Human antibodies against spores of the genus *Bacillus*: A model study for detection of and protection against anthrax and the bioterrorist threat

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Communicated by Sydney Brenner, Molecular Sciences Institute, Berkeley, CA, March 1, 2002 (received for review February 19, 2002)

A naïve, human single-chain Fv (scFv) phage-display library was used in bio-panning against live, native spores of *Bacillus subtilis* IFO 3336 suspended in solution. A direct *in vitro* panning and enzyme-linked immunosorbent assay-based selection afforded a panel of nine scFv-phage clones of which two, 5B and 7E, were chosen for further study. These two clones differed in their relative specificity and affinity for spores of *B. subtilis* IFO 3336 vs. a panel of spores from 11 other *Bacillus* species/strains. A variety of enzyme-linked immunosorbent assay protocols indicated these scFv-phage clones recognized different spore epitopes. Notably, some spore epitopes markedly changed between the free and microtiter-plate immobilized state as revealed by antibody-phage binding. An additional library selection procedure also was examined by constructing a Fab chain-shuffled sublibrary from the nine positive clones and by using a subtractive panning strategy to remove crossreactivity with *B. licheniformis* 5A24. The Fab-phage clone 52 was improved compared with 5B and was comparable to 7E in binding *B. subtilis* IFO 3336 vs. *B. licheniformis* 5A24, yet showed a distinctive crossreactivity pattern with other spores. We also developed a method to directly detect individual spores by using fluorescently labeled antibody-phage. Finally, a variety of ''powders'' that might be used in deploying spores of *B. anthracis* were examined for antibody-phage binding. The strategies described provide a foundation to discover human antibodies specific for native spores of *B. anthracis* that can be developed as diagnostic and therapeutic reagents.

The proliferation of biological weapons should be of grave concern. Notably, some disease-causing agents have apparently been used in isolated instances during warfare (1). It is generally agreed that spores of *Bacillus anthracis*, the primary infectious agent causing anthrax, is perhaps the most likely candidate for a biological assault (2, 3). Indeed, in the aftermath of September 11, 2001, anthrax was intentionally disseminated on the American population via spores in the postal system resulting in sickness, death, and societal disruption (4–8). Clearly, virtually any nation, terrorist organization, or individual has access to the materials and facilities necessary to develop and deploy simple, extremely deadly biological weapons with moderate effort.

Effective antidotes and convenient, rapid means of detection will be invaluable during future events. In this regard, development of antibodies against infectious organisms and biological toxins has become an increasingly significant endeavor. However, because of the limitations of immunization procedures in which bacteria, viruses, or toxins must be inactivated before immunizing an animal and in which spore germination processes occur *in vivo*, the antibodies obtained often lack affinity and specificity against the desired native target. Moreover, the polyclonal or monoclonal immunoglobulins obtained are not human, further limiting application for therapeutic purposes. Over the past decade, we and others have refined and used antibody phage-display and associated antibody engineering techniques that bypass the immune system, invoking selection from *in vitro* combinatorial human antibody libraries often followed with *in vitro* maturation refinement (9–16). Current molecular methods should allow for the controlled evolution of antibody-binding sites to satisfy demands of affinity and specificity against virtually any biological material.

In this study, we investigated procuring human antibodies that bind viable, native spores from the aerobic, spore-forming genus *Bacillus.* As a test case, spores of *B. subtilis* suspended in solution were used for bio-panning with a phage-displayed naïve human antibody repertoire. Some of the selected antibodies were highly specific vs. other species/strains of the genus. Furthermore, it was possible to visualize antibody-phage complexed with spores of *B. subtilis* with high resolution and sensitivity by using fluorescence microscopy. The direct selection method demonstrated the possibility of discovering human antibodies specific for antigens on the bio-active spore surface. Antispore human antibodies could be useful as immunodiagnostic and immunotherapeutic reagents. Significantly, the strategy serves as a paradigm for obtaining human antibodies against spores of *B. anthracis*.

Materials and Methods

Bacteria. *B. subtilis* IFO 3336 (3336) was kindly provided by Dr. Makoto Ashiuchi (Research Institute of Molecular Genetics, Kochi University, Japan; ref. 17). *B. licheniformis* 5A24 (5A24), *B. licheniformis* 5A36 (5A36), *B. cereus* T (6A1), *B. cereus* ATCC 14579 (6A5), *B. thuringiensis* subsp. *Kurstaki* HD1 (4D1), *B. megaterium* QMB1551 (7A16), *B. pumilus* Meyer and Gottheil ATCC 7061 (8A3), *B. polymyxa* ATCC 842 (25A1), *B. circulans* ATCC 4513 (16A1), *B. sphaericus* ATCC 14577 (13A6), and *B. globigii* SB512 (11A1) were acquired from the *Bacillus* Genetic Stock Center (BGSC; http://bacillus.biosci.ohio-state.edu). The number-letter designation in parentheses following the name of each *Bacillus* species/strain is used as a shorthand code in the text and figures.

