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Facile Generation of Heat Stable Antiviral and Antitoxin Single Domain Antibodies from a Semi-synthetic Llama Library

Ellen R. Goldman[1,*], **George P. Anderson**[1], **Jinny L. Liu**[1], **James B. Delehanty**[1], **Laura J. Sherwood**[2], **Lisa E. Osborn**[2], **Larry B. Cummins**[3], and **Andrew Hayhurst**[2,*]

1*Center for Bio/Molecular Science and Engineering, U.S. Naval Research Laboratory, Washington, DC 20375*

2*Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, TX 78227-5301*

3*Department of Comparative Medicine, Southwest Foundation for Biomedical Research, San Antonio, TX 78227-5301*

Abstract

Llamas possess a class of unconventional immunoglobulins that have only heavy-chains; unpaired heavy variable domains are responsible for antigen binding. These domains have previously been cloned and expressed as single domain antibodies (sdAbs); they comprise the smallest known antigen binding fragments. SdAbs have been shown to bind antigens at >90°C and to refold after being denatured. To take advantage of the remarkable properties of sdAbs we constructed a large, semi-synthetic llama sdAb library. This library facilitated the rapid selection of binders to an array of biothreat targets. We selected sdAb specific for live vaccinia virus (a smallpox virus surrogate), hen egg lysozyme, cholera toxin, ricin, and staphylococcal enterotoxin B. The selected sdAb possessed high specificity as well as enhanced thermal stability in comparison to conventional IgG and scFv antibodies. We also determined equilibrium dissociation constants as well as demonstrated the use of several anti-toxin sdAbs as effective capture and reporter molecules in sandwich assays on the Luminex instrument. The ability to rapidly select such rugged antibodies will enhance the reliability of immunoassays by extending shelf-life, and the capacity to function in hostile environments.

Introduction

Environmental surveillance is being strengthened by advances in accurate, timely and reliable immunoassays for contaminants ranging from harmful microorganisms and their toxins, to herbicides, pesticides and poisonous industrial byproducts [1-4]. Many of the same immunoassay formats are now being applied to the monitoring of air, food and water sources for deliberate contamination with biothreats [5, 6]. Immunoassays can be applied to high throughput multiplex analyses on microarrays [7, 8], bead based arrays [9] and portable multi-channel mass sensors which are capable of directly monitoring the presence of several threats in real time [10]. Just as important, immunoassay platforms can be simple, effective and affordable field portable ELISA screens [11] and lateral flow type assays [12]. In all applications, it is essential that the contaminant specific antibodies are not only exquisitely sensitive and specific but also very

*Corresponding authors: Mailing address for E. R. Goldman: CBMSE Code 6920, Naval Research Laboratory, 4555 Overlook Ave. SW., Washington DC 20375. Phone: 202-404-6052. Fax: 202-767-9594. E-mail: ellen.goldman@nrl.navy.mil. Mailing address for A. Hayhurst: Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, TX 78227-5301. Phone: 210-258-9530. Fax: 210-670-3329. E-mail: ahayhurst@sfbr.org.

durable possessing prolonged assay shelf life and the ability to withstand extended periods of operation in extreme temperatures.

Antibodies are unparalleled in their capacity to bind a diverse array of antigens with high specificity and high affinity. Most rapid environmental diagnostic assays rely on monoclonal or polyclonal antibodies (IgG) as their recognition elements. These antibodies are large complex 150 kDa molecules made up of 2 heavy chains and 2 light chains with the antigen binding site formed by combinations of amino acids in both the variable (V) light and heavy domains. Their multi-domain complexity is their Achilles heel since at high temperatures >60-70°C the heavy and light chains unfold and aggregate, causing the antibody to precipitate irreversibly [13]. Furthermore, IgG are time-consuming and costly to produce, requiring large amounts of antigen to immunize animals to deliver polyclonal sera or hybridomas for monoclonal antibodies. While *in vitro* derived libraries of IgG fragments composed of the antigen binding arms (Fab) or V domains alone (scFv) can rapidly bypass the requirement for immunizations and high antigen concentrations [14] the final molecules are usually more unstable than an equivalent IgG. Consequently, immunoassays relying on conventional immunoglobulins or their recombinant derivatives often require refrigeration to extend shelf-life and may have limited lifespan in the field before needing replacement.

In the mid 1990s it was found that certain animals, such as camelids (i.e. camels and llamas) and sharks, can naturally make antibodies that consist of heavy chains only [15, 16]. The V domains of these antibodies represent the smallest naturally occurring antigen binding domains known and have 3 (camels and llamas) or 2 (sharks) recognizable hypervariable regions or complementarity determining regions (CDRs) that mediate antigen contact and are borne on a relatively conserved scaffold of framework regions (FRs). These V domains have been cloned and expressed as 12-15kDa proteins known as single domain antibodies (sdAb) (see figure 1a). SdAbs have been found to be inherently thermostable, with antigen binding of llama sdAbs being demonstrated at 90°C [17], which suggests they will be well suited for long-term field applications where refrigeration is often not possible. SdAbs have also shown to be extremely plastic in that when they do eventually undergo denaturation, they are often capable of quantitative refolding [18, 19]. Such beneficial properties have already been utilized for an immunoaffinity chromatography column that withstood >2000 regenerations [20] indicating sdAb are well suited for the establishment of recyclable immunoassays.

SdAb genes can be RT-PCR cloned from the mRNA of peripheral lymphocytes of animals immunized with the antigens of interest to generate biased or immune libraries. A display methodology such as phage [21] is then used to isolate antigen binding clones from the large background of non-binders. Although camels [22], llamas [20] and nurse sharks [23] have all been immunized to yield binders, this route is often unavailable when targeting potentially lethal and transmissible agents. The process is also costly in terms of animal housing, husbandry and antigen quotas with the process often taking several months for the animals to raise a sufficiently high immune response.

Alternatively, non-immunized animals can be used to generate unbiased or non-immune libraries from which antigen binders can be selected. Since the range of germline sequences is somewhat restricted and no antigen specific *in vivo* affinity maturation has occurred, these libraries tend to yield μM affinity clones [24, 25]. CDRs may also be randomized during assembly of sdAb genes to confer more diversity at the antigen binding surfaces than occurs naturally to increase the probability of the libraries containing clones with higher affinities [26-28]. Once made, such “single-pot” non-immune libraries can be stored indefinitely and used to generate binding clones against an unlimited number of antigens since the library can be reamplified and the “pot” replenished with relatively simple techniques. However, the dual role that several CDR3 amino acids play in both antigen binding and structural interaction with

neighboring FR residues, can make it difficult to expand antigen binding diversity using typical CDR randomization strategies without losing sdAb ruggedness unless consideration is paid to each and every position [29].

We have constructed a novel type of llama sdAb single pot library of 10^9 members that was assembled from hyperdiversified framework/CDR combinations of a 10^6 member non-immune library. Our approach was geared towards having sufficient antigen binding diversity yet retaining ruggedness. First, we gently diversified the CDR and local framework amino acid sequences by error prone PCR. Second, we spliced these sequences together through the common FR overlaps to randomly shuffle them. From this novel library, we could isolate sdAb proteins to a range of targets in a matter of days. Our targets included live vaccinia virus (a smallpox virus surrogate), the model protein hen egg lysozyme (HEL), and the toxins cholera toxin (CTX), ricin, and staphylococcal enterotoxin B (SEB). The panel of sdAb proteins was evaluated for their target specificity and heat stability in comparison to conventional antibodies and scFv. In addition, the equilibrium dissociation constants for the toxin-specific sdAbs were determined, their ability to bind soluble target was evaluated, and their potential as both capture and recognition molecules in Luminex fluid array sandwich immunoassays was demonstrated.

Experimental Section

All experiments at SFBR were performed in a CDC certified BSL2 laboratory with virus sample manipulations being performed in an approved class II biosafety cabinet. All experiments at NRL were performed following applicable federal safety regulations; protocols for the handling of the toxins were approved by the NRL biosafety review committee.

