Evaluation of llama anti-botulinum toxin Heavy chain Antibody

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Abstract: Lama immunoglobulins consist of conventional antibody (IgG1) and unique forms that lack light chains, called heavy chain antibodies (IgG2 and IgG3). These unusual antibodies possess unique properties ideal for diagnostics and therapeutics. To evaluate the IgG from a llama immunised with botulinum complex toxoids A through F each IgG subclass was tested as capture

and recognition ligand in xMAP fluid array immunoassays. The optimal combination, IgG3 capture and IgG2 tracer, detected as low as 64 pg/ml of BoNT/A complex toxoid. Also, heavy chain antibodies were shown to bind BoNT as effectively as conventional IgG1, while possessing much greater thermal stability. [Received 15 October; Accepted 28 December 2007]

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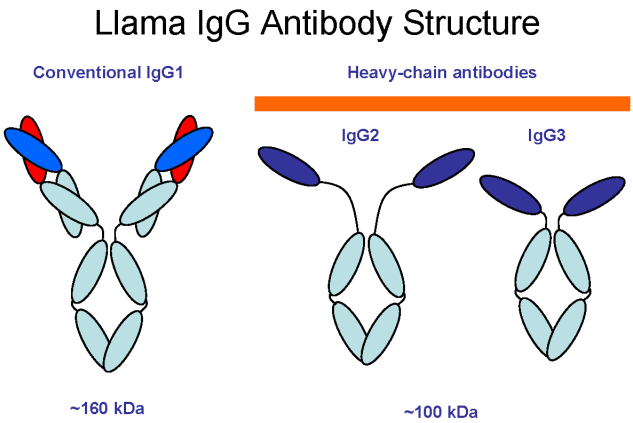
1 Introduction

Antibodies belong to the class of proteins called immunoglobulins; they are produced by the immune system in response to foreign antigens. Numerous immunoassays take advantage of the high affinity and selectivity of in vivo matured antibodies derived from animals. The primary class of antibody used for diagnostic applications is the IgG. For most mammals, IgG is a large, 160 kDa, protein made up of two pairs of different polypeptide chains: two heavy and two light chains connected by disulphide bonds. Each chain is composed of one variable domain plus either one constant domain for the light chain or three constant domains for the heavy chain. A variable domain from a light chain combines with a variable domain from a heavy chain to form one of two antigen-binding sites, which upon binding help to neutralise and eliminate pathogens and their toxins. Advances in recombinant DNA technologies have made possible the in vitro production of just these variable regions (scFv). However, these fragments can be problematic due to limitations in solubility or stability of the recombinant product (Brockmann et al., 2005).

Remarkably, the members of the Camelidae family (i.e., camels and llamas) have IgG subclasses that possess a unique structural arrangement (Figure 1). The IgG2 and IgG3 subclasses consist of only two heavy chains. The heavy chains lack the first constant domain and thus they fail to pair with light chains, yielding a molecular weight of ~110 kDa; meanwhile, the IgG1 subclass maintains the conventional structure (~160 kDa) (Hamers-Casterman et al., 1993). The Heavy chain Antibody's (HcAb) two antigen-binding sites are each formed by only a single variable domain, and thus the binding interaction has only half the normal number of Complementarity Determining Regions (CDRs), three as opposed to six. Also, the structure of this domain is altered by the replacement of select surface amino acids to increase its hydrophilicity to compensate for the lack of light chain (Muyldermans et al., 1994). The binding site being formed from a single domain that is small, highly stable, and able to refold properly after denaturation, makes the HcAb's variable domain a valuable source of alternative recombinant binding ligand for many applications (Daley et al., 2005; Goldman et al., 2006; Ladenson et al., 2006; Sherwood et al., 2007).

Previous studies have shown that HcAbs can act as effective high affinity binding ligands for foreign target molecules (Hamers-Casterman et al., 1993; Huang et al., 2005; Maass et al., 2007). However, other studies have found no added benefit from these unusual molecules; one group failed to detect any specific HAb binding following immunisation (Lange et al., 2001), while another found HcAb ineffective as an enzyme inhibitor (Ferrari et al., 2007). To ascertain the situation that prevails for a llama immunised against botulinum neurotoxin (BoNT) and to better evaluate HcAb capabilities found in the natural immune repertoire, we purified and tested the llama IgG subclasses obtained from a llama that had been immunised for BoNT.

Figure 1 Schematic of llama IgG subclass structures. IgG 1, conventional antibody, contains both a heavy and light chains. IgG2 and IgG3 consist of only heavy chains and have a long or short hinge region respectively



BoNT is the most potent of the known biological toxins, consisting of an approximately 100 kDa heavy chain and a 50 kDa light chain linked together via a single disulphide bond. It is secreted by bacteria of the genus *Clostridium*, which has the potential to form very tough spores, allowing them to survive in a dormant state until exposed to conditions that support their growth. Species of Clostridia are found worldwide in soil, sea and freshwater sediments. There are seven unique serotypes of BoNT, ranging from A through G, which represent potential biothreat agents. These serotypes are categorised on the basis of serological non-cross reactivity of neutralising antisera. BoNT can cause disease in humans through ingestion, inhalation, or wound infection. Botulinum intoxication constitutes a medical emergency, which requires prompt provision of an effective antitoxin that destroys or inhibits its toxic effects. Owing to its extreme toxicity, there is an urgent need to produce rapid diagnostic assays for BoNT (Merson and Dowell, 1973; Arnon et al., 2001).

