Journal of Immunological Methods 334 (2008) 1 – 10

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

Real time detection of anthrax spores using highly specific anti-EA1 recombinant antibodies produced by competitive panning

Tracey E. Love *, Caroline Redmond, Carl N. Mayers

Detection Department, Dstl Porton Down, Salisbury, Wiltshire, SP4 OJQ, United Kingdom

Received 10 October 2005; accepted 1 March 2006
Available online 29 February 2008

Abstract

We describe a targeted approach for the production of biological recognition elements capable of fast, specific detection of anthrax spores on biosensor surfaces. The aim was to produce single chain antibodies (scFvs) to EA1, a *Bacillus anthracis* S-layer protein that is also present, although not identical, in related to *Bacillus* species. The aim of the work was to produce antibodies that would detect *B. anthracis* EA1 protein and intact spores with a high degree of specificity, but would not detect other *Bacillus* species. Existing monoclonal antibodies were evaluated and found to recognise *B. anthracis* EA1 and S-layer proteins from other closely related *Bacillus* species. Recombinant anti-EA1 scFvs were isolated from *B. anthracis* immune library that contained antibody genes raised against *B. anthracis* spores and purified exosporium. Two approaches for scFv selection were used; standard (non-competitive) panning, and competitive panning. The non-competitive biopanning strategy isolated scFvs that recognised EA1 from *B. anthracis*, but also cross-reacted with other *Bacillus* species. In contrast, the competitive panning approach used S-layer proteins from other *Bacillus* species to generate scFvs that were highly specific to *B. anthracis* EA1 and demonstrated apparent nanomolar binding affinities. Specific, real time detection of *B. anthracis* spores was demonstrated with these scFvs using an evanescent wave biosensor, the Resonant Mirror. The approach described can be used to generate specific antibodies to any desired target where homologous proteins also exist in closely related species, and demonstrates clear advantages to using recombinant technology to produce biological recognition elements for detection of biological threat agents.

Keywords: EA1; *Bacillus anthracis*; Biosensor; Biodetection; scFv; S-layer

1. Introduction

The first step in developing an immunoassay for the detection of a pathogen is usually the development of a highly specific antibody that will recognise the live pathogen. These antibodies can be either poly- or monoclonal, and are usually produced against the whole organism. Although often successful, it can prove difficult to obtain antibodies with sufficient specificity by this approach. In the case of polyclonal antibodies raised against whole cells or spores, the antibody will recognise a range of undefined antigens and epitopes. Furthermore, these epitopes may be conserved between closely related species, particularly if unique targets are rare or are not immunodominant. In the case of a

Abbreviations: EA1, extractable antigen; scFv, single chain fragment variable/single chain antibodies; S-layer, surface layer; PBS, phosphate buffered saline; HBS, HEPES buffered saline; RM, resonant mirror; CMD, carboxymaethylated dextran.

* Corresponding author. Tel.: +44 1980 613942; fax: +44 1980 613912.
E-mail address: telove@dstl.gov.uk (T.E. Love).

0022-1759/$ - see front matter.
doi:10.1016/j.jim.2007.12.022

monoclonal antibody only one epitope, often of an unknown protein, is recognised. Monoclonal antibodies, although directed against a single epitope, may still not be able to discriminate between highly conserved epitopes in closely related species. The use of antibodies against unidentified and uncharacterised proteins or epitopes gives significant limitations to the sensitivity and specificity of a detection assay, as well as reducing confidence in the results obtained.

In addition to the problems observed with lack of specificity or the recognition of conserved target proteins, there are other drawbacks to producing antibodies by traditional methods. Antibody production is reliant on the mammalian immune response, making it difficult to produce antibodies to toxic substances, rare epitopes, or non-immunodominant epitopes that could be suitable targets for detection assays (Emanuel et al., 2000). In the case of polyclonal antibodies, a specific target can be identified and specific antibodies purified through the use of affinity purification (Petrenko and Volyanoy, 2003). This requires considerable knowledge of suitable targets and requires a large amount of purified target, often a recombinant protein. It is an expensive and time consuming procedure, particularly for large scale production.

Production of antibodies by traditional methods has provided a platform that is the basis of many detection and diagnostic technologies. However, production of these natural molecules has often required the modification of detection technologies to optimise performance. For example, this may involve the use of additional reagents (Fratamico et al., 1998), optimisation of assay conditions (Studentsov et al., 2002), development or modification of new or existing technologies (Perkins and Squirrel, 2000). Advances in recombinant DNA technology and computational molecular biology have allowed the production of alternatives to traditional antibodies that can be modified to suit the requirements of the detector and assay design (Lindner et al., 1997; Irving et al., 1996; Piervincenzi et al., 1998 and Jung et al., 1999). One of the distinct advantages of recombinant antibodies is that they can be designed or selected to discriminate between very similar proteins. This can be done either by experimental methods or by a process of rational design (Glasner et al., 1992; Miyazaki et al., 1999; McCarthy and Hill, 2001; Cai and Garen 1995; Kirkham et al., 1999 and Skerra, 2000). The use of a carefully selected target antigen that contains highly specific, non-conserved epitopes, allows an increase in the specificity, sensitivity and confidence of a detection assay (Labadie and Desnier, 1992). The aim of the work described here was the production of highly specific single chain antibodies to *Bacillus anthracis* spores. It is extremely easy to produce antibodies to the highly antigenic *Bacillus* spores; however, making antibodies that are specific only to *B. anthracis* is much more challenging.

