilution of alkaline phosphatase-conjugated mouse anti-c-Myc mAb (Roche Applied Sciences, #11667203001) in 10% CB in $1\times$ TBST was added and incubated for 1 h at RT. Wells were washed 3 times with $1\times$ TBST. The signal for fluorescence was obtained by adding Attophos substrate (Roche Applied Sciences, #1681982). After 10 min of incubation at RT, the plates were read at an excitation wavelength of 435 nm and an emission wavelength of 530 nm. Fab clones that had fluorescence signal 5-fold (Ab1) or 10-fold (Ab2) higher when binding to their Abs compared to no Fab lysate control were chosen for further study.

In the specificity ELISA, the chosen Fab cell lysates were cross-screened against a framework-matched IgG, human serum (20% in $1\times$ TBS), and biotinylated BSA. Fab clones that bound specifically to Ab1 or Ab2 were picked for sequence analysis to identify unique binders. Fab inserts for these clones were amplified by PCR, and the PCR amplicon was used as template in the Big Dye Terminator v3.1 protocol (Applied Biosystems, #4336919).

2.4. Fab production

Based on the screening assays, 5 unique clones for each Ab target were purified. Fabs were cultured in 10 ml $2\times$ YT/CAM35/Glu at 37 °C shaking at 250 rpm overnight. 10 ml of the overnight cultures were used to inoculate 1 liter cultures and was grown at 37 °C until OD_{600 nm} was 1.0. Fab expression was induced by the addition of 0.5 mM IPTG, and the cultures were incubated at 26 °C with shaking overnight. Cultures were centrifuged, and cell pellets were resuspended with 50%PBS/50%BBS (24.7 g/L boric acid, 18.7 g/L NaCl, pH 8.0) containing Complete EDTA-free protease inhibitor cocktail (Roche

Applied Sciences, #11873580001). Cells were lysed in a microfluidizer, and the cell debris was removed by centrifugation. The His₆-tagged Fabs were purified using Talon resin (BD Biosciences, #635504) by batch mode and gravity flow through 25 mL columns. Approximately 10 mL of rinsed Talon resin was added to the spent cell lysate. A column was packed with the Fab-bound resin, and the unbound material was removed by gravity flow. The Fab-bound resin was washed 3 with 3 column volumes (~25 mL total) of PBS containing 200 mM NaCl, 0.05% Tween20, and 10 mM imidazole, and the purified Fab was eluted with 150 mM EDTA and dialyzed against PBS. The recoveries of Fab 3 (anti-Ab1) and Fab 6 (anti-Ab2) were low and they were not characterized further.

2.5. ELISA assays

For the detection format, Maxisorb plates were coated with the Ab1 or Ab2 and blocked as for the Fab screening above. Serial dilutions of each purified Fab in 10% CB in 1× TBST were added with and without 10% pooled human serum to the Ab-coated wells and incubated for 1 h at RT. Biotinylated mouse anti-HIS antibody (R&D Systems, #BAM050) (1:3000 dilution in 10% CB in 1× TBST) was added to wells and incubated for 1 h at RT. A 1:5000 dilution of alkaline phosphate-conjugated streptavidin (Zymed, #438322) in 10% CB in 1× TBST was allowed to bind to the biotinylated mouse anti-HIS for 20 min at RT. The fluorescence signal was obtained by adding Attophos reagent. The plates were washed between each step and finally read as stated in the Fab screening section.

For the capture assay format, Maxisorb plates were coated with a solution of Fab at 20 nM overnight at 4 °C. The plates were blocked with Chemiblocker (CB) and serial dilutions of biotinylated Abs in 10% CB in 1× TBST were bound to the Fabs for 1 h at RT. Biotinylated Ab1 or Ab2 was detected with a 1:5000 dilution of alkaline phosphate-conjugated streptavidin in 10% CB in 1× TBST and Attophos reagent as described above.

For the Fab competition assays, Maxisorb plates were coated with a solution of Ab1 or Ab2 at 7 nM and blocked as above. Serial dilutions of competing Fab, starting at 100 nM, were mixed with 667 pM of a biotinylated Fab, and the mixture of Fabs was applied to the Ab-coated plate and incubated for 1 h. The biotinylated Fabs were detected with streptavidin–alkaline phosphatase and Attophos as above.

