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RECOMBINANT ANTIBODIES FOR THE DETECTION OF BACTERIOPHAGE MS2 AND OVALBUMIN

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Antibodies are currently field-deployed as the recognition component of sensors that detect biological threat agents. Antibodies that detect simulants of biothreat agents are also incorporated into biodetection platforms for testing and evaluating new devices and materials. Previously, we developed an anti-botulinum toxin antibody using a powerful genetic technology known as phage display, in which a very large library of immunoglobulin (antibody) genes are expressed on the surface of bacteriophage (bacterial virus) particles. We describe here the isolation of additional recombinant antibodies that bind two simulants of biothreat agents: bacteriophage MS2, and the protein ovalbumin (simulants for viruses and protein toxins, respectively). The phage display method allows the rapid selection of genes encoding specific antibodies out of an enormous population of candidates, and the subsequent production of large amounts of the recombinant antibody in bacterial fermentations. A single anti-MS2 antibody was isolated. The screen for anti-ovalbumin antibodies yielded two independent clones with unique nucleotide and amino acid sequences. The antibodies demonstrated specificity for the molecule against which their selections were targeted. A key feature of the method was the use of a recombinant antigen, cloned MS2 coat protein, for the affinity enrichment of clones in place of the intact bacteriophage target.

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PREFACE

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RECOMBINANT ANTIBODIES FOR THE DETECTION OF BACTERIOPHAGE MS2 AND OVALBUMIN

1. INTRODUCTION

Antibodies are the essential component in immunological sensors that detect BW (biological warfare) agents, giving them both sensitivity and selectivity for BW agents. The Army and the Joint Program Office for Bio-Defense (JPO-BD) purchase and field biosensor platforms (JBIDS, JPBDS, JBREWS, and PORTAL SHIELD systems) that incorporate antibodies produced in whole animals (polyclonal) and mammalian cell cultures (monoclonal) for BW agent detection.

There is considerable lot-to-lot variability in the current production of antibodies. This is especially true for polyclonal antibodies, which require the injection of a disarmed BW agent into animals. The individual response of each animal to an agent can vary dramatically. The process of developing antibodies in animals or in mammalian cell culture is also time-consuming, which limits the capacity for "just-in-time" or surge production in time of conflict.

A recent advance in antibody production technology is the cloning of antibody genes and their expression in bacterial fermentations. This technology has been proven capable of producing antibodies for BW agent detection that are of higher quality and greater uniformity from lot to lot; therefore, their inclusion in fielded bioassays would result in greater reliability. Recombinant antibodies are also faster and potentially less expensive to produce and acquire in quantity; therefore, establishing a process for their production would improve the maintainability and supportability of fielded biodetection systems. The successful cloning and properties of a recombinant antibody that binds botulinum toxin has been described (Emanuel *et al.*, 1996).

In addition to antibodies that bind and detect BW agents, there is a need for antibodies that bind and detect organisms and substances that simulate BW agents. A panel of BW agent simulants that are non-toxic and non-pathogenic is widely used for the development and testing of biosensors and environmental samplers, in a work setting without the need for high levels of biological containment. Two such simulants are bacteriophage MS2 (a non-pathogenic virus of the bacterium *Escherichia coli*, which is used to simulate viruses) and ovalbumin (a benign protein which is used to simulate protein toxins, such as ricin). To meet the need for high-quality, inexpensive antibody reagents that bind these BW simulants, we have used a powerful technique called phage display to isolate antibody genes from immunized mice. The resulting antibody molecules are called Fabs, indicating that they are comprised of heavy and light chain antibody sequences which form the antigen-binding variable region, but do not contain the IgG constant region. We describe here the cloning and initial characterization of three new recombinant reagents, antibodies that bind biothreat simulants.

