Development and Implementation of a Single-Chain Fv Antibody for Specific Detection of Bacillus anthracis Spores

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A single-chain Fv (scFv) antibody was developed and applied for efficient and specific detection of Bacillus anthracis spores. The antibody was isolated from a phage display library prepared from spleens of mice immunized with a water-soluble extract of the outer membrane of the B. anthracis spore (exosporium). The library (7 × 10⁶ PFU) was biopanned against live, native B. anthracis ATCC Δ14185 spores suspended in solution, resulting in the isolation of a unique soluble scFv antibody. The antibody was affinity purified and its affinity constant (3 × 10⁸ ± 1 × 10⁶ M⁻¹) determined via flow cytometry (FCM). Preliminary characterization of scFv specificity indicated that the scFv antibody does not cross-react with representatives of some phylogenetically related Bacillus spores. The potential use of scFv antibodies in detection platforms was demonstrated by the successful application of the soluble purified scFv antibody in enzyme-linked immunosorbent assays, immunofluorescence assays, and FCM.

Bacillus anthracis spores, the primary infectious agents causing anthrax, are probably the most likely candidates for a biological terrorist assault. Therefore, rapid detection of spores is critical for a timely response and successful treatment of the disease. Various immunoassays based on the high specificity of antibodies have been developed for the rapid detection of several pathogens. Over the past decade, recombinant technology enabled the production of engineered antibody fragments such as Fabbs or single-chain Fv (scFv) antibodies. An scFv antibody is a small engineered antibody in which the variable heavy chain and light chain of the antibody molecule are connected by a short, flexible polypeptide linker. Phage display technology enables the presentation of scFv antibody on the phage surface and has been used successfully for the isolation of specific scFv antibodies from animal repertoire libraries via several enrichment cycles.

Using scFv antibodies for antigen detection has several advantages. scFv antibodies can be produced in large quantities in bacterial expression systems, with high reproducibility at low cost, and can be manipulated genetically for improved specificity and affinity (5, 15, 17). The recombinant antibody can also be fused to marker molecules for detection purposes (25). Recombinant antibodies have been developed for treatment of anthrax infection (26) and disease detection in clinical applications (25). However, the use of recombinant antibodies for detection of B. anthracis spores has not been described. In this study, we describe the construction of an scFv antibody and its successful application for B. anthracis spore detection.

MATERIALS AND METHODS

Bacteria. B. anthracis Δ14185 is a nontoxigenic, nonencapsulated (Tox⁻ Cap⁻) derivative of ATCC 14185 (4) (Bacillus Genetic Stock Center); Bacillus subtilis DSM 675 and Bacillus cereus 569 are from the Israel Institute for Biological Research collection. Escherichia coli TG1 was part of the RPAS expression module (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, United Kingdom).

Bacillus cultures and sporulation. Spores of all strains were produced in SSM sporulation medium, as previously described (6).

Immunization. Female BALB/c mice were immunized subcutaneously with 1 × 10⁸ CFU of irradiated (15 min at maximum intensity in a microwave oven) B. anthracis spores or with 10 μg of a soluble exosporium fraction (4). Immunizations were carried out every 2 weeks in incomplete Freund’s adjuvant until no change in antibody titer was observed (four or five injections). Enzyme-linked immunosorbent assay (ELISA) was carried out against live spores. Mice were sacrificed (4 days after the last booster), and spleens were removed directly to liquid nitrogen.

ELISA for immunized mouse antibody titer determination. ELISA plates were coated with 1 × 10⁸ CFU/ml urogalin-purified (20) B. anthracis Δ14185 spores in carbonate-bicarbonate buffer (C-3041; Sigma-Aldrich, St. Louis, MO) and incubated overnight at 4°C. Plates were then washed three times with PBS-T (phosphate-buffered saline [PBS] containing 0.05% [vol/vol] Tween 20) and blocked for 1 h with PBS-2% bovine serum albumin (BSA)-0.05% Tween 20 at 37°C. After additional washes, immunized mouse serum, diluted in blocking buffer, was applied for another hour, to be detected by alkaline phosphatase–anti-mouse immunoglobulin G antibodies (A-4312; Sigma). Antibody titers were calculated as reciprocal geometric mean titers. Values of at least twice the background signal were considered positive.