***Bacillus* Cultures and Sporulation.** The procedure used for the growth of *Bacillus* cultures and spore purification for all twelve strains was as described (18). See supporting information, which is published on the PNAS web site, www.pnas.org.

Abbreviations: scFv, single-chain Fv antibody; V_H, heavy chain variable region; V_L, light chain variable region; ELISA, enzyme-linked immunosorbent assay; cfu, colony-forming unit.

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Bio-Panning Against Spores of *B. subtilis* IFO 3336. A human single-chain Fv (scFv) library, constructed in our laboratory, was amplified and rescued, as described (19, 20). The library was panned against spores suspended in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) and the positive scFv-phage were selected against immobilized spores by enzyme-linked immunosorbent assay (ELISA). For details, see supporting information.

Selection of Positive Antibody-Phage Clones by ELISA. A solution of 1×10^7 colony-forming units (cfu)/ml prepared from the spore stock solution was washed twice with PBS and centrifuged; the spore pellet was resuspended in PBS at a concentration of 1×10^7 cfu/ml. An aliquot of spore suspension (25 μl per well) was added to Maxisorp (Corning) microtiter plates and incubated overnight at 4°C. The spore-coated plates were washed twice with PBS and blocked with Blotto solution (5% skimmed milk in PBS) for 1 h at room temperature. Then, 25 μl per well of scFv-phage were added at different concentrations (2-fold serial dilution) and incubated 1 h at room temperature. After the plate was washed 8–10 times with PBS, 25 μl per well of horseradish peroxidase-conjugated mouse anti-M13 antibody (Amersham Pharmacia) diluted 1:1,000 in Blotto was added and incubated for 1 h at room temperature. The plate was washed 10 times with PBS, then 50 μl per well of TMB/H_2O_2 substrate solution (Pierce) was added and incubated at room temperature until an adequate signal was reached. The reaction was stopped by the addition of 50 μl per well of 2 M H_2SO_4, and the absorbance was measured at 450 nm with a Thermomax microplate reader (Molecular Devices). Different time points in the sporulation of strains 3336, 5A24, and 5A36 also were assayed by using the above procedure. All experiments were done in triplicate.

Chain Shuffling and Subtractive Panning. The heavy chain variable region (V_H) and light chain variable region (V_L) fragments from nine positive scFv clones against spores of 3336 were individually amplified by PCR; V_H and V_L then were separately pooled. A Fab sublibrary was constructed (see supporting information) from these pooled V_H and V_L fragments, and the Fab-phage were rescued, as described above. Two rounds of regular panning were carried out against 3336 to enrich all of the possible binders. For the third round of panning, purified Fab-phage were first incubated with 1×10^9 spores of *B. licheniformis* 5A24 for 2 h at room temperature to subtract binders from the pool that recognize a common epitope on spores of both 3336 and 5A24. The spores of 5A24 were spun down, and the supernatant was transferred to 1×10^7 spores of 3336 and incubated for another 2 h at room temperature. The washing, elution, and amplification procedure was as described above for bio-panning. Additional rounds of subtractive panning were performed until the output titer reached 10^9–10^{10} cfu/ml. The positive clones were selected by ELISA, as described above.

Characterization of Antibody-Phage by Competition ELISA. The specificity of individual scFv-phage 5B and 7E and Fab-phage 52 for free spores in solution was assessed by various types of competition ELISA. The concentration or dilution factor of each antibody-phage was determined by titration on a microtiter plate coated with spores of 3336. Spore competition ELISA using plates coated with various spore strains or plates only coated with 3336 was performed as follows. The purified spores of 3336, 5A24, 5A36, 6A1, 6A5, 4D1, 7A16, 8A3, 25A1, 16A1, 13A6, and 11A1 were prepared in PBS at the concentration of 1×10^7 cfu/ml. Each column on the plate was coated with a different spore strain for experiments using the various spores, or only 3336 for experiments using 3336-coated plates, and then was washed and blocked as described above for ELISA. Then, antibody-phage from the stock was prepared at twice the assay concentration (determined above by serial dilution) in PBS. In a 0.50-ml tube, equal volumes of a given spore suspension (1×10^7 cfu/ml) and antibody-phage solution were mixed and incubated at room temperature for 1 h with occasional shaking. The spores were spun down at full speed in a microcentrifuge for 5 min, then 25 μl per well of the supernatant was added to the spore-coated ELISA plates (25 μl per well of VCSM13 helper phage was used as a control) and incubated at room temperature for 1 h. The remainder of the ELISA procedure was as described. The relative binding capacity and specificity of the different antibody-phage for free spores of 3336 was determined by using 3336 coated plates as follows. The plate was coated with 1×10^7 cfu/ml spores of 3336 in PBS, washed, and blocked as described above. A diluted antibody-phage solution was prepared at the assay concentration (determined by serial dilution) in PBS. A 0.50-ml tube containing 1×10^7 cfu/ml spores of 3336 was washed with PBS, spun down, and the spore pellet was resuspended in 400 μl of a high concentration of antibody-phage stock solution (not diluted) and incubated at room temperature for 1 h with occasional shaking. The spores were washed several times with PBS and spun down; the spore pellet was resuspended in 100 μl of diluted antibody-phage solution and incubated at room temperature for 1 h with occasional shaking. The spores were spun down, then 25 μl per well of supernatant was added to a 3336 spore-coated plate and incubated at room temperature for 1 h. The remainder of the ELISA procedure was as described above.

