Antibody affinity maturation in vitro using unconjugated peptide antigen

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Selection of antibody library in vitro is almost always performed on a certain solid-phase with immobilized antigen. However, for the selection of small molecule binders, conjugation of the antigen to a carrier molecule is indispensable, which often leads to the selection of unwanted binders such as conjugate-binders or those with insufficient specificity. Here we describe a rapid and efficient way to improve the affinity of an anti-small molecule antibody without antigen derivatization. The method is based on the open-sandwich (OS) principle, which utilizes the antigen-dependent stabilization of antibody variable domain Fv. We used an anti-osteocalcin C-terminal peptide Fv that showed a good response but with moderate sensitivity in OS ELISA as a model. By selecting PCR-randomized VH-displaying phages for superior binders to the immobilized VL fragment in the presence of limited amount of antigen peptide, VH mutants that show superior detection sensitivity in OS ELISA were obtained, and were characterized to retain improved antigen-binding affinity. Furthermore, saturation mutagenesis of a mutant resulted in further improvement in sensitivity. This ‘OS-selection’ will be the first to select anti-small molecule antibodies without using conjugated antigens, and especially useful in the affinity maturation of antibodies whose Fv has limited stability in the absence of antigen.

Keywords: affinity maturation/antibody library/hapten/open sandwich selection/phage display

Introduction

The immune system uses somatic hypermutation to generate sequence variants of rearranged immunoglobulin genes. High and low affinity antibodies binding to a certain immunogen are discriminated by clonal B-cell selection, thus favoring immunoglobulins with a lower dissociation rate (Nossal, 1992; Batista and Neuberger, 1998; Noia and Neuberger, 2007). However, acquisition of high-affinity binders to small molecules is generally more difficult, because of the limited surface area of the paratope (Sheedy et al., 2007). This is also the case for the selection in vitro, and has been a problem especially when naive phage-displayed antibody library is used for the selection. Although a number of synthetic or focused libraries on small molecules have been studied (Barbas III et al., 1993; Söderlind et al., 2000; Persson et al., 2006; Cobaghan et al., 2008), the dissociation constants attained for small molecules were mostly in 10–100 nM range.

As a way to improve the antigen-binding affinity, affinity maturation of the lead antibody by phage library selection has been utilized as a powerful means to optimize antibodies with inferior affinity and specificity for proteins (Clackson et al., 1991; Marks et al., 1992; Hawkins et al., 1992; Yang et al., 1995; Irving et al., 1996; Low et al., 1996; Steidl et al., 2008; Finlay et al., 2009) and for smaller molecules (Clackson et al., 1991; Gram et al., 1992; Hawkins et al., 1992; Kobayashi et al., 2008; Marks et al., 1992; Miyazaki et al., 1999; Sheedy et al., 2007).

For the selection of antigen-specific phage-displayed antibody fragments, in most cases biopanning on immobilized antigen on certain solid phase is performed. However, while larger protein antigens can be immobilized directly or by straightforward coupling methods, smaller molecules such as haptens or peptides are unsuitable for direct adsorption, where the adsorption efficiency is low or leads to inefficient display of its epitope. Hence, these small molecules require site-specific chemical conjugation to either solid surface or carrier proteins through appropriate linkers. However, if the site of derivatization for conjugation is inappropriate, troubles like concentration of less-specific binders or selection of conjugate-binders could happen. Furthermore, when such conjugates are immobilized on a solid phase, the potential to select phage antibodies for improved display (or homodimerization/diabodies) and avidity cannot be ignored, while the monovalent binding constant does not change. If we can select antibodies for a small molecule with their unmodified antigen, we will not have to worry about these consequences.