SdAb library construction

A schematic figure of library construction is shown in figure 1b. Small blood samples (6 ml) were drawn from the left jugular vein of three research naïve mature llamas (1 male, 2 female) and combined with RNALater (Ambion, TX). RNA was extracted using RiboPure blood extraction kit (Ambion, TX), and cDNA synthesized using oligo-dT primed reverse transcription with a RETROscript kit (Ambion, TX). Primers for the PCR amplification of llama heavy chain only V domains were based on the findings of Ghahroudi and van der Linden and colleagues [17, 30] and were modified to allow unidirectional cloning using *Sfi*I sites. SdAb genes were cloned into a chloramphenicol resistant open reading frame (ORF) selection vector which fuses inserts to B-lactamase and confers conditional ampicillin resistance to transformants plated on IPTG containing media. Clones were selected on media containing IPTG and ampicillin to make an initial “megalibrary” of approximately 10^6 sdAb ORFs which was scraped, combined with glycerol and frozen. Forty eight clones of the megalibrary were sequenced and found to be unique with diverse CDRs. Highly conserved regions within FR2 and FR3 were used to design forward and reverse primers to segment the sdAb gene. These primers in concert with primers flanking the sdAb inserts were used in error prone PCR [31] of phagemid DNA extracted from the megalibrary to amplify each segment of the sdAb insert. These segments were gel purified and used in splice overlap extension (SOE) PCR to create hyperdiversified full-length sdAb gene repertoires. The sdAb repertoire was cloned into pecan21 which is a phage display vector with the low expression capacity of pAK100 [32] borne on the ampicillin resistant pMoPac10 [33]. The vector has a full length gene III protein (g3p) PCR amplified from M13KO7 with two tobacco etch virus (TEV) protease cleavage sites encoded between insert and g3p to allow protease elution of phage. Approximately 10^9 individual transformants were obtained in XL1-Blue to create a “gigalibrary” Nomad#1. Following rescue with M13KO7 and phagemid purification [34], the library was resuspended in PBS, combined with an equal volume of glycerol, aliquoted and stored at -80°C.

SdAb selection

Vaccinia (strain Western Reserve) was grown in Vero-E6 cells (12×225 cm^2 flask scale), harvested when the cytopathic effect (CPE) was maximal by freeze thaw lysing. Virus was purified by centrifugation onto a sucrose cushion and then through one sucrose gradient essentially as described by Moss and colleagues [35]. Virus was plaque titrated in 6 well plates using crystal violet staining. Gamma irradiated SARS-CoV (severe acute respiratory coronavirus) was kindly provided by Matthias Niedrig, Robert-Koch Institute, Berlin. Influenza A strain PR/8/34 was purchased from ATCC. HEL was purchased from Sigma (St. Louis, MO), CTX from Calbiochem (San Diego, CA), ricin from Sigma (St. Louis, MO) and Vector (Burlingame, CA), and SEB from Toxin Technology, Inc (Sarasota, FL).

Selection was carried out essentially as described previously (Griffiths *et al*, 1994). Briefly, targets were immobilized overnight at 4°C on the wells of high binding ELISA plates using the following conditions: 10^6 pfu of vaccinia in 5 × 100 μl PBS, and 50 μg of protein target in 5 × 100 μl PBS. The next day plates were washed with PBS and blocked with PBS containing 2% (w/v) non-fat powdered milk (PBSM), and 10^{11} cfu of Nomad#1 phage in PBSM were applied to each antigen containing well and incubated an hour on a microtiter plate shaker. Excess phage were washed with 20 washes of PBS plus 0.1% Tween-20 (PBST) and 20 washes of PBS. Phage from the vaccinia virus selection were eluted with 100 μl of a TEV protease cocktail (Invitrogen, Carlsbad, CA) which cleaves the sdAb from the phagemid particles. In the cases of the protein selections, a five-minute base elution with 100 mM triethylamine followed by neutralization with 0.5 volumes of 1M Tris-HCl pH 7.5 was used to isolate binding phage. In all cases, phage eluted from each antigen were combined and were then used to infect *E. coli* (XL1-Blue or TG-1). The polyclonal phage populations were amplified and rescued by M13K07 helper phage (New England Biolabs, Beverly MA) to generate phage displaying sdAb to be used for the next round of panning.

After 3 rounds of panning, we performed polyclonal phage ELISA to monitor the success of the selection. Pools of phagemid from each round (about 10^{10} cfu in PBSM) were incubated on wells coated with target and irrelevant antigen. Binding was detected using an anti-M13-HRP conjugate (GE Healthcare, Piscataway, NJ). We then used monoclonal phage ELISA to identify individual positive clones, which were then sequenced to identify unique sdAb genes.

SdAb protein production

Representatives of unique sdAb genes were mobilized to pecan22, a high level periplasmic expression vector based on pMoPac10 [33] but encoding only a C-terminal His6 tag instead of His-myc tag. Constructs were transformed into *E. coli* Tuner + pRARE (Novagen, Madison, WI). The sdAb proteins were isolated from the periplasmic compartment of 500mL scale shake flask cultures by osmotic shocking, IMAC and gel filtration on a Superdex 200 column (GE-Healthcare) [33]. Proteins were made to 50% glycerol and stored at -80 °C or kept at 4 °C short term prior to analysis. Proteins were quantified using micro-BCA assay (Pierce, Rockford, IL).

ELISA

Wells of high binding ELISA plates were coated overnight with cognate antigen as well as an irrelevant antigen. Antigen coated wells were blocked with PBSM for one hour. Duplicate serial dilutions of sdAb were applied in PBSM to wells coated with both target and control and binding allowed to occur for 1 hour. Excess sdAb was washed off with PBST/PBS and anti-His6 HRP conjugate (Sigma, St. Louis MO) was added at the recommended dilution for 1 hour in PBSM. Excess conjugate was washed off with PBST/PBS and HRP activity determined by colormetric OPD substrate (Sigma) or chemiluminescent Super Signal Pico substrate (Pierce, Rockford, IL).

SdAb thermal stability by ELISA

SdAb, conventional antibody or scFv were heated for 5 minutes in 100 μL of HEPES buffered saline in 200 μL volume PCR tubes in a DNA engine (MJ Research). Samples were allowed to cool to room temperature (22-25 °C) for 20min before applying to ELISA plates coated with target or control protein and proceeding with our standard ELISA protocol as described above. In these experiments, the sdAb proteins were at 10 μM, IgG were diluted 10^{-3} and scFv was at 0.07μM. Anti-vaccinia antibodies (mouse monoclonal and rabbit polyclonal) were purchased from the Critical Reagents Program (CRP). The gene encoding anti-HEL scFv, D1.3 [36] was provided by the Medical Research Council, England and was subcloned to vector pecan22, mobilized to Tuner + pRARE, expressed and purified as scFv-His6 as above.

Preparation of Luminex immuno-reagents

Polyclonal rabbit anti ricin antibodies, and mouse monoclonal antibodies specific for ricin (Mab Ric-03-A-G1, Mab Ric-07-A-G1) and SEB (Mab SEB03b2a) were the kind gifts of Dr. Robert Bull (Naval Medical Research Center, Silver Spring, MD). Both goat and rabbit anti-CTX were purchased from Biogensis (Kingston, NH). The affinity-purified sheep anti-SEB was purchased from Toxin Technology (Sarasota, FL). Antibodies utilized as fluorescent reporters were labeled with Cy3 (GE Healthcare) by addition of 1 vial of Cy3 dye as supplied to 3 mg of antibody in PBS with 1/2 volume of sodium borate + 100 mM sodium chloride pH 9.1. After 1 hour labeling at room temperature, the Cy3-labeled antibody was separated from free dye by gel filtration on a Bio-gel P10 column (Bio-Rad, Hercules, CA). We determined the antibody concentration and the dye-to-protein ratio (which varied between 2 to 4) by UV-Vis absorption as described by the manufacturer. Antibodies were biotinylated using NHS-LC-Biotin (Pierce) dissolved in dimethyl sulfoxide (1.4 g/L). The antibodies were reacted with a 5:1 molar excess of the NHS-LC-Biotin; the pH was increased by the addition of ½ volume of 100 mM sodium borate + 100 mM sodium chloride pH 9.1. After 1 hour at room temperature, the biotinylated antibodies were separated from free biotin by gel filtration and concentrations determined by absorbance at 280 nm.

