INTRODUCTION

pIII-based phage display has been used to identify antibody fragments specific to various antigens (1–5). To enhance the level of the displayed antibody, Rondot et al. used a gene-III-deleted helper phage called Hyperphage to rescue the phagemid encoding antibody-pIII fusion protein (6). As a result, the number of single-chain variable fragments (scFvs) presented on the consequent phagemid particles could increase by over two orders of magnitude. However, the production of Hyperphage was so cumbersome that it was necessary to construct an Escherichia coli packaging cell line (DH5α/pIII) to supply pIII for the phage assembly in order to avoid the presence of a plasmid during helper phage production. Furthermore, the yield of Hyperphage (about 1.34 × 10^9/mL) was rather low. Also, most phagemids used in constructing a phage display scFv library produced pIII under the control of a wild-type Lac promoter, which is not absolutely stringent, thus pIII could be produced in cells in the absence of isopropyl β-D-1-thiogalactopyranoside (IPTG) to mediate pIII resistance to the helper phage superinfection (7). Therefore, glucose, a pLac repressor, is often added to the culture medium to repress pIII expression (8).

To solve the problem, we employed a novel method of pIII-based antibody phage display by applying F^{+} Escherichia cells bearing a gene-III-lacking helper phage genome (VCSM13d3). The modified cells were named Hpd3cells, and the concept of Hpd3cells is shown in Figure 1.

MATERIALS AND METHODS

Plasmid Constructs

Standard cloning procedures were carried out according to Sambrook et al. (9). VCSM13d3 was kindly provided by Dr. Jasna Rakonjac and Prof. Peter Model (10); pCANTAB5E-T was derived from pCANTAB-5E (GE Healthcare, Piscataway, NJ, USA) by inserting oligonucleotides encoding thrombin cleavage site behind NotI restriction enzyme recognition site; pET28a-EGFP was obtained by inserting

Figure 1. The concept of Hpd3cells. TG1 cells bearing gene-III-deleted helper phage plasmid VCSM13d3 was transformed with phagemid encoding scFv-pIII to produce phagemid particles displaying several copies of scFv. scFv, single-chain variable fragment.
enhanced green fluorescent protein (EGFP) DNA from pEGFP-N1 (Clontech Laboratories, Mountain View, CA, USA) into pET28a (EMD Biosciences, San Diego, CA, USA); and dpET-EGFP-S2 was constructed by inserting coding sequence of S2 domain of epidermal growth factor receptor (EGFR) into pET28a-EGFP.

Immunoblots

Approximately 10^{10} phages were loaded per lane on a 10% polyacrylamide gel. Blocking was done with 2% skim milk powder in phosphate-buffered saline (PBS) for 2 h at room temperature, and immunostaining was conducted with mouse MAb anti-m13 pIII (New England Biolabs, Ipswich, MA, USA) recognizing the C terminus of pIII coat protein of M13KO7 and goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-streptavidin conjugate (ImmunoLabs, Sunnyvale, CA, USA), and visualized with a SuperSignal® West Pico kit (Pierce Biotechnologies, Rockford, IL, USA).

Determination of Antigen Binding Reactivity by Phage ELISA

To determine antigen binding specificity of recombinant phage particles, 100 ng bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO USA) or recombinant EGFP in coating buffer (0.1 M NaHCO_3, pH 9.1) were coated in microtiter plates at 4°C overnight. Recombinant EGFP protein was produced by growing BL21(DE3) cells harboring pET28-EGFP and affinity purifying with Ni-NTA resin (Qiagen GmbH, Hilden, Germany). The plate was blocked with 1% BSA in PBS, and 10^{10} scFv phages packaged with either M13KO7 or Hpd3cells, ELISA was performed as previously described, except that serial dilutions of purified recombinant EGFP protein were applied to the coating of microtiter plates.