2. METHODS AND RESULTS

2.1 Immunization.

Antibody genes for immunoglobulin library construction were obtained by isolating RNA from B cells (antibody-producing cells) in the spleens of immunized BALB/c mice. Mice were immunized with MS2 and ovalbumin to increase the numbers of B cells producing anti-MS2 and anti-ovalbumin antibodies, thereby increasing the likelihood of isolating the corresponding genes. Primary immunizations were followed with three additional immunizations bi-weekly, with concurrent monitoring of antibody production in the mice by ELISA.

2.2 Antibody gene amplification and cloning.

Following the immunization of BALB/c mice with a suspension of intact MS2 virions, RNA was extracted from the spleens of immunized mice by standard methods (Chomczynski and Sacchi, 1987). Complementary DNA (cDNA) was synthesized from total immunized mouse spleen RNA. The resulting cDNAs were processed and amplified by polymerase chain reaction (PCR) amplification (Hogrefe and Shopes, 1994) in order to isolate individual sets of immunoglobulin genes. PCR-amplified heavy chain genes are shown Fig. 1. Heavy and light chain gene PCR fragments were assembled by ligation (MS2) or by PCR assembly (ovalbumin) and subjected to digestion with the restriction enzymes *Not*I and *Spe*I for cloning into the phage display vectors. The primary antibody library was transformed by electroporation into cells of *E.coli* strain XL-1 Blue and then amplified by plating the library on solid Luria medium containing carbenicillin.

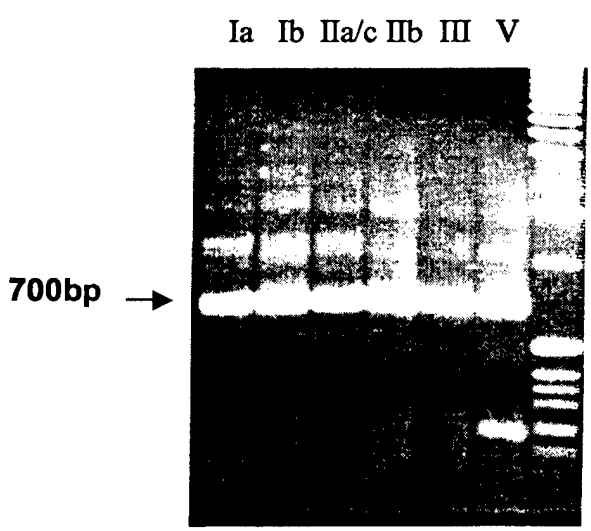


Fig. 1. Amplification of heavy chain genes. Antibodies consist of heavy and light chain proteins; both are required to bind antigen. Mammals contain several families of both heavy and light chain proteins in their antibody repertoire. To obtain the most diverse collection of antibodies from which to select desirable clones, each family of mouse immunoglobulin heavy and light chain was amplified separately using PCR primers specific to that family. Shown are amplified gene fragments of six heavy chain families (Roman numerals). The desired gene fragments are 700 bp long. Other bands represent PCR artifacts. Right lane, DNA size marker.

2.3 Display of antibodies on the surface of phage particles.

The resulting primary antibody library was then expressed on filamentous phage particles by infection of the *E. coli* host cells with the helper phage VCSM13 (Stratagene, La Jolla, CA). Infection of the cells with the VCSM13 helper virus induced production of filamentous bacteriophage that express the heavy and light chain polypeptides on the surface of the virus (Parmley and Smith, 1988). The filamentous phages that were produced by the *E. coli* culture displayed recombinant antibodies on their surfaces, as determined by the detection of mouse kappa light chains in dot blots of the expressed phage. Filamentous phages displaying antibodies and containing the cloned antibody gene library were separated from *E. coli* cells by centrifugation, and phages were concentrated from the supernatant solution by centrifugation (Fig 2).

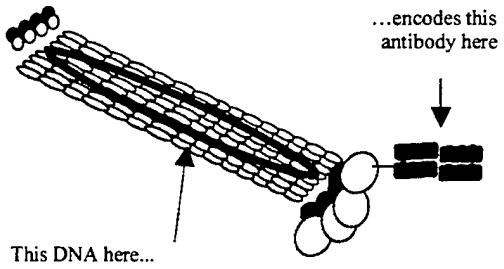


Figure 2. Schematic of antibody-displaying particle. A phage particle in the display library contains DNA encoding an antibody from the immunized mouse, and displays that same antibody on its surface. The entire repertoire of antibodies cloned from the mouse is thereby converted to a recombinant form that can be screened by applying the population to a surface coated in the antigen of interest.