In this study, our objective was to evaluate the effectiveness of llama polyclonal IgG as recognition ligands to BoNT. The llama IgG was fractionated into its various subclasses, the conventional antibody (IgG1) and HcAbs (IgG2 and IgG3), and evaluated for BoNT target specificity and heat stability in comparison with polyclonal antibody from rabbits or mouse monoclonals. In addition, their capabilities as both capture and recognition molecules in Luminex fluid array sandwich immunoassays were investigated. Native llama antibodies may provide unique capabilities for BoNT detection in field conditions, where a heat stable rugged molecule is not only desirable but a necessity.

2 Materials and methods

2.1 Buffers and reagents

The Llama serum was provided by the Naval Medical Research Center (Silver Spring, MD); the llama had been immunised with a mixture of botulinum toxoided complexes A, B, E, and F for a period of three years followed by immunisation with a mixture of toxoided complexes A,B,C,D and E for a period of two years. PhycoLink®

Streptavidin-R-Phycoerythrin PJ31S (SA-RPE) was purchased from Prozyme (San Leandro, CA). Phosphate buffered saline (PBS), Tween 20, and Bovine Serum Albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). BoNT toxoids, toxins, toxin complexes, and BoNT toxin-coated Luminex beads were purchased from Metabiologics (Madison, WI). The toxin-coated microspheres were prepared as a custom product, washed extensively to remove unbound toxin; the Luminex microsphere final wash verified nontoxic using a standard mouse biosassay. Rabbit anti-botulinum toxin A/B (R-anti Bot A/B), rabbit anti-botulinum toxin A (R-anti-Bot A), and monoclonal anti-botulinum toxin A (Mab-anti-Bot A), and botulinum A toxoid complex (BoNT/A) (certified inactive) were purchased from the US Department of Defense Critical Reagents Program. The monoclonal antibody (Mab-anti-Bot B) was provided by Tetracore (Rockville, MD). Experiments involving native toxins and toxin complexes were carried out in accordance with all applicable Federal policies as part of the Centers for Disease Control and Prevention Select Agent Program, and Southwest Foundation for Biomedical Research biohazard safety committee guidelines.

2.2 Antibody purification and subclass fractionation.

A crude purification of the llama IgG was first obtained using Caprylic Acid (CA) (McLaren et al., 1994). Llama serum (10 mL) was diluted with 20 mL of sodium acetate 60 mM, and pH adjusted to 4.8. While stirring, 0.5 mL of CA was slowly added. After incubating for 30 min at room temperature, the solution was centrifuged at 10,000 rpm for 10 min, then supernatant transferred to dialysis tubing and dialysed vs. three changes of PBS.

The CA purified IgG was further purified using MEP HyperCel hydrophobic charge induction chromatography (Pall, East Hills, NY).

The CA supernatant was loaded to a 10 mL column. The column was then washed to background OD using PBS. The antibody was eluted using 50 mM Sodium Citrate (pH 4.0). After dialysis vs. PBS, antibody concentrations were determined by absorbance at 280 nm.

IgG subclass were fractionated by a combination of affinity chromatography on protein G and protein A as described previously (Hamers-Casterman et al., 1993; van der Linden et al., 2000; Meddeb-Mouelhi et al., 2003; Daley et al., 2005). Briefly, either llama IgG purified by CA – MEP HyperCel or serum diluted 5-fold with PBS was first loaded onto 5 mL Protein G column (Pierce). After washing to background with PBS the IgG3 was eluted with 150 mM NaCl–0.58% acetic acid (pH 3.5), then the IgG1 fraction was eluted with 100 mM glycine–HCl (pH 2.7). The fraction not adsorbed on protein G was loaded onto a 5 mL Protein A column (Pierce). After washing with PBS, the IgG2 was eluted using 150 mM NaCl-0.58% acetic acid (pH 4.5). All collected IgG fractions were immediately neutralised using 1 M sodium bicarbonate (pH 8.31) and transferred to dialysis tubing to dialyse vs. three changes of PBS.

Fast Protein Liquid Chromatography (FPLC), using Superdex G200 column (Pharmacia), was then performed to evaluate the resultant antibody purity. For the IgG1, the FPLC was also used as the final purification step to remove antibody, which aggregated upon exposure to the low pH elution conditions of the Protein G column.

2.3 Biotinylation of antibodies

Antibodies were biotinylated using NHS-LC-Biotin (Pierce) dissolved in dimethyl sulphoxide (1.4 g/L). The antibodies were reacted with a 10 : 1 molar excess of the NHS-LC-Biotin. To enhance the rate of reaction, the pH was increased by the addition of a half-volume of 100 mM sodium borate + 100 mM sodium chloride (pH 9.1). After incubation for 1 h at room temperature, the biotinylated antibodies were separated from free biotin by gel filtration on a Bio-gel P10 column (BioRad, Hercules, CA).

2.4 Enzyme linked immunosorbant assays

96-well plates were coated with toxoided toxins and toxin complexes or native toxins and toxin complexes, 100 µl/well at 1 µg/ml in PBS overnight at 4°C. The next day, wells were washed with PBS + 0.05% Tween 20 (PBST) and blocked for an hour at room temperature with 2% non-fat powdered milk in PBS. After washing with PBST, dilutions of the IgG fractions were added to the wells and incubated for an hour. Wells were washed and Horse Radish Peroxidase (HRP) conjugated rabbit anti-llama (Bethyl lab, Montgomery, TX) was added for 45 min at a 1/10,000 dilution. After a final wash, the results were visualised using sigmafast OPD substrate or TMB Ultra (Pierce), stopped with 4M H_2SO_4, and the absorbance at 490 nm or 450 nm, respectively, recorded on a plate reader.

