Multiplexed fluid array screening of phage displayed anti-ricin single domain antibodies for rapid assessment of specificity

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Phage display is a well-known technique that facilitates the selection of peptides or proteins that bind to a desired target. Using this tool, binding elements contained in the natural immune repertoires of the source animal or from a synthetically generated collection may be selected. The unpaired variable domain of the llama's heavy-chain-only classes of immunoglobulins represents an ideal source of genetic material to create phage display libraries. Initial panning of a semi-synthetic llama library yielded only one binder to the toxin ricin. In an effort to increase the number of monoclonal phage binders selected, the Luminex xMAP technology (Luminex, Austin, TX, USA) was used in addition to the enzyme-linked immunosorbent assay (ELISA) to screen clonal populations of phage after three rounds of selection. The xMAP technology detected phage displayed single domain antibody (sdAb) bound to ricin immobilized on the surface of microspheres under equilibrium conditions. This enhanced capability led directly to the identification of additional single domain antibodies of interest. The selected sdAbs were expressed, purified, and then evaluated for their specificity as well as enhanced thermal stability in comparison to conventional immunoglobulin G (IgG). We determined equilibrium dissociation constants and demonstrated their use as effective capture molecules in sandwich immunoassays.

INTRODUCTION

A variety of methods have been developed for the presentation and subsequent selection of binding elements from a library of candidates. These include techniques such as phage display (1–3), yeast display (4,5), *Escherichia coli* display (6), baculovirus/insect cell display (7), and ribosome display (8). While all useful, our group chose phage display, a common method for selection of binders from single domain antibody (sdAb) libraries (9–13). sdAbs, the smallest functional antibody binding unit, originated with the discovery that the entire Camelidae family possesses unique classes of heavy chain immunoglobulins (14). These antibodies, a result of a fortuitous genetic mutation, lack light chains. The variable domain of the heavy chain has several altered surface amino acids to increase its hydrophilicity to compensate for the lack of the light chain and forms

effective high-affinity binding sites for a wide variety of target molecules.

Many sdAbs have been derived using phage display libraries created from immunized camels or llamas, which allow the animal to enrich the phage pool for high-affinity binders (11,13). Others have focused on creating naive phage display libraries from which it is theoretically possible to extract binders for a nearly boundless number of targets (15,16); however, naive libraries may possess only low-affinity binders. While most applications require high-affinity binders, lower affinity binders may provide the starting point for affinity maturation. Affinity maturation has resulted in the attainment of binders having hundreds- to thousands-fold higher affinity than the starting antibody fragment (17,18). Unfortunately, identifying low-affinity clones using the standard enzyme-linked immunosorbent assay (ELISA) methodology can be highly problematic; since numerous wash steps are involved, low-affinity binders

can be easily lost. Thus, the ideal selection method would avoid wash steps and allow one to evaluate ligand-target interactions under equilibrium binding conditions. This would increase the likelihood of isolating sdAbs that possess the selectivity and stability desired, while absolute affinity could be addressed downstream. Recently, approaches that offer this avoidance of the washing steps required in the ELISA format have been demonstrated; namely, the use of surface plasmon resonance and standard flow cytometry (19,20). The method we chose to explore is a multiplexed flow cytometric approach using the Luminex 100 platform and xMAP technology. Using this specialized system, 100 different microsphere sets at a time can simultaneously be evaluated, thereby allowing us to rapidly examine the various phage display sdAb for their binding to our target, ricin, as well as to monitor for nonspecific binding to a number of nontarget proteins, in a high-throughput manner.

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MATERIALS AND METHODS

Materials

Ricin, ricin A chain, and ricin B chain were from Vector (Burlingame, CA, USA), cholera toxin (CTX) from Calbiochem (San Diego, CA, USA), and staphylococcal enterotoxin B (SEB) from Toxin Technology (Sarasota, FL, USA). Phosphate-buffered saline (PBS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal rabbit anti-ricin antibody and mouse monoclonal antibodies specific for ricin (MAb Ric-03-A-G1, MAb Ric-07-A-G1) were the kind gifts of Robert Bull (Naval Medical Research Center, Silver Spring, MD, USA). The anti-M13 antibody was purchased from GE Healthcare (Piscataway, NJ, USA). This antibody was fluorescently labeled using bis functional NHS-Cy3 ester (GE Healthcare) as described by the manufacturer.