2.4 Expression and purification of recombinant target protein.

To minimize the possibility of contaminating bacterial cultures with intact MS2 and to obtain the purest antigen possible for affinity screening, we expressed and purified a recombinant version of the MS2 coat protein. A clone of the coat protein (Dr. L. Lo, Scripps Institute, San Diego, CA) in the expression vector pET15b, contained in *E. coli* strain BL21(DE3) was induced by addition of IPTG to a three-liter batch fermentation. Cells were harvested after an overnight incubation at 37°C, and disrupted to release the coat protein by sonication. After centrifugation to remove cell debris, cell lysate was passed over a nickel affinity column. The recombinant coat protein included six histidine residues (a "6xHis" tag) on the C-terminal end, which preferentially bind metal ions. This technique gave >90% purification of the target protein in a single purification step (data not shown).

After purification, the recombinant coat protein was used to screen the antibody library for clones that bind MS2. Ovalbumin for both immunization and biopanning is was obtained commercially (Pierce Co, Rockford, IL) in highly purified form.

2.5 Affinity enrichment ("biopanning") of antibody clones.

The display of the recombinant antibodies on the surface of the phage allowed for the enrichment of antigen specific clones through biopanning against recombinant MS2 coat protein immobilized in the wells of microtiter plates. Phage particles displaying anti-MS2 or anti-ovalbumin antibodies (and containing the corresponding cloned antibody genes) were obtained by a form of affinity purification called "biopanning"(Fig. 3).

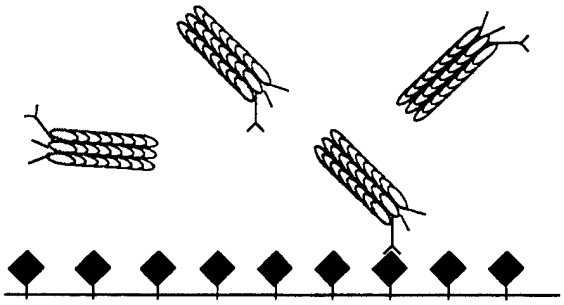


Figure 3. Biopanning. The antigen against which antibodies are desired (diamonds, above) is immobilized on a solid surface. A population of phage particles displaying a repertoire of antibodies is washed across the surface, and those phages displaying antibodies that bind the target are retained; the rest are washed away.

Small amounts of each antigen were immobilized on a solid surface (a well of a microtiter plate), and a population of phages displaying antibodies cloned from the corresponding immunized mice were washed across the surface. Those phage particles displaying antibodies that bind the antigen were retained on the surface, while non-specific phages were washed away. Because of the way in which the antibodies were cloned, each phage bound to antigen contains the genes that encode the antibody on the surface of that phage. Bound phages were removed from the surface, used to infect fresh cultures of *E. coli*, and reinfected with helper phage to start the next round of enrichment. Three to four rounds of biopanning were typically performed.

2.6 Subcloning and expression of the antibody genes.

Following the last round of biopanning, individual phage clones containing the desired antibodies were identified by culturing individual clones and screening for binding of the appropriate antigen by ELISA. Verified positive clones were sequenced fully and from the sequence, the IgG subclass of each clone was determined. Genes for each Fab antibody were then removed from the phage display vectors (Fig. 4) and cloned into expression vectors which incorporate a 6x histidine tag on each heavy chain for subsequent expression and purification in fermentations of *E. coli*.