The Ni-streptavidin phycoerythrin (Ni-SA-PE) was prepared by addition of a >10 molar excess of biotin-X NTA (Biotium, Hayward, CA) to SA-PE (Molecular Probes, Eugene OR). This material was then charged by addition of 50 mM $NiSO_4$. The excess $NiSO_4$ and biotin-X NTA were removed from the Ni-SA-PE by gel filtration on a Bio-gel P10 column equilibrated with PBS.

Preparation of Luminex microspheres

Luminex microspheres (Lx) were coated with NeutrAvidin (Pierce, Rockland, IL), CTX, ricin, SEB, or the various sdAbs using a modification of the protocol provided by Luminex for two-step carbodiimide coupling. Briefly, 0.1 mL of the carboxylated microspheres were diluted with 0.1 mL of 0.1 M sodium phosphate buffer pH 6.2 (PB), and washed by centrifugation in a Eppendorf microfuge at 14krpm for 5 minutes. The supernatant was discarded, and the microspheres were vortexed in 0.2 mL PB to resuspend, then washed as before. After centrifugation, the beads were resuspended in 0.1 mL PB. The carboxyl groups were then activated by addition of 10 μL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce) (50 g/L in DMSO) and 10 μL of N-hydroxysulfosuccinimide sodium salt (Pierce) (50 g/L in water). After incubating for 30 minutes at room temperature, the beads were then washed as above in 0.1 mL PB, followed by 0.5 mL PBS. After a final centrifugation, the beads were resuspended in 0.1 mL PBS containing the coating protein (0.1 - 5 g/L), and were allowed to couple overnight at 4°C in the dark. After coupling, unbound protein was removed by adding 0.4 mL of PBS with 0.05% Tween-20 (PBST), and centrifuging as before. The beads were again washed with 0.5 mL PBST and finally resuspended in 0.1 mL PBST + 1 g/L bovine serum albumin (PBSTB). Between steps the tubes were vortexed briefly or placed

in a bath type sonicator to disperse the microspheres as needed. The protein coated microspheres were stored at 4°C in the dark until use.

Luminex sdAb assays

The signal for all Luminex experiments is reported as the median fluorescence intensity (MFI). For the sdAb direct binding assays the sdAb to be tested were added to a 96 well microtiter plate and serial dilutions prepared using PBSTB. To each well a mixture of toxin coated microspheres was added in sufficient quantity to provide > 100 beads of each type being used. For most assays, we utilized a mixture of four different bead sets: two coated with CTX, one with ricin, and one with SEB. Thus for each experiment, one (SEB and ricin assays) or two (CTX assay) bead sets generated a positive signal and the remaining non-target coated sets acted as negative controls. After the Luminex microspheres had been added, they were allowed to incubate with the sdAb for 30 minutes to permit the initial binding to approach equilibrium. Then the fluorescent reporter, Ni-SA-PE, was added (10 mg/L) and permitted to bind for 30 minutes prior to measuring the bound fluorescence using the Luminex100. For the competitive assays, dilutions of target toxin in PBSTB were prepared in columns of the microtiter plate, to which the mixture of toxin-coated Luminex microspheres were added. 1 mg/L of each sdAb was then added to each well, incubated 15 minutes prior to addition of Ni-SA-PE (10 mg/L), followed by a 30 minute incubation and measurement by the Luminex100.

For the thermal stability tests, 30 μl sdAb (100 mg/L) or conventional antibody (10 mg/L) were heated for the times specified in a thermal cycler (Tetrad 2, MJ Research). This material was allowed to cool to room temperature and tested at 3 and 1 μg/ml, for direct binding to toxin-coated microspheres as described above.

For the sandwich assays where the sdAb were the recognition molecules, capture antibody coated bead sets were prepared by incubating 20 μg biotinylated capture antibody (goat anti-CTX, Mab Ric-03-A-G1, Mab Ric-07-A-G1, Mab SEB03b2a anti-SEB) with 20 μL NA coated Lx beads at 4°C in the dark for at least 1 hour. The beads were centrifuged, the supernatant discarded to remove the unbound antibodies, resuspended in 200 μL of PBST and stored at 4° C in the dark until use. This protocol typically provided approximately 100 – 300 beads/μL as determined by the Luminex100. In a 96-well format antibody-coated beads were incubated with dilutions of soluble toxin for 30 minutes at RT followed by the addition of the appropriate anti-toxin sdAb (final concentration 1 μg/ml) or Cy-3 labeled antibody (sheep anti-SEB, rabbit anti-ricin, rabbit anti-CTX, final concentration 10 μg/ml). After 15 minutes, Ni-SA-PE was added to the wells with sdAb reporter and incubated at RT for an additional 30 minutes before evaluating the signal on the Luminex.

For the assays using sdAb as the capture reagent, dilutions of the various toxins were prepared in PBSTB in columns of a microtiter plate. A mixture of sdAb coated microspheres was added to each well and incubated for 30 minutes. Then the recognition antibody and SA-PE, final concentration 10 μg/ml each, was added and incubated 30 minutes prior to measuring.

Results and Discussion

Library construction and panning

We set out with the intention of creating a rapid and straightforward route to isolate heat stable single domain antibodies, each specific for one of a range of diverse targets. Starting with small blood samples taken from 3 research naïve llamas, we constructed a 10^6 member mega-library of diverse sdAb open reading frames. Forty eight randomly chosen clones were sequenced and shown to have residues characteristic of heavy chain only antibodies at positions 42, 49, 50 and 52 [37]. The sdAb amino acid residues at these positions compensate for the lack of a pairing

to a VL domain. The sequence results indicated that the library could serve as a rich source of sdAbs. Only a single clone had an extra cysteine pair that is known to link CDR1 with CDR3, yet this has been shown to be a minor component of llama heavy chain only subfamilies [37] and is not required for thermostability and plasticity. While all of the sdAbs sequenced had unique and diverse CDR1, 2 and 3 sequences, attempts to select antigen binding clones from a phage displayed version of this initial megalibrary failed. Limited size and diversity is known to be a problem afflicting even large naïve based sdAb libraries [24, 25, 38]. In the absence of an enormous diversity of germline variants and the recombinatorial diversity afforded by VH:VL pairing, affinity maturation in camelids relies to a larger extent on somatic hypermutation geared towards fine tuning the CDRs to the immunogen [39, 40]. In an effort to mimic such *in vivo* diversification to yield a diverse enough library capable of generating sdAb to any given antigen, we hyperdiversified the mega-library using a combination of error-prone PCR and SOE-PCR assembly of the various sequence combinations (Figure 1b). Our strategy was aimed at trying to conserve the framework regions surrounding the CDRs which may be crucial in conserving the structural integrity of sdAb, especially for CDR3 [29]. Through this unique combinatorial strategy, we created Nomad#1, a library containing over 10^9 sdAb members.

The library was first panned against a model 14kDa protein (lysozyme), and a smallpox virus live surrogate (vaccinia) at SFBR in San Antonio, Texas. After successful isolation of binders, and characterization by ELISA of sdAb isolated against these two initial targets, the Nomad#1 library was sent to NRL in Washington DC, where it was panned against three toxins. Toxin binding sdAb were characterized by the Luminex 100.

We were able to isolate panels of unique sdAb specific for all chosen targets for which we performed selections, the acid test of any single pot antibody library. These binders were isolated in a matter of days using inexpensive and portable phage display selection techniques. To our knowledge this is the first time that llama sdAbs have been selected against an enveloped virus and protein toxins. The ability to isolate rugged, specific recognition reagents in a fraction of the time required to produce conventional antibodies could be important in the generation of immuno reagents towards new and emerging pathogens. Initially selected sdAb could act as interim reagents as conventional antibodies were developed and the first generation sdAb affinity matured for the production of superior reagents.