For the antigen competition assays, Maxisorb plates were coated with the Ab1 or Ab2 and blocked as described above. Serial dilutions of human IL-13

(produced at Centocor in CHO cells) or human IL-6 (R&D Systems, #206-IL-050/CF) were mixed with a fixed concentration of Fab (equal to the EC₅₀ determined in the detection ELISA). The mixture of antigen and Fab was applied to the Ab-coated plate and incubated for 1 h. The binding Fabs were detected with a biotinylated mouse anti-HIS antibody followed by the streptavidin–alkaline phosphatase as for the Fab detection ELISA.

For the sandwich assay, Maxisorb plates were coated overnight at 4 °C with a 20 nM solution of the purified Fab. The plates were blocked with Chemiblocker (CB) and then serial dilutions of Ab1 or Ab2 in 10% pooled human serum/10% CB in 1× TBST were bound for 1 h at RT. The biotinylated Fabs with the lowest EC₅₀ in the detection ELISA and with distinct binding activity in the capture ELISA were added at 20 nM and incubated for 1 h at RT. Binding of biotinylated Fab was detected with streptavidin conjugated alkaline phosphatase as described above.

2.6. Affinity determination

Binding kinetics for the anti-id Fabs were determined on a BIAcore 3000 (BIAcore, Piscataway NJ). The sensor surface was prepared by immobilizing Protein A onto flow cell 2 of a CM5 sensor chip, using the protocol described in the amine coupling kit (BIAcore, #BR-1000-50). Approximately 3000 RU of protein A was immobilized. A reference surface was prepared using flow cell 1 following the above procedure, but without the injection of protein A. In all studies, the dilution and running buffer used in BIAcore was 10 mM sodium phosphate and 150 mM sodium chloride, pH 7.4 with 3 mM EDTA and 0.05% Tween-20 (filtered through a 0.1 µm filter and degassed).

A 7 nM solution of the Ab1 or Ab2 was passed over the protein A surface for 1 minute at 30 µl/minute, capturing approximately 100 RU of antibody. Running buffer was passed over the surface at 30 µl/min until the new baseline stabilized. Anti-id Fab was then passed over both the reference and captured antibody surfaces for three minutes at 30 µl/min, and then 6 min of dissociation data were collected. The surfaces were regenerated using a 30-second injection of 100 mM glycine.

The resulting sensorgram were evaluated by subtracting the reference data from the antibody data. The association and dissociation rate constants were determined using BIAevaluation 3.0 provided with the BIAcore. The equilibrium dissociation constant was calculated from the ratio of the dissociation and association rate constants.

2.7. Converting Fab to mAb

The V_H and V_L regions from the selected Fabs were cloned into the MorphoSys human IgG1/ κ mammalian expression vectors (Krebs, 2001) using restriction sites EcoRI/BlpI and EcoRV/HpaI, respectively. The IgGs were produced by transient expression in HEK293-E cells, following the manufacturer's protocol for Lipofectamine 2000 (Invitrogen, # 11668-019) with 300 μ g of DNA per 10⁸ cells in Optimem media (Invitrogen, # 31985-070). 48 h after switching to expression media the spent supernatant was collected and the IgG was purified following the manufacturer's recommendation using Mab Select resin (Amersham Biosciences, # 17-5199-01).

2.8. Immune response assay

The anti-Ab1 immune response assay is a bridging ELISA format. Microtiter plates were coated with Ab1 at 0.5 μ g/mL and blocked with buffered casein solution (Pierce). Varying amounts of mAb4, two murine hybridoma antibodies specific to Ab1 (C188, C189), or anti-serum from a monkey immunized with Ab1 (poly-35) were prepared in 3% BSA in 1 $\times$ PBS (assay diluent) and incubated for 60 min at 25 °C. Biotinylated Ab1 was added at 1 μ g/mL and incubated at 25 °C for 60 min. Streptavidin-HRP was added at a 1:7500 dilution and incubated at 25 °C for 30 min. Signals were produced by addition of Tetramethylbenzidine (TMB) solution followed by 4 N H₂SO₄ and captured on a spectrophotometric microplate reader.

To determine the optimal coating condition, various concentrations of Ab1 were applied to the plate surface. The plates were blocked as previously described. A fixed amount of mAb2, mAb4, C188, C189 and poly-35 were applied to the blocked plates. Biotinylated Ab1, Streptavidin-HRP, TMB, and H₂SO₄ were added as described for the basic assay format.