As a strategy for conjugate-free selection of phage-displayed antibody fragments, we previously reported a selection of VH-displaying phage on an immobilized VL fragment, based on the antigen-driven Fv stabilization mechanism (open-sandwich selection, OS selection) (Tsumoto et al., 1997). By the OS selection, in the presence of unmodified human lysozyme, the selection successfully converted the recognition specificity of anti-hen egg lysozyme (HEL) antibody to human lysozyme. Although the method has an apparent limitation with respect to the library size, where only one of two variable domain fragments can be diversified, it also has several merits. First, since the method handles smaller size of gene than scFv or Fab, it is less subject to gene deletion during the selection. Second, the fragments selected can be directly applicable to open-sandwich immunoassay (OS-1A), which we proposed previously as a novel type of noncompetitive immunoassay.
approach (Ueda et al., 1996; Suzuki et al., 1999; Ueda, 2002). Briefly, this assay exploits re-association of the antibody variable region fragments V H – V L by a bridging antigen. By OS-ELISA (OS-IA performed in microplates) using immobilized V L and enzyme-tagged V H fragments, we could measure HEL concentration in a sample in a shorter time period than using a conventional sandwich assay, due to the omission of an incubation/washing cycle. Also, the assay was found to be compatible with a number of anti-hapten antibodies, and could attain similar or a lower detection limit as well as a wider working range (Suzuki et al., 2000; Aburatani et al., 2003; Ihara et al., 2009). Lastly, the selection requires neither antigen immobilization nor modification, which distinguishes this method from any other in vivo or in vitro antibody selection methods, especially if it is applied to the selection of small molecule binders. From these points altogether, we reasoned that the method might be suitable to improve the property of anti-small molecule antibodies, especially to improve the detection sensitivity in OS-IA.

In this study, we applied the OS selection to the affinity maturation of an anti-peptide antibody whose molecular weight is less than 1000. As a target, we chose an anti-osteocalcin C-terminal peptide antibody KTM219 that shows a good response but with moderate sensitivity in OS ELISA (Lim et al., 2007). Human osteocalcin (also known as bone g-carboxyglutamic acid (Gla)-protein or BGP), a 49-amino acid peptide that is major non-collagen protein of bone, is considered to reflect bone metabolism and is currently investigated in clinical settings (Twist and Parfitt, 1996). The g-carboxyglutamic acid ligand, which distinguishes this method from any other antibody selection methods, especially if it is applied to the selection of small molecule binders. From these points altogether, we reasoned that the method might be suitable to improve the property of anti-small molecule antibodies, especially to improve the detection sensitivity in OS-IA.

Materials and methods

Materials

The plasmid pIT2-13CG2 (de Wildt et al., 2000) was kindly provided by Dr Ian Tomlinson, while pMALp2 was obtained from New England Biolabs (Ipswich, MA, USA). Escherichia coli strains used in this study were XL1-Blue (Stratagene, La Jolla, CA, USA) for general cloning, TG-1 (GE Healthcare, Tokyo, Japan) for phage display, HB2151 (GE Healthcare) for soluble V H protein expression and BL21(DE3, pLysS) (Novagen, Takara-Bio) for the expression of MBP-V L proteins. Restriction and modification enzymes were either from Takara-Bio (Chiba, Japan), Toyobo (Osaka, Japan), Roche Diagnostics (Tokyo, Japan) or New England Biolabs. Oligonucleotides synthesized either by Texas Genomics Japan (Tokyo, Japan) or Invitrogen (Tokyo, Japan) are listed in Supplementary Table S1. BGP-C8 (NH2-VEAYRFYGPV-COOH) and biotinylated BGP-C11 (bio-NH2-QEAYRFYGPV-COOH) peptides were synthesized by Genescript (Piscataway, NJ, USA). Other chemicals, reagents and antibodies, unless otherwise indicated, were obtained from Sigma (St Louis, MO, USA) or Wako Pure Chemicals (Osaka, Japan).