Detection of Spores by Fluorescence Microscopy. Direct and indirect fluorescence labeling methods were examined for 5B, 52, and 7E. The antibody-phage (direct method) and murine anti-M13 mAb (Amersham Pharmacia; indirect method) were labeled with FITC or rhodamine-*N*-hydroxysuccinimide, according to the instructions (Pierce). In brief, for example, purified scFv-phage 7E (2×10^{12} cfu/ml) was dialyzed against 100 mM sodium carbonate, pH 9.0 overnight at 4°C. The FITC was freshly prepared at 1 mg/ml in the same buffer, and 10 μl was immediately added to the scFv-phage solution and incubated for 1 h at room temperature in the dark. The unreacted FITC was removed by dialysis against PBS overnight at 4°C. A 1-μl aliquot of the scFv-phage-FITC was mixed with 50 μl of spore solution at different concentrations in PBS containing 5 mg/ml BSA and incubated at room temperature for 1 h. The spores were spun down, the supernatant was discarded, and the spores were washed several times with PBS-BSA. The spore pellet was resuspended in 50 μl of PBS, and 1 μl of the suspension was used to make a slide for fluorescence microscopy (Nikon Optiphot-2 fluorescent microscope; 100× oil-immersion lens). For the indirect method, for example, a washed spore pellet bound to unlabeled scFv-phage, prepared in a similar fashion as above, was resuspended in 50 μl of a 1:500 dilution of FITC-labeled anti-M13 mAb in PBS-BSA and incubated for 1 h at room temperature. Then, the spore pellet was washed, prepared, and examined as described above.

Binding of Antibody-Phage to Powder Substrates. Wheat starch, green bean starch, baby, tooth whitening, baking, and yeast powders were individually prepared as suspensions in PBS at a concentration of $OD_{600} \approx 0.5$ and coated onto microtiter plates at 4°C overnight. Spores of 3336, as a positive control, and spore debris (prepared by disruption by using a MiniBeadBeater; Biospec Products, Bartlesville, OK) also were coated onto plates. The antibody-phage 5B, 7E, and 52 were assayed for binding by using ELISA as described above.

Results

Selection of Anti-Spore Antibody-Phage. The overall selection process resulted from a combination of solution-phase bio-panning followed by solid-phase ELISA. Direct panning of a naïve human

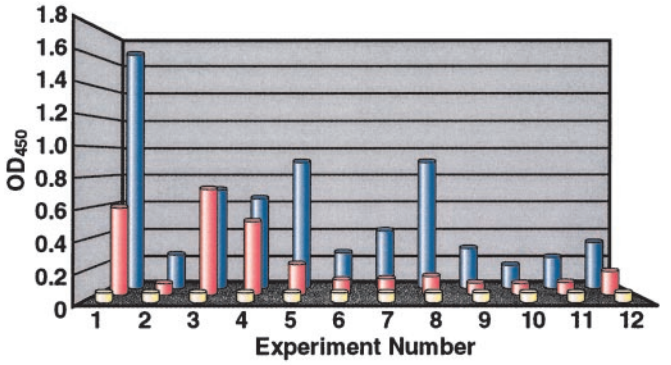

Fig. 1. Crossreactivity of scFv-phage with *Bacillus* strains at different life-cycle stages (1) 3336 spores; (2) 3336 vegetative cells, 16 h; (3) 3336 vegetative cells, 40 h; (4) 3336 vegetative cells, 64 h; (5) 5A24 spores; (6) 5A24 vegetative cells, 16 h; (7) 5A24 vegetative cells, 40 h; (8) 5A24 vegetative cells, 64 h; (9) 5A36 spores; (10) 5A36 vegetative cells, 16 h; (11) 5A36 vegetative cells, 40 h;3 (12) 5A36 vegetative cells, 64 h.