Genetic analysis has revealed that *B. anthracis* is very closely related to *Bacillus cereus* and *Bacillus thuringiensis* (Read et al., 2003). Helgason et al. (2000) have suggested that all three could be regarded as the same species and that *B. anthracis* and *B. thuringiensis* evolved from a common ancestral species (*B. cereus*) through the acquisition of plasmids, such as pXO1 in the case of *B. anthracis*. Many of the proteins that have been identified for *B. anthracis* spores also have homologues within other members of the *B. cereus* group (Ivanova et al., 2003; Todd et al., 2003 and Redmond et al., 2004). Completely unique targets for detection and diagnosis may be very rare and/or of low abundance, making identification of these proteins and production of antibodies by traditional methods difficult. An approach that would allow for the production of antibodies that can discriminate between closely related species is vital for sensitive and specific detection of *B. anthracis* spores. Zhou et al. (2002) reported an approach to obtain antibodies to *B. anthracis* spores from a naïve human scFvs library, but cross-reactivity with closely related *Bacillus* species was observed. Williams et al. (2003) report the development of peptide ligands to *B. anthracis* spores, but the ligand target was unknown. Both of these approaches used whole spores where the detection target was not defined. The work described here uses a targeted approach to ligand production. We chose to produce single chain antibodies that can specifically detect *B. anthracis* spores through recognition of an individual, well characterised protein target.

The protein target selected from *B. anthracis* is the surface layer protein EA1. EA1 is a vegetative cell protein; however, results from other work suggest that it is also present in spore preparations (Redmond et al., 2004 and Williams and Turnbough, 2004). It has been suggested that EA1 was a contaminant within spore preparations and could be removed through the use of Urografin purification (Williams and Turnbough, 2004), although this does not match our own observations. In this instance EA1 has been used as a model system to demonstrate a methodology that can be employed to rapidly select for specific antibodies to difficult targets. EA1 is known to have homologues in other *Bacillus* species. The work described here selected for recognition elements specific to *B. anthracis* EA1 that could demonstrate specific detection of *B. anthracis* spores without cross-reactivity with other *Bacillus* species.

2. Materials and methods

2.1. Bacterial strains and plasmids

Strain RBA91 (PXO1$^-$, PXO2$^-$ *B. anthracis* Sap mutant) was kindly provided by the Pasteur Institute (25,28 rue du Docteur Roux, Paris). *B. cereus* (NCTC 11143, NCTC 9946 and NCTC 11145), *B. pumilus* (NCTC 10337), *B. brevis* (NCTC 2611), *B. coagulans* (NCTC 10334), *B. subtilis* var. *niger* (NCTC 10073), *B thuringiensis* var. *kurstaki* and *B. thuringiensis* var. *israelensis* were obtained from NCTC (PHLS, 61 Colindale Avenue, London). Plasmids pAK100 (used for phage display) and pAK300 (used for production of soluble scFvs) were a kind gift from Dr A. Plückthun (University of Zürich, Switzerland), and were used as previously described (Krebber et al., 1997) Fig. 1.

2.2. Preparation of S-layer proteins

The S-layer protein was extracted as described previously (Baillie et al., 2003) and the presence of the S-layer proteins was confirmed by SDS-PAGE. Soluble S-layer protein was concentrated by ultrafiltration and filtered using a 0.45 µm filter. Further purification of S-layer proteins was achieved using a HiLoad 1660 Superdex 200 preparatory grade column (Amersham, Amersham Place, Little Chalfont, Buckinghamshire, UK) and an AKTA FPLC system (Amersham), monitoring purity by SDS-PAGE. Fractions that contained pure S-layer protein were pooled and the concentration determined by BCA Assay (Pierce, Century House, High Street, Tattenhall, Cheshire, UK.), according to the manufacturer's instructions.

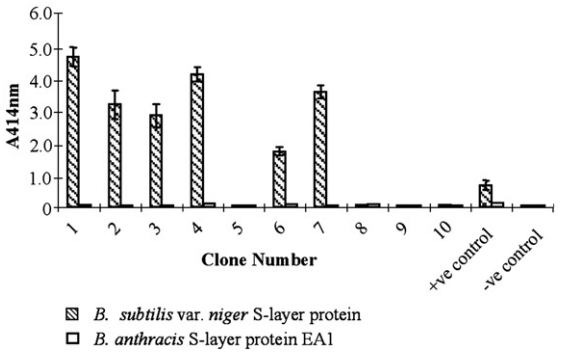

Fig. 1. ELISA results demonstrating binding of ten monoclonal scFvs to *B. anthracis* EA1 and to *B. subtilis* var. *niger* S-layer protein. Monoclonal phage-displayed scFvs isolated from the third round of EA1 panning were prepared from 2 ml of supernatant by PEG precipitation and resuspended in 0.4 ml 2% (w/v) MPBS. An anti-ovalbumin scFv was used as a negative control (labelled −ve control), and a polyclonal scFv known to bind to *B. anthracis* spores were used as a positive control (labelled +ve control). Bound phage were detected using an anti-M13 HRP conjugated antibody. Assays were performed in triplicate, error bars show two standard deviations from the mean. A positive result was defined as being higher than the average of the background signal plus three standard deviations of the mean background sample.

2.3. Spore production

B. anthracis UM23CL2 spores were prepared and washed as described previously (Redmond et al., 2004). All other *Bacillus* spores were prepared using Isolation agar (Oxoid nutrient broth No. 2 CM67 6.0 g/l; manganese sulphate 0.3 g/l; KH2PO4 0.2 g/l Oxoid technical agar No. 3 CL13 12.0 g/l) and incubated at 37 °C until the cultures contained >95% phase bright spores. Spores were harvested and washed as described for *B. anthracis* UM23CL2 spores.