Sequences of selected sdAb

Predicted amino acid sequences of sdAb specific for each antigen are shown in Figure 2. Out of 24 vaccinia binding representatives identified through monoclonal phage ELISA, we found seven unique sequences designated clones A, B, C, D, F, G, and H. Sequences A, C, F, and H were each found once, D and B were found twice, and G was found 16 times out of the 24 representatives sequenced. Similarly, we isolated 2 unique sequences out of 12 HEL binding representatives examined with clone A found 7 times and clone B found 5 times. We isolated 5 unique CTX binding clones from 5 sequenced, 1 unique ricin specific clone from 2 positives sequenced, and 2 unique SEB clones from 3 sequenced. Interestingly, all 5 of the CTX clones shared the same CDR3 but were variant in CDRs 1 (5/5 unique) and CDRs 2 (4/5 unique), demonstrating the capacity of the library to deliver diverse target specific binders.

Binding specificity

For each of the targets used in this work, we first examined direct binding to ensure the specificity of the isolated sdAb and to rank binding of the sdAb isolated in the selections (Fig. 3). In these experiments we compared direct binding of dilutions of sdAb to both their cognate antigen and an irrelevant antigen.

The sdAb isolated in Texas towards vaccinia and HEL were analyzed by chemiluminescent ELISA. Clones G and D showed significant signal against vaccinia yet not on control antigen ovalbumin (Fig. 3a). It is often the case that lower affinity antibodies, while recognized as positives by monoclonal phage ELISA fail to be demonstrably positive when tested as soluble proteins due to the massive signal amplification in phage ELISA afforded by the phage particle [33, 34]. The two best clones (G and D) were assayed for lack of cross-reactivity towards control viruses SARS and an influenza A strain. In both cases, signal on the irrelevant virus was less than 1% of the specific signal (data not shown) indicating that the clones were vaccinia specific. As assessed also by chemiluminescent ELISA, both HEL clones demonstrated HEL antigen specific binding (Fig. 3b).

The toxin binding sdAb isolated in Washington DC were analyzed by the Luminex100 instrument. An advantage of the Luminex system is that it uses just a fraction of the purified protein required to perform an ELISA. Luminex also allows up to 100 simultaneous multiplexed assays. Consequently, all sdAbs selected on toxin proteins were monitored for binding to all three toxin conjugated bead sets simultaneously. With one exception, toxin binding sdAb proved to be specific showing negligible signal on the control bead sets. The only case where a high background was observed on beads coated with irrelevant toxin was the CTX binder LCTC3. This sdAb showed the highest signal of the CTX binders, however the background signal on ricin and SEB coated beads was 10-20% of the CTX signal at the highest sdAb concentrations tested. Figure 3c includes traces of the LCTC3 signal on both the CTX and ricin bead sets. In the case of the other 4 CTX binders, the signal on ricin and SEB was negligible, at maximum 3% of the CTX signal at the highest sdAb concentrations. The ricin selected clone LRE7 was shown to be specific for ricin holotoxin and not control CTX (Fig. 3d). In addition to showing the toxin specificity of sdAb LRE7 we demonstrated LRE7 was able to bind the B chain and not the A chain. Both SEB binding sdAb also demonstrated target specific binding (Fig. 3e).

After ranking, the toxin binding sdAb were tested for competition with soluble toxin to show that they were able to recognize antigen in solution as well as immobilized on a surface. It is important for the development of sandwich immunoassays to ensure that the selected sdAbs are not specific for partially denatured toxin on a surface, and that they can actually bind soluble native target. These assays showed that soluble CTX inhibited binding of CTX binding sdAb to immobilized toxin to at least 40% (Fig. 4a). Similarly, soluble ricin competed off the LRE7 sdAb (Fig. 4b), and soluble SEB inhibited the binding to SEB coated beads (Fig. 4c). Although the concentrations of toxins required for competition are quite high compared to those of conventional toxin specific monoclonal antibodies (data not shown) we are aware that primary clones isolated from large antibody libraries often require further affinity maturation to reach the same equilibrium dissociation constants and sensitivities. The sdAb appeared to bind much better to immobilized target than to the soluble toxin. One possible explanation is that our selection process may have led to the isolation of binders to certain epitopes preferentially exposed on immobilized toxins.

Thermal stability

An important property of sdAb that has been reported in the literature is their impressive stability at elevated temperatures [17] and extreme plasticity which allows the denatured sdAbs to refold in contrast to IgG based antibodies [18, 19]. These properties should improve the shelf life of immunoassays and also permit the real time analysis of hot samples e.g. checking for caffeine in coffee [41]. We first assayed for the ability of sdAb proteins to survive heat exposure by heating the sdAb for 5 minutes at elevated temperatures, side by side with conventional IgG antibodies or scFv antibody fragments (Table 1). With one exception, the sdAb retained ~ 90% of the unheated antigen specific binding signal after being heated to a temperature between

95 and 100 °C. Only one of the conventional antibodies, a ricin monoclonal, exhibited significant thermostability (~50%), while heating rapidly degraded all the others binding ability. Several of the conventional antibodies, as well as the scFv, even lost binding activity after heating for 5 minutes to lower temperatures, 75 and 60 °C respectively. Each experiment incorporated a binding step to target or control antigen after the heating to account for non-specific stickiness due to unfolding [17]. Importantly, signals from the binding of heat-treated sdAb on control antigens was negligible except in the case of LCTC3 which, as in the direct binding assay, had an appreciable signal on the SEB and ricin control bead sets (not shown).

Exposure to high temperatures for longer periods has been used to try and predict the long term stability of food products [42, 43] and more modest temperatures have been used to predict long-term immunoassay stability for parasites for example [44, 45]. To determine if our sdAbs had the potential to ruggedize immunoassays, we heated representative sdAbs to 95 °C for varying lengths of time, and checked for their ability to bind target. The anti-vaccinia clone G retained ~40% of its activity after heating for 40min decreasing to around 3% of original activity after 80min, in contrast to the CRP monoclonal antibody which lost all activity within 5min (Fig 5a). Signal from the anti-vaccinia clone G as well as the anti-vaccinia Mab on ovalbumin coated wells yielded less than 1% of the specific signal over all time points (data not shown). The anti-CTX sdAb LCTG3 was examined along with polyclonal rabbit anti-CTX for binding to CTX and irrelevant toxins after longer term heating (Fig 5b). After heating for an hour, the sdAb still retained at least 30% of its target binding ability where as the antibody was down to ~10% binding activity after only 5 minutes at elevated temperature. The anti-SEB sdAb LSEBE3 and polyclonal sheep anti-SEB were heated to 95 °C for varying lengths of time and checked for their ability to bind target (Fig. 5c). The conventional antibody had 90% reduced binding after 5 minutes and lost all binding after 10 minutes at 95 °C. In contrast the sdAb retained 100% of its binding activity for 20 minutes before loosing all activity by 45 minutes. Both LCTG3 and LSEBE3 retained specificity during the heating, showing negligible signal on the control bead sets (data not shown). We speculate that loss of activity may be due to an aggregation event perhaps nucleated by small amounts of contaminating proteins, or proteolytically nicked sdAb which is unable to refold correctly, in the preparations. The durability of sdAbs to extreme temperatures is unlike any other antibody so far discovered. Our ability to rapidly select such rugged antigen binding clones should help to increase the shelf-lives and operating periods of immunoassays.

Determination of binding constants

We determined equilibrium binding constants for the toxin binding sdAb (Table 1) from the equilibrium binding curves[46, 47] shown in Figure 3. These determined binding constants give a relative estimate of the sdAb in comparison to conventional antibodies. The binding constant for each sdAb was determined from 2 data sets, and both curves gave similar values. These results show that while the isolation of these sdAb from the Nomad#1 library was much faster than the development of conventional antibodies, their binding affinities were often not as good as traditional antibodies. These isolated sdAb could be the starting point for affinity maturation with the goal of isolating sdAb with both plasticity and improved thermal stability as well as affinities comparable to IgG.