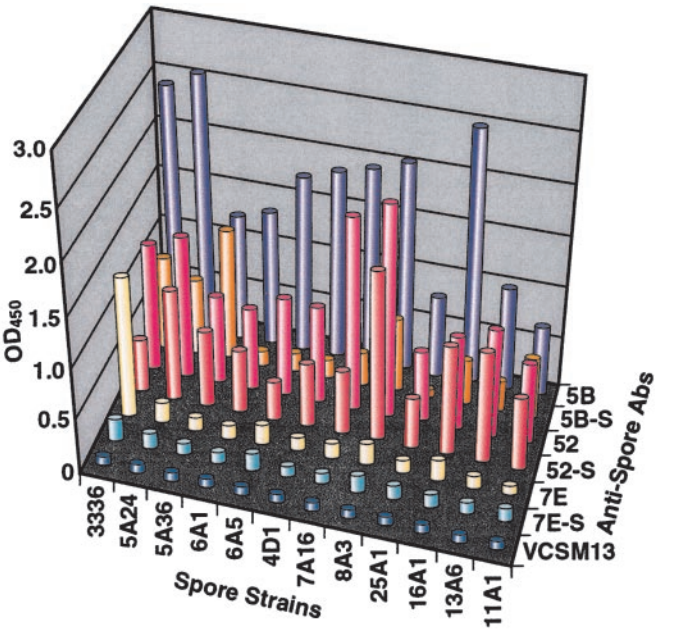

Fig. 2. Competition ELISA using various free and immobilized spore strains. 5B, 7E, and 52 denote adding only the respective antibody-phage; 5B-S, 7E-S, and 52-S denote first incubating a spore strain with the respective antibody-phage and then adding the separated supernatant to the immobilized strain.

antibody library against spores of 3336 afforded a 3,000-fold clonal enrichment from 1×10^6 cfu up to 3×10^9 cfu over four rounds. In each round, the recombinant phage was produced by helper-phage rescue and selectively enriched by panning against live spores in solution; the eluted phage pool was reamplified and taken through the next round of panning. To verify the binding activity of individual scFv-phage, 48 clones were randomly selected and tested for binding to spores of 3336 coated on a microtiter plate by ELISA. Of the 48 clones, 12 clones, in which the ELISA signal was at least 10-fold higher than background, were identified as positive. The sequences were determined, and nine clones possessed intact human antibody V_H and V_L genes. By comparison to the human antibody germline sequence at V BASE (from Ian Tomlinson at http://www.mrc-cpe.cam.ac.uk/imt-doc/restricted/ALIGNMENTS.html), the corresponding canonical structure, the alleles that belong to functional loci, and the particular subgroups were identified (see Table 1, which is published as supporting information on the PNAS web site). In addition, the complete sequences were aligned by using CLUSTALW (MACVECTOR V.6.5, Oxford Molecular Group/Accelrys, San Diego) and the CDR regions of the V_H and V_L genes compared (see Tables 2 and 3, which are published as supporting information on the PNAS web site). The sequence alignments revealed that none of the clones shared the same V_H or V_L genes.

Two clones, 5B and 7E, were selected for further analysis. First, it was shown that VCSM13 helper phage did not bind spores and so, as anticipated, the scFv-phage was endowed with specificity for spores that resulted from the scFv antibody displayed on the phage coat (Fig. 1). Second, a number of specificity differences were evident between 5B and 7E and their interactions with the three *Bacillus* strains 3336, 5A24, and 5A36, as well as their interactions with various stages of *Bacillus* development (Fig. 1). The scFv-phage 5B was specific for spores of 3336 and showed moderate crossreactivity with spores of 5A24 and 5A36, whereas the scFv-phage 7E was also most specific for 3336 and showed very little crossreactivity. Although the greatest specificity was for 3336, as expected, because this strain was used for selection during panning, both scFv-phage showed detectable binding to these other spore strains. In all cases, excellent specificity was observed for the completed spore stage of *Bacillus* development as opposed to the vegetative stage and intermediate stages of sporulation.

To test whether crossreactivity with other strains could be decreased, a chain-shuffled Fab library was constructed and used in conjunction with a subtractive panning method. We chose the Fab format concomitant with these studies to demonstrate the viability of producing a fully human anti-spore Fab that may have advantages for future applications. The Fab sublibrary contained all of the V_H and V_L genes from nine positive scFv-phage clones and, after chain shuffling, the maximum gene number or diversity was 81. Initially, after two rounds of panning against spores of 3336, the clone number of the eluted Fab-phage was raised from 1.6×10^7 cfu to 1.1×10^{10} cfu. Then, two rounds of subtractive panning were performed against 5A24, each followed by selection against the target 3336, in which the clone number dropped to 6.0×10^8 cfu in the third round and increased to 2.4×10^9 cfu in the forth round. A new clone was identified—designated as 52—which showed much lower crossreactivity with free spores of 5A24 than the scFv-phage 5B and even the weakly crossreactive 7E, as determined by competition ELISA (see *Materials and Methods* and below). As confirmed by the DNA sequence, the V_H and V_L fragments of this Fab were derived from the V_H of clone 4C and V_L of clone 6B (see supporting information). Interestingly, many of the positive clones in this chain-shuffled library possessed the V_H fragment from 4C rather than from 5B or 7E, which suggests that the epitope on spores of 3336 recognized by Fab-phage 52 may be different from that recognized by 5B or 7E.