2.5 Luminex fluid array immunoassays

Luminex carboxylated microspheres (Lx) were crosslinked with a variety of proteins using the two-step carbodiimide coupling protocol provided by the manufacture. Typically, 100 µl of microspheres were used per protein, 50–100 ug of each protein at a concentration of 1 mg/ml or greater was used for conjugation to the microspheres. For toxin conjugation, 0.5 mg of toxin (1 mg/mL) was coupled to 1 ml of microspheres.

The signal for all experiments is reported as the median fluorescence intensity (MFI) of at least 100 separate microspheres. For direct binding assays, the various llama anti-botulinum toxin antibodies were serially diluted in a 96-well microtiter plate, 60 µl/well. To each well, a mixture of toxin-coated microspheres was added, 5 µl/well; they were allowed to incubate for at least 30 min. Then, SA-RPE (5 µl/well) was added (10 mg/L) and incubated an additional 30 min prior to measuring using the Luminex 100 flow analyser. For the thermal stability testing, 30 µL of the Llama IgGs at 100 mg/L were heated for various times at 85°C in a thermal cycler (Tetrad 2, MJ Research). After cooling to room temperature, each sample was tested at 10 mg/L for direct binding to toxin-coated microspheres as described above.

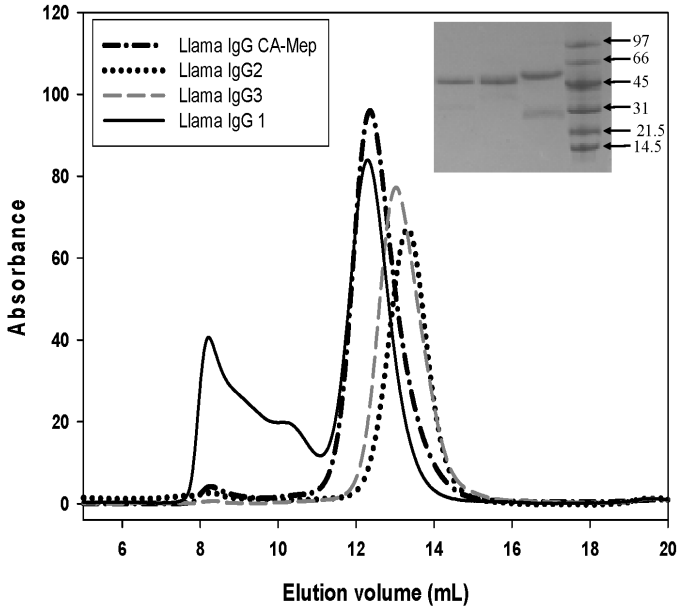
For the sandwich immunoassays, selected antibody-coated microspheres were incubated, in wells of a 0.45 µm multiscreen filter plate (Millipore, Billerica, MA) with different amounts of BoNT/A for 30 min at room temperature. The BoNT/A was removed by filtration, and selected anti-Bot antibodies added at 10 mg/L. After 30 min of incubation, the antibody was removed by filtration and SA-RPE (2.5 mg/L) was added and incubated at room temperature in the dark for 30 min. Binding was then evaluated using the Luminex 100 flow analyser.

3 Results

3.1 Characterisation of purified llama IgG subclasses

The IgG was successfully fractionated into its various subclasses using Protein G and Protein A affinity chromatography. As observed previously, we found that llama IgG was primarily IgG1 (~80% by wt.) and lesser amounts of IgG2 and IgG3 (~10% each) (Hutchison et al., 1998). FPLC Superdex G200 chromotography (Figure 2) verifies that llama IgG1 has a higher molecular weight than IgG3, and both are of higher molecular mass than IgG2. The elution profile of the IgG1 also showed that significant aggregation had occurred during the Protein G purification, since the peak that elutes at the void volume was not observed from the MepHypercel purified IgG. Thus, the IgG1 used for this study was further purified by FPLC to remove the aggregated material. The nature of the IgG subclasses was further confirmed by polyacrylamide gel electrophoresis under reducing conditions (Figure 2, Insert). The IgG1 (purified by FPLC) showed the expected heavy and light chains while both the IgG2 and IgG3 showed only the presence of heavy chains, with slightly lower molecular weights when compared with the IgG1 heavy chain.

Figure 2 Llama IgG subclass elution volumes from FPLC Superdex G200 column. The MEP HyperCel purified IgG mix as well as the IgG1, IgG2, and IgG3 subclasses are shown. Insert: 8–25% gradient gel run under reducing conditions. Lanes left to right; IgG3. IgG2, IgG1, MW markers



3.2 Direct binding immunoassays

The ability of the llama serum antibodies to bind to both live toxin and toxin complex was verified by ELISA (Figure 3). The serum showed specific binding to all purified BoNT types as well as BoNT complex types; however, the titer appeared highest for the BoNT/A,B, and F complexes. Selectivity of binding was then tested for the llama IgG

subclasses using both standard ELISA (Figure 4) and xMAP assays on the Luminex 100 (Figure 5). The ELISA results showed all the IgG subclasses bound well to both BoNT/A and B as either purified toxin or in the complex (tested on toxoided material). Binding to F, D, and E toxoid was observed but in deceasing amounts for each, respectively; this pattern was even more apparent for the HcAbs. Similar results were observed by xMAP protocol; however, binding to BoNT/A complex was significantly favoured by all subclasses using this method. A rabbit anti-Bot A/B, Mab anti-Bot A, and Mab anti-Bot B were also tested to show examples of specificity demonstrated by other conventional antibodies (Figure 5, Panels F, G and H). All toxin- and toxoid-coated microspheres had been tested with a specific antibody to confirm the success of their immobilisation (not shown). Even though this assay was performed on a filter plate, the monoclonal antibodies as well as the llama IgG subclasses 2 and 3 hooked at the highest concentration tested, while the rabbit polyclonal and the llama IgG1 subclass did not; one possibility is that this difference is related to a relatively higher titer of the monoclonals vs. the polyclonals. This higher titer of the HcAbs implies that these subclass populations expand in greater proportion upon immunisation than does the IgG1, which point towards the importance of HcAbs for the llama immune response to pathogens.