To determine the recovery of ADA signal from NHS, various concentrations of each ADA were prepared in NHS or assay diluent. Each was then diluted ten-fold in assay diluent so that the final NHS concentration was zero or 10%. ADA samples with and without NHS were then analyzed using the basic assay format described above.

3. Results

3.1. Selection and screening for anti-Ids

Fabs specific to a human IL-13 mAb (Ab1) or to a human engineered IL-6 mAb (Ab2) were isolated by

targeting their unique CDR sequences using antibody phage display. To isolate CDR-specific Fabs a solution panning protocol was performed using the HuCAL GOLD® Fab phage library (Kretzschmar and von Ruden, 2002). Three rounds of panning were performed in the presence of human serum (10%) to eliminate binding to shared sequences in human IgG and fortuitous cross-reactivity with other serum proteins. For Ab2, a specific framework-matched mAb was added to the human serum to further ensure a specific selection to the CDR regions. Following selection, recovered phagemid was cloned *en masse* into a Fab expression vector and individual clones were screened for binding to the respective target Abs, followed by DNA sequencing to determine hit diversity. For Ab1, 51 of 185 chosen screened hits were unique by sequence analysis and for Ab2, 5 out of 26 were unique. These unique candidates consisted of a diverse group of V-region sub-families and all had lambda light chains.

To assess specificity, selected unique Fab clones were produced as cell lysate supernatants and screened for binding to human serum, biotinylated BSA, and a framework-matched antibody (Fig. 1). None showed reactivity to pooled human serum or the framework-matched antibody, demonstrating that the competition selection was effective in eliminating Fabs that bind framework and constant regions of the Abs or other abundant serum proteins. The selected Fabs also showed no binding to biotinylated bovine serum albumin and thus do not recognize the biotin label on the target Abs.

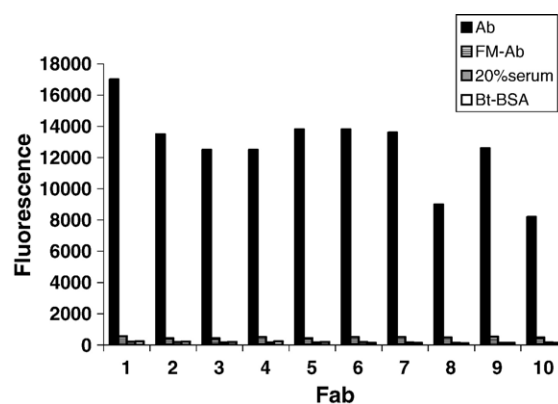


Fig. 1. Fab binding specificity. Ab (solid black), framework-matched antibody (horizontal hatch), 20% human serum (solid grey), and biotin-bovine serum albumin (solid white) were coated on the plate surface. Cell lysates containing Fabs 1–5 selected from the Ab1 (IL-13 mAb) project and Fabs 6–10 selected from the Ab2 (IL-6 mAb) project were tested for binding to the above reagents. Results represent an average of 2 experiments.

Table 1
BIAcore analysis

Fab	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (pM)
1	1.1×10^6	8.7×10^{-5}	81
2	1.2×10^6	1.9×10^{-4}	100
4	7.4×10^5	1.7×10^{-4}	230
5	6.8×10^5	7.4×10^{-4}	1000
7	1.4×10^6	5.0×10^{-4}	360
8	4.7×10^5	5.6×10^{-4}	1200
9	8.5×10^5	6.6×10^{-4}	780
10	1.3×10^5	8.5×10^{-4}	6500

Purified Fabs were applied to BIAcore chips coated with the Ab. The association (k_a) and dissociation (k_d) rates along with the calculated affinity constants (K_D) are given for each Fab.

3.2. Fab characterization

Fabs1, 2, 4, and 5 (for Ab1) and Fabs7-10 (for Ab2) were selected for further characterization based on having the highest signals in the primary screening assays. The Fabs were produced and purified to >90% homogeneity as estimated by SDS-PAGE (data not shown).