Competition ELISA. To further characterize antibody-phage clones 5B, 7E, and 52, different competition assays were used. The results of competition ELISA using various strains of spores free in solution vs. their counterparts immobilized on microtiter plates (Fig. 2) revealed (*i*) scFv-phage 5B bound to all free and immobilized spores except for 5A36 and 11A1, (*ii*) scFv-phage 7E bound significantly only to free or immobilized 3336, and (*iii*) Fab-phage 52 bound to all twelve spore strains when immobilized but could not be competed or showed very weak competition by 5A24, 5A36, 6A1, 8A3, 16A1, 13A6, and 11A1.

The results of competition ELISA using various strains of spores free in solution vs. spores of 3336 immobilized on plates (Fig. 3) revealed the following. (*i*) The scFv-phage 5B could be inhibited by all spore strains that suggested the epitope recog-

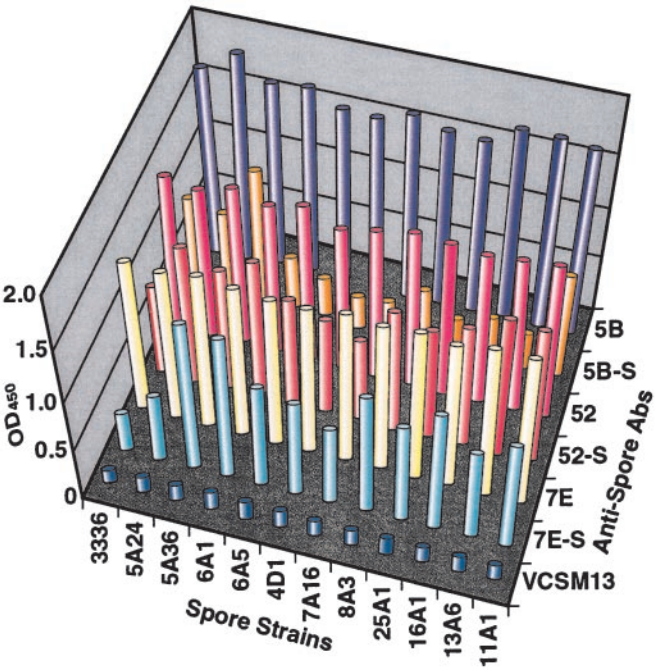

Fig. 3. Competition ELISA using various free-spore strains and immobilized spores of 3336. 5B, 7E, and 52 denote adding only the respective antibody-phage; 5B-S, 7E-S, and 52-S denote incubating first a spore strain with the respective antibody-phage and then adding the separated supernatant to the immobilized 3336.

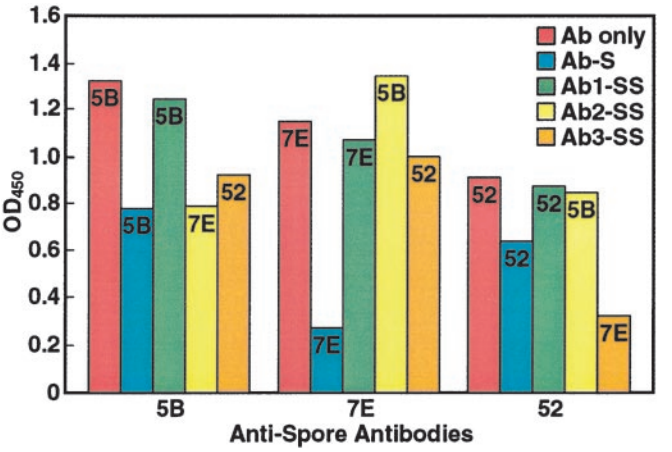

Fig. 4. Competition between antibody-phage using free and immobilized spores of 3336. Ab denotes adding diluted antibody-phage alone; Ab-S denotes first incubating diluted antibody-phage with 3336, then adding the separated supernatant to the immobilized 3336; Ab1-SS, Ab2-SS, and Ab3-SS denote first using concentrated antibody-phage (*x* axis) to saturate 3336, washing the saturated spores, incubating diluted antibody-phage (shown inside bars) with the saturated spores, and then adding the separated supernatant from the last incubation to the immobilized 3336.

nized by 5B is universal to the spore surface of all *Bacillus* species. The results from this experiment were consistent with those obtained above (Fig. 2). (*ii*) The scFv-phage 7E could be competed significantly by all of the strains except 5A36 and 6A1, and so the result was much different from the assay performed using plates coated with the various strains. (*iii*) The results for Fab-phage 52 were consistent with those obtained from competition ELISA using plates coated with the various strains. Notably, the ELISA signal of a particular antibody-phage was always within a consistent range. For example, the average signal shown for 5B was OD ≈1.5, which reflects a consistency in the concentration of spores of 3336 used in coating plates and the concentration of each antibody-phage used in this assay. Hence, it was possible to conclude that, if there were no competition by a tested spore strain against 5B, the signal for 5B-S also should be ≈1.5. On the contrary, if 5B were able to bind a tested spore strain in solution, then the signal for 5B-S should be <1.5.