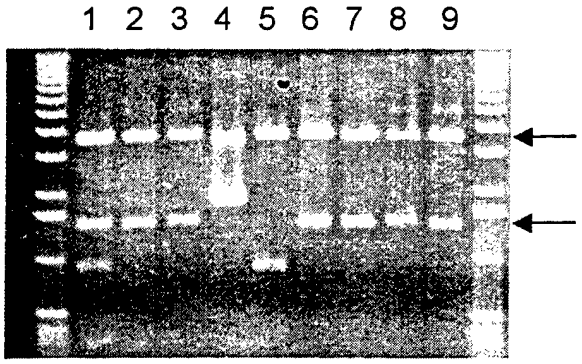


Figure 4. Subcloning of antibody genes. Nearly all clones obtained after several rounds of biopanning retained intact antibody genes (represented by clones 1-3, 6-9; **lower arrow** indicates 1400 base pair gene inserts, 700 bp light chain gene plus 700 base pair heavy chain gene). Some clones (4 and 5) underwent mutation or genetic rearrangement and were discarded. The remainder were used to produce antibody and were individually screened for the ability to bind ovalbumin or MS2 (depending on which antigen was used in panning). The 1400 base pair gene inserts of clones that passed successfully were removed from the phage display DNA vector (**top arrow**) and cloned into a vector that added a 6xHis tag to the carboxyl terminal of the heavy chain gene. Subsequent expression in *E. coli* fermentation and purification gives a pure recombinant antibody product. Left and right lanes, DNA size markers.

2.7 Expression and purification of recombinant anti-MS2 antibody.

The introduction of antibody genes into the expression vector pHis1.1 fused the heavy chain genes with a 6xHis tag. The pHis1.1 expression vector (carrying the anti-MS2 antibody genes) was introduced into an *E. coli* strain constructed to optimize protein expression (TOPP I, Stratagene). Cells were grown in a 20-liter fermentor and the compound IPTG was added to induce expression of the antibody genes. After fermentation, cells were disrupted with a sonicator to release a crude lysate containing the recombinant anti-MS2 antibodies. The crude cell lysate was applied to a column packed with Talon metal affinity resin according to the manufacturer's instructions, and eluted with imidazole buffer. Eluted anti-MS2 antibody was further purified by passage over Sephadex gel filtration columns to greater than 90% purity. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC for purity and protein content. Proteins were detected by staining with Coomassie Brilliant blue and by immunoblotting (Figure 5).

2.8 Expression and partial purification of recombinant anti-ovalbumin antibodies.

Genes for two anti-ovalbumin antibodies were cloned into the expression vector pHis1.1 as described above, and expressed in small-scale (3-liter) fermentations of *E.coli* strains TOPP I and TOPP II to begin optimizing a production scheme. After growth of the cultures, induction of gene expression with IPTG and cell disruption by sonication, crude lysates were passed over Talon metal affinity columns as described in section 2.7. Samples of the crude lysate, material that passed through the column, and eluted 6xHis-tagged anti-ovalbumin antibodies were obtained during purification and analyzed by SDS-PAGE and immunoblotting (Figure 6).