Construction of scFv library. Total RNA was extracted from homogenized spleen tissues (homogenization was carried out under liquid nitrogen), using TRI reagent (TR118; MRC Molecular Research Center) according to the manufacturer’s instructions. cDNA templates were prepared using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), applying a two-step reverse transcription-PCR protocol recommended by the manufacturer. Primers were used for the amplification and assembly of heavy- and light-chain DNAs as described previously (3). scFv DNA was purified, digested with SfI and NotI, and ligated (T4 DNA ligase; NEB, Beverly, MA) into NotI/SfiI-linearized phagemid pCANTAB-5E (Pharmacia) containing an E tag sequence in frame. The recombinant phagemid was introduced into competent E. coli TG1 cells by electroporation, and library size was determined by plating. Diversity was analyzed by fingerprinting (BstNI digestion).

Biopanning against live spores in suspension. The scFv phage library was biopanned for binders against live spores in suspension as follows. The library was amplified and rescued as described previously (5). Phage particles (1 × 10¹⁴) were challenged with 1 × 10¹⁰ CFU of live avirulent B. anthracis Δ14185 spores suspended in 1 ml PBS containing 2% skim milk (Difco-Becton Dickinson, Sparks, MD). After 1.5 h at room temperature with gentle agitation, the spores were spun down (5 min, 4°C, 12,000 × g) and washed 10 times (PBS containing 0.05% [vol/vol] Tween 20). The spore-phae complex was then allowed to infect...
E. coli TG1 host cells directly, with no elution step, in order to amplify the selected spore-binding phenotype. The enriched library was then plated on ampicillin (100 μg/ml) and glucose (0.4%)–containing plates, rescued, and used for further panning cycles (no B. anthracis contamination of the resulting enriched libraries was observed). Further cycles were carried out essentially as described above, with decreasing spore titers (down to 1 × 10^4 CFU) and increasing wash cycles (up to 10x PBS plus 0.05% Tween 20 and 5× PBS). For cycle n, the phage enrichment factor was calculated as follows: (Out_{n-1}/In_{n-1}). Spore-binding activity following library enrichment was monitored by phage ELISA as described in the subsequent section.

Screening for specific binders by phage ELISA. Rescue of individual phage colonies and testing for spore-binding activity was carried out as described previously (3). The same ELISA was also used for monitoring enrichment of specific binders following panning cycles. ELISA plates were coated with B. anthracis DSM 614185 spores. Blocking was carried out with PBSM (PBS plus 2% skim milk) for 2 hours at 37°C. BSA was used as the control antigen. Anti-M-13 horseradish peroxidase (HRP)-conjugated antibodies (Pharmacia) were used as reporting antibodies (1:5,000) in the assay. Clones were considered positive if they demonstrated at least twice the signal developed with the control antigen.

Production and screening of soluble scFv antibody fragments by ELISA. Soluble scFv antibodies were produced in E. coli TG1 cells as described previously (3). Screening for spore-binding soluble scFv antibodies was carried out by ELISA, after cold osmotic shock of each clone (RPAS purification module; Amersham Pharmacia Biotech, Buckinghamshire, England). The ELISA was carried out essentially as described in the previous section, with the following modifications: anti-E-tag HRP-conjugated antibodies (Pharmacia) were used as reporting antibodies (1:2,000) in the assay. Clones were considered positive if they demonstrated at least twice the signal developed with the control antigen.

Purification of soluble scFv antibody fragments. Large-scale production of positive scFv antibody clones as E. coli periplasmic extracts was carried out as outlined for the RPAS purification module (Amersham Pharmacia Biotech, Buckinghamshire, England). Isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 0.75 mM) was given at an optical density at 600 nm of 1.5, and the culture was harvested after 20 h at 30°C. The resulting pellet was resuspended in 0.5 M phosphate buffer, pH 8, and sonicated on ice (eight pulses of 30 seconds each, at maximum capacity) in a Vibra Cell sonicator (Sonics and Materials Inc., Newtown, CT). Purification of scFv antibodies from the periplasmic extract of E. coli was carried out using a commercial anti-E-tag affinity column (Amersham Pharmacia Biotech, Buckinghamshire, England) according to the manufacturer’s instructions.

Western blotting for antigenic determinant (epitope) evaluation. A soluble exosporium fraction was resolved in 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels. Gels were either stained with Coomassie blue or blotted onto a nitrocellulose filter (polyvinylidine difluoride; Roche Diagnostics GmbH, Mannheim, Germany). The filter was probed with the soluble scFv antibody, which in turn was detected with an anti-E-tag HRP-conjugated antibody (Pharmacia) (1:1,000). An E. coli periplasmic extract was used as a negative control to demonstrate at least twice the signal developed with the control antibody.