The Luminex was also exploited to give information about on and off rate binding kinetics of several of the isolated sdAbs (LCTC11 and LSEBE3, data not shown). We found that the on rates of the sdAb and conventional antibodies looked comparable (~5×10^5), indicating that the binding of the sdAb is diffusion limited. However, on looking at dissociation, the sdAb lost ~50% of their signal after about 35 minutes versus several hours for conventional antibodies. This indicates that while the sdAb bind onto the target rapidly, they also come off fast, leading to reduced overall affinity.

SdAb as capture and reporter molecules in sandwich assays

The performance of the sdAb in sandwich assays is important for their integration into many classes of biosensor systems. For these initial tests we utilized the sdAb as either the capture or the reporter molecule, while maintaining a conventional antibody as the assay partner. Eventually, we hope to evaluate sdAb only assays; for some targets such as SEB or ricin this will require two different sdAb each specific for a distinct epitope to establish a sandwich assay. When we utilized sdAb as the reporter molecule, we found that when combined with highly fluorescent Ni-SA-PE as secondary conjugates the resulting complex functioned as highly effective reporter molecules in sandwich assays (Fig. 6). When paired with goat anti-CTX, the anti-CTX sdAb LCTA9 functioned well as a reporter, detecting levels of toxin comparable to rabbit anti-CTX reporter (Fig. 6a). Similarly, we showed the ricin binding sdAb LRE7 to be an effective reporter molecule in a sandwich assay when paired with two separate A-chain binding monoclonal antibodies, detecting ricin down to 4 ng/ml toxin (Fig. 6b). Competition assays indicated that the 2 anti-SEB sdAbs isolated have overlapping epitopes and would not function as a pair in a sandwich assay (data not shown). We then screened available anti-SEB monoclonal antibodies to identify clones that did not overlap with our sdAb and used these as the capture motif. SEB concentrations down to 0.05 ng/ml and 0.5 ng/ml could be detected using either the LSEBE3 or LSEBG6 reporter respectively while the conventional antibody reporter Sh-anti-SEB, was able to detect 0.01 ng/ml toxin (Fig. 6c).

We also evaluated the effectiveness of several sdAb as capture molecules. The sdAb were covalently immobilized to the luminex microsphere surface using a two step carbodiimide chemistry. This method however was found to be effective for many of the sdAb, however more orientationally directed methods maybe required for universal application. Figure 7 shows that LCTG4, LRE7, and LSEBE3 were each effective as capture molecules for their respective targets, cholera toxin, ricin, and SEB, while at the same time showing minimal cross reactivity with irrelevant toxins. The limit of detection for each of the toxins was about 130 ng/mL, a reasonable limit considering the Kds determined. While not as sensitive as conventional antibodies, the sdAb gave limits of detection that would be useful in all but trace detection scenarios. For development of sdAb only assays towards monomeric and hetero-oligomeric proteins such as SEB and ricin will require sdAb targeting non-overlapping epitopes for capture and detection. Detection of CTX, which contains 5 copies of the B subunit, may be possible using the same sdAb as both a capture and reporter reagent. We are assembling second generation libraries that will allow us to deliver a broader range of sdAb so that we may use them as both capture and reporter elements in sandwich assays and ruggedize the overall assay to extreme temperatures. Furthermore, we are applying *in vitro* evolution [26, 48] and multimerisation strategies [49, 50] to affinity mature the sdAbs isolated here to drive the limits of detection lower.

Conclusion

We have shown that we are able to select panels of heat stable single domain antibodies to a broad range of target antigens within days that can perform well as both capture or reporter molecules in standard immunoassay formats. Advances in phage selection technologies will allow us to speed up the isolation of suitable serviceable sdAbs to a matter of hours [51]. Since phage selection is very portable it is allowing us to select sdAb from the Nomad#1 library specific for SARS coronavirus and Marburg hemorrhagic fever virus in high containment facilities to quickly deliver durable immunoassay reagents to emerging threats (LS, LO and AH, in preparation). The delivery speed and the sdAb protein characteristics of small size, ruggedness and ability to be engineered for optimal orientation [52] and patterning [53] means that sdAbs are likely to be high performance yet low maintenance substitutes for any antibody based biosensor.

Acknowledgements

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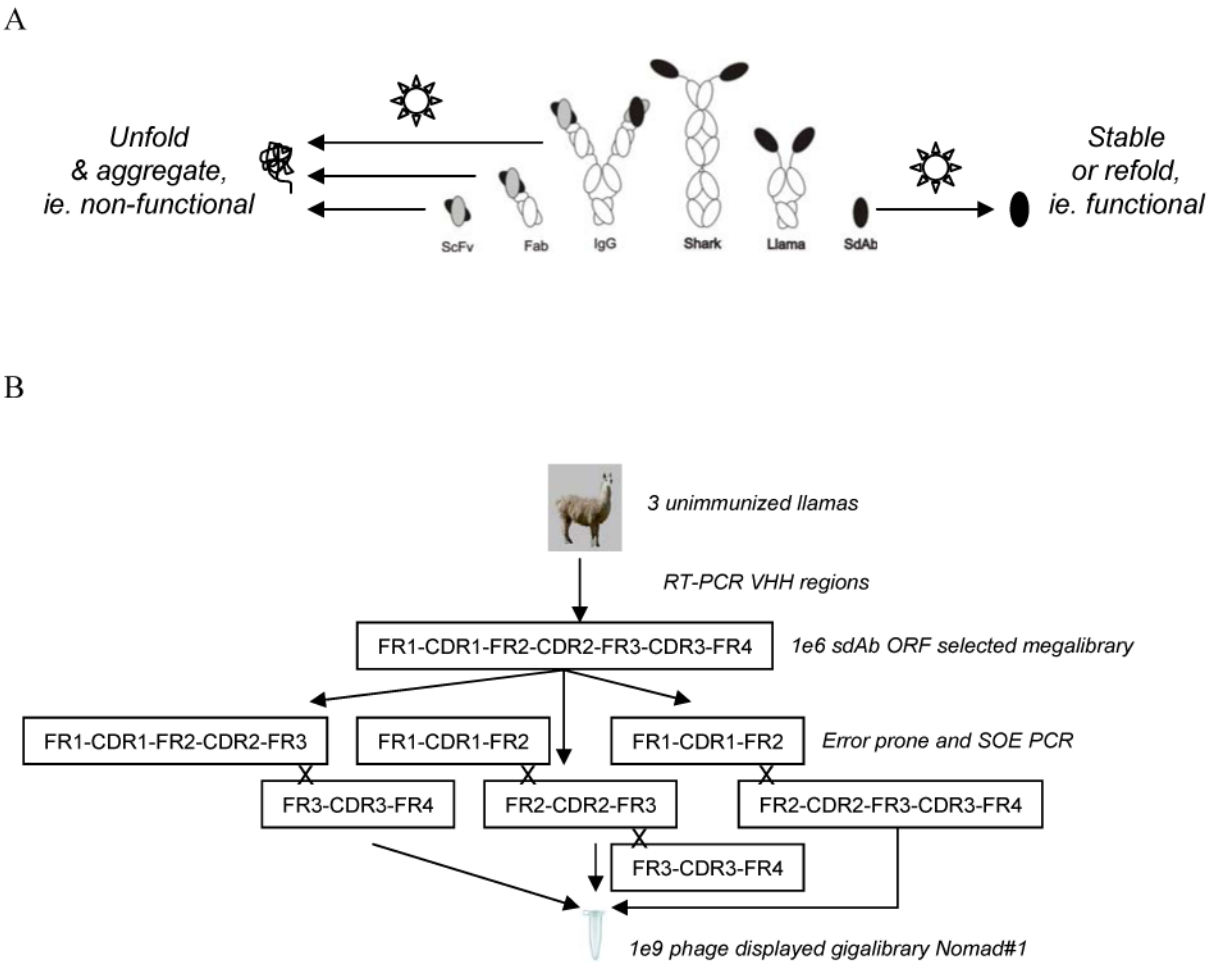


FIG. 1.