Preparation of Antigen and Immunization

The recombinant protein EGFP-S2 was produced in BL21(DE3) and then affinity purified with Ni-NTA resin. With 100 μL Freud’s adjuvant (Sigma-Aldrich), 100 μg EGFP-S2 proteins in 100 μL 0.9% NaCl were mixed and injected subcutaneously into each mouse. Immunization was repeated three times with the same amount of EGFP-S2 protein injected subcutaneously every 2 weeks. Two weeks following the third time of immunization, 50 μg EGFP-S2 were injected intravitreally into the mouse. Four days later, the mice were sacrificed, the spleens obtained.

Construction of the Single-Chain Variable Fragment Phage Display Library

Total RNA was extracted from the spleen, and the purified mRNA was used to amplify the light chain and heavy chain. The scFv encoding fragments were obtained by overlap assembly PCR with a linker and then digested with SfiI and NotI. The primers were derived according to Reference 11 with some modification (see Supplementary Table S1 available online at www.BioTechniques.com). The consequent fragments were purified and ligated with SfiI/NotI backbone of pCANTAB-5ET. To obtain Hpd3cells, TG1 cells were transformed with VCSM13d3 and then plated on agar plates containing 50 μg/mL kanamycin. With the preparation of the electrocompetent Hpd3cells, the transformation was performed according to the user’s manual of the MicroPulser™ Electroporation Apparatus (Bio-Rad Laboratories). For titration, 1 μL Hpd3cells transformed with ligation was serially diluted and plated on agar plates containing 100 μg/L ampicillin/50 μg/L kanamycin. The remaining transformed Hpd3cells were transferred into a flask containing 200 mL LB and shaken at 37°C for 16 h. Consequently, the phage library was collected, purified by polyethylene glycol (PEG)/NaCl, and filtered through a 0.45-μm filter. The phages were quantified by ELISA as previously described (6). As for the preparation of the phage library using
M13KO7 helper phage, TG1 cells were transformed with the pCANTAB-5ET phagemids encoding scFv gene libraries and then plated on agar plates containing ampicillin in the presence of 1% glucose. Following the growth in LB containing ampicillin, the cells were superinfected by M13KO7 helper phage with a multiplicity of infection (MOI) of 50, with the consequent phages collected and purified by PEG/NaCl.

**Panning**

The immunotube was coated with 50 μg antigen in PBS at 4°C overnight and blocked with 1% BSA in PBS at 37°C for 2 h. Washed with PBS three times, the tube was filled with 5 × 10¹¹ scFv phage library in 1% BSA and 0.1% Triton® X-100 in PBS and incubated at 37°C for 2 h. The solution was discarded completely, and the tube was washed 20 times with 0.5% Tween-20 in PBS, then 20 times with PBS. The bound phages were eluted with 4 U thrombin (Sigma-Aldrich) in PBS at 37°C for 1 h, and an equal volume of log-phage Hpd3cells or TG1 cells was added to the solution, which was then incubated at 37°C for 1 h with shaking. The cells were centrifuged and washed with fresh LB two times to remove the thrombin. The cell pellet was resuspended with 100 μL LB. One microliter cell suspension was serially diluted and plated on LB agar plates containing ampicillin/kanamycin (Hpd3cells) or ampicillin alone (TG1), then incubated overnight at 30°C. The remaining cell suspension was added to culture medium containing ampicillin/kanamycin to produce antibody phages used for the next round of panning; for the infected TG1 cells, the cell suspension was added to culture medium containing ampicillin, then superinfected with M13KO7 helper phage. Phage ELISA was performed by coating 100 ng/well EGFP-S2 protein at 4°C overnight. For polyclonal phase ELISA, 10¹⁰ of amplified phage after panning were added onto microtiter plates; for monoclonal phase ELISA, TG1 or Hpd3cells were infected with eluted phage for 20 min in room temperature and spread onto LB/ampicillin plates or LB/ampicillin/kanamycin plates, respectively. The plates were incubated overnight at 37°C. As for Hpd3cells, E. coli colonies were randomly picked and inoculated to 200 μL LB/ampicillin/kanamycin in sterile 96-well plates at 30°C overnight (Corning Life Sciences, Lowell, MA, USA); and as for TG1, E. coli colonies were randomly picked and inoculated to 200 μL LB/ampicillin in sterile 96-well plates, then superinfected with M13KO7 at 30°C overnight. To microtiter plates, 50 μL culture supernatant containing phage particles from the 96-well plates were added, and phage ELISA was performed as previously described. BSA was used as an antigen negative control, and M13KO7 helper phage was used as a phage negative control.