Figure 3 ELISA results of direct binding of Llama serum dilutions to BoNT toxin (Panel A) or BoNT toxin complex (Panel B). These data verify the live toxin and toxin complex binding capability of the Llama serum antibodies

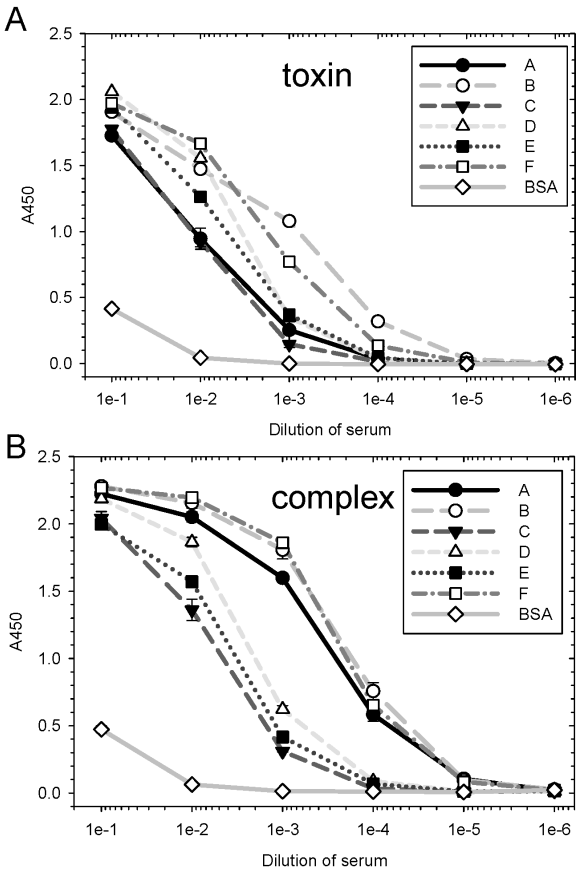


Figure 4 ELISA results of direct binding Llama IgG subclasses to BoNT types. Panels A and B show conventional IgG1; panels C and D show HcAb IgG2; and panels E and F show HcAb IgG3