2.4. Construction and use of immune mouse scFv library

Six 12 week old female BALB/c mice were immunised with irradiated *B. anthracis* Ames spores. Each immunisation consisted of 1×10^7 spores in Freund's incomplete adjuvant. Mice were immunised 4 times at intervals of three weeks, and killed by cervical dislocation once they showed a sufficiently high titre (> 1/100 000) to the spores by endpoint ELISA. Spleens were removed from the killed mice and splenic mRNA isolated using Trizol regeant (Invitrogen, Fountain Road, Inchinnan Business Park, Paisley, UK.). The total RNA from the immunised mice was used to produce the immune scFv library; PCR amplification of antibody sequences, overlap extension PCR, cloning of the assembled scFv sequences into pAK100 and production of phage-displayed scFvs was carried out as described by Krebber et al. (1997).

2.5. Biopanning with EA1

Immunotubes (Nunc, BRL, Life Technologies Ltd., Trident House, Washington Road, Paisley, UK) were coated with 1 ml of purified EA1 at 10 µg/ml in PBS overnight at 4 °C and blocked with 2% (w/v) milk powder PBS (MPBS). A volume (100 µl) of scFv-phage were mixed with 900 µl MPBS, incubated for one h at room temperature, and added to the coated immunotubes. After a 2 h room temperature incubation the immunotubes were washed 10 times with PBS 0.1% (v/v) Tween 20. Bound phage was eluted with 100 mM triethylamine and neutralised with 500 µl 1 M Tris HCl, pH 7.5. Eluted phage were infected into log phase XL1-

Blue *E. coli*, and plated on a 24 cm square 2×YT 1% (w/v) glucose 30 μg/ml chloramphenicol agar plate and incubated overnight at 30 °C. This procedure was repeated for each round of panning carried out. Competitive panning was carried out in an identical fashion, adding S-layer extracts to the scFv-phage MPBS solution for 1 h before panning. The concentrations of antigen used for competitive panning were 50 μg/ml *B. cereus* 11145 S-layer protein, and 25 μg/ml of *B. cereus* 11143, *B. cereus* 9946 and *B. pumilus* S-layer protein.

2.6. DNA fingerprinting of scFv clones

scFv sequences from selected clones were amplified by PCR primers surrounding the scFv sequence (scfor and scback; Krebber et al., 1997) and subjected to BstN1 digestion to determine the diversity of the original library and each consecutive round of selection. Restriction digest products were resolved on 4% E-gels (Invitrogen) using 25 bp markers. PCR and DNA fingerprinting were carried out on 10 randomly selected scFv clones from rounds 1 and 2 and 50 scFv clones from round three.

2.7. ELISA

A volume of 100 μl of EA1, S-layer extract (10 μg/ml in PBS) or *Bacillus* spores (1×10^6 spores/ml in water) and appropriate control antigens were coated onto Immulon2 plates (Nunc.) and incubated overnight at 5 °C. PEG-purified phage-displayed scFvs were diluted with MPBS, and an anti-M13 HRP conjugated antibody (Sigma, Fancy Road, Poole, UK) used to detect bound phage. Bound phage were quantified by measuring the conversion of ABTS substrate to coloured product based on A405 nm readings in an automated ELISA reader (Anthos 2001, Anthos Labtec Instruments, Salzburg, Austria).

2.8. Western blot analysis

Proteins were prepared in LDS sample buffer (Invitrogen) according to the manufacturer's instructions and separated by SDS-PAGE in MES buffer (Invitrogen) using precast 10% NuPAGE® BIS gels at 200 V for 40 min Proteins were then blotted onto 0.2 μm nitrocellulose membranes in NUPAGE® transfer buffer (Invitrogen) at 30 V for 1.5 h. For determination of molecular mass MagicMark™ Western protein standard was used (Invitrogen). Blots were prepared according to the manufacturer's instructions (ECL Detection reagent kit, Amersham BioSciences, Chalfont, Bucks, UK.) and probed using the appropriate scFv antibody at 2.5 μg/ml in 3% (w/v) milk powder PBST and an anti-rabbit HRP conjugate (Amersham) antibody at 1/1000 dilution. Protein bands recognised by the antibody were visualised by enhanced chemiluminescence (ECL Detection reagent kit and ECL hyperfilm, Amersham).

2.9. Kinetic analysis of scFv binding

The kinetic data for scFvs binding purified EA1 were obtained using the BIAcore 3000 (BIAcore, Rapsgatan 7, Uppsala, Sweden) with EA1 immobilised onto a CM5 sensor chip, and *B. cereus* 11145 S-layer protein as a negative control. ScFv were passed over the immobilised protein at concentrations varying from 5–400 nM in HBS EP buffer at a flow rate of 10 μl/min. To examine cross-reactivity, the antibody was immobilised onto a surface, and S-layer proteins from *B. cereus* 11145, *B. cereus* 11143, *B. cereus* 9946, *B. thuringiensis* var. *israelensis, B. thuringiensis* var. *kurstaki, B. pumilus, B. brevis, B. coagulans* and *B. subtilis var. niger* passed over at final concentration of 400 nM.

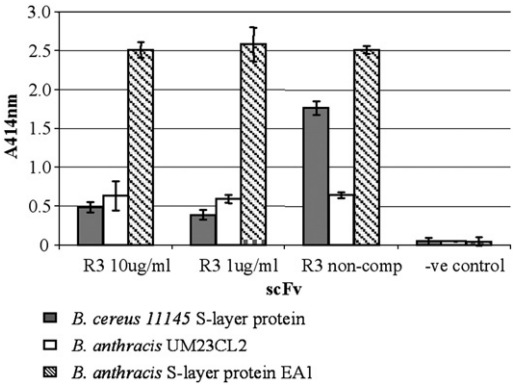

Fig. 2. ELISA results showing binding of polyclonal anti-EA1 from round 3 of competitive and non-competitive biopanning procedures. Polyclonal phage-displayed scFvs from round 3 of the biopanning procedure were diluted 50% (v/v) in MPBS for detection of *B. anthracis* EA1, *B. cereus* 11145 S-layer protein and *B. anthracis* UM23CL2 spores. Three biopanning procedures were used; competitive panning with *B. anthracis* EA1 at 10 μg/ml (labelled R3 10 μg/ml) or 1 μg/ml (labelled R3 1 μg/ml), or non-competitive panning with *B. anthracis* EA1 at 10 μg/ml (labelled R3 non-comp). An anti-ovalbumin scFv was used as a negative control (labelled −ve control). Bound phage were detected using an anti-M13 HRP conjugated antibody. Assays were performed in triplicate, error bars show two standard deviations from the mean. A positive result was defined as being higher than the average of the background signal plus three standard deviations of the mean background sample.