ELISA and xMAP Monoclonal Phage Selections

The sdAb library was constructed and panned for ricin binders as described previously (9). The phage display vector is based upon an ampicillin-resistant derivative of the low-level expression phagemid pAK100 (9,21,22) with sdAb fused to full-length g3p with no intervening amber codon. Individual colonies of *E. coli* were randomly selected following the third round of panning. These were then grown in wells of a microtiter plate; afterwards crude phage preparations were prepared in the same wells. Meanwhile, wells of high-binding ELISA plates were coated overnight with ricin or 3 µg/mL bovine serum albumin (BSA). The next morning, wells were blocked with PBS with 2% low-fat dry milk for 1 h, then each phage was added to both target- and BSA-coated wells and allowed to incubate for 1 h. Excess phage was washed off with PBS with 0.5% Tween-20 (PBST) and the binding was detected using an anti-M13-horseradish peroxidase (HRP) conjugate (GE Healthcare). Excess conjugate was washed off with PBST/PBS, and HRP activity was determined by colorimetric o-phenylenediamine dihydrochloride

Figure 1. Signals from xMAP and enzyme-linked immunosorbent assay (ELISA) screens. xMAP data shown for signal on intact ricin, ricin B chain, and ricin A chain. The data was adjusted for nonspecific binding of phage to bovine serum albumin (BSA) and standardized to a positive control (microspheres coated with the anti-M13 antibody). ELISA data are shown as the ratio between the signal on toxin and the signal on BSA, averages of three wells. Clones with a ratio over 2 were considered positive.

(OPD) substrate (Sigma-Aldrich) (Figure 1, lower panel).

Microspheres (Lx) were coated with a variety of proteins using the two-step carbodiimide coupling protocol provided by the manufacturer (Luminex, Austin, TX, USA). To screen for ricin binding phage display proteins using the Luminex xMAP technology, 10 µL phage from each well were added to a mixture of 50 µL microspheres coated with either ricin, ricin A chain, ricin B chain, Mab anti-M13, BSA, SEB, and CTX. After 1 h incubation, 10 µg/mL Cy3-labeled anti-M13 antibody were added to each well and incubated for an additional 1 h prior to measurement. Clones that showed high binding by either method were sequenced.

sdAb Protein Production

Representatives of unique sdAb genes were mobilized to pecan22, a high-level periplasmic expression vector based on pMoPac10 (22) but encoding only a C-terminal His6 tag instead of His-myc tag. Constructs were transformed into *E. coli* Tuner (Novagen, Madison, WI, USA). The sdAb proteins were isolated from the periplasmic compartment of 500-mL scale shake flask cultures by osmotic shocking, immobilized metal-ion affinity chromatography, and gel filtration on a Superdex G75 column (GE Healthcare) (22). Proteins were quantified using micro-bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and kept at 4°C prior to analysis.

Figure 2. Sequences of selected binders. Sequences were aligned using the Multalin program (a multiple alignment method with hierarchical clustering) (27). LRE7, LR10F, and LR10B were identified by both enzyme-linked immunosorbent assay (ELISA) and xMAP screening. The remaining sequences were found through use of the xMAP screening method. The three hypervariable regions are underlined.

sdAb Binding and Stability Assays

The signal for all xMAP fluid array binding experiments is reported as the median fluorescence intensity (MFI). For sdAb direct binding assays, sdAb was added to the top row of a 96-well microtiter plate and diluted serially down the plate using PBST with 1 mg/mL BSA (PBSB). To each well, a mixture of toxin-coated microspheres was added (>100 of each type); they were allowed to incubate with the sdAb for at least 30 min to permit the initial binding to approach equilibrium. Then 10 mg/L fluorescent reporter, Ni-streptavidin-R-phycoerythrin (Ni-SA-PE), were added and permitted to bind for 30 min prior to measuring.

For the thermal stability tests, 30 μ L sdAb (100 μ g/mL) or conventional antibody (10 μ g/mL) were heated for the times specified in a thermal cycler (Tetrad 2; MJ Research, Bio-Rad Laboratories, Hercules, CA, USA). This material was allowed to cool to room temperature and tested at 3 and 1 μ g/mL for direct binding to toxin-coated microspheres as described above.

For the assays using sdAb as the capture reagent, ricin dilutions were prepared in PBSTB in columns of a microtiter plate. A mixture of sdAb covalently coated microspheres was added to each well and incubated for 30 min. Then the recognition antibodies, MAb Ric-03-A-G1, MAb Ric-07-A-G1, and SA-PE, final concentration 10 μ g/mL each, were added and incubated 30 min prior to measuring.