Construction of randomized V H library

The V H gene of KTM219 was randomly mutated by error-prone PCR employing unequal concentrations of dNTPs and MnCl2 (Ueda et al., 2004). A 200 μl reaction mix containing 16 U Taq polymerase, 10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100, 0.5 mM MnCl2, 2.5 mM MgCl2, 0.35 mM dATP, 0.40 mM dCTP, 0.20 mM dGTP, 1.35 mM dTTP and 500 nM each of primers M13RV and VH1For2Not (pH 9.0) was subjected to PCR with 30 cycles of 94°C 1 min, 55°C 1 min and 72°C 1 min. The amplified fragment was digested with NcoI and NotI, and ligated with pT2 digested with the same enzymes at 16°C for 6 h. Ethanol precipitated DNA was dissolved in sterile water, and used for the electroporation of freshly prepared TG-1 competent cells. A portion of cells was plated to YTAG plates (YT agar plates containing 100 μg/ml ampicillin and 1% glucose, where YT agar is 15 g/l Bacto-Agar, 8 g/l NaCl, 8 g/l tryptone and 5 g/l yeast extract, pH 7.6) and incubated at 37°C for 16 h. Resultant colonies were counted to estimate the size of the library, and used for plasmid preparation and DNA sequencing. Rest of the cells was cultured in 1 L of 2YT medium (2YT medium containing 100 μg/ml ampicillin and 1% glucose, where 2YT medium is 8 g/l NaCl, 16 g/l tryptone and 10 g/l yeast extract, pH 7.6) and used for the phage preparation.

Dideoxy reactions and DNA sequencing analysis were performed using CEQ 8000 Genetic Analysis System (Beckman-Coulter) according to the protocol supplied by the manufacturer.

Phage display of randomized V H library

Escherichia coli TG-1 harboring the plasmid library was cultured in 2YTAG medium at 37°C with shaking at 200 rpm until OD 600 reached ~0.5, when helper phage KM13 (Kristensen and Winter, 1998) was added with a multiplicity of infection (m.o.i.) of 20. After incubation at 37°C for 30 min without shaking, the culture was centrifuged at 2300 g for 15 min. Then the pellet was resuspended in 2YTAG (2YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin) and incubated for 16 h with vigorous shaking at 30°C. After the overnight culture was centrifuged at 2300 g for 30 min, 0.2 vol PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) was added to the supernatant and the mixture was incubated at 4°C for 1 h. After incubation, the mixture was centrifuged at 6000 g for 30 min. The pellet was resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

Preparation of soluble V L fragments

The V L fragment of KTM219 was expressed as a fusion protein with E.coli maltose binding protein (MBP-V L) as follows. To obtain an expression vector for tag-less MBP-V L, pMAL-VL(219) (Lim et al., 2007) was digested with NdeI, treated with T4 DNA polymerase, and self-ligated to yield pMAL-VL(219)ΔTag. Escherichia coli BL21(DE3, pLysS) cells were transformed with pMAL-VL(219) or pMAL-VL(219)ΔTag, grown on YTAC agar plates (YT agar plates containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol) at 37°C for 16 h, and single colony was...
When 0.4 mM of isopropyl-β-D-galactopyranoside (IPTG) was added, the culture was incubated for 16 h with shaking at 16°C. His-myc-tagged MBP-V_L or tag-less MBP-V_L was purified from the cell lysate with Talon Co²⁺-immobilized resin (Clontech, Takara-Bio) or amylose-immobilized resin (New England Biolabs), respectively. The purified proteins were analyzed by SDS–PAGE (Supplementary, Fig. S1), and confirmed to retain sufficient activity. The purified proteins were analyzed by SDS–PAGE and confirmed to retain sufficient activity. The purified proteins were analyzed by SDS–PAGE and confirmed to retain sufficient activity.

Selection of specific V_H-displaying phage from a model library

A model library was made by mixing phages displaying the V_H of KTM219 (1 × 10⁷ cfu) and those displaying V_H for a neonicotinoid pesticide imidacloprid (ICP) (1 × 10⁷ cfu) as a nonspecific V_H. The model library was mixed with 0.1 or 0.5 µg/ml bio-MBP-V_L and 0–100 ng/ml BGP-C8 peptide in 250 µl of MPBS (PBS containing 2% skim milk). The mixture was rotated for 30 min and then incubated for 1 h without rotation at 25°C. After incubation, 125 µg/ml of streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin C1, Dynal, Invitrogen) were added and rotated for 30 min, before the beads were washed three times with 1 ml of PBST (PBS containing 0.1% Tween 20). The bound phages were eluted by adding 200 µl of trypsin solution (1 ng/ml in PBS). Escherichia coli TG-1 cells (900 µl, OD₆₀₀ = 0.5) were infected with 100 µl of eluted phage solution, and the cells were plated to YTAG plates and incubated at 37°C for 16 h. Resultant colonies at each condition were counted to estimate the titers (Supplementary, Table S2), and used to analyze the fingerprints of the inserted DNA by checking the AffIII-digested pattern of colony PCR products (Supplementary, Fig. S3).