Screening of specific binders. The first step in the preparation of a high-quality immunized phage display library involves the optimization of an immunization strategy for high antibody titers. To this end, two immunization strategies were evaluated. The first utilized the intact antigen inactivated B. anthracis spores. The second consisted of a partial spore extract, i.e., a watersoluble fraction of the outer membrane of B. anthracis spores (exosporium). The two reagents were used for immunization of two BALB/c mouse groups. Antibody titers, determined by ELISA with live spores, were 10,000 and 100,000, respectively, indicating a higher humoral response induced by the soluble fraction. Hence, RNAs, used as a template for library construction, were extracted from the spleens of mice immunized with a soluble exosporium fraction. An scFv antibody phage display library was constructed from cDNA templates essentially as described previously (3). After primary and secondary amplifications of the heavy- and light-chain genes by PCR, the linker-containing fragments were assembled by overlap extension PCR, resulting in scFv DNA fragments with an expected length of ~750 bp. The fragments were NotI/SfiI digested and ligated into NotI/SfiI-linearized phagemid pCANTAB-5E (Pharmacia). The antibodies were fused to the pIII phage surface protein and expressed with an E tag sequence in frame. The recombinant phagamids were introduced into competent E. coli TG1 cells, with a transformed rate of 7 × 10^6. Library quality was assessed by PCR for full-length inserts and fingerprinting (BstNI digestion of full-length inserts) of 30 randomly selected antibodies. The library was found to contain >90% intact (~750-bp) unique (different digestion patterns) antibodies.

Biopanning. The biopanning process, outlined in Fig. 1, included the introduction of the entire phage library into live B. anthracis spores in solution. Thus, antibodies were challenged by the native antigen. After four panning cycles, with decreasing spore concentrations and increasing wash cycles, an enrichment factor (calculated as described in Materials and Methods) of 2,000 was obtained. The antibody population consisted of 90% intact (as determined by full-length insert PCR) and 60% diverse (fingerprinting of the antibody fragments) antibodies. Phage, after each enrichment cycle, were tested for spore-binding ability by phage ELISA as follows. Phage rescue was carried out with adhered spores and detected with anti-M13 HRP-conjugated antibodies. The final enriched library...
exhibited an enhanced (14 times the signal of the original library) specific response to \textit{B. anthracis} spores.

**Selection of anti-spore binding antibodies.** Individual clones of the enriched library were rescued and examined for spore-binding activity by phage ELISA, and 80\% of them were found to be positive. All binders were induced (IPTG) for soluble expression of the scFv antibody, and a periplasmic extract was prepared by cold osmotic shock. ELISA for spore-binding activity was performed against adhered spores. Soluble scFv antibodies were detected with an anti-E-tag HRP-conjugated antibody. Fingerprinting (BstNI digestion) of the resulting antibodies revealed four unique patterns. The expression of intact antibodies in the periplasmic fraction of \textit{E. coli} was verified by Western blotting. A clear and strong signal for the putative scFv protein (\(\sim 30\) kDa) was detected, using anti-E-tag antibodies, in the induced TG1 periplasmic samples carrying the selected scFv antibody clones. No band was observed for the uninduced \textit{E. coli} periplasmic extract (results not shown).

The four selected clones exhibited similar responses with \textit{B. anthracis} spores. Consequently, only one of the antibodies was further characterized. Expression of the selected scFv antibody was carried out in \textit{E. coli} TG1 cells. The scFv antibody was purified from 1 liter of cell culture on a commercial anti-E-tag column, with a yield of about 0.2 to 0.3 mg/liter. The purity of the resulting scFv protein was verified with a Coomassie blue-stained SDS-polyacrylamide gel (results not shown).

**Characterization of scFv antibody.** The soluble scFv antibody was incubated with the exosporium fraction in order to elucidate an antigen recognition pattern. To this end, the soluble exosporium was resolved in 10\% SDS–polyacrylamide gels that were either stained with Coomassie blue or blotted onto a nitrocellulose filter. The filter was then probed with the purified scFv antibody included a preliminary specificity profile determination. To this end, two other \textit{Bacillus} species, the phylogenetically closely related species \textit{B. cereus} and the somewhat distant relative \textit{B. subtilis} DSM 675, were subjected to FCM analysis. An affinity constant of \(3 \times 10^8 \pm 1 \times 10^8 \text{ M}^{-1}\) was attained, in accordance with affinity constants obtained by other groups for immune phage display libraries (1, 19). Affinity constants in this range are characteristic of secondary immune response antibodies. Further characterization of the purified scFv antibody included a preliminary specificity profile determination. Application of the purified scFv antibody to \textit{B. anthracis} detection. The scFv antibody was applied in a “sandwich” ELISA for detection of \textit{B. anthracis} spores. To this end, different concentrations of \textit{B. anthracis} spores were captured by anti-exosporium antibodies and detected with the purified scFv antibody followed by an HRP-conjugated anti-E-tag antibody (Fig. 4A). The soluble scFv antibody afforded a strong ELISA signal with live spores, with a detection limit of 2 \(\times 10^6\) CFU/ml (background plus 3 standard deviations), no less than...
the one obtained when polyclonal antibodies, previously prepared by our group (27), were implemented in the same ELISA format (11). No signal was observed with *B. subtilis* DSM 675 spores, which were used as control spores in the experiment. The binding of the soluble scFv antibody to *B. anthracis* spores was also demonstrated with immunofluorescence microscopy. *B. anthracis* spores were strongly detected (Fig. 4B), whereas no signal was observed on *B. subtilis* DSM 675 spores. No detectable binding to either of the spores was observed with a control unrelated scFv antibody produced from an unbiased clone of the original library (results not shown). Visualization of spores by IFA validated our previous results, indicating that the scFv antibody interacts with an immunodominant extracellular protein on the spore surface, which is probably the known protein Bcl1.