RESULTS

The Nomad #1 library was subjected to three rounds of panning for the selection of ricin binders (9); the success of the panning was confirmed by polyclonal

phage ELISA. Ninety-six clones from the third round were grown, and phage were prepared for monoclonal phage ELISA and xMAP evaluation (Figure 1). Monoclonal phage ELISA (Figure 1, lower panel) showed five positive clones giving a target to BSA signal ratio of over 2. The positive phage clones from the plate along with clones from the same microtiter plate column, as well as other random clones, were then examined by xMAP detection (Figure 1, upper panel) for binding to ricin, BSA, CTX, and SEB. xMAP evaluation of the 40-phage supernatant showed at least three times more positive clones. None of the phage tested showed significant binding to either CTX or SEB (not shown). Since such a large number of positive clones were obtained, we did not screen the other 56 clones.

Seventeen clones (fifteen ricin/ricin B chain binders and two potential ricin A chain binders) were selected for sequencing (Figure 1). Six unique sequences were identified: LR10F (onerepresentative), LR3D (one representative), LRE7 (our previously isolated sequence, four identified in this screening, LR3F, LR8F, LR10G, and LR12B), LR10B (eight representatives), LR12D (one representative), and LR10H (two representatives) (Figure 2). Three of these sequences, LRE7 (LR3F, LR8F, and LR10G), LR10F, and LR8E (LR10B) were identified by both ELISA and xMAP, and the remaining three sequences were found through use of the xMAP screening method. LR12D has the same complementarity determining regions (CDRs) as the LR10B sequences with five differences in framework regions.

Binding Specificity of the Anti-Ricin sdAb

Increasing concentrations of each sdAb were added to mixed sets of micro-

spheres that had ricin, ricin B chain, ricin A chain, BSA, or SEB covalently attached. After reaching equilibrium, the amount bound to each bead type was evaluated. All the sdAb tested showed a degree of specificity for ricin, specifically the ricin B chain, and little-to-no binding to BSA-coated microspheres was observed (not shown). However, their selectivity fell into two groups: three clones (LR10H, LR10F, LR10B) showed excellent specificity (Figure 3, B, D, and F), while the other three clones (LR3D, LR12D, LRE7) showed significantly elevated signal on the irrelevant proteins at higher sdAb concentrations (Figure 3, A, C, and E). For each of the new sdAb ricin binders, we determined equilibrium binding constants for the binding to both intact toxin and ricin B chain (Table 1) from the equilibrium binding curves shown in Figure 3 (23,24).

Thermal Stability of Anti-Ricin sdAb

sdAb and conventional monoclonal and polyclonal anti-ricin antibodies were heated at 95°C for various time points up to 60 min. After cooling to room temperature, their binding activity was assessed (Figure 4). Half of the binders (LR10F, LR10H, LR10B) maintained both their binding ability as well as specificity after heating (Figure 4, A, C, and D). The other clones (LR3D, LR12D, LRE7) greatly increased their binding to irrelevant targets after an extended 95°C incubation (Figure 4, B, D, and F). Both polyclonal antibodies lost ricin binding ability within the first few minutes of the incubation, while the best ricin monoclonal lost binding ability after 20 min (Figure 4G); nonspecific binding for all the conventional antibodies was minimal (not shown).

sdAb as Capture and Recognition Molecules in Sandwich Assays

Each sdAb was covalently attached to a different set of microspheres and tested in conjunction with sdAbs (also attached to beads) that were not directed toward ricin. Figure 5A shows the earlier obtained clone as a capture reagent in the detection of ricin. A limit of detection for ricin of approximately 12 ng/mL was obtained. Figure 5B shows an assay testing all the other clones (solid symbols) as capture reagents along with previously isolated clones for CTX and SEB (open symbols). Three of the clones (LR12D, LR10F, LR10B) showed significant signal over background down to as little as 1 ng/mL. Only LR3D failed to show clear binding versus the control bead sets. Of note was the fact that at high concentrations of ricin, >100 ng/mL, measurable nonspecific binding occurred on the control microspheres.