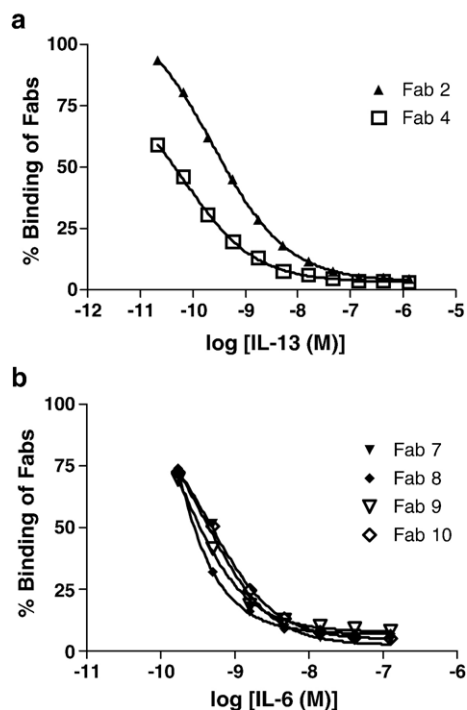


Fig. 2. Antigen competition ELISA. Serial dilutions of human IL-13 or IL-6 were used to compete with a fixed amount of the indicated Fabs for binding to the immobilized Abs. Fabs derived against Ab1 (IL-13) and Ab2 (IL-6) are represented in Fig. 4a and b, respectively. Results represent an average of 2 experiments.

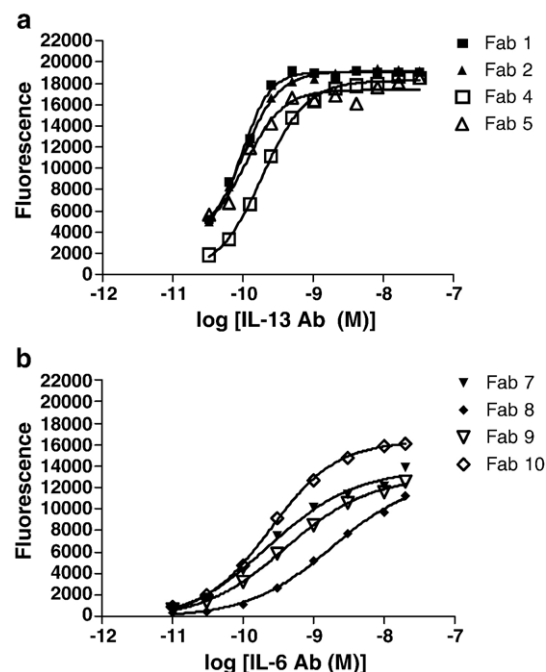


Fig. 3. Capture ELISA. Plates were coated directly with Fab to capture serial dilutions of biotinylated Ab. Fabs derived against Ab1 and Ab2 are represented in Fig. 3a and b, respectively. Results represent an average of 2 experiments.

The affinity of interaction between the Fabs and their respective target Ab was determined by BIAcore analysis. Ab1 or Ab2 were captured on protein A-coated sensor chips, and the kinetic constants for Fab binding were determined and used to calculate dissociation constants (Table 1). The affinities of the Fabs ranged from 0.08 to 1 nM for Ab1 and 0.3 to 6.5 nM for Ab2.

The Fabs were tested in an antigen competition assay to evaluate their specificity for the idiotypes of the Abs. For Ab1 only Fabs 2 and 4 were tested, based on their affinity and slightly distinguished activities in either capture or detection assays (see below). Varying amounts of the soluble cytokine antigens (IL-6 or IL-13) were used to compete with a fixed concentration of the respective Fabs for binding to Ab1 or Ab2 coated on the plate surface. For both Abs, antigen fully competed with the binding of the Fabs (Fig. 2). For Ab1, although of comparable affinity, Fab 2 gave a higher IC₅₀ than Fab 4, indicating that these Fabs recognize similar, but not identical, sites on Ab1 (Fig. 2a). For Ab2, IL-6 protein competed equally with all of the Fabs (Fig. 2b). These results, together with the lack of binding to serum or the framework-matched antibody, localize the binding site of all the Fabs to the idiotypic region of the CDRs of their respective target Abs.