**pIII-Resistance Assay**

TG1 scFv library was superinfected with M13KO7 and shaken in ampicillin medium at 30°C for 16 h. The phagemid particles produced from the library were precipitated by PEG/NaCl and used to infect TG1 cells. The infected TG1 cells were then plated in ampicillin plate in the presence or absence of 1% glucose with overnight incubation at 37°C; then 96 individual clones were picked out and grew in the presence or absence of 1% glucose at 30°C for 1 h. Next, the infected TG1 cells were superinfected with M13KO7 helper phage at about 50 MOI and grew for another 20 h. The Hpd3cells infected with phages from 96 individual phage clones from TG1/M13KO7 scFv library were plated in ampicillin/kanamycin plate in the absence of glucose with overnight incubation at 37°C, and the same procedure was performed on their colonies. The A₆0₀ values of the culture were measured. The phages in the supernatant were also collected, purified with PEG/NaCl and then quantified by measuring A₂₆₅.
Glucose Positive Clones Production of Phages

RESULTS

Application of Hpd3cells to Construct Phage Display Single-Chain Variable Fragment Library

Since phagemid particles are conventionally made by superinfecting cells containing phagemid with helper phages, we reason that phagemid particles could be produced by cells containing phagemid and helper phage genome simultaneously. Previously, gene-III-lacking helper phages VCSM13d3 and R408d3 were applied to package phagemid encoding pIII (10). R408d3 has also been demonstrated to help the high-level display of EGFP in the rescued phagemid particles (12). However, R408d3 has no antibiotic-resistance marker. Therefore, the VCSM13d3 genome, which contains kanamycin-resistance marker, was employed to make Hpd3cells. A phagemid pCANTAB-5ET-19 encoding a scFv fragment binding to the EGFP (the sequence of the scFv shown in the Supplementary Data S1) was employed to make phagemid particles. The application of Hpd3cells yielded apparently high titers of phagemid particles, 1.2 × 10^{11} cfu/mL, which is similar to what the conventional method produces. The levels of scFv display were further analyzed by Western blot analysis. As expected, scFv-pIII fusion protein was the major form of pIII in Hpd3cells, whereas most of pIII was wild-type in pCANTAB-5ET-19/M13KO7, demonstrating dramatic increase of display level by Hpd3cells (Figure 2A). The effects of pCANTAB-5ET-19/Hpd3cells packaging on antigen binding specificity and sensitivity were analyzed by phage ELISA. pCANTAB-5ET-19/Hpd3cells and pCANTAB-5ET-19/M13KO7 phage particles specifically did not react to BSA protein, but only to recombinant EGFP protein (Figure 2B). In addition, the former phages presented a signal more than two times to the EGFP protein in comparison with the latter phages, indicating that an increase of display level enhanced antigen binding signal through avidity effect. In order to determine antigen binding sensitivity of recombinant phages, different concentrations of human recombinant EGFP protein (0–10 μg) were coated onto microtiter plates, and 10^{10} of pCANTAB-5ET-19/Hpd3cells phages or pCANTAB-5ET-19/M13KO7 were tested for antigen binding reactivity by phage ELISA. The former bound at 100-fold lower concentration of the antigen than the latter, generating the same ELISA signal (A_{405} = 0.37), and produced a positive signal