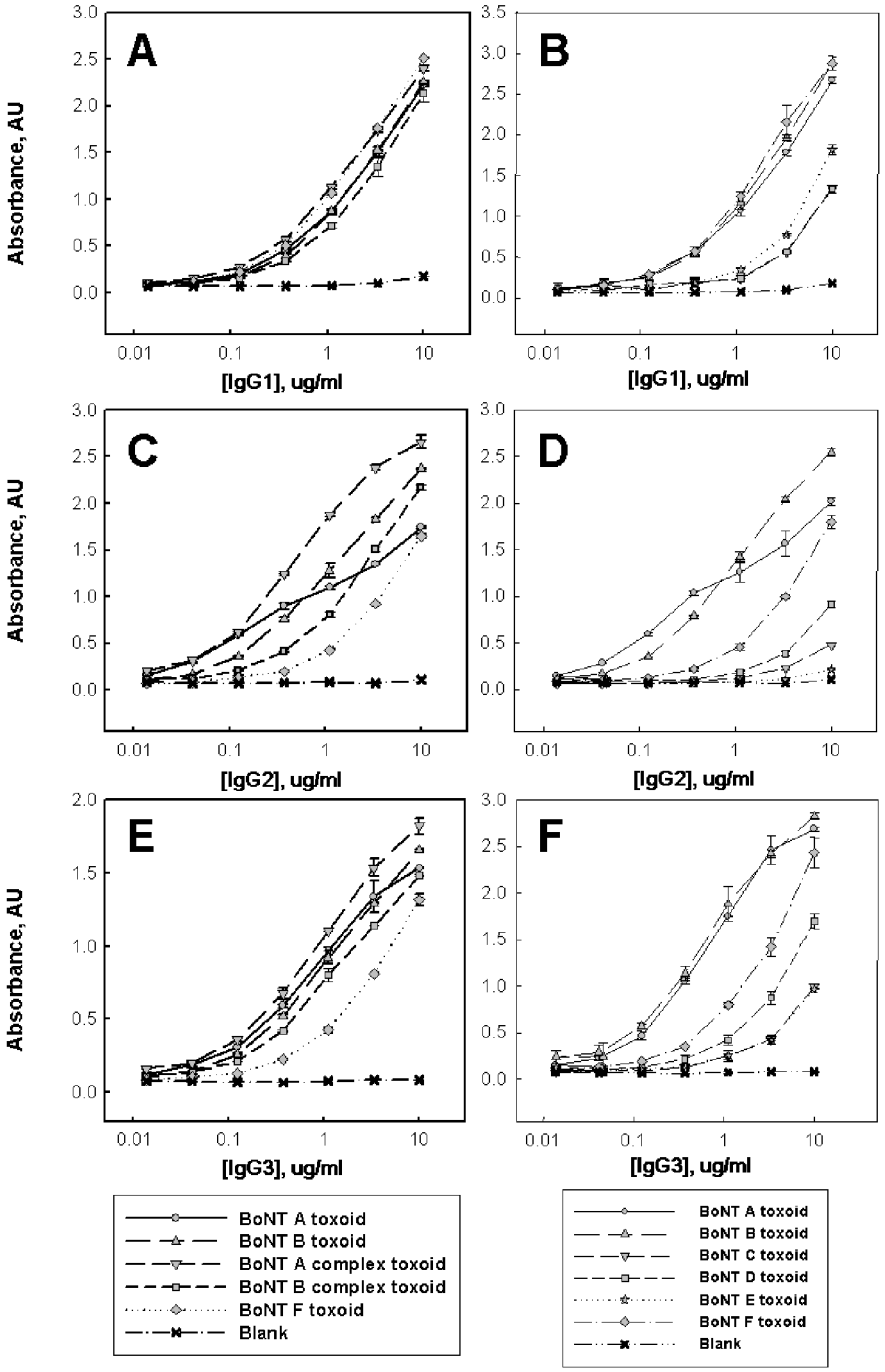
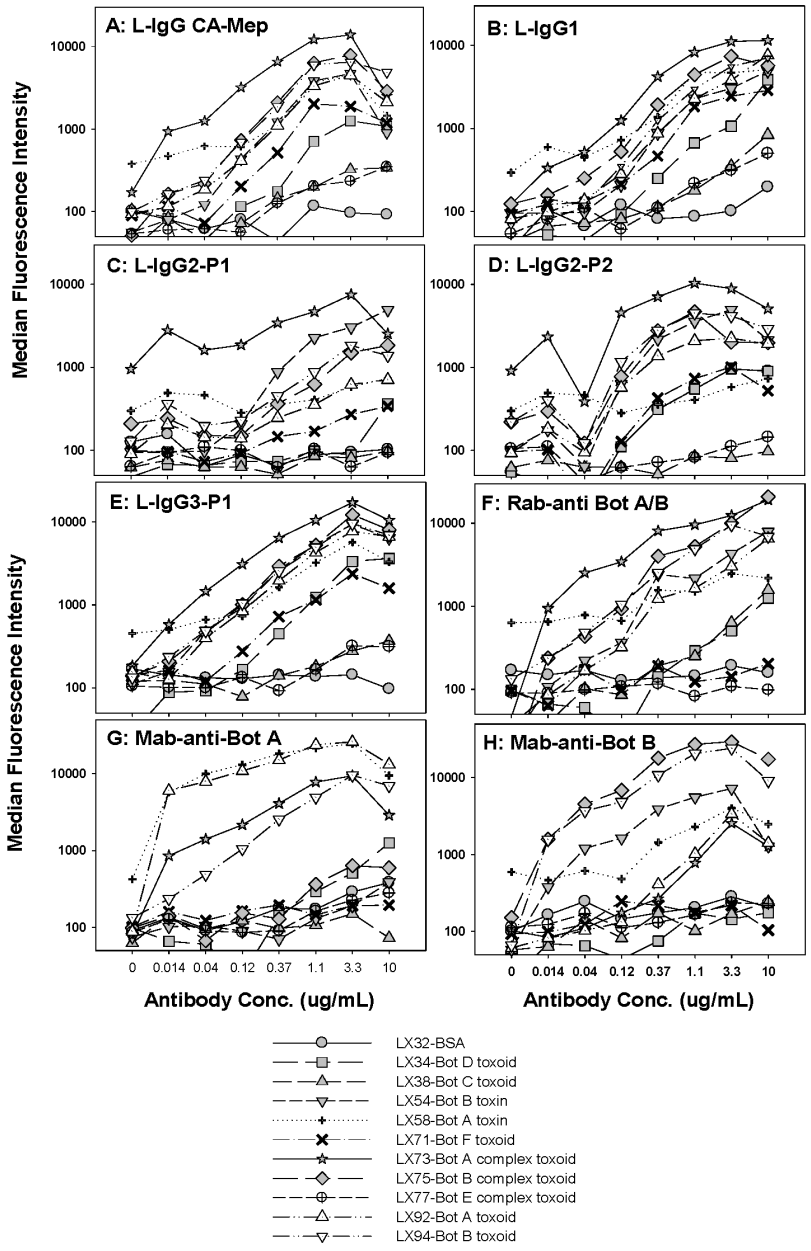


Figure 5 xMAP test of direct binding of the llama IgG subclasses or monoclonal antibodies to various BoNT types. P1 indicates CA/MEP Hypercel purified prior to Protein G/Protein A fractionation; P2 indicates direct fractionation of the llama serum

