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Research paper

Comparison of the efficiency of antibody selection from semi-synthetic scFv and non-immune Fab phage display libraries against protein targets for rapid development of diagnostic immunoassays

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ABSTRACT

Rapid development of diagnostic immunoassays against novel emerging or genetically modified pathogens in an emergency situation is dependent on the timely isolation of specific antibodies. Non-immune antibody phage display libraries are an efficient *in vitro* method for selecting monoclonal antibodies and hence ideal in these circumstances. Such libraries can be constructed from a variety of sources e.g. B cell cDNA or synthetically generated, and use a variety of antibody formats, typically scFv or Fab. However, antibody source and format can impact on the quality of antibodies generated and hence the effectiveness of this methodology for the timely production of antibodies. We have carried out a comparative screening of two antibody libraries, a semi-synthetic scFv library and a human-derived Fab library against the protective antigen toxin component of *Bacillus anthracis* and the epsilon toxin of *Clostridium botulinum*. We have shown that while the synthetic library produced a diverse collection of specific scFv-phage, these contained a high frequency of unnatural amber stops and glycosylation sites which limited their conversion to IgG, and also a high number which lost specificity when expressed as IgG. In contrast, these limitations were overcome by the use of a natural human library. Antibodies from both libraries could be used to develop sandwich ELISA assays with similar sensitivity. However, the ease and speed with which full-length IgG could be generated from the human-derived Fab library makes screening this type of library the preferable method for rapid antibody generation for diagnostic assay development.

1. Introduction

Antibody phage display is an *in vitro* screening technique that allows for rapid selection of multiple monoclonal antibodies of high affinity and specificity (Hoogenboom, 2005). It involves the display of a polyclonal collection of antibody fragments containing the variable regions, numbering up to 10^{11} different clones, on the surface of filamentous phage carrying the genetic sequence of the displayed fragment. Antibody library repertoires can be obtained primarily by two methods, either from cDNA antibody sequences derived from the B cells of immunized or non-immune animal or human donors, or synthetically generated using random nucleotide sequences within selected CDRs (complementarity-determining regions) in combination with one or multiple framework regions to replicate the diversity of a natural antibody repertoire (Conrad and Scheller, 2005). These sequences are then fused to the sequence encoding the gene III (gIII) coat protein of the phage, enabling expression and incorporation of the antibody fragment on its surface. This collection of phage-displayed antibodies is then panned repeatedly against the antigen of interest, producing a polyclonal phage collection enriched for antigen-specific antibodies, from

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which individual monoclonal clones can then be identified and characterized.

The rapidity of this technique makes it ideal for generating novel antibodies for diagnostic and potentially therapeutic responses to outbreaks of emerging infectious disease such as SARS coronavirus and H5N1 Avian Influenza or genetically modified pathogens released in a bioterrorism incident. Since antibodies are highly specific and are capable of recognizing virtually every class of pathogen, including toxins, viruses, bacteria and fungi, they enable easy and rapid identification of pathogens (Nowakowski et al., 2002; Hayhurst et al., 2003; Paoli et al., 2004; Steiniger et al., 2007; Cabezas et al., 2008). Indeed, sandwich ELISAs utilizing high affinity mouse monoclonal antibodies have been developed against likely bioterrorist threats such as epsilon toxin of *Clostridium botulinum* and protective antigen, a toxin component of anthrax, with demonstrated toxin detection limits of 1–2 ng/ml (el Idrissi and Ward, 1992; Mabry et al., 2006). In addition, their effectiveness against all classes of pathogens, low toxicity and minimal severe or long-term side effect make them ideal as prophylactic or therapeutics drugs that have to be rushed into use (Casadevall et al., 2004).

However, while traditional hybridoma techniques isolate clones that secrete full-length immunoglobulins, the initial phage library screen typically utilizes bacterially expressed clones that are in the scFv or Fab format. Therefore for functional use as an antibody, the clone must be transferred to an appropriate vector for expression of recombinant full-length immunoglobulin in eukaryotic cell culture (Kohler and Milstein, 1975; Jostock et al., 2004). However, some antibody clones may fail to express properly or lose affinity during this conversion from a bacterial to a eukaryotic expression system, as well as a change of antibody structural format. This may be caused by the presence of amber stops within coding sequences generated from random synthetic nucleotide sequences (Marcus et al., 2006), or improper folding. This is a particular problem for synthetic libraries as they have not undergone positive selection within the immune system to remove antibodies that are unable to express or fold properly, unlike libraries generated from natural repertoires. On the other hand, synthetic libraries may have advantages over natural ones in that they possess non-natural sequences that may code for antibodies with unusual specificities that are not present in natural repertoires (Griffiths et al., 1994).