2.10. Detection of whole spores and evaluation of sensitivity using an optical biosensor

The Resonant Mirror (RM, Thermo Labsystems, Saxon Way, Bar Hill, Cambridge, UK.) was used to demonstrate detection of whole *B. anthracis* spores. Antibodies were immobilised onto a RM T70 low molecular weight carboxymethylated dextran (CMD) cuvette surface by standard EDC/NHS coupling methods. The spores were passed over at various concentrations for 10 min and the surface regenerated using 20 mM KOH for 3 min.

3. Results and discussion

3.1. Single chain antibodies produced by non-competitive panning

The non-competitive panning strategy required three rounds of biopanning against an immobilised target antigen, EA1. Binding to EA1 was confirmed by ELISA using a polyclonal population of scFv present after each round of selection. This library would be expected to contain scFvs to a range of spore and exosporium antigens, and has been used to produce antibodies to several other spore surface proteins in addition to EA1 (data not shown).

After round 3 of non-competitive biopanning 50 scFv clones were selected and their ability to bind EA1 assessed by ELISA. A sample result from ten of these clones is shown in Fig. 2. Of the 50 scFv clones analysed 43 were found to bind to EA1, with 7 clones not showing any binding to EA1. Only 2 scFv clones demonstrated any cross-reactivity to *B. subtilis* var. *niger* S-layer protein, with the remaining 41 showing no detectable cross-reactivity to *B. subtilis* var. *niger* S-layer protein. A total of 13 unique clones were identified by BstN1 fingerprinting,. These clones were all confirmed to be different by DNA sequencing (data not shown), and are referred to as scFv1 to scFv 13.

The scFvs produced were to be used in a biosensor assay that would detect *B. anthracis* with the exclusion of all other *Bacillus* species. Therefore, it was important to demonstrate that the anti-EA1 scFvs would not react with S-layer proteins from other *Bacillus* species. It is often argued that any antibody can cross-react with another unrelated target, leading to the observation that "an antibody is only truly specific in the background in which it is

Table 1
Summary of ELISA results demonstrating the binding of unique monoclonal scFvs to EA1 and the cross-reactivity to other S-layer proteins, determined by ELISA

	EA1	*B. thuringiensis* var. *israelensis*	*B. thuringiensis* var. *kurstaki*	*B. cereus* 11143	*B. cereus* 9946	*B. cereus* 11145	*B. pumilus*	*B. brevis*	*B. coagulans*	*B. anthracis* UM23CL2 spores
scFv1	+++	−	−	−	++	−	−	−	−	++
scFv2	+++	−	−	−	−	+++	+++	−	−	++
scFv3	+++	−	−	−	−	+++	+++	−	−	++
scFv4	+++	−	−	−	−	++	++	−	−	++
scFv5	+++	−	−	−	−	+++	++	−	−	++
scFv6	+++	−	++	++	++	+++	+++	++	−	++
scFv7	+++	−	−	−	++	+++	++	−	−	++
scFv8	+	−	−	−	−	+++	+++	−	−	++
scFv9	+++	−	−	−	−	++	−	−	−	+
scFv10	+	−	−	−	−	−	+++	−	−	++
scFv11	+	−	−	−	−	−	+++	−	−	++
scFv12	+++	−	−	+++	−	−	−	−	−	++
scFv13	+	−	−	++	−	+++	−	−	−	++
EA1.1	+++	−	−	−	−	−	−	−	−	++
EA1.23	+++	−	−	−	−	−	−	−	−	++
EA1.10	+++	−	−	−	−	−	−	−	−	+
EA1.20	+++	−	−	−	−	−	−	−	−	++
EA10.1	+++	−	−	−	−	−	−	−	−	++
EA10.4	+++	−	−	−	−	−	−	−	−	++

Soluble scFvs were produced, purified by IMAC and used at 5 μg/ml in ELISA. Bound phage were detected using an anti-his tag HRP conjugated antibody (Sigma). Each assay was performed in triplicate. A summary of these ELISA assays is presented here; results are expressed in terms of the percentage of the maximum signal seen in the assay (+++ indicates 60–100% of the maximum, ++ indicates 20–59%, + indicates a signal greater than detection threshold, defined as the background signal plus 3 standard deviations from the mean of the background signal, and − indicates a signal below the detection threshold.). Clones given the designation scFvs or EA1 resulted from the non-competitive or competitive panning strategy respectively.

tested" (Holt et al., 2000). In a biodetection context the relevant background is the background that the sample would have been taken from, usually an environmental air sample. For this reason only a selection of *Bacillus* species were tested for cross-reactivity. An organism of particular concern was *B. thuringiensis*, used (and sprayed) widely as an insecticide. BLAST searches demonstrate a very high degree of similarity between the EA1 protein sequence of *B. thuringiensis* and *B. anthracis*. The other organism of concern was *B. cereus*, another closely related species (Helagason et al., 2000).