To determine whether the Fabs bound to overlapping sites on the target Abs, a competition ELISA was performed. In this assay, a representative Fab was labeled with biotin, and the binding of the biotinylated Fab to plate-bound Ab1 or Ab2 was determined in the presence of serial dilutions of unlabeled Fabs. In all cases, the unlabelled Fabs fully competed with the binding of the biotinylated Fab to its respective target Ab, indicating overlapping recognition of a common site in the idiotype region (data not shown).

3.3. Sandwich detection assays

A typical pharmacokinetic assay is a sandwich ELISA that relies on the capture of the administrated Ab in serum samples by an immobilized anti-idiotypic antibody and detection using the same or different anti-idiotypic antibody. Although the detection antibodies may be cross-competitive, this format succeeds because of the bivalency of the therapeutic antibody. We screened the anti-id Fabs to identify binding. To evaluate capture efficiency, the Fabs were immobilized in an ELISA format followed by addition of serial dilutions of biotinylated Ab1 or Ab2 and detection with streptavidin-HRP (Fig. 3). All Fabs were active in capturing their

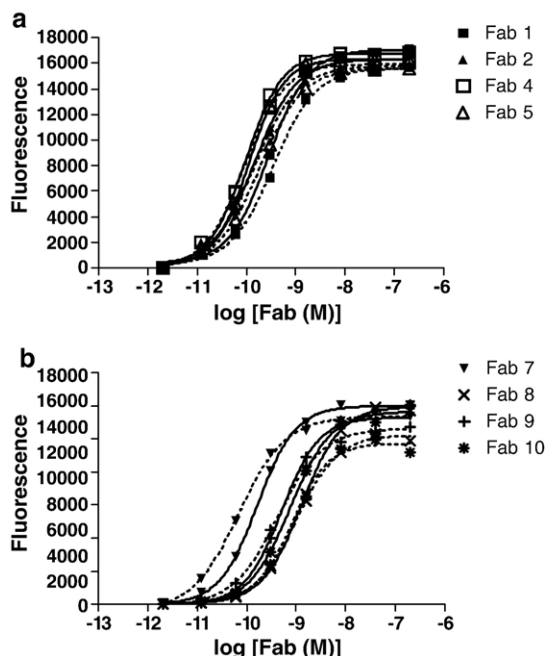


Fig. 4. Detection ELISA. Serial dilutions of purified Fab were added to immobilized Ab with 10% serum (dotted lines) and without 10% serum (solid lines). a) Ab1-binding Fabs; b) Ab2-binding Fabs. Results represent an average of 2 experiments.

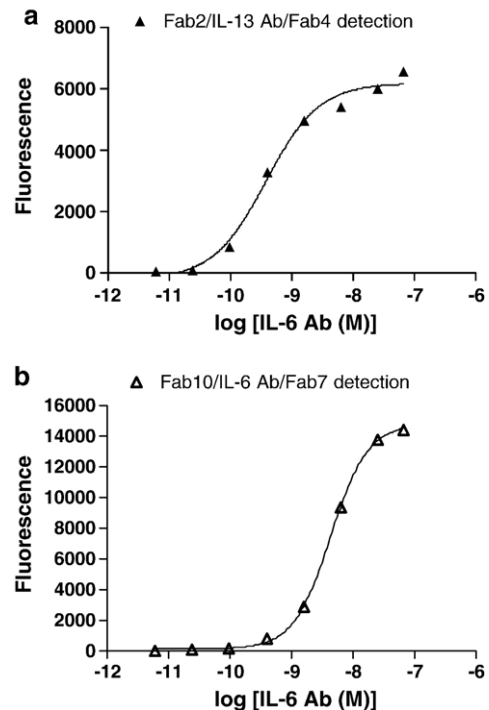


Fig. 5. Sandwich ELISA. For Ab1 (a), Fab 2 was coated directly on the plate, followed by serial dilutions of Ab1 in 10% human serum, and then detection with biotinylated Fab 4. For Ab2 (b), Fab 10 was coated directly on the plate, followed by serial dilutions of Ab2 Ab in 10% human serum, and then detection with biotinylated Fab 7. Results represent an average of 2 experiments.

target Abs, with Fabs1, 2, 5 and Fab10 showing the greatest sensitivity for Ab1 and Ab2, respectively. To evaluate detection efficiency, the rank order of binding of the soluble Fabs was measured to immobilized Ab1 or Ab2 in the presence of 10% human serum (Fig. 4). All of the Fabs from the Ab1 project had comparable binding activities, and Fab 7 from the Ab2 project showed the best binding in this assay format. Serum had a negligible effect on the Fab binding to Ab1, further demonstrating that the inclusion of serum in the phage selection process was effective.