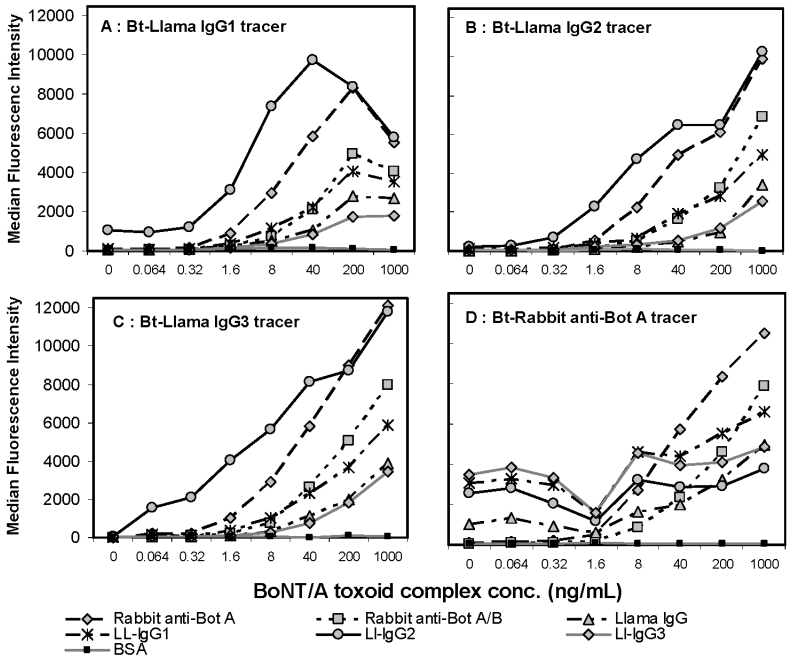


3.3 Sandwich immunoassay

To evaluate the capability of the llama IgG subclass, each was covalently attached to a different Luminex bead set and tested as the capture molecule in a sandwich immunoassay for BoNT/A complex toxoid (Figure 6). In addition to the microspheres

with the llama IgG subclasses, microspheres with Rab anti-Bot A, Rab anti Bot A/B, and MEP Hypercel purified llama IgG were tested. For these assays, each of the biotinylated llama IgG subclasses and a biotinylated Rab anti Bot A was evaluated as the recognition molecule. While all the capture antibodies were functional, the llama IgG2 coated microsphere provided the most sensitive detection regardless of the tracer molecule being tested. Surprisingly, while the IgG3 coated microspheres performed poorly, the Bt-IgG3 was the superior recognition molecule, providing at least a 5-fold enhancement in sensitivity, detecting BoNT/A complex toxoid at concentrations as low as 64 pg/mL.

Figure 6 xMAP sandwich immunoassay for BoNT/A complex toxoid using the llama IgG subclasses or rabbit polyclonal antibodies as the capture molecule and different recognition molecule in each panel. Assays were preformed as described in the methods

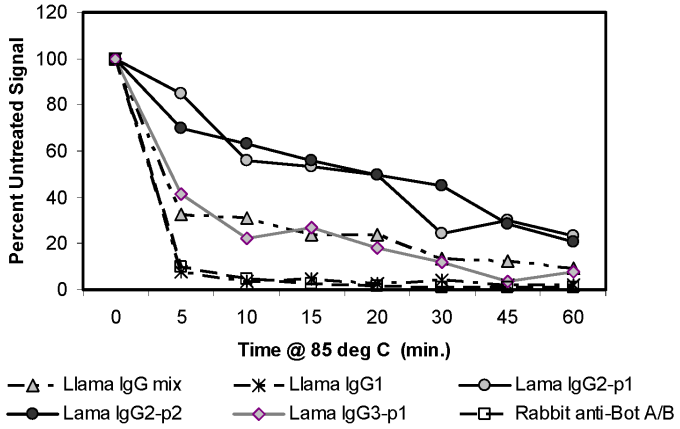


3.4 Thermal stability

The llama IgG subclasses were also subjected to high heat conditions to evaluate their thermal stability. The llama IgG subclasses as well as conventional antibodies were heated at 85ºC for various time points up to 60 min (Figure 7). Antibodies were then cooled to room temperature and assayed on the Luminex for binding to BoNT/A toxoid complex. The conventional antibody from llama (IgG1) lost approximately 93% of its binding ability within the first 5 min of incubation. However, llama HcAbs (IgG2 and IgG3) maintained a greater percentage of both their binding ability as well as specificity after heating. After 5 min at 85ºC, the percent binding of llama HcAbs (IgG2 and IgG3) decreased only 35% and 60%, respectively, and only slowly lost additional activity over the hour at 85ºC. The heat stability of the IgG2 and IgG3 explains why the IgG mix

retained residual toxin binding after heating. The conventional antibody Rabbit anti-Bot A/B as expected rapidly lost activity upon heating.

Figure 7 Thermal stability of the llama IgG subclasses. Aliquots (30 µl) of each biotinylated antibody were heated for various lengths of time at 85ºC. They were then diluted to the final test concentration of 10 µg/mL and their binding to BoNT/A complex toxoid evaluated using the Luminex xMAP assay



4 Discussion

The goal of this project was to evaluate the BoNT binding capability of antibodies obtained from an immunised llama, and detail the capabilities of llama HcAbs. Earlier work had shown that llama HcAb was active towards pathogens, and the ability to create single domain antibodies from immunised libraries attests to their antigen recognition capability. However, llama HcAbs had not been evaluated for their applicability for immuno-diagnostics, nor had the thermal stability of intact HcAb been reported.

To achieve this goal, we obtained serum from a llama that had been repeatedly immunised with botulinum toxoid mixtures including serotypes A through F over a number of years. The titer of this serum was evaluated for binding to both toxin and toxin complex (Figure 3), and found that the llama did develop antibodies to toxin in response to toxoid immunisation. Next, the serum IgG was purified and fractionated into subclasses. This was done by a series of steps. First, CA precipitation, which was followed by hydrophobic charge induction chromatography to purify the IgG from other serum proteins. Then, a combination of affinity chromatography on protein G and protein A was used to separate the subclasses. CA precipitates many serum proteins, primarily unwanted hydrophobic proteins. An initial trial utilised 0.7 mL of CA per 10 mL of serum; however, this was found to result in excessive loss of IgG. When the CA added was reduced to 0.5 mL, a much better IgG yield resulted. This initial crude purification was followed by hydrophobic charge induction chromatography. These two steps yielded a highly purified IgG preparation, with only minimal amount of contaminants (Figure 2). To fractionate IgG into its constituent subclasses, adsorption and selective elution to Protein G and Protein A columns was performed. Llama IgG isolated from this animal is approximately 80% IgG1 and 10% each IgG2 and IgG3. These antibodies were evaluated