Open sandwich biopanning

The V_H-displaying phage library (1 × 10¹⁰ cfu) was mixed with 0.5 µg/ml bio-MBP-V_L and 1 ng/ml BGP-C8 peptide in 250 µl of MPBS. The mixture was rotated for 30 min and then incubated for 1 h without rotation at room temperature. After incubation, 125 µg/ml of streptavidin-magnetic beads was added and rotated for 30 min, before the beads were washed with PBST for three (first round) or five (later rounds) times. Bound phages were eluted by adding 200 µl of trypsin solution (1 mg/ml in PBS), incubated for 10 min and 100 µl of the solution was used to infect TG-1 cells (900 µl, OD₆₀₀ = 0.5), which were plated to YTAC plates and incubated at 37°C for 16 h. The number of clones on the plates was counted at each stage. Resultant colonies were cultured in 1 L of 2YTAC medium and used for phage preparation for the next rounds of biopanning.

In the case of conventional biopanning for the comparison, the phage library (1 × 10¹⁰ cfu) was mixed with 0.5 µg/ml MBP-V_L-his-myc and 1.7 ng/ml (the same molar concentration of BGP-C8 as used for OS biopanning) of biotinylated BGP-C11 peptide in 250 µl MPBS. Subsequent procedure of biopanning is the same as above.

Open-sandwich phage ELISA

The antigen-binding activity of V_H displaying phage was tested by OS-ELISA. BD Falcon 353912 microplates (Becton Dickinson, Franklin Lakes, NJ, USA) were coated overnight with 100 µl of 1 µg/ml MBP-V_L-His-Myc or 1 µg/ml anti-c-myc 9E10 antibody in PBS at 4°C. After removing the solution, 200 µl of blocking buffer (PBS containing 20% Immunoblock (DS Pharma, Osaka, Japan)) was added to each well. After incubation for 2 h at room temperature, each well was washed three times with PBST, and added with 100 µl of V_H-phage (10⁹ cfu) mixed with antigen in a series of concentrations in IPBS (PBS containing 5% Immunoblock) and incubated for 1 h at 25°C. After washing three times with PBST, 100 µl of 5000-fold diluted HRP/anti-M13 monoclonal conjugate (GE Healthcare) in IPBS was added and incubated at 25°C for 1 h. After washing three times with PBST, 100 µl of TMBZ solution (100 mg/ml 3,3′,5,5′-tetramethylbenzidine (Sigma), 0.04 µl/ml H₂O₂, in 100 mM NaOAc, pH 6.0) was added to each well. After incubation for 5–30 min, the reaction was stopped by adding 50 µl/well of 1 M sulfuric acid, and the absorbance was read using a Model 680 microplate reader (Bio-Rad, Tokyo, Japan) at 450 nm with 655 nm as a control.

Construction of O2AG2 chimeric mutants

Chimeric V_H genes between the wild-type and O2AG2 mutant were constructed as follows. First, four DNA fragments which divide four mutation sites were amplified from each V_H gene with primer pairs M13RV and VHBGP01for, VHBGP01back and VHBGP02for, VHBGP02back and VHBGP03for, and VHBGP03back and pHENseq (Supplementary, Table S1). PCR was carried out under the following conditions: 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 18 s with ExTag DNA polymerase. Chimeric genes were assembled from these fragments by the overlap extension PCR with 15 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 40 s, followed by a normal PCR with primer pairs M13RV/pHENseq with 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 40 s. The amplified fragments were digested with NeoI and NotI, and ligated with pIT2 digested with the same.