The cross-reactivity of our 13 unique scFvs was determined by direct ELISA against S-layer proteins isolated from other *Bacillus* species. All of the 13 unique scFvs tested showed cross-reactivity with S-layer proteins from the other *Bacillus* tested (for brevity these ELISA results are summarised in Table 1). The highest degree of cross-reactivity was observed with *B. cereus* NCTC 11145 and *B. pumilus* S-layer proteins.

A high degree of cross-reactivity of the scFvs produced by non-competitive biopanning was observed with *B. pumilus, B. subtilis* var. *niger* and *B. cereus* 11145 S-layer. This suggests that these proteins demonstrate the greatest degree of similarity to EA1 or that a highly conserved or immunodominant epitope exists within these species (Holt et al., 2000). Irrespective of the mechanism, none of these anti-EA1 scFvs were of use for specific detection of *B. anthracis*.

3.2. Single chain antibodies produced by competitive panning

After failing to isolate *B. anthracis* specific anti-EA1 scFvs a competitive panning strategy was employed. This involved negative selection of cross-reacting scFvs by binding to S-layer proteins from species that cross-reacted with the original anti-EA1 scFvs. The panning procedure was repeated, but this time a mixture of competitive S-layer extracts (50 μg/ml *B. cereus* 11145 S-layer protein, and 25 μg/ml of *B. cereus* 11143, *B. cereus* 9946 and *B. pumilus* S-layer protein) was added to the solution of panning phage at the first round. The amount of EA1 used to coat the immunotube was also varied (1 or 10 μg/ml). Binding to EA1 by the polyclonal population of scFv present after each round of panning was again confirmed by ELISA. There was no significant difference in signal observed between the polyclonal scFvs selected using 1 or 10 μg/ml of EA1 (Fig. 3). After round 3 there was still some cross-reactivity with *B. cereus* 11145 S-layer protein, however this was much lower as that observed in round 3 scFv-phage using the non-competitive approach (Fig. 3).

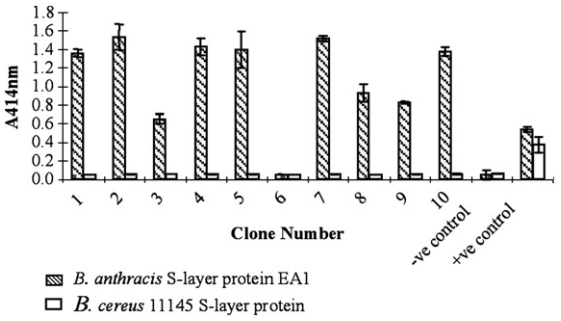

Fig. 3. An example of ELISA results showing binding of anti-EA1 monoclonal scFvs selected by a competitive biopanning procedures that show no cross-reactivity with *B. cereus* 11145 S-layer protein. Monoclonal phage-displayed scFvs isolated from the third round of EA1 competitive panning were prepared from 2 ml of supernatant by PEG precipitation and resuspended in 0.4 ml 2% (w/v) MPBS. An anti-ovalbumin scFv was used as a negative control (labelled −ve control), and a polyclonal scFv known to bind to *B. anthracis* spores were used as a positive control (labelled+ve control). Bound phage were detected using an anti-M13 HRP conjugated antibody. Assays were performed in triplicate, error bars show two standard deviations from the mean. A positive result was defined as being higher than the average of the background signal plus three standard deviations of the mean background sample.

These ELISA results indicate some scFvs that cross-react with *B. cereus* 11145 S-layer protein are still selected, even though a competitive selection procedure was used. This may be because the cross-reactive antigens from closely related species were not sufficiently in excess (although 10 fold excess was used) leaving cross-reactive scFvs to bind to the target. Furthermore, the cross-reactive epitope within the S-layer protein that binds to the scFv may be immunodominant, ensuring that a large proportion of the scFvs bind to this site (Holt et al., 2001). It may also be due to selective pressures imposed by growth or expression, or a combination of these factors.

After round 3 of competitive panning 50 scFvs (25 rom each of the 1 μg/ml and 10 μg/ml selections) clones were selected and analysed by ELISA. Ten of these are shown as an example in Fig. 4. In total, 18 scFvs cross-reacted with *B. cereus* 11145 S-layer protein (6 from the 1 μg/ml strategy and 12 from the 10 μg/ml strategy). Three of the selected scFvs did not bind EA1, all of which were taken from the 1 μg/ml EA1 panning. 29 of the 50 scFvs analysed were found to be specific for *B. anthracis* EA1; 16 of these were selected using 1 μg/ml EA1 and 13 were selected using EA1 at a concentration of 10 μg/ml. This result demonstrates the utility of the competitive panning method. It was not possible to obtain scFvs antibodies specific to *B. anthracis* EA1 by conventional non-competitive panning, while the competitive method rapidly isolated non cross-reactive scFvs antibodies.