by both PAGE and FPLC. Each subclass was found to elute from the G200 column at a different volume; and the heavy chain make up of the IgG2 and IgG3 was confirmed by the PAGE. The unfractionated IgG and the IgG1 have similar retention volume on the G200 column, as expected since IgG1 is 80% of the llama IgG. Of interest, the low pH elution of the IgG1 from the Protein G column appears to have caused significant aggregation. Thus, all experimental work utilised llama IgG1 that had been further purified by the FPLC. To confirm subclass yields, llama serum was also fractionated directly by Protein G and Protein A chromatography, similar amounts of the IgG1 was obtained, but the recovery of IgG2 was worse, and the IgG3 was contaminated with IgG1, thus the initial purification of the IgG appeared to be of significant benefit.

With the Llama IgG subclasses of anti-botulinum toxin antibody purified, we proceeded to evaluate the activity and specificity of each. Much of this work was performed using the Luminex flow cytometer, which is a specialised system that performs multiplexed assays by discriminating up to 100 different bead sets, each of which can be coated with a different antibody. The instrument discriminates bead sets based on the relative amount of two fluorescent dyes embedded in the latex particles. The bead sets are mixed together during the assay, and then the flow cytometer separates each bead to identify it and quantify tracer fluorescence. The Luminex platform has become a common platform for a variety of immunoassay applications (Yan et al., 2005; Fouda et al., 2006; Anderson et al., 2007).

Our initial studies used microspheres coated with a variety of BoNT toxin, toxoids, or complex toxoids. Direct binding of each IgG subclass was tested to estimate the relative affinities and specificities of the llama HcAbs in comparison with conventional antibodies. The llama IgG subclasses appeared to have fairly comparable target specificities, all binding most strongly to BoNT/A complex toxoid, indicative of the strong immune response induced by the BoNT/A complex that includes hemagglutinin. Further work on purified hemagglutinin will be necessary to confirm this possibility. Most interesting was the fact that the HcAbs appear to possess titers with similarities to the monoclonal antibodies. This implies that in this instance, the HcAb population is highly responsive to immunisation, possibly due to either the nature of the toxin or perhaps due to the long duration over which immunisation took place.

In addition, llama HcAbs were demonstrated to be effective as both capture and recognition molecules in sandwich immunoassays. Analysis of the results obtained show that IgG2 performed better as a capture molecule than the others llama IgGs. Furthermore, Bt-Llama IgG3 provided the best BoNT/A limit of detection when compared with Bt-Llama IgG1 and Bt-Llama IgG2 recognition antibody. This may imply that the IgG2 and IgG3 favour different epitopes on the toxin, as this would lead to an improved sandwich assay response. On the Luminex flow analyser using the best antibody pair (llama IgG2 capture and IgG3 tracer) we detected as low as 64 pg/ml BoNT/A, this approaches the sensitivity of the mouse bioassay (LD50 ≅ 50 pg/ml), but is achieved much faster (less than 2 h).

Conventional IgG antibodies are composed of two identical heavy and light chains, each chain having a variable and a constant region. The interaction of the heavy and light variable regions forms the site of antigen binding. This can be irreversibly disrupted by denaturating conditions, such as high temperatures (Garber and Demarest, 2007). While it has been well documented that the recombinant single domain antibodies can withstand high heat conditions, less is known about the native heavy chain molecules (van der Linden et al., 1999; Ladenson et al., 2006; Goldman et al., 2006). Our results

indicate that HcAbs just like their derived fragments possess impressive thermal stability, losing activity only slowly upon heating at 85°C, while conventional antibodies lost nearly all binding activity in the first 5–10 min (Figure 7). Such enhanced thermal stability suggests that llama HcAbs may provide more rugged and reliable reagents for immunodiagnostic applications.

Summarising, we have demonstrated that llama HcAbs are not only effective immuno-reagents, in many ways they are superior to conventional llama IgG1. The primary limitation to utilisation of the purified llama heavy chain IgG is that they represent a minor proportion of the total serum IgG. One route would be to use camel as the source of immune sera, as heavy chain IgG has been reported to be greater than 50% of the camel serum IgG (Meddeb-Mouelhi et al., 2003). The more common route has been the use of molecular biology, where using nucleic acid primers to conserved regions of the llama HcAb's variable domain, the entire immune repertoire of the animal can be captured and then displayed as a coat protein-fusion on a filamentous phage (Holliger and Hudson, 2005). Such a phage display library has been constructed from the same animal used in these studies and single domain antibodies are currently being evaluated. These single domain antibodies should provide all the advantages observed for the native heavy chain IgG, but will be even smaller in size, more stable, and easily produced for the improving the reliability of biothreat detection in field situations.

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References

Anderson, G.P., Lamar, J.D. and Charles, P.T. (2007) 'Development of a Luminex based competitive immunoassay for 2,4,6-trinitrotoluene (TNT)', *Environ. Sci. Technol.,* Vol. 41, pp.2888–2893.

Arnon, S.S., Schechter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Swerdlow, D.L. and Tonat, K. (2001) 'Botulinum toxin as a biological weapon: medical and public health management', *JAMA*, Working Group on Civilian Biodefense, Vol. 285, pp.1059–1070.

Brockmann, E.C., Cooper, M., Strömsten, N., Vehniäinen, M. and Saviranta, P. (2005) 'Selecting for antibody scFv fragments with improved stability using phage display with denaturation under reducing conditions', *J. Immunol. Methods*, Vol. 296, pp.159–170.