Poor conversion of the isolated antibody fragment-phage clones to a soluble antibody format impairs the suitability of phage display as a method for the rapid generation of antibodies. Therefore, in this study, we compared the relative ability of a semi-synthetic scFv library and a natural non-immune Fab library to generate useful diagnostic antibodies that can be converted and expressed as soluble recombinant antibodies to be used as a detection reagent. Our choice of antigens was two commonly cited bioterrorism threats, epsilon toxin (Etox) and protective antigen (PA). While screening of the scFv library produced multiple clones that bound as phage, only a small percentage of clones were successfully expressed as either soluble scFv or full-length IgG and retained specificity and functionality. On the other hand, screening of the Fab library produced multiple high affinity clones against Etox and PA, all of which functioned as soluble Fab. Upon conversion to full-length IgG, they were capable of sub-microgram levels of detection in a sandwich ELISA. Many of the synthetic scFv clones were found to contain amber stops in their CDRs or N-linked glycosylation sites. However, removal of these sequences did not render the majority of these clones amenable to expression as soluble recombinant antibody, suggesting that improper folding may also be a factor. The results of the study suggest that non-immune naturally derived antibody libraries may be preferable to synthetic scFv libraries for rapid isolation of antibodies against typical foreign or pathogenic protein antigens.

2. Material and methods

2.1. Panning of scFv library

The Tomlinson I and J scFv-phage library (Goletz et al., 2002) (Source BioScience LifeSciences , UK) was panned as follows. Briefly, 80 μg of purified protein in 4 ml of PBS was coated onto Maxisorb Immunotubes (Nunc, Denmark) overnight at 4 °C then blocked with 2% skim milk in 1× PBS (MPBS) for 2 h at room temperature, followed by 3 washes with 1× PBS. The phage library (in 4 ml of MPBS) was applied for 2 h then discarded. The immunotube was washed with PBS/0.1% Tween 10 times for the first pan and 20 times for all subsequent pans. Elution of the remaining bound phage was carried out by digestion with 500 μl of 1 mg/ml trypsin solution for 10 min at room temperature. Eluted phage was re-infected into 1.75 ml of TG1 *E. coli* at OD_{600} 0.4 for 30 min at 37 °C and grown up on a 2TYE plate overnight. To recover the library, the plate was scraped and grown up in 50 ml 2TYAG broth (100 μg/ml Ampicillin, 2% glucose) until log growth phase (OD_{600} 0.5); The phage library was rescued by adding 5×10^{10} pfu of KM13 helper phage to 10 ml of this culture followed by incubation for 30 min at 37 °C and grown up in 50 ml 2TYAK (100 μg/ml Ampicillin and 50 μg/ml Kanamycin) liquid culture overnight at 30 °C. PEG precipitation was used to recover the phage the next day.

2.2. Panning of Fab library

The Fab phage display library was obtained from Humanyx Pte Ltd, Singapore which was constructed from the peripheral blood lymphocytes of non-immune human donors by the method of de Haard (de Haard et al., 1999). The library was enriched for binders to antigen as previously described via selection using biotinylated PA bound to Stretavidin-coated magnetic Dynabeads (Invitrogen, US) or immunotubes for Etox (Harrison et al., 1996). Briefly, 100 pmol of full-length PA previously biotinylated using sulfo-NHS-Biotin (Thermo Scientific, US) was incubated for 1 h at room temperature with 70 μl of Dynabeads in 500 μl of casein block (Thermo Scientific, US). Unbound PA was removed by 3 washes of casein block. For Etox, the antigen was coated onto immunotubes as described for screening of the scFv library.

To select for positive binders, 1 ml (~10^{13} phage) of pre-blocked phage library was incubated with either pre-coated beads or immunotube for 2 h at room temperature followed by 3 washes of 2% MPBS/T (containing 0.05% Tween-20), 3 washes with PBS/T and 2 washes with 1× PBS. For subsequent pans, beads or immunotube was washed 7 times with 2%MPBS/T, 7 times with PBS/T and twice with PBS. Bound phage was eluted