Table 1. pIII-Resistance Assay

<table>
<thead>
<tr>
<th>Helper Phage/Cells</th>
<th>Glucose</th>
<th>Positive Clones</th>
<th>Production of Phages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hpd3cells</td>
<td>-</td>
<td>100% (96 of 96)</td>
<td>100% (96 of 96)</td>
</tr>
<tr>
<td>M13KO7</td>
<td>+</td>
<td>98% (94 of 96)</td>
<td>98% (94 of 96)</td>
</tr>
</tbody>
</table>

The phages from 96 individual phage clones from TG1/M13KO7 single-chain variable fragment (scFv) library were used to infect TG1 or Hpd3cells, and the infected TG1 cells were rescued by M13KO7. The (absorbance) A_{405} values < 0.5 were considered to be positive; otherwise were negative.

Figure 3. Hpd3cells increased panning efficiency from an immunized single-chain variable fragment (scFv) library. (A) Titers of phage eluted per panning round. cfu, colony-forming units. (B) Polyclonal phage enzyme-linked immunosorbent assay (ELISA) on EGFP-S2 plasmids using the enriched sublibraries after one or two rounds of panning. M13wt represents M13KO7 phage used as a negative control for antigen binding. (C) Binding capacity of individual clones to EGFP-S2 after second round of panning. Ninety-six randomly picked single clones were analyzed by ELISA.
at much smaller amounts of the antigen, indicating that the increase of displaying scFv-pIII fusion by Hpd3cells directly enhanced antigen binding sensitivity of recombinant phage particles (Figure 2C). In accordance with Figure 2B, packaging pCANTAB-5ET-19 with Hpd3cells led to an increase of ELISA signal by more than two times.

Enhanced Selective Enrichment of Antigen-Specific Phage by Hpd3cell Packaging

Since the proteins encoded by the VCSM13d3 may affect the cell growth and transformation, we first compared the growth rate of Hpd3cells with that of TG1 cells and found that the Hpd3cells had a slower growth rate than TG1, with the doubling time of the Hpd3cells being about 30 min. pCANTAB-5ET-19 was used to transform electropotent TG1 cells and Hpd3cells, whose transformation efficiency was 3.76 × 10^6/μg DNA and 2.58 × 10^6/μg DNA, respectively. Therefore, some of the proteins expressed by VCSM13d3 did affect the transformation efficiency. However, the effect was still tolerable because it was easy to increase the total repertoire with more frequencies. To demonstrate the feasibility of Hpd3cells in selecting high-affinity binders, we constructed a phage display scFv library by transforming the phagemids encoding scFv-pIII gene library derived from mice immunized with fusion protein EGFP-S2 into competent Hpd3cells or TG1 cells. For the present phage display scFv library constructed by Hpd3cells, the complexity was 3.75 × 10^7, while that constructed by TG1 cells was 4.38 × 10^7.

Following panning against the antigen, we employed the thrombin cleavage site at the scFv-pIII fusion junction to elute the bound phage from antigen and to recover wild-type phenotype phage. The eluted phages were used to infect Hpd3cells or TG1 cells. Phage titers bound in each panning round were also analyzed. We found that the Hpd3cells phage was enriched 935-fold, showing a significant enrichment following the second panning round (Figure 3A). The analysis of the antigen binding activity of the enriched sublibraries revealed that phages packaged by Hpd3cells bound in a polyclonal phage ELISA using EGFP-S2 as antigen with an A_405 >1.0 following the second panning. In contrast, M13KO7-packaged phage showed no obvious specific antigen binding activity following the second panning (Figure 3B). To assess the yield of antibody clones specifically enriched to EGFP-S2, 96 individual clones were randomly picked up following the second panning round. It was shown that <10% of clones from M13KO7-packaged phages were positive for EGFP-S2 binding (Figure 3C). In contrast, >80% of the clones obtained with Hpd3cells were positive for EGFP-S2 binding in ELISA assay. Furthermore, A_405 values from 19% (18/96) of the clones were >1.0, indicating a high level of binding efficiency. The phagemid DNA from the clones with A_405 values >1.0 was sequenced, with different scFv sequences observed, demonstrating that the employment of Hpd3cells could increase the chance of obtaining more diverse antibodies specific for target antigens.