Construction of second library

Saturation mutagenesis was performed for the four mutated residues of O2AG2 by PCR amplification of the V_H gene using primer pairs containing degenerate codons (NNK) at the corresponding positions. Four DNA fragments were amplified with primer pairs M13RV and BGFL01for, BGFL01back and BGFL02for, BGFL02back and BGFL03for, and BGFL03back and pHENseq, and then assembled into the fragment encoding entire V_H. Following procedure was the same as described earlier.

Expression and purification of soluble V_H fragments

Escherichia coli HB2151 cells were transformed with pIT2-VH, grown on YTAC agar plates at 37°C for 16 h and single colony was cultured in YT medium containing
100 μg/ml carbenicillin at 37°C with shaking at 200 rpm until OD₆₀₀ reached ~0.5 when 0.4 mM of IPTG was added. Then, the culture was incubated with shaking at 16°C for 16 h. Culture supernatant was added with ammonium sulfate at 65% saturation, and the precipitates were collected by centrifugation at 10 000 g for 10 min. The protein precipitates were dissolved in 50 mM phosphate buffer containing 300 mM NaCl (pH 7.0), and the VH fragments were purified with Talon resin. SDS–PAGE was performed to confirm their purity (data not shown).

**ELISA with soluble VH and VL fragments**

Each well of microplate was coated overnight with 100 μl/well of 10 μg/ml streptavidin type II (Wako) in PBS at 4°C. After removing the solution, 200 μl of blocking buffer was added to each well and incubated for 2 h at 25°C. After incubation, each well was washed three times with PBST, added with 100 μl of 1 μg/ml biotinylated BGP-C11 in IPBS and incubated for 1 h at 25°C. Each well was washed three times with PBST, added with 100 μl of IPBS containing 1 μg/ml VH-His-Myc and 1 μg/ml MBP-VL-His-Myc, and incubated for 1 h at 25°C. The wells were washed three times with PBST and incubated with 100 μl/well of 4000-fold diluted HRP/anti-His₆ monoclonal conjugate in IPBS. After washing three times with PBST, the bound VH/VL was detected by adding TMBZ solution as above.

To perform OS ELISA, the microplate wells were coated overnight with 100 μl/well of 1 μg/ml MBP-VL-DTag in PBS at 4°C. After removing the solution, 200 μl of blocking buffer was added to each well and incubated for 2 h at room temperature. After incubation, each well was washed three times with PBST, and added with 100 μl of IPBS containing 1 μg/ml VH-His-Myc and 0 or 100 ng/ml BGP-C8 and incubated for 1 h at room temperature. The wells were washed three times with PBST and incubated at room temperature with 100 μl/well of 4000-fold diluted HRP/anti-His₆ monoclonal conjugate in IPBS. After three times of washing with PBST, the bound VH was detected as described earlier.

**SPR analysis**

Antigen-binding kinetics of soluble VH and VL fragments were determined by surface plasmon resonance analysis using Biacore 2000 (GE Healthcare). Streptavidin type II (Wako pure chemicals) was immobilized to the two flow cells of a CM5 sensor chip, and then 1 μg/ml of biotinylated BGP-C11 was injected to one flow cell, leaving another cell as a reference. HBS-ET (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4) containing 800 nM each of VH-his-myc and VL-his-myc was injected over the sensor chip at a flow rate of 10 μl/min. The kinetic analysis was performed with BIAevaluation software 4.1 (GE Healthcare).

**Results**

**Construction of randomized VH library**

The scheme of OS biopanning is depicted in Fig. 1. To construct VH gene library, the anti-BGP VH gene was randomly mutated by error-prone PCR, and the amplified fragments were ligated to a phagemid vector pIT2. As a result, 1.3×10¹¹ independent transformants were obtained. When colony PCR was performed with 46 clones from the transformants, 20 of these clones were sequenced, 15 clones were confirmed to have mutated VH genes without detectable biases, deletions and insertions. Consequently, actual library size was...
estimated to be $9.3 \times 10^7$. In average, 3.3 mutations were found per VH gene.