The best capture and detection Fabs were used to establish sandwich assays for both target antibodies. For Ab1, Fab 2 was coated on the assay plate to capture Ab1 in the presence of serum, and the captured Ab1 was detected with biotinylated Fab 4. The binding EC_{50} in this study was 0.3 nM, which is within the sensitivity range estimated for clinical studies (Fig. 5a). For the Ab2, Fab10 was used as the capture agent and Fab 7 as the detection agent. This pair gave an EC_{50} of 3 nM (Fig. 5b). Although selective, this assay may not be sufficiently sensitive for clinical monitoring. Other Fab

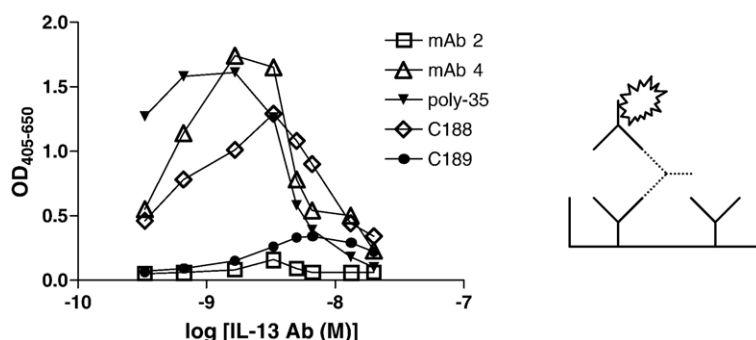


Fig. 6. Immunoresponse bridging assay. ADAs derived from mouse hybridomas (C188, C189), a cynomolgus monkey (poly-35), and phage display (mAb2, mAb4) were tested at constant concentrations. The immune response assay signal varied with the concentration of solid phase Ab1. Schematic depicts the bridging assay (Ab1 coated ELISA well, ADA binding (dotted line), capture of solution phase Ab1 with detection label). Results represent an average of 2 experiments.

combinations were configured for the sandwich assay but resulted in a lower strength of signal or a higher EC₅₀ (data not shown). Thus, the individual capture and detection assays identified a suitable pair and orientation for the sandwich ELISA.

3.4. Bridging assay for detection of an immune response to the Ab1

The clinical development of a therapeutic antibody requires measurement of the immune response (IR) to the administered therapeutic Ab, as measured by the occurrence of an anti-drug antibody (ADA). One approach to measuring ADA is a bridge ELISA, in which the therapeutic antibody is coated on an ELISA well followed by addition of test serum and detection with a labeled form of the therapeutic antibody. Because different reference antibodies may show optimal binding under different assay conditions, it is essential to establish the bridge assay using a suitable reference standard reflective of the native immune response. We tested five different reference antibodies, two murine anti-ids (C188 and C189), two of the phage-derived human anti-ids in a mAb format (mAb2 and mAb4) and a monkey polyclonal anti-serum (poly-35). Fig. 6 shows the binding of a fixed concentration of reference antibody to a variable amount of Ab1 coated on the plate. The optimal concentration of Ab1 coated on the solid phase was 0.5 to 1.0 µg/ml for both murine mAbs and 0.25 to 0.5 µg/ml for the phage-derived anti-ids and the monkey polyclonal serum. Phage mAb4, C188 murine mAb and the monkey serum gave the best sensitivity and 0.5 µg/ml of coated Ab1 was chosen for further assays.

To evaluate sensitivity and serum effects, the reference mAbs were titrated against coated Ab1 in the