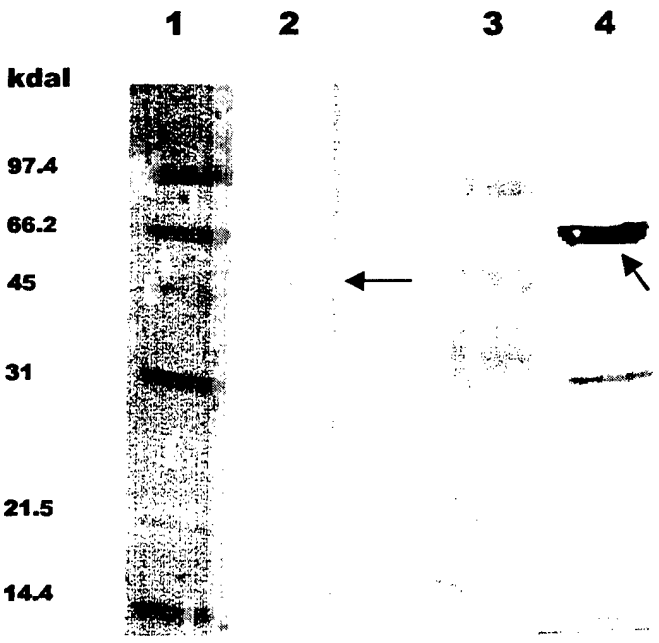


Figure 5. Purified recombinant anti-MS2 antibody. After purification as described in section 2.7, anti-MS2 antibody (lanes 2 and 4, arrows) was >90% pure as determined by SDS-PAGE and staining with Coomassie Brilliant blue (left image). Lane 1, molecular weight standard; lane 2, purified anti-MS2 antibody. Right image, experiment identical to lanes 1 and 2, but demonstrating the identity of the protein as mouse antibody by immunological detection. Lane 3, molecular weight marker; lane 4, purified anti-MS2 antibody. The anti-MS2 antibody was transferred to a nitrocellulose membrane after SDS-PAGE and detected with goat anti-mouse antibody conjugated to alkaline phosphatase, using NBT/BCIP as substrate.

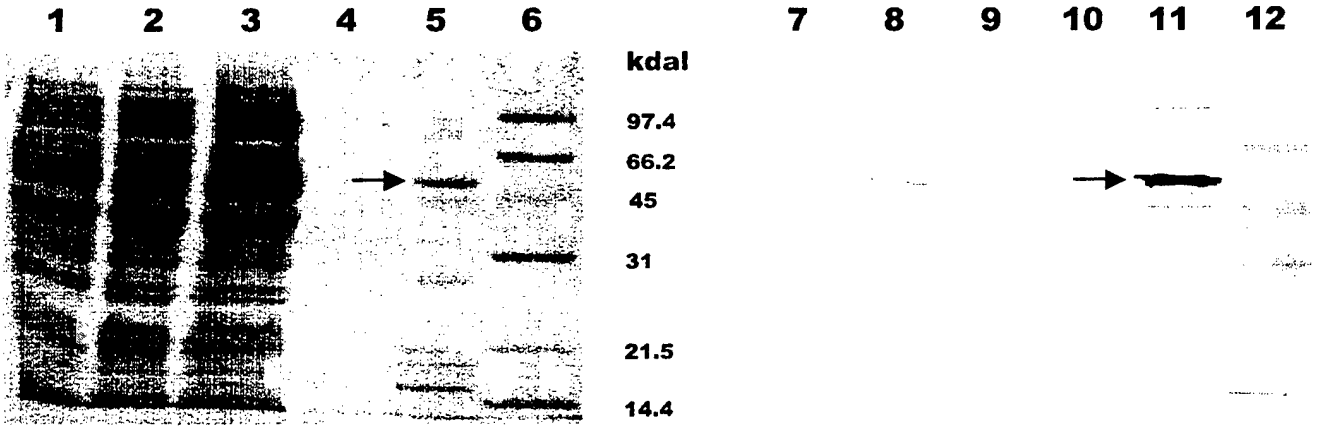


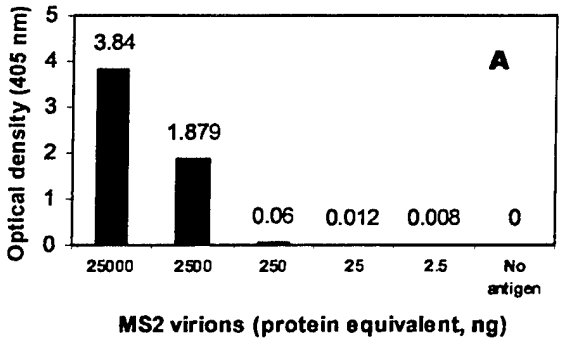
Figure 6. Partial purification recombinant anti-ovalbumin antibody (OVA-3) by metal affinity chromatography. Left image, Coomassie blue-stained gel. Lane 1, crude lysate; lanes 2 and 3, cellular proteins unbound by column; lane 4, fraction immediately preceding antibody during elution; lane 5, pooled fractions containing recombinant antibody OVA-3; lane 6, molecular weight marker. Right image, experiment identical to left image except that proteins were blotted to a nitrocellulose membrane after SDS-PAGE and detected with goat anti-mouse antibody conjugated to alkaline phosphatase, using NBT/BCIP as substrate. Lanes 7-12 identical to lanes 1-6. **Arrows** indicate position of recombinant antibody.