DISCUSSION

Anthrax is an acute infectious disease caused by the spore-forming organism *B. anthracis*. The potential use of *B. anthracis* spores as a biological weapon and the success of biological attacks in the past highlight the need for rapid detection of spores. In this work, we report the isolation of a specific, highly reactive scFv antibody suitable for specific spore detection from a phage display library. The purified soluble antibody was successfully implemented in several detection platforms.

A phage display library was constructed from spleens of mice immunized with a soluble exosporium fraction. This soluble fraction, comprised of proteins from the outermost layer of the spore, was found to induce a greater humoral response in mice than that induced by irradiated spores. This may indicate an advantage for the native, soluble, more accessible antigen versus its particulate form (irradiated spores) for immunization. In fact, the use of selected fractions of viral or bacterial lysates, consisting of membrane-bound or membrane-associated proteins as immunogens, was applied by other groups for efficient detection of intact live agents in clinical and environmental specimens (12). The same exosporium fraction was applied in the past for the successful preparation of polyclonal anti-*B. anthracis* spore antibodies in rabbits (27), resulting in highly reactive antibodies. The constructed library contained $7 \times 10^6$ independent scFv recombinants, a population characteristic of immunized phage display libraries (9, 24).

Following library construction, rescued phage were challenged with live *B. anthracis* spores in solution and used to infect *E. coli* logarithmic-phase cells with no elution step. This methodology enabled the isolation of four unique scFv antibodies which were highly reactive against *B. anthracis* spores. Solution-based screening was applied in the past by several groups for the successful isolation of scFv antibodies (2, 23). Previous observations indicate that solution-based screening has some advantages over solid-based screening, particularly for the selection of monomeric over dimeric scFv antibodies (15) and for better recognition of the native antigen (16). One of the unique isolated scFv antibodies was expressed, purified, and characterized. The purified scFv antibody demonstrated an affinity

![FIG. 3. FCM analysis of scFv antibody specificity profile. Spores of *B. anthracis*, *B. subtilis*, or *B. cereus* ($5 \times 10^6$ CFU/ml) were challenged with the purified scFv antibody (1 nM). The scFv antibody was detected by anti-E-tag followed by an anti-mouse–FITC antibody. Plot histograms were gated to the spore population, as determined by light scatter parameters (side and forward scatter), and were analyzed by the FlowJo program.](http://aem.asm.org/)

![FIG. 4. scFv antibody application in specific detection of *B. anthracis* spores. Analysis of *B. anthracis* and *B. subtilis* spores was done by “sandwich” ELISA (A) and IFA (B).](http://aem.asm.org/)
constant of $3 \times 10^8 \pm 1 \times 10^8 \text{M}^{-1}$ for B. anthracis spores (determined by FCM). Affinity constants in this range are characteristic of antibodies produced in the secondary immune response and were determined (by surface plasmon resonance) for other scFv antibodies isolated from immune phage display libraries (1, 19). The scFv antibody produced in this work was implemented successfully in several detection platforms, including IFA, ELISA, and FCM. Preliminary characterization has shown that the antibody has no cross-reactivity with other phylogenetically related Bacillus spores (Fig. 3), demonstrating improved specificity compared to that of polyclonal antibodies generated in our group by means of the same exosporium fraction (27). As demonstrated in our previous work, implementation of the polyclonal antibodies for specific detection of B. anthracis was achieved solely by using a double-staining method (fluorescence resonance energy transfer), whereas the scFv antibody enabled direct specific detection. Additional comparison of the specificity profile via ELISA (11) revealed a similar specificity profile for other scFv antibodies isolated from immune phage display constructs, p. 10.19B.1–10.19B.31.

In conclusion, to our knowledge, this work is the first in immunology. John Wiley & Sons, Inc., New York, NY.

**References**