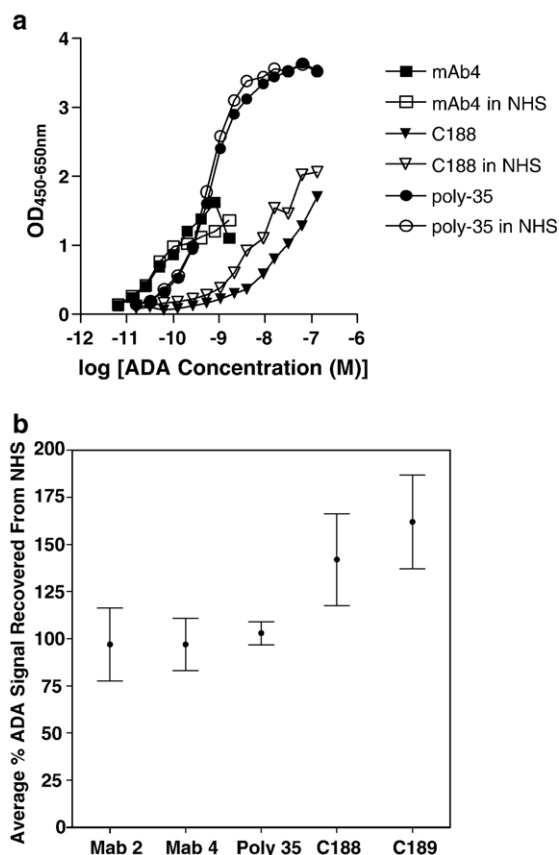


Fig. 7. ADA sensitivity and serum recovery. 7a) A titration of the ADAs derived from mouse hybridomas (C188, C189), a cynomolgus monkey (poly-35), and phage display (mAb2, mAb4) were prepared in assay diluent or NHS. Analysis of ADA sensitivity in the immune response assay indicated total and serum adsorbed ADA reactivity. Results represent an average of 2 experiments. 7b) The average ratio of binding signal with and without NHS for all five ADAs.

presence and absence of serum. All five reference antibodies were tested and results for the most sensitive antibodies (mAb4, C188 and the monkey serum) are shown in Fig. 7a. Mab4 was the most sensitive in terms of its lower EC₅₀ and the polyclonal serum in terms of total signal. An important property of a reference standard is its uniform recovery from varying concentrations of serum. Serum had little effect on the binding of mAb4 or the monkey serum, but substantially altered binding of murine mAb C188 by increasing the EC₅₀ approximately 50%. The effect of serum was further quantified by averaging the ratio of the binding signal in the presence and absence of serum over the full titration range of the reference antibody. These results are shown for all five test antibodies in Fig. 7b. The two phage-derived mAbs and the monkey polyclonal serum showed nearly equivalent signals in the presence or absence of serum, indicating that human serum had little or no effect on binding of these antibodies. However, both murine mAbs showed substantially higher binding ratios in the presence of serum. This enhanced binding ratio may reflect the formation of murine mAb aggregates with human antibodies other than Ab1. These results indicate that selection of anti-idiotypic antibodies by phage display successfully identified reference antibodies that mimic a primate ADA.

4. Discussion

Immune response testing is an aspect of the clinical safety evaluation of biological therapeutics required by FDA (Geng et al., 2005). As the technology has advanced from generation of murine to humanized or fully human therapeutic Abs, the challenge of detecting a humoral response, or ADAs, in clinical specimens is drastically increased. It is not possible to employ a simple sandwich ELISA using a secondary anti-human-antibody detection reagent to test for human ADAs when the drug itself is a human Ab. This issue is overcome with a bridging assay format in which the multivalent ADA is detected through its ability to cross-link a capture and detection form of the drug. To date, much consideration has been given to optimizing and validating assays intended to detect human immunogenicity (Mire-Sluis, 2004), however, there has been limited consideration of the reference analytes. For example, human anti-ids that specifically react to the therapeutic antibody during a trial are used to optimize and validate the assays (Geng et al., 2005). This source of analyte is rare and not constantly available making the assay validation process continual throughout clinical development.

In the past, typically non-primate anti-ids (principally rabbit, mouse, or rat) served as surrogate analytes during development, optimization and validation of immune response assays for a murine or a chimerized murine/human therapeutic mAb. Although these permit the development of bridge-type assays, one can no longer be certain of proper assay optimization for clinical use because a murine mAb cannot always accurately model a human ADA. Phage display technology provides a new solution to the problem of generating surrogate human immune response analytes. Our results showed that panels of high-affinity, human anti-ids to two therapeutic antibodies in pre-clinical and clinical development were readily generated by competition selection of a human Fab library, HuCAL GOLD®. The phage derived human anti-ids do not bind to serum proteins or to the framework-matched mAb. In addition, all the anti-ids isolated against a specific target were competitive with each other and with antigen binding to the Ab, indicating recognition of a common surface on the CDRs. Similarly, Goletz et al., 2002 reported the isolation of anti-ids by phage display as shown by competition with antigen binding to the target antibody.