Both antibodies were purified to approximately 30% of total collected protein after one pass over the Talon resin (antibody OVA-3 shown in Figure 6; antibody OVA-4, data not shown).

2.9 Detection of MS2 and ovalbumin with recombinant antibodies.

Recombinant anti-MS2 and anti-ovalbumin antibodies were used in enzyme-linked immunosorbent assays (ELISAs) to detect their corresponding antigens. Density-gradient-purified bacteriophage MS2 (Dugway Proving Grounds, UT) and ovalbumin (Sigma, St. Louis, MO) were serially diluted and bound by adsorption to wells of Maxisorp microtiter plates (Nunc). After incubation with blocking agent (3% bovine serum albumin in Tris-buffered saline, pH 7.4), recombinant antibodies were applied to antigen coated wells and incubated for 2 h, followed by washing in TBS-Tween 20 (0.05%). Goat-anti-mouse IgG (Fab-specific) conjugated to alkaline phosphatase (Sigma; working dilution 1:2500) was added to each well and incubated for 1-3 hours. Following another wash step, AP enzyme substrate solution (PNPP; para-nitrophenylphosphate, 2 mg/ml) was added to each well. Color development was measured spectro-photometrically after one hour, at 405 nm.

Purified MS2 was detectable down to a level of 250 ng (protein equivalent) in this experiment (Figure 7). The recombinant coat protein used for biopanning and screening, however, was detected to a level of approximately 20 ng. This apparent greater sensitivity for the recombinant coat protein over the intact virus may reflect the isolation of the antibody using the recombinant protein as the biopanning target. It may also indicate that the epitope bound by the anti-MS2 Fab is partly obscured when the coat protein is in the three-dimensional context of the intact viral coat. Use of intact virions as the target, as well as performing the affinity capture of anti-body-displaying phage in solution will allow the isolation of Fabs with even greater affinity for intact MS2. Anti-MS2 did not bind phage M13, BSA, or ovalbumin (data not shown). Using partially purified recombinant antibodies OVA-3 and OVA-4, ovalbumin was detectable down to a level of 1 microgram (Figure 8).



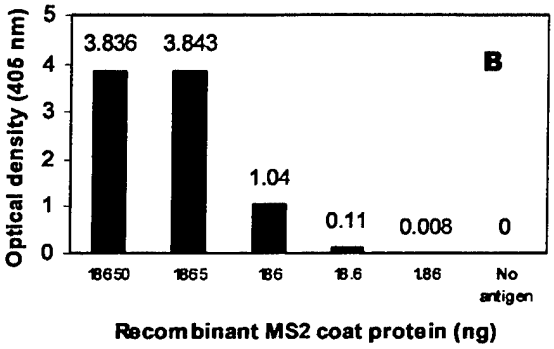
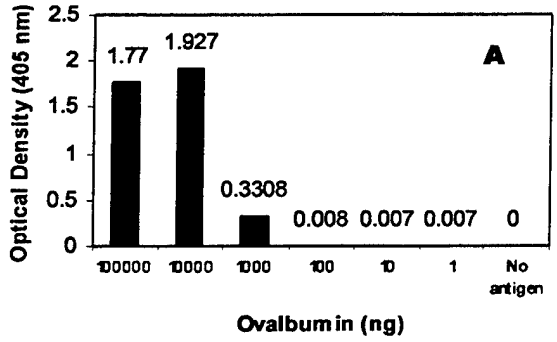


Figure 7. ELISA detection of (A) MS2 virions and (B) recombinant MS2 coat protein using purified anti-MS2 Fab antibody. Values represent the mean of duplicate measurements, adjusted by subtracting the absorbance measured in the no antigen control. The antibody appears to be more sensitive for the detection of the recombinant coat protein.