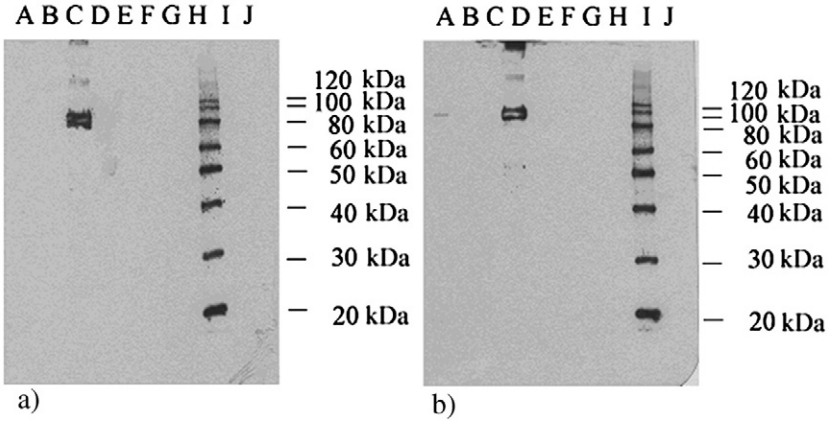

Fig. 4. Cross-reactivity of monoclonal scFvs selected by competitive biopanning to *Bacillus* S-layer proteins by Western blot. The cross-reactivity of two different antibodies is shown here; scFv EA1.1 (blot a) and scFv EA10.4 (blot b). 5 μg of each S-layer protein extract was run per lane on a 10% BIS TRIS NuPAGE gels (Invitrogen) and blotted onto nitrocelluose membranes (Invitrogen). Lane loading was as follows: A*) B. pumilus* S-layer protein, B) *B. brevis* S-layer protein, C) *B. coagulans* S-layer protein, D) *B. anthracis* S-layer protein EA1, E*) B. cereus* 11145 S-layer protein, F) *B. cereus* 11143 S-layer protein, G) *B. cereus* 9946 S-layer protein H) *B. subtilis* var. *niger* S-layer protein I) MagicMark™ Western standard, J) ovalbumin negative control. After probing, bound scFvs was visualised using an anti-his tag HRP conjugated antibody (Sigma) followed by enhanced chemiluminescent detection.

ScFv antibodies from rounds 1 and 3 of the competitive panning were examined by BstN1 fingerprinting to identify unique clones. In total six unique scFvs were identified in total from both the 1 μg/ml and 10 μg/ml competitive panning strategies and were confirmed unique by DNA sequence analysis (EA1.1, EA1.23, EA1.10, EA1.20, EA10.1, EA10.4; data not shown). This suggests that competitive panning rapidly lead to the elimination of a large percentage of the population of scFv clones by negative selection. Therefore, some binders may have been lost through further rounds of selection, either due to low affinity or growth or expression selection pressures. The scFvs produced by the competitive approach showed less diversity in the CDRs than those produced by non-competitive selection. As expected, variations in CDR-H3 gave the main source of diversity between different antibodies, as CDR-H3 is mainly responsible for specificity (McCarthy and Hill, 2001).

The cross-reactivity of the six unique scFvs isolated by competitive panning was determined by direct ELISA against S-layer proteins isolated from other *Bacillus* species. None of these scFvs showed any cross-reactivity with S-layer proteins from the other *Bacillus* tested (Table 1). This indicates that all six scFvs recognise epitopes that are unique to *B. anthracis* EA1. To verify this result the scFvs were used to probe purified *Bacillus* S-layer extracts on Western blots. Detection of purified *B. anthracis* EA1 was demonstrated with all six scFvs generated (two shown as examples in Fig. 5). EA1.1, EA1.23, EA1.10 and EA10.1 showed no cross-reactivity with any other *Bacillus* S-layer proteins tested, even in grossly overexposed blots. EA1.20 and EA10.4 did show low levels of cross-reactivity with *B. pumilus* S-layer protein (only visible after 30 min exposure; example shown in Fig. 5. This cross-reactivity with *B. pumilus* S-layer protein was never observed by ELISA, Resonant Mirror or BIAcore analyses (data not shown).

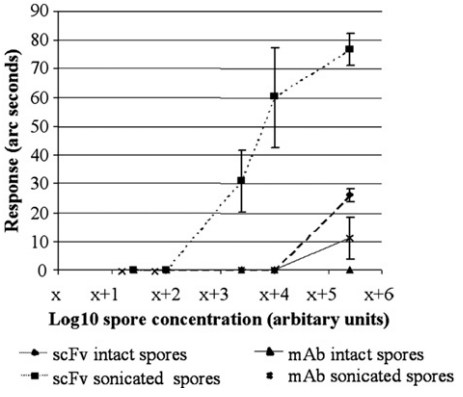

Fig. 5. Detection of *B. anthracis* UM23CL2 spores on a real time biosensor using a competitively selected scFvs and a monoclonal anti-EA1 antibody. Antibodies were immobilised using standard EDC/NHS coupling onto a T70 CMD surface (Labsystems, Affinity sensors). Intact or sonicated spores were passed over immobilised scFv EA1.1 (scFv intact or sonicated spores) or anti-EA1 monoclonal (mAb intact or sonicated spores) antibody in PBS 0.05% (v/v) Tween 20. Spores were sonicated as described previously (Borthwick et al., 2005).

3.3. Use of specific EA1 antibodies on biosensors

Analysis of the affinity constants of the six *B. anthracis* EA1-specific scFvs demonstrated that the highest affinity constants were seen in the scFvs selected using 1 μg/ml of EA1 compared to those isolated using 10 μg/ml EA1 (Table 2). No binding was observed on the BIAcore with any of these six specific scFvs for any other S-layers evaluated (*B. pumilus, B. cereus* 11145, *B. cereus* 11143, *B. cereus* 9946, *B. coagulans, B. brevis, B. subtilis* var. *niger, B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *kurstaki;* data not shown).

These results revealed that these *B. anthracis* specific scFvs had apparent nanomolar affinities for EA1; very satisfactory values for antibodies to be used for sensitive detection. As absolute specificity was used as the original criteria for success, other stronger binders (albeit cross-reactive) may have been eliminated through the competitive panning process. It is likely that the affinities of these antibodies could be improved by further maturation techniques if desired (for example, Mossner and Pluckthun, 2001, Jermatus et al., 2001).