A. Representations of a whole IgG antibody and the antibody binding derivatives Fab and scFv (variable heavy chain, black, variable light chain, unshaded) are shown on the right, shark and llama IgG like molecules and the sdAb (variable domain, black) are shown on the left. SdAb provide rugged recognition elements as their non-multimeric domain structure is heat tolerant and able to refold if they do denature making them very durable and reusable. Multimeric paired structures such as scFv, Fab and IgG are relatively fragile and will unfold and irreversibly aggregate.

B. Schematic diagram showing the assembly of the Nomad#1 library through hyper diversification of a pool of sdAb genes isolated from small blood samples, designed for rapid delivery of heat stable recognition elements.

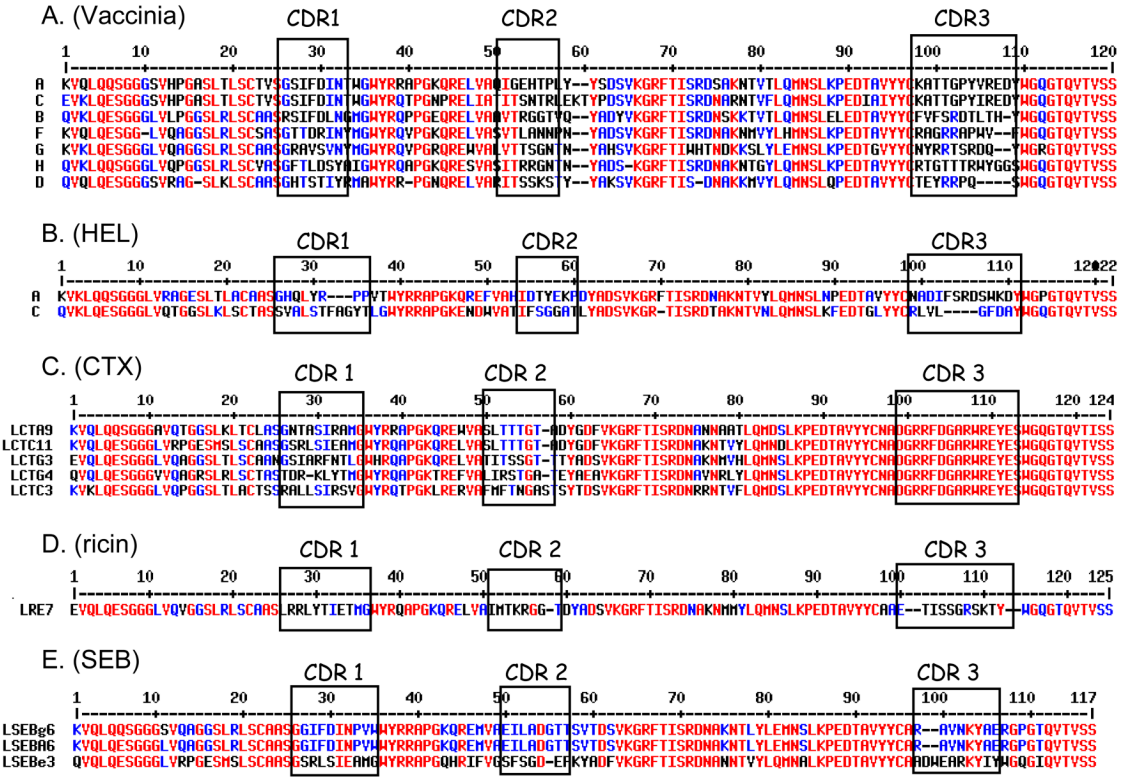
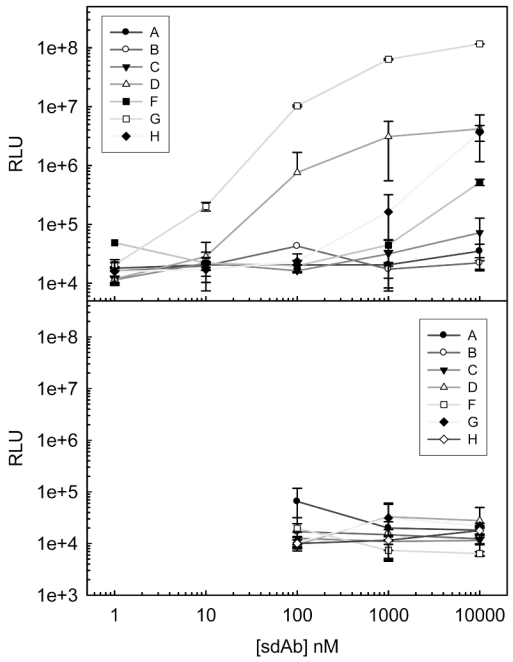


FIG. 2.

Predicted amino acid sequences of selected sdAb. Sequences were aligned using the Multalin program [54]. CDR regions indicated in boxes. A, vaccinia; B, HEL; C, CTX; D, ricin; E, SEB.

A



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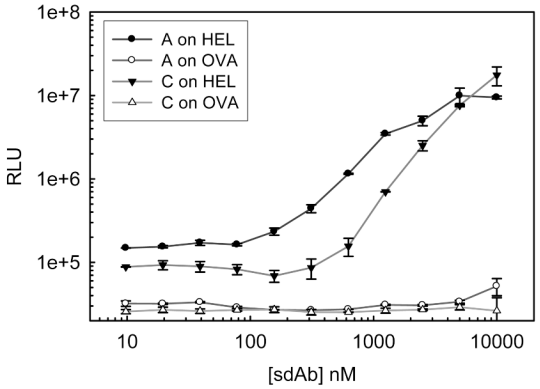

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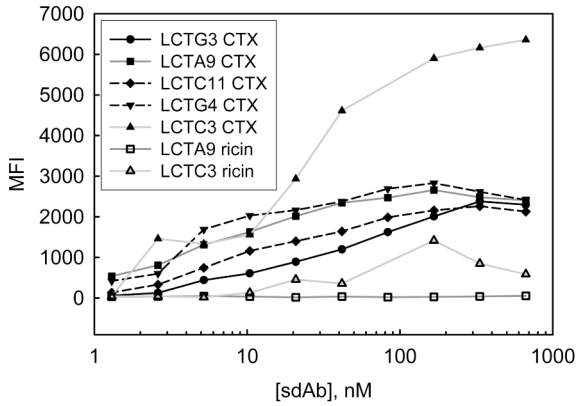
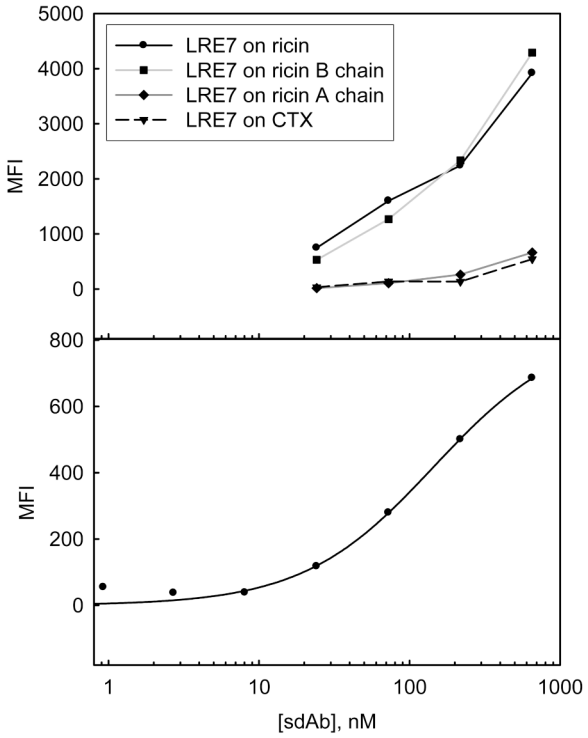


D



E

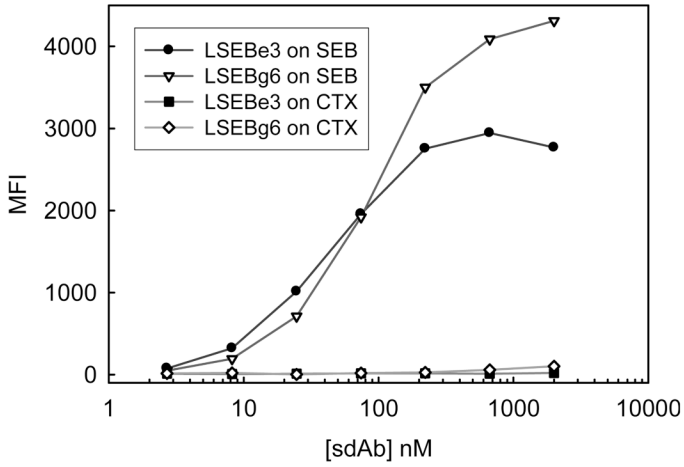


FIG. 3.