The results of a measure of the relative binding capacities and specificities of the different antibody-phage using a competition assay together with immobilized 3336 (Fig. 4) revealed the following. (*i*) From the data for scFv-phage 5B, it was evident that scFv-phage 7E (OD ~ 0.787 for 5B-Ab2-SS) was still able to bind to spores saturated with scFv-phage 5B (OD ~ 1.15 for 7E-Ab). However, Fab-phage 52 (OD ~ 0.925 for 5B-Ab3-SS) could not bind to spores saturated with scFv-phage 5B (OD ~ 0.907 for 52-Ab). (*ii*) From the data for scFv-phage 7E, it was evident that scFv-phage 5B (OD ~ 1.35 for 7E-Ab2-SS) could not bind to spores saturated with scFv-phage 7E (OD ~ 1.32 for 5B-Ab). Also, Fab-phage 52 (OD ~ 0.998 for 7E-Ab3-SS) could not bind to spores saturated with scFv-phage 7E (OD ~ 0.907 for 52-Ab). (*iii*) From the data for Fab-phage 52, it was evident that scFv-phage 5B (OD ~ 0.845 for 52-Ab2-SS) was still able to bind to spores saturated with Fab-phage 52 (OD ~ 1.32 for 5B-Ab). Also, scFv-phage 7E (OD ~ 0.325 for 52-Ab3-SS) was still able to bind to spores saturated with Fab-phage 52 (OD ~ 1.15 for 7E-Ab). Hence, a particular spore surface epitope could be saturated by a high concentration of a given antibody-phage, as indicated by the fact that the subsequent addition of a diluted antibody-phage did not bind. In these cases, no inhibition was evident in the data for Ab-SS compared with Ab.

Fluorescence Detection of Spores. The binding of antibody-phage 5B, 7E, and 52 to spores of 3336 was visualized with fluorescence microscopy. All permutations of two labeling methods (direct and indirect) and two fluorescent dyes (FITC and rhodamine) were investigated. The principal experiments used (*i*) antibody-phage labeled with FITC, (*ii*) antibody-phage labeled with rhodamine, (*iii*) an anti-VCSM13 phage mAb labeled with FITC, and (*iv*) an anti-VCSM13 phage mAb labeled with rhodamine. The direct method was less expensive, faster, and more convenient, but both methods gave comparable results in visualizing spores by using fluorescence microscopy. As an example, two experiments were photographed (Fig. 5).

Antibody-Phage Binding to Various Powders. The binding of 5B, 7E, and 52 against a panel of powder-like substances revealed that 5B could bind to all substrates to varying extents, whereas 7E and 52 only showed activity with wheat starch powder. In addition,

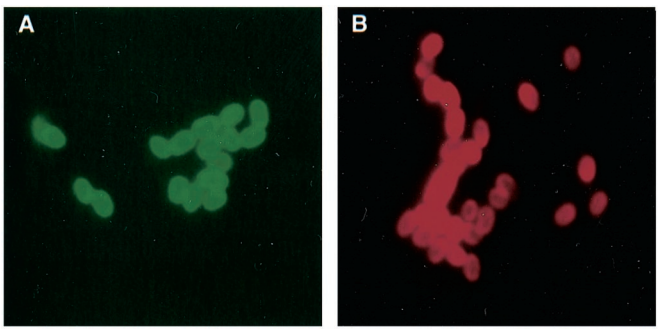

Fig. 5. Fluorescence microscopy of spores of *B. subtilis* IFO 3336 bound to scFv-phage 7E labeled with FITC (*A*) and rhodamine (*B*).