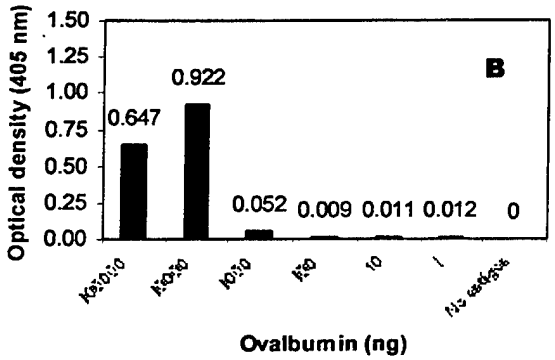


Figure 8. ELISA detection of ovalbumin using (A) recombinant antibody OVA-3 and (B) recombinant antibody OVA-4. Values represent the mean of duplicate measurements, adjusted by subtracting the absorbance measured in the no antigen control.

3. DISCUSSION

This work is part of an overall strategy to develop an improved suite of biodetection reagents by establishing the methods for cloning, isolation and large-scale production of recombinant polypeptides. The antibodies being developed in this program complement another ongoing effort in our group to isolate and characterize small peptide aptamers that bind and detect BW agents.

The DNA and amino acid sequences of the three antibodies described here have been determined and a detailed analysis will be reported elsewhere. Each antibody displays canonical sequences flanking each of the six CDRs (complementarity determining regions) per clone. The CDRs are the portions of the antibody chains in which the greatest sequence variability is present. CDRs also form the antigen-binding pockets that determine the affinity and specificity of each antibody for its antigen.

A recombinant products approach to biodefense reagent development has several technical, logistical, and animal use advantages over traditional methods of isolating and producing antibodies.

The technical advantages include the potential to produce antibody diversity greater than that obtainable by the immune systems of mammals. Mammalian immune systems do not normally recognize "self" epitopes, in order to avoid targeting tissues of the host animal for destruction. While beneficial for the animal, it eliminates a large number of potentially useful molecular structures as targets when making antibodies in animals, either polyclonal (the serum of immunized animals) or monoclonal (the living antibody-producing cells, fused to mouse cancer cells to make them immortal, the so-called hybridomas). Removing antibody diversity from the body of an animal allows antibody genes to be assorted and altered to increase antibody diversity by means of modern DNA manipulation techniques, and therefore increases the chances of isolating useful antibodies from a population of clones. In addition, monoclonal antibodies are generally produced by a laborious manual sorting and screening of hybridoma cells, a process that cannot handle more than several hundred clones at a time. The biopanning procedure by its nature allows the library to self-select by allowing particles displaying a desirable antibody to bind to an immobilized target for subsequent retrieval.

Logistically, producing antibodies in bacterial fermentation offers several advantages over production in mammalian cell culture (hybridomas). Bacteria are much easier to grow, store, and manipulate genetically than mammalian cells. Microbial contamination is much less of a problem and much more easily dealt with when encountered. In general, bacterial fermentations can be scaled up more easily than mammalian cell cultures and their much more rapid growth and harvest provides the capacity for surge production. When using bacteria to produce products to be used in humans, there is no danger of viral contaminants being passed from the mammalian cell culture to human users, as such viruses cannot multiply in bacteria.

Lastly, recombinant antibody production in bacteria is in step with a growing consensus that current methods of producing monoclonal antibodies in quantity are inhumane. The production of monoclonal antibodies in quantity has traditionally been achieved by injecting hybridoma cells into animal hosts, and subsequently collecting

abdominal fluids (ascites) that contain the essentially pure antibody. This method produces great discomfort in animals and is strongly discouraged for large-scale antibody production (National Research Council, 1999). Phage display library construction and subsequent cloning of antibody genes, minimizes animal use by using only those animals initially immunized in the process. All subsequent manipulation and manufacture takes place in bacterial cells, reducing the numbers of animals used from hundreds to fewer than ten per antibody cloned.

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