Specific binding of selected sdAb to cognate antigens.

A. Binding of vaccinia selected sdAb (clones A,B,C,D,F,G,H, see Fig. 2 for sequences) as determined by chemiluminescent ELISA shown on either vaccinia coated wells (top panel) or on ovalbumin (bottom bottom panel).

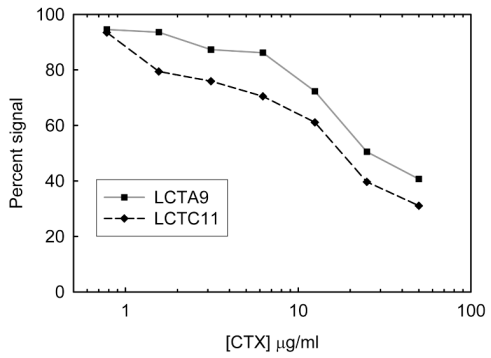
B. Binding of HEL selected sdAb (clones A and C see Fig. 2 for sequences) as determined by ELISA shown on either HEL or ovalbumin (OVA) coated wells.

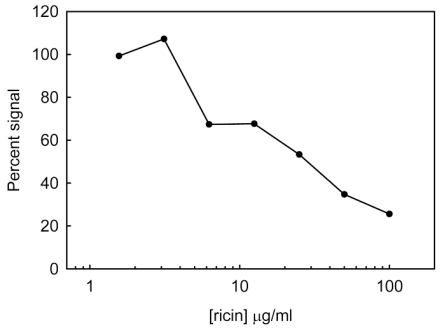
C. Binding of CTX selected sdAb (see Fig 2. for sequences) as determined by Luminex 100. Curves are shown for binding of all anti-CTX sdAb on CTX-coated beads, compiled from separate experiments. Binding to SEB and ricin coated control beads was negligible except for LCTC3. The ricin control is shown both for LCTC3 and LCTA9, the latter is representative of the other sdAb. Binding of LCTC3 to SEB coated beads was essentially the same as on the ricin coated beads and is not shown. Binding of LCTA9 to ricin coated beads was identical to the binding on SEB coated beads and is representative of the traces found on control beads for LCTG3, LCTC11, and LCTG4. The majority of the curves on control beads have been omitted for clarity.

D. Binding of ricin selected sdAb (see Fig. 2 for sequence) as determined by Luminex Top panel shows binding of sdAb LRE7on intact ricin, ricin A chain, ricin B chain, and CTX coated control beads. Binding on SEB control beads was the same as on the CTX control and is not shown for clarity. Bottom panel shows a binding curve of the sdAb to ricin fit with sigmaplot software using the standard binding equation: $y = (Bmax)x/(Kd+x)$.

E. Binding of SEB selected sdAb (see Fig. 2 for sequences) as determined by Luminex on SEB and CTX coated beads, compiled from two separate experiments. Binding on ricin coated beads was negligible and not shown for clarity.

A





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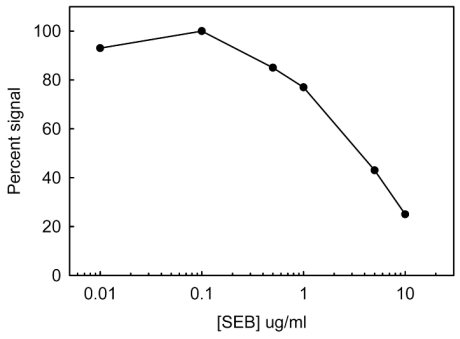
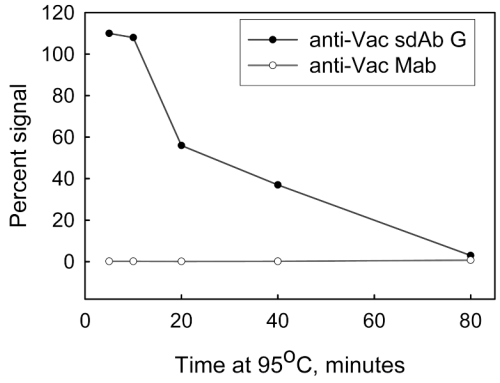


FIG. 4.

Ability of soluble toxins to inhibit binding of sdAb proteins to bead immobilized toxins. Increasing amounts of each toxin was added to the toxin coated bead set mixture prior to addition of the sdAb. After 15 min. incubation to approach equilibrium, Ni-SA-PE (10 mg/L) was added to generate the signal. Then after an addition 30 minutes, inhibition of binding of sdAb to toxin coated beads were assessed. Panel A, CTX; B, ricin; C, SEB.

A



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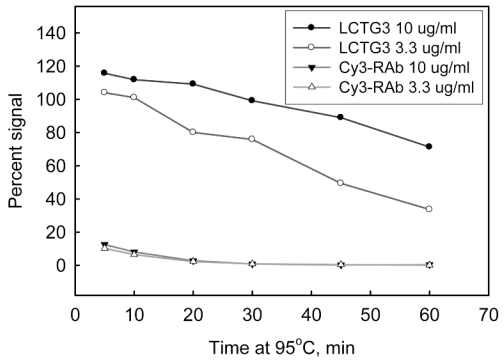

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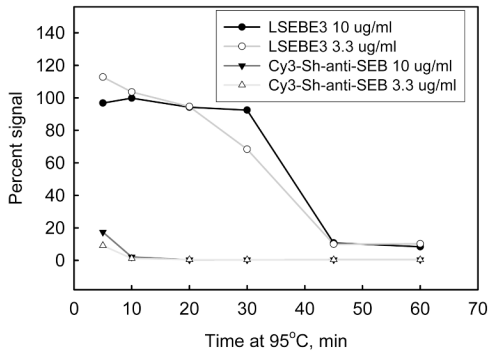


FIG. 5.

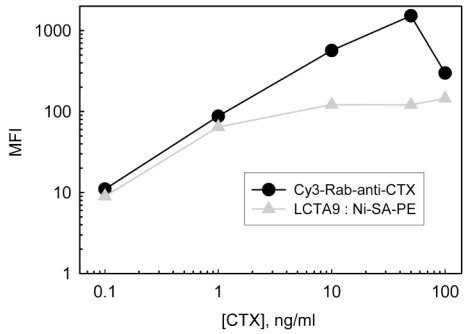
Thermal stability trials showing activity of selected sdAb after extended heating to 95°C. Percent signal is compared to the signals of unheated sdAb.

A. Time-course of heat exposure at 95°C examining the binding activity of sdAb clone G and CRP Mab on vaccinia and control ovalbumin antigen. At all time points binding on control ovalbumin coated surfaces was negligible (less than 1%) for both the sdAb and Mab.

B. Time course examining the activity of the anti-CTX sdAb LCTG3 and a rabbit anti-CTX on heating to 95°C. At all time points binding to control SEB and ricin bead sets was less than 3%.

C, Time course examining the activity of the anti-SEB sdAb LSEBE3 and a sheep anti-SEB on heating to 95°C. At all time points binding to control CTX and ricin bead sets was less than 5%.