We compared the performance of primate anti-serum, murine hybridoma-derived, and human phage-derived anti-ids in a bridging ELISA assay. For this assay format, balancing immobilized and solution phase drug antibody concentrations is often an important aspect of optimization, because it is typical for each ADA to bind optimally at a unique ratio of immobilized and solution phase Ab. The optimized assay conditions should be able to detect a wide variety of ADAs, or at least be able to detect ADAs representative of the study population. Frequently, ADAs from closely related species bind optimally under similar conditions. For example, we showed that two human phage-derived mAbs and a non-human primate anti-serum to Ab1 were best detected under similar assay conditions. By comparison, somewhat different conditions were ideal for the detection of two murine ADAs. If more than one reference analyte is available for immune response, it is best to have a pair that can show high and low reactivity profiles in the bridge ELISA as shown in Fig. 6, because they represent the heterogeneity of possible human responses to the therapeutic antibody. In this regard, the anti-ids mAb2 and mAb4 to Ab1 showed high (mAb4) and low (mAb2) reactivity to Ab1, and more closely mimicked the monkey polyclonal serum reactivity to the Ab1 than the two murine ids. Phage technology can purposely generate a panel of ids that have high and low reactivity to the target antibody by adjusting the anti-id selection

criteria; whereas, murine anti-ids usually have high affinity to the drug Ab due to hyperimmunization.

Another key characteristic of a desired reference human ADA for a clinical immune response assay is the drug Ab CDR binding exclusivity, with no cross-reactive binding to endogenous human serum antibodies and proteins. Phage derived anti-ids were selected in the presence of human serum and a framework-matched mAb to assure the CDR binding exclusivity. For example, the phage derived mAb2 and mAb4 could be detected much like non-human primate anti-serum in an immune response assay to Ab1. However, the murine hybridoma-derived mAbs gave spurious results, likely due to interaction with endogenous human Abs in the NHS matrix. In addition, because of its exquisite binding specificity, the human anti-id can be spiked into a human serum matrix in order to develop an assay that accounts for the performance of both the analyte and the matrix, or for use as a mock quality control sample in a validated assay.

For pharmacokinetic sandwich assays, the phage display Fabs showed good selectivity but borderline sensitivity, relative to murine anti-ids generated against the same human IL-13 mAb (data not shown). This reduced sensitivity most likely is due the lower affinity of the Fabs, which is typical for antibodies isolated from most primary phage libraries. However, affinity can be improved substantially by applying affinity maturation techniques or directed evolution to these primary antibodies and continuing the phage selection process (Hoogenboom, 2005; Wark and Hudson, 2006).

Another important application of anti-ids, is their potential for cognate antigen mimicry, applicable to protein therapy or vaccines (Jerne, 1974; Bona and Köhler, 1984; Fields et al., 1995; Langone, 1989; Nisonoff, 1991). Several results indicate possible structural, immunological, and functional native protein mimicry by anti-ids, including structural comparisons between antibody–anti-id complexes and those of the antibody with its cognate antigen (Williams et al., 1989; Bentley et al., 1990; Ban et al., 1994; Poljak, 1994; Goldbaum et al., 1997; Sege and Peterson, 1978; Kennedy and Dreesman, 1986; Bharracharya-Chatterjee et al., 2001). We evaluated ligand mimicry of the anti-ids against the IL-6 Ab in a cell proliferation assay and did not observe any IL-6 like activity. Similarly, anti-ids developed against a neutralizing antibody to a human chemokine failed to show ligand activity (unpublished). This lack of mimicry may be due to our focus on the selection of high-affinity, CDR-specific anti-ids, without regard for antigen mimicry. Together, the functional assay results suggest that functional mimicry of the original target antigen is at best restricted to a small

subset of anti-idiotypic antibodies (Amzel et al., 1994). Nonetheless, competition phage selection and the characterization assays were highly efficient for our intended purpose of obtaining selective human anti-ids. The phage derived anti-ids proved to be particularly suited as reference analytes for the development and validation of an immune response bridge ELISA, offering advantages over murine hybridoma-derived antibodies.

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