Demonstration of the detection of intact *B. anthracis* spores was conducted using the Resonant Mirror (Thermo Labsystems). Three of the specific scFvs (EA1.1, EA1.23 and EA1.10) were taken forward for evaluation of the sensitivity to whole *B. anthracis* UM23C12 spores. For comparison an anti-EA1 monoclonal antibody was also evaluated. All scFvs demonstrated detection of untreated *B. anthracis* spores (Fig. 5), although the sensitivity of the assay improved significantly when the spores were sonicated as has been demonstrated with other *Bacillus* spores (Borthwick et al., 2005). In comparison the mAb could not detect intact *B. anthracis* UM23CL2 spores at any of the concentrations tested, and only a small amount of binding was observed after sonication (Fig. 5). The scFvs showed no cross-reactivity to any other *Bacillus* species spores tested (both untreated or sonicated) while the monoclonal antibody showed detection of sonicated *B. cereus* 11145 spores.

Table 2
Equilibrium association (KA) and dissociation (KD) constants for scFv antibody clones produced using competitive panning strategies

Single chain	KA (1/M)	KD (M)
EA1.1	5.85E+10	1.71E−LI
EA1.23	4.48E+10	2.23E−11
EA1.10	1.72E+08	5.81E−09
EA1.20	1.89E+10	5.28E−11
EA10.1	1.01E+09	9.91E−10
EA10.4	3.55E+08	2.81E−09

Equilibrium constants were derived using the BIAevaluation software (Biacore) using a simple Langmuir 1:1 binding model and the association (K_a) and dissociation (K_d) rate constants calculated for each set of data. The equilibrium constant KA was calculated from the ratio K_a/K_d and KD from K_d/K_a.

3.4. Competitive biopanning: a significant advantage?

We routinely produce scFv libraries from immunised mice in order to obtain scFvs that show high affinities for the desired targets. While we have used naive libraries successfully, we find that higher affinities and a wider diversity of antibodies can be obtained from an immune library. Competitive panning is extremely useful to reduce the complexity of these immune libraries by eliminating cross-reactive antibodies. Immune libraries have the advantage of having undergone significant affinity maturation in the mouse; the antibodies evolve *in vivo* in the B cell by hypersomatic mutation within the hypervariable regions to enhance specificity and affinity (Borrebaeck and Ohlin, 2002).

The immune library used here was created from mice immunised with a complex antigen (whole *B. anthracis* spores) not specifically against EA1, so this library would have contained a selection of antibodies that bound to a range of target antigens. The diversity of this library may be limited with respect to those that recognise a range of EA1 epitopes. If an anti-EA1 library had been utilised, this may have enhanced the probability of isolating an EA1 specific scFvs by non-competitive selection, although in practice producing a single library for each complex target allows a range of antibodies to be isolated while minimising animal use. It is possible that this method may only be applicable to highly immunogenic antigens; EA1 is a major antigen associated with the vegetative cells of *B. anthracis* (Mesnage et al., 1997). Western blot analysis of a polyclonal goat antiserum raised against whole *B. anthracis* spores also showed binding to EA1, demonstrating that it is present and immunogenic in spore preparations (data not shown). It is important to remember that specific EA1 antibodies must have been lost during non-competitive panning, perhaps due to competition from non-specific antibodies with higher affinities for the target or other selection pressures such as slow growth.

The use of a competitive panning procedure (sometimes termed pre-adsorption, subtractive antibody screening or negative selection) has been used for other targets; Krebs et al. (2001) described a method by which pre- and post-adsorption steps could be utilised to select out scFvs that bound to cross-reactive targets to produce specific scFvs. Specific anti-melanoma antibodies have also been prepared using pre-absorbing with melanocytes 10 times (Cai and Garen, 1995). The much simpler procedure of

one step negative selection demonstrated here shows that a complex mixture of competitors can be used in the first round of selection to remove the majority of non-specific binders and isolate a number of scFvs specific for the original target.

The generation of specific recognition elements for EA1 and consequently *B. anthracis* spores in this case was only possible using the competitive strategy. The resultant recombinant antibodies can be used successfully across a wide range of detection techniques, from conventional laboratory analysis methods such as ELISA to evanescent wave based biosensors. When used on the Resonant Mirror the sensor was able to signal specific detection of anthrax spores in real time, with no false positives even when exposed to very high backgrounds of closely related *Bacillus* species that commonly cross-react with anti-anthrax polyclonal and monoclonal antibodies. We have not found any non-recombinant antibodies that are able to perform to this high specification, and believe that this method of competitive panning gives us a major advantage over conventional monoclonal and polyclonal approaches, especially for critical diagnostic and detection applications.

Acknowledgements

We thank the Pasteur Institute for provision of the *B. anthracis* strain RBA91, Dr. A. Plückthun (University of Zürich, Switzerland) for the pAK 100 and 300 plasmids, John Dewey (Dstl Porton Down, Wiltshire, UK) for production of EA1 and Dr. I. Atkin for provision of anti-ovalbumin scFv. This work was funded by the UK Ministry of Defence.

References

Baillie, L., Hebdon, R., Flick-Smith, H., Williamson, D., 2003. Characterisation of the immune response to the UK human anthrax vaccines. FEMS Immunol. Med. Microbiol. 36, 218.

Borrebaeck, C.A.K., Ohlin, M., 2002. Antibody evolution beyond nature. Nat. Biotechnol. 20, 1189.

Borthwick, K.A., Love, T.E., McDonnell, M.B., Coakley, W.T., 2005. Improvement of immunodetection of bacterial spore antigen by ultrasonic cavitation. Anal. Chem. 742.

Cai, X, Garen, A, 1995. Anti-melanoma antibodies from melanoma patients immunised with genetically autologous tumour cells: selection of specific antibodies from single-chain Fv fusion phage libraries. Proc. Natl. Acad. Sci. USA. 92, 6537.