A



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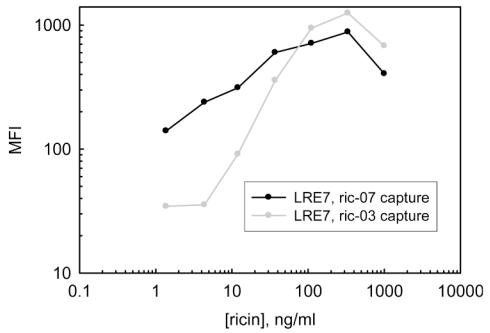

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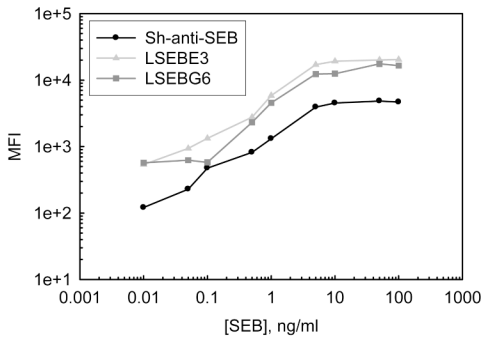

FIG. 6.

Performance of sdAb as a reporter molecule in sandwich assays. In all these experiments a conventional antibody was used on the capture surface (microsphere). The microspheres were allowed to bind various amounts of toxin for 30 minutes. This binding was then detected by either use of a Cy3 labeled conventional antibody (10 μg/ml) or by addition of sdAb (1 μg/ml) detected through Ni-SA-PE (10 μg/ml).

A. Anti-CTX sdAb LCTA9 and rabbit anti-CTX as reporters in a sandwich assay. Goat anti-CTX was used as the capture.

B. Anti-ricin sdAb LRE7 as a reporter when paired with two different anti-ricin mouse monoclonal antibodies (Mab Ric-03-A-G1, Mab Ric-07-A-G1).

C. Comparison of anti-SEB sdAbs (LSEBE3, LSEBG6), and sheep anti-SEB acting as reporters in a sandwich assay. Mouse monoclonal Mab SEB03b2a was used as the capture.

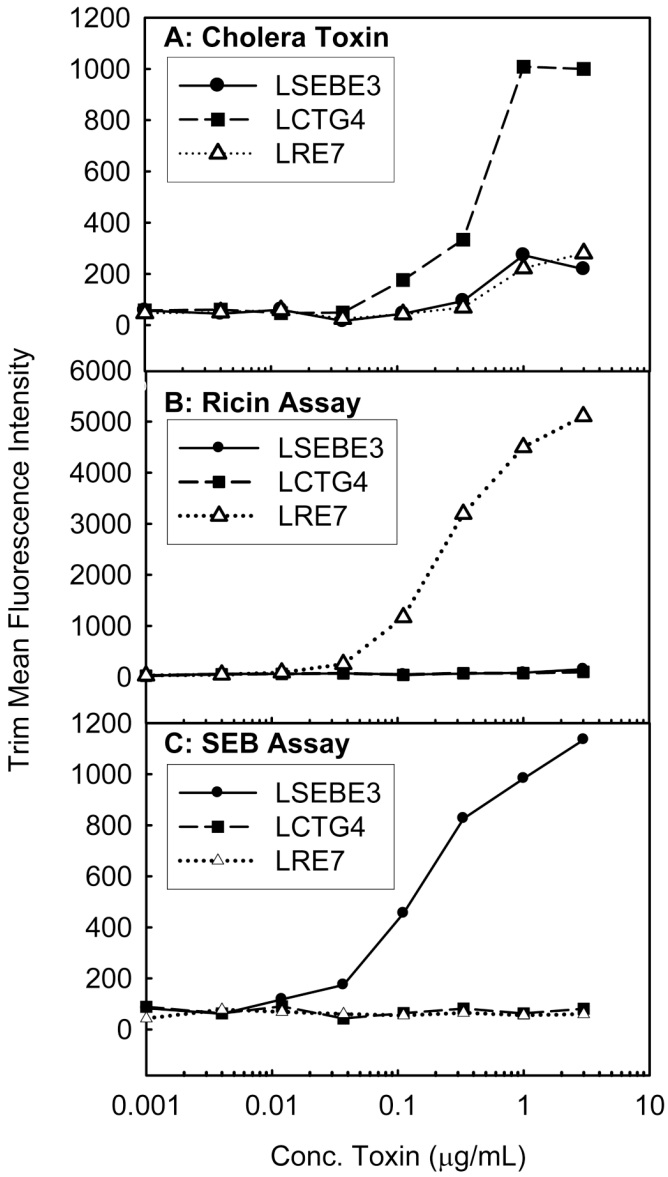

FIG. 7.

Performance of sdAb as a capture molecule in sandwich assays. In all these experiments a biotinylated conventional antibody and SA-PE was as the reporter complex. SdAb, LCTG4, LRE7, and LSEBE4, were covalently attached to Luminex microsphere sets. Mixtures of microspheres were allowed to bind various amounts of toxin for 30 minutes. This binding was then detected by a biotinylated conventional antibody (10 μg/ml) and detected through SA-PE (10 μg/ml). After an additional 30 minute incubation the samples were interrogated by the Luminex 100.

A. Microspheres coated with anti-CTX sdAb LCTG4 and biotinylated rabbit anti-CTX as the recogntion molecule in a sandwich assay.

B. Microspheres coated with anti-ricin sdAb LRE7 and biotinylated Mab Ric-07-A-G1 as the recognition molecule in a sandwich assay.

C. Microspheres coated anti-SEB LSEBE3 and biotinyated Mab SEB03b2a as the recognition molecule in a sandwich assay.

Table 1

Thermostability and equilibrium dissociation constants

SdAb	**Temperature °C (5 min)**			
	Percent of RT Signal			
Vaccinia Ab	Temp 50	70	100	
sdAb - D	**130**	**169**	**117**	
sdAb - G	**112**	**119**	**119**	
Mab-IgG	***88***	***107***	***0***	
Rab-IgG	***96***	***97***	***0***	
HEL Ab	Temp 60	80	100	
SdAb – A	**106**	**80**	**33**	
ScFv – D1.3	***5***	***5***	***3***	
CTX Ab	Temp 42	75	95	**Kd (nM)**
sdAb – G3	**102**	**110**	**101**	**41** ± 5.3
sdAb – A9	**100**	**99**	**107**	**5.6** ± 0.4
sdAb – C11	**100**	**105**	**94**	**18** ± 1.5
sdAb – G4	**104**	**109**	**116**	**5.2** ± 1.0
sdAb – C3	**119**	**146**	**86**	**26.5** ± 9
Cy3-Rab-anti-CTX-IgG	***104***	***88***	***3***	**1.5** ± 0.4
Bt-Gt-anti-CTX-IgG	***101***	***51***	***1***	**14.5** ± 1.8
BT-Mab-3D11	***79***	***5***	***3***	**24.5** ± 2.9
Ricin Ab	Temp 42	75	95	
sdAb - E7	**150**	**168**	**113**	**168** ± 18
Cy3-Rab-anti-Ricin-IgG	***90***	***58***	***4***	**50** ± 7.5
Cy3-Mab-Ric07AG1	***100***	***73***	***53***	**1.2** ± 0.1
Bt-Mab-Ric07AG1	***83***	***64***	***46***	**0.3** ± 0.1
Bt-Mab-Ric03AG1	***93***	***6***	***11***	**0.5** ± 0.1
SEB AB	Temp 42	75	95	
SdAb – E3	**134**	**106**	**112**	**18** ± 3
SdAb – G6	**108**	**105**	**88**	**45** ± 6
Cy3-Sh-anti-SEB-IgG	***99***	***36***	***10***	**3.6** ± 3.6
Cy3-Mab-02b3a	***100***	***37***	***33***	**0.1** ± .04
Bt-Mab-02b3a	***96***	***13***	***4***	**0.03** ± .02

In all cases, after the 5 minute incubation, the sdAb, conventional antibodies and scFv were also examined for binding to control surfaces. Binding to controls was always less than 3% of the room temperature (RT) signal except for the anti-CTX sdAb C3, where binding on controls was ~10% of the room temperature signal.