Hpd3cells to Overcome pIII Resistance

Previous studies have demonstrated that pIII produced in the host cells can mediate resistance to filamentous phage infection (7). Since pIII cannot be expressed in pIII-lacking Hpd3cells, we believed that application of Hpd3cells could overcome pIII resistance. Cell growth assay was performed to examine the pIII resistance, because if pIII resistance occurs, cells will not be infected with phages harboring antibiotics marker and its growth will be inhibited by the corresponding antibiotics. The phages from 96 individual phage clones from TG1/M13KO7 scFv library were used to infect TG1 or Hpd3cells. As expected, all the A_600 values of the infected Hpd3cells clones reached 2.0. However, in infected TG1 cell clones followed by M13KO7 superinfection, the A_600 values below 0.5 accounted for 17% (16/96) in the absence of glucose and 2% of in the presence of glucose (2/96), respectively (Table 1). To further examine whether the phages were produced, the supernatant from the clones was precipitated with PEG/NaCl, respectively, and the titers were determined by ELISA. Phages were produced from all the Hpd3cells clones. Phages were also produced from the TG1 clones except from those with A_600 value <0.5. These results further demonstrated that pIII resistance did exist in the previous method, even when glucose was supplemented to the medium. This might be due to the fact that glucose could not completely abolish the expression of pIII (8). In contrast to the previous method, the application of Hpd3cells in phage display could overcome pIII resistance completely, even in the absence of glucose. Therefore, Hpd3cells may increase the panning efficiency in part by overcoming pIII resistance, which might lead to the loss of some phagemids encoding specific binding antibodies.

DISCUSSION

Rescue of phages from a phagemid-based phage display library using conventional helper phages like VCSM13 generally results in a high frequency of phages not displaying the protein of interest, which thus form a significant background during selection procedures. In order to circumvent this problem, phages from a phagemid-based phage display library was rescued by Hyperphage (6) or Ex-phage (13). But the yield of Hyperphage (about 1.34 × 10^8/μL) was rather low, and perhaps the pIII resistance existed in the phage rescue procedure of phagemid-based phage display library (7,8). Thus, we employed a novel method of pIII-based antibody phage display by applying F+ Escherichia cells bearing gene-III-lacking helper phage genome (VCSM13d3). From the data presented, we concluded that Hpd3cells not only could function as Hyperphage in helping high-level display of scFv, but also overcome pIII resistance in comparison with the conventional phage display. And Hpd3 cells may increase the chance of obtaining scFv specific to an antigen by overcoming pIII resistance, which may lead to loss of some phagemids encoding specific binding antibodies. Therefore, Hpd3cells should be powerful for efficient enrichment of specific binding antibodies from a phage display library. In addition to the application for scFv display, Hpd3cells...
can be used conveniently for high-level ligand or antigen display. Furthermore, the concept of Hpd3 cells can be simply extended to the modification of other capsid genes. For instance, we have already used this method to display some peptides on pVI by cotransforming gene VI-lacking mutant helper phage plasmid with phagemid encoding pVI peptide fusion protein (data not shown). In addition, this new technique can conveniently produce multiple copies of scFvs on the phagemid surface, which potentially may lead to the discovery of antibodies with lower affinity but with unique biological activities and the stable multivalent phagemid can produce enough avidity to be selected by phage display. Avidity effects can easily be avoided by performing the first selection step in solution with a very short subsequent panning, and then the second and subsequent panning rounds can be performed using conventional M13KO7 helper phage (6). Taken together, this new technique provides a new concept for ligand, antibody, and antigen phage display.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


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