Emanuel, P.A., Dang, J., Gebhardt, J.S., Aldrich, J., Garber, E.A.E., Kulaga, H., Stopa, P., Valdes, J.J., Dion-Scultz, 2000. Recombinant antibodies: a new reagent for biological detection. Biosens. Bioelectron. 14, 751.

Framatico, P.M., Strobaugh, T.M., Medina, M.B., Gehring, A.G., 1998. Detection of *Escherichia Coli* 0157:h7 using a surface plasmon resonance biosensor. Biotechnol. Tech. 12, 571.

Glasner, S.M., Yelton, D.E., Huse, W.D., 1992. Antibody engineering by codon-based mutagenesis in a filamentous phage vector system. J. Immunol. 15, 3903.

Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., 2000. *Bacillus anthracis, Bacillus cereus* and *B. thuringiensis* — one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66, 2627.

Holt, L.J., Büssow, K., Walter, G., Tomlinson, I.M., 2000. By-passing selection: direct screening for antibody–antigen interactions using protein arrays. Nucleic Acids Res. 28 (15), E72.

Irving, R.A., Kortt, A.A., Hudson, P.J., 1996. Affinity maturation of recombinant antibodies using *E. coli* mutator cells. Immunotechnology. 2, 127.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A, et al., 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *B. anthracis*. Nature 423, 87.

Jermatus, L., Honeggar, A., Schwesinger, F., Hanes, J., Plückthun, 2001. Tailoring in vitro evolution for protein affinity or stability. Proc. Natl. Acad. Sci. 98 (1), 75.

Jung, S., Honegger, A., Plückthun, A., 1999. Selection for improved protein stability by phage display. J. Mol. Biol. 294, 163.

Kirkham, P.M., Neri, D., Winter, G., 1999. Towards the design of an antibody that recognises a given protein epitope. J. Mol. Biol. 285, 909.

Krebber, A., Bornhauser, S., Bumester, Bumester, J., Honegger, A., Willunda, J., Bosshard, H.R., Plückthun, A., 1997. Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J. Immunol. Methods. 201, 35.

Krebs, B., Rauchenberger, R., Reiffert, S., Rothe, C., Tesar, M., Thomassen, E., Cao, M., Dreier, T., Fischer, Höß, A., et al., 2004. High-throughput generation and engineering of recombinant human antibodies. J. Immunol. Methods 254, 67.

Labadie, J., Desnier, I., 1992. Selection of cell wall antigens for the rapid detection of bacteria by immunological methods. J. Appl. Bacteriol. 72 (3), 220.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mockikat, R., Plückthun, A., 1997. Specific detection of His-tagged proteins with recombinant anti-His tag scFv-phosphatase or scFv fusions. Biotechniques 22, 140.

McCarthy, B.J., Hill, A.S., 2001. Altering the fine specificity of an anti-Legionella single chain antibody by a single amino acid insertion. J. Immunol. Methods. 251, 137.

Mesnage, S., Tosi-Couture, E., Mock, M., Gounon, P., Fouet, A., 1997. Molecular characterisation of the *Bacillus anthracis* main S-layer component: evidence that it is the major cell associated antigen. Mol. Microbiol. 23 (6), 1147.

Miyazaki, C., Iba, Y., Yamada, Y., Takahashi, H., Sawada, J., Kurosawa, Y., 1999. Changes in the specificity of antibodies by site specific mutagenesis followed by random mutagenesis. Prot. Eng. 12 (5), 407.

Mössner, E., Plückthun, A., 2001. Directed evolution with fast efficient selection technologies. Chimia. 55, 324.

Perkins, E.A., Squirrel, D.J., 2000. Development of instrumentation to allow the detection of microorganisms using light scattering in combination with surface plasmon resonance. Biosens. Bioelectron. 14, 853.

Petrenko, V.A., Vodyanoy, V.J., 2003. Phage display for detection of biological threat agents. J. Microbiol. Methods, 53, 253.

Piervincenzi, R.T., Reichart, W.M., Hellinga, H.W., 1998. Genetic engineering of a single-chain antibody fragment for surface immobilisation in an optical biosensor. Biosens. Bioelectron. 13 (3 and 4), 305.

Read, T., Peterson, S.N., Tournasse, N., Baillie, L.W., Paulsen, I.T., Nelson, K.E., Tettelin, H., Fouts, D.E., Eisen, J.A., Gill, S.R., et al., 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature 423.

Redmond, C., Baillie, L.W.J., Hibbs, S., Moir, A.J.G., Moir, A., 2004. Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology. 150, 355.

Skerra, A., 2000. Engineered protein scaffolds for molecular recognition. J. Mol. Recognit. 13, 167.

Studentsov, Y.Y., Schiffmann, M., Strickler, H.D., Ho, G.Y.F., Pang, Y.S., Schiler, J., Herrero, R., Burk, R.D., 2002. Enhanced enzyme-linked immunosorbent assay for detection of antibodies to virus like particles of Human Papillomavirus. J. Clin. Microbiol. 40 (5), 1755.

Todd, S.J., Moir, A.J., Johnson, M.J., Moir, A., 2003. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. J. Bacteriol. 185 (11), 3373.

Williams, D.D., Benedeck, O., Turnbough, C.L., 2003. Species-specific peptide ligands for the detection of *Bacillus anthracis* spores. Appl. Env. Microbiol. 69 (10), 6288.

Williams, D.D., Turnbough, C.L., 2004. Surface layer protein EA1 is not a component of *Bacillus anthracis* spores but is a persistent contamination in spore preparations. J. Bacteriol. 186 (2), 556.

Zhou, B., Wirsching, P., Janda, K.D., 2002. Human antibodies against spores of the genus *Bacillus:* A model study for detection of and protection against anthrax and the bioterrorist threat. Proc. Natl. Acad Sci. 99 (8), 5421.