Daley, L.P., Gagliardo, L.F., Duffy, M.S., Smith, M.C. and Appleton, J.A. (2005) 'Application of monoclonal antibodies in functional and comparative investigations of heavy-chain immunoglobulins in new world camelids', *Clin. Diagn. Lab. Immunol*, Vol. 12, pp.380–386.

Ferrari, A., Rodríguez, M.M., Power, P., Weill, F.S., De Simone, E.A., Gutkind, G. and Leoni, J. (2007) 'Immunobiological role of llama heavy-chain antibodies against a bacterial beta-lactamase', *Vet. Immunol. Immunopathol*, Vol. 117, pp.173–182.

Fouda, G.G., Leke, R.F., Long, C., Druilhe, P., Zhou, A., Taylor, D.W. and Johnson, A.H. (2006) 'Multiplex assay for simultaneous measurement of antibodies to multiple Plasmodium falciparum antigens', *Clin. Vaccine Immunol.*, Vol. 13, pp.1307–1313.

Garber, E. and Demarest, S.J. (2007) 'A broad range of Fab stabilities within a host of therapeutic IgGs', *Biochem. Biophys. Res. Com.*, Vol. 335, pp.751–757.

Goldman, E.R., Anderson, G.P., Liu, J.L., Delehanty, J.B., Sherwood, L.J., Osborn, L.E., Cummins, L.B. and Hayhurst, A. (2006) 'Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library', *Anal Chem.*, Vol. 78, pp.8245–8255.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N. and Hamers, R. (1993) 'Naturally occurring antibodies devoid of light chains', *Nature*, Vol. 363, pp.446–448.

Holliger, P. and Hudson, P.J. (2005) 'Engineered antibody fragments and the rise of single domains', *Nat. Biotechnol*., Vol. 23, pp.1126–1136.

Huang, L., Reekmans, G., Saerens, D., Friedt, J.M., Frederix, F., Francis, L., Muyldermans, S., Campitelli, A. and van Hoof, C. (2005) 'Prostate-specific antigen immunosensing based on mixed self-assembled monolayers, camel antibodies and colloidal gold enhanced sandwich assays', *Biosens Bioelectron.*, Vol. 21, pp.483–490.

Hutchison, J.M., Salman, M.D., Garry, F.B., Johnson, L.W. and Keefe, T.J. (1998) 'Characterisation of plasma immunoglobulin G concentrations of llamas', *Am. J. Vet. Res*., Vol. 59, pp.406–409.

Ladenson, R.C., Crimmins, D.L., Landt, Y. and Ladenson, J.H. (2006) 'Isolation and characterisation of a thermally stable recombinant anti-caffeine heavy-chain antibody fragment', *Anal Chem*., Vol. 78, pp.4501–4508.

Lange, I.G., Daxenberger, A. and Meyer, H.H. (2001) 'Studies on the antibody response of Lama glama – evaluation of the binding capacity of different IgG subtypes in ELISAs for clenbuterol and BSA', *Vet. Immunol. Immunopathol.*, Vol. 83, pp.1–9.

Maass, D.R., Sepulveda, J., Pernthaner, A. and Shoemaker, C.B. (2007) 'Alpaca (Lama pacos) as a convenient source of recombinant camelid heavy chain antibodies (VHHs)', *J. Immunol. Methods*, Vol. 324, pp.13–25.

McLaren, R.D., Prosser, C.G., Grieve, R.C. and Borissenko, M. (1994) 'The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chickens immunised with ovine alpha-lactalbumin', *J. Immunol. Methods*, Vol. 177, pp.175–184.

Meddeb-Mouelhi, F., Bouhaouala-Zahar, B., Benlasfar, Z., Hammadi, M., Mejri, T., Moslah, M., Karoui, H., Khorchani, T. and El Ayeb, M. (2003) 'Immunised camel sera and derived immunoglobulin subclasses neutralising *Androctonus australis hector* scorpion toxins', *Toxicon*, Vol. 42, pp.785–791.

Merson, M.H. and Dowell Jr., V.R. (1973) 'Epidemiologic, clinical and laboratory aspects of wound botulism', *N. Engl. J. Med.*, Vol. 289, pp.1005–1010.

Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J.A. and Hamers, R. (1994) 'Sequence and structure of VH domain from naturally-occurring camel heavy chain immunoglobulins lacking light chains', *Protein Eng.*, Vol. 7, pp.1129–1135.

Sherwood, L.J., Osborn, L.E., Carrion Jr., R., Patterson, J.L. and Hayhurst, A. (2007) 'Rapid assembly of sensitive antigen-capture assays for Marburg virus, using in vitro selection of llama single-domain antibodies, at biosafety level 4', *J. Infect. Dis.*, Vol. 196, pp.s213–s219.

van der Linden, R.H.J., Frenken, L.G.J., de Geus, B., Harmsen, M.M., Ruuls, R.C., Stok, W., de Ron, L., Wilson, S., Davis, P. and Verrips, C.T. (1999) 'Comparison of physical chemical properties of llama V-HH antibody fragments and mouse monoclonal antibodies', *Biochim. Biophys. Acta*, Vol. 1431, pp.37–46.

van der Linden, R., de Geus, B., Stok, W., Bos, W., van Wassenaar, D., Verrips, T. and Frenken, L. (2000) 'Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama', *J. Immunol. Methods*, Vol. 240, pp.185–195.

Yan, X., Zhong, W., Tang, A., Schielke, E.G., Hang, W. and Nolan, J.P. (2005) 'Multiplexed flow cytometric immunoassay for influenza virus detection and differentiation', *Anal. Chem.*, Vol. 77, pp.7673–7678.