DISCUSSION

Screening of phage clones using equilibrium binding conditions resulted in the identification of many more positive phage than found in ELISA screening. After determining the sequences of those selected, most of the phage were found to be duplicates of clones picked up by ELISA; however, we did double the number of unique clones obtained by use of the xMAP system from three to six. One of these clones, LR10H, is among the most specific and thermally stable representatives identified. In addition, the xMAP assays use only a small fraction of the phage needed for the ELISA and allow simultaneous screening for binding to a number of proteins. Screening phage for binding to irrelevant proteins (BSA, SEB, and CTX), as well as intact ricin and its subunits (ricin A chain and ricin B chain), provided an early indication of specificity. The multiplexing capability also allows inclusion of a positive control, microspheres coated with anti-M13 antibody; this allows one to adjust for phage number in the crude supernatants, a refinement no other method currently includes. Of the clones screened, two clones appeared to weakly bind ricin A chain, LR10D and LR12B, and the sequence of those clones proved identical

Figure 3. Binding specificity of the six unique single domain antibody (sdAb) clones on ricin (black squares), ricin B chain (black circles), ricin A chain (gray upside down triangles), and staphylococcal enterotoxin B (SEB; gray triangles). (A) LRE7, (B) LR10B, (C) LR3D, (D) LR10F, (E) LR12D, (F) LR10H. The data had an average SD of 9.1%.

Table 1. Equilibrium Dissociation Constants

Binder	K _d Ricin (nM)	K _d Ricin B Chain (nM)
sdAb-LRE7	291 ± 56	2424 ± 1000
sdAb-LR10F	30 ± 6	20 ± 3
sdAb-LR10H	140 ± 20	98 ± 10
sdAb-LR3D	18 ± 2	8 ± 2
sdAb-LR12D	12 ± 2	10 ± 2
sdAb-LR10B	24 ± 4	10 ± 1
Cy3-Mab-Ric07AG1	1.2 ± 0.1	N/A ^a
Bt-Mab-Ric07AG1	0.3 ± 0.1	N/A ^a
Bt-Mab-Ric03AG1	0.5 ± 0.1	N/A ^a

^aThe monoclonal antibodies bind to the ricin A chain.
K_d, affinity constant.

to sdAbs that were confirmed B chain binders. When the affinity constants (Kds) of the new sdAbs were evaluated, all the clones possessed Kds lower than the previously obtained LRE7. Thus, while the xMAP provides the ability to identify lower affinity interactions and monitor for nonspecific binding, it is still just an initial screen. The true nature of that clone is still best evaluated following production of the soluble protein.

One of the key advantages that sdAbs offer over conventional antibodies is their unique thermal stability. However, not all sdAbs are created equal, with

some being more stable than others (9,10,25,26); this was found to be equally true for our anti-ricin sdAb. While some clones showed remarkable thermal stability, being able to refold nearly quantitatively even after extended heating, others rapidly lost their specificity; only giving the appearance of thermal stability if one failed to monitor the appropriate controls. This contrasts with data from other clones isolated from this library, which clearly lose binding activity over time without showing an increase in nonspecific binding (9). The ultimate desire in the

selection of sdAbs that bind toxins such as ricin is to replace conventional antibodies in immunoassays that have limited shelf lives with these robust small protein molecules. Toward that end, we also tested this current generation of sdAbs as capture elements. In the experiments shown, the sdAbs were covalently attached to microspheres. Most of the sdAbs were able to capture ricin at reasonably low concentrations, aided by the fact that the monoclonal antibodies used as the recognition molecules bind to epitopes on the ricin A chain, permitting completion of the sandwich. The use of affinity maturation to obtain higher affinity binders and the addition of sequences to permit the oriented deposition of the sdAb should aid considerably in their utility as capture immunoassay elements.

In conclusion, we have demonstrated the utility of xMAP-based phage binding analyses as an alternative method for the screening of monoclonal phage displayed proteins. This method allowed us to obtain significantly more binders to the target of interest, thereby enhancing the

Figure 4. Thermal stability of representative conventional antibodies and single domain antibodies (sdAbs). Panels A through F show the binding of the six sdAbs on ricin B chain, cholera toxin (CTX), bovine serum albumin (BSA), and staphylococcal enterotoxin B (SEB) after heating to 95°C for various time points. (A) LR10F, (B) LR3D, (C) LR10H, (D) LR12D, (E) LR10B, (F) LR12D. Panel G shows the binding activity of two polyclonal antibodies and two monoclonal antibodies on ricin after extended heating at 95°C.

Figure 5. Anti-ricin single domain antibodies (sdAbs) as capture reagents. (A) The previously isolated LRE7 as a capture (solid circle) along with control anti-staphylococcal enterotoxin B (SEB) capture (open symbols) in sandwich assays for ricin using an anti-ricin monoclonal antibody as a reporter. (B) The newly isolated anti-ricin clones (solid symbols) as capture reagents along with control anti-cholera toxin (CTX) and anti-SEB (open symbols) in sandwich assays for ricin using an anti-ricin monoclonal antibody as a reporter. The data had an average SD of 7.2%.

odds that one with suitable characteristics could be obtained.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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