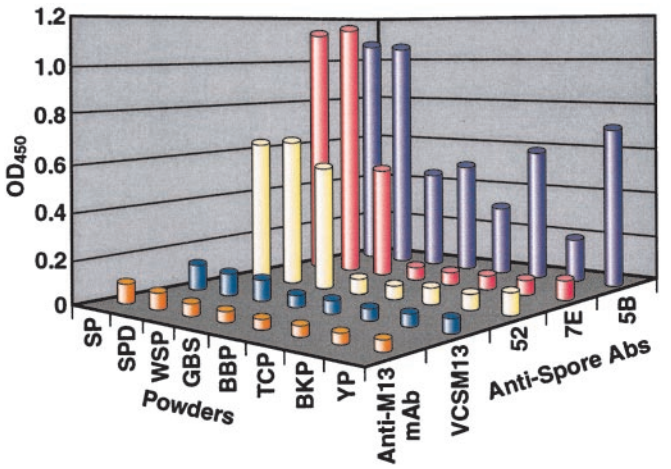

Fig. 6. Binding of antibody-phage to various powder suspensions. SP (3336 spores, control), SPD (3336 spore debris), WSP (wheat starch), GBS (green bean starch), BBP (baby powder), TCP (tooth cleaning powder), BKP (baking powder), YP (yeast).

all antibody-phage could bind to mechanically disrupted spores (Fig. 6).

Discussion

The primary aim of this investigation was to demonstrate that an *in vitro* naïve human antibody repertoire could be used to directly select antibodies that bind viable, native spores of the genus *Bacillus*. Within the genus, all species of *Bacillus*, of which there are greater than 20 exclusive of a variety of strains, are very closely related, although a subdivision of the vegetative morphology into several broad groups is possible (21). However, the spore morphology throughout the genus is even more highly conserved (22). Yet, despite the similarities, it was possible to use a straightforward, solution panning process against one spore strain and select relatively specific antibodies for that strain vs. vegetative cells, as well as other spore strains. We chose a strain of the well studied *B. subtilis* for our initial purposes because it is (*i*) avirulent, which greatly facilitated extensive handling for exploring the methodology, (*ii*) classified into the same morphological group-1 as *B. anthracis* (21), and (*iii*) known to form encapsulated vegetative cells crucial to anthrax virulence (23) and so, along with *B. cereus*, can be considered a near neighbor within the group. Subsequently, a variety of other species/strains, all of which (except *B. cereus*) are generally considered relatively innocuous, were entered into the study. Given the results, we believe our preliminary investigations serve as an excellent model for addressing the problem of obtaining specific human antibodies against spores of *B. anthracis.*

After panning against spores of *B. subtilis* IFO 3336, nine positive clones were selected from the scFv-phage pool. DNA analysis indicated a high diversity among the gene sequences. The lack of a dominant sequence is contrary to expectations and suggests that there are a variety of distinct and accessible epitopes on the spore surface. In other words, the complexity of the spore surface was reflected by the diversity of the scFvs. However, some of the sequences do derive from the same germline V gene (e.g., V_H of 6B and 9E are derived from the 4–34 locus, and V_L of 8C and 9G are derived from the 2B2 locus; see Table 1). Consequently, although the sequences are different, some antibodies may bind to the same epitope but perhaps with different affinities. Two clones, 5B and 7E, were further characterized for crossreactivity vs. spores of *B. licheniformis* 5A24 and 5A36. In general, mAbs elicited against spores of a particular species via immunization have shown significant cross-reactivity with vegetative cells of that species, spores and cells of related species (near neighbors in the same morphological group), and a number of isolated antigens from the spore coat and cells (24, 25). On the contrary, from our *in vitro* selection, we found that clone 7E was highly specific for the spore 3336 of interest, but that the other clone 5B showed a significant crossreactivity vs. spores of 5A24. Moreover, 7E was specific only for 3336 when tested against 11 other spore strains immobilized on microtiter plates. This finding is remarkable, given the relatedness of spore-coat morphology and the fact that the scFv-phage were derived from only four rounds of standard bio-panning. Interestingly, the combinations V_H 1–3/Vκ 2–1, V_H 1–3/Vλ 14–7(A), and V_H 1–3/Vλ 2–1 used for 52, 7E, and 5B, respectively, show a relatively frequent representation in antibody structures (Table 1; ref. 26). This result may suggest that spore or spore-like antigens are prevalent in the human environment and, so, are recognized by the largest subset of antibodies in the immune repertoire, thus accounting, perhaps, for the quality of the clones selected from the library.

We examined the efficacy of an advanced library panning protocol for obtaining a clone with reduced crossreactivity against 5A24 relative to 3336 and for comparison vs. a panel of other strains by using competition ELISA. The antibody variable region of both light and heavy chains from the nine positive clones were used to construct a Fab sublibrary by chain shuffling followed by a subtractive panning protocol. The resulting clone 52 had almost no crossreactivity with 5A24 and was substantially improved, compared with the parent clone 5B and similar to clone 7E. However, unlike 7E, Fab-phage 52 does show crossreactivity with both several other spore strains in solution and can bind to all spore strains that are immobilized on polystyrene plates (Fig. 2, Fig. 3). Together with the fact that the V_H and V_L of 52 are different from both 5B and 7E, this fact suggests that 52 recognizes a unique epitope universal to all of the *Bacillus* species but which is accessible in only a few in the native state and then becomes highly exposed upon hydrophobic interactions with polystyrene. The chain-shuffling results indicate that it is possible to increase the diversity of heavy and light chain pairings, and that shuffling a greater number of clones should further enhance this procedure. Also, subtraction panning can be used effectively to reduce or cancel out undesirable antibody crossreactivity against selected spore species. Indeed, higher-order subtraction panning involving stepwise or combination removal of various species, especially near neighbors, could ultimately allow the selection of an antibody with a very narrow range of crossreactivity or even stringent, singular specificity.

Based on the results from the various competition ELISA experiments (Figs. 2 and 4), clone 5B, similar to clone 52, strongly recognizes an epitope common to the 12 strains, but both in the free and immobilized state, suggesting that the epitope is likely different from that recognized by 52. This finding is also in accord with the observation that 5B can completely block the binding of 52 to spores of 3336, but this is not so for the converse situation, implying that a specific domain within a single epitope or one of several epitopes recognized by 5B is more exposed—in essence, that the epitope has a higher effective "concentration" than the epitope recognized by 52. Perhaps one epitope recognized by 52 is among a cluster of several epitopes recognized by 5B. It also is evident that the epitopes recognized by 5B and 52 are quite distinct from the epitope recognized by 7E. Moreover, the epitope recognized by 7E is probably the most prevalent epitope on the spore surface, because spore saturation with 7E can prevent the binding of either 5B or 52. We expect that improved binding results and more quantitative data can be obtained by using antibodies in free, soluble form.

One advantage of using a phage-displayed antibody is that the phage particle can serve as an amplifier, affording a convenient

and enhanced sensitivity of spore detection. The high-copy number labeling of the phage-coat protein VIII (>2,000 copies; ref. 27) with a fluorophore produces a greater signal than can be achieved by using a labeled, soluble anti-spore antibody. Our fluorescence microscopy results demonstrate readily observable "single spore" resolution available from such a format. Antibody-phage that bind spores could be integrated further into other detection systems (28–31) and, combined with strategies herein, the sensitivity optimized to one or a few spores with exclusive specificity.

The results also showed the substantial differences that can arise for some antibodies in recognizing free and immobilized spores. In particular, 7E showed virtually singular specificity for 3336 vs. 11 other strains when all are immobilized, yet showed measurable binding to all spore strains in the free state. Apparently, only in 3336 does the critical epitope or domain thereof, bound during both the panning and selection steps, remain intact or accessible when immobilized on polystyrene. A method that can both bio-pan and select antibodies against spores directly from solution, without relying on selection from the immobilized phase, should be placed under development and should afford enhanced specificity and affinity for free spores. Regardless of the outcome, the data emphasize that the spore coat is a complex antigenic environment, and that the use of various binding experiments can be valuable in assessing the specificity and relative affinity of antibody binding.

A high affinity, specific antibody will be a prerequisite for the rapid detection of and protection against anthrax. Our *in vitro* technology invoking human antibodies and native spores offers advantages compared with the nonhuman antibodies from immunization protocols arising from *in vivo* spore germination or heat/formaldehyde-inactivated spores that have altered epitopes (32–34). The preliminary investigations herein have already afforded improved spore-binding characteristics. In addition, we showed that protein adherence or nonspecific binding of the antibody-phage to extraneous powders could be minimal (7E and 52 only with wheat starch) and are likely nullified by using a subtraction procedure. These experiments await further elaboration and are important in detection systems, given that real or fraudulent threats of anthrax spores will be associated with powder-like materials. Also significant will be the use of improved antibodies to map spore epitopes of *B. anthracis* that could yield information for the design of a more effective, less toxic anthrax vaccine, because spore antigens contribute to protection against anthrax (35–37). On the other hand, passive administration of spore-specific human antibodies might afford protection, because some anti-spore antibodies can stimulate spore uptake by phagocytes and markedly inhibit spore germination critical to the early stages of spore infection (38). The possibility also exists that the whole IgG format of a library-selected antibody could destroy spores by recruiting complement and/or antibody-dependent cytotoxic pathways. Because inhalation anthrax is almost invariably fatal (39, 40), passive antibodies might be especially effective when present at the point of entry in the mucosal lining of the respiratory tract. Unquestionably, a number of areas remain to be investigated with regard to antibodies and their potential role in solving the anthrax problem.

At this time, more than ever, there is a need for additional countermeasures against harmful biological materials. There are dramatic weaknesses in our diagnostic and medicinal capabilities. The availability of refined antibody-based methodologies for clinical analyses to gather intelligence data and protect against biological weapons such as anthrax would be of great significance with regard to addressing the terrorist threat and the health of military and civilian personnel.

We gratefully acknowledge the support of The Skaggs Institute for Chemical Biology.

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