**Selection of specific VH-displaying phage from a model library**

To test the concept of OS biopanning for the selection of antigen-specific antibodies and to investigate the most appropriate selection condition, the selection for the anti-BGP (KTM219) VH was performed with a model library consisting of KTM219 VH phage ($1 \times 10^7$ cfu) and the phage ($1 \times 10^7$ cfu) displaying VH that is specific to a neonicotinoid pesticide imidacloprid (HU in preparation). The identification of the recovered phages was performed by the colony PCR of the infected colonies and subsequent restriction digestion, since VH gene of KTM219 has no AflII site whereas that of imidacloprid-specific VH has one. As shown in Supplementary, Fig. S3 and Table S2, KTM219 VH-displaying phages were obtained only under the conditions where 100 ng/ml BGP-C8 was mixed with VH-phage and biotinylated VL (five out of eight clones with 0.1 µg/ml bio-MBP-VL, or seven out of eight with 0.5 µg/ml bio-MBP-VL). Without antigen, no enrichment of KTM219 VH phage was observed. In spite of the competitive binding of heterologous VH-phage, the result showed the utility of OS biopanning for the selection of antigen-specific antibodies.

**Open sandwich biopanning**

According to the result of the model OS selection, we decided to use 0.5 µg/ml of biotinylated MBP-VL for subsequent rounds of biopanning. Also, to perform affinity maturation, we decided to use reduced amount of antigen (BGP-C8 peptide) not to concentrate the phage displaying the wild-type KTM219 VH but to select for mutants with improved affinity. The phage library ($10^{10}$ cfu) was reacted with the mixture of 0.5 µg/ml biotinylated MBP-VL and 0, 1 and 100 ng/ml of intact BGP-C8 peptide in PBS containing blocking agent, and the VH-phages bound to VL via ternary complex formation were recovered through bio-MBP-VL with streptavidin-paramagnetic beads. At the first round, we also tried to add a negative selection to remove strong VL binders in the absence of antigen. In this case, an additional incubation with biotinylated MBP-VL without BGP-C8 and removal of the complex by the SA beads were performed before the positive selection. However, since we could not observe any differences in recovered phage titers (Table I, A and B), we decided to omit this step. After three rounds of biopanning using the three antigen concentrations, we decided to use the phages selected with 1 ng/ml of antigen, because we could get the largest increase in titer (Table IB). When the phages selected from each round were analyzed by polycional OS phage ELISA, concentration of specific binders was confirmed even after the first round (Fig. 2A). In addition, its efficiency was better than that of conventional biopanning with the same molar concentration of biotinylated BGP-C11 peptide (Table IC and Fig. 2B). Then the clones isolated from the phages recovered from the second or third round of biopanning were used to prepare monoclonal VH-phages, and screened by monoclonal OS-ELISA (Supplementary, Fig. S4). According to the result, 11 clones that showed higher binding signals were selected, and determined for their dose-dependency and for their DNA sequence. As shown in Supplementary, Fig. S5, at least the clones O2A2G2, O2B2B6, O2C6 and O3A7E7 had lower detection limit (~0.05 ng/ml) than that of the wild-type VH.

As summarized in Table II, the clones had 2–4 mutations not only at their CDRs, but also at whole VH gene including FR regions.

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**Table I.** Titers of the phage recovered from each round of first OS-biopanning at several different conditions

<table>
<thead>
<tr>
<th>(BGP C8 conc.)</th>
<th>(0 ng/ml)</th>
<th>(1 ng/ml)</th>
<th>(100 ng/ml)</th>
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</thead>
<tbody>
<tr>
<td>(A) Biopanning with negative selection</td>
<td>Recovered phage (cfu) Round 1 $5 \times 10^3$ $2 \times 10^4$ $&gt;10^5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B) Biopanning without negative selection</td>
<td>Recovered phage (cfu) Round 1 $4 \times 10^3$ $2 \times 10^4$ $&gt;10^5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C) Conventional biopanning using immobilized antigen</td>
<td>Recovered phage (cfu) Round 1 $3 \times 10^3$ $3 \times 10^4$ $2 \times 10^5$</td>
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<td></td>
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**Fig. 2.** The results of polycional phage ELISA. (A) OS Phage ELISA of the phages before (Library) and after the OS selection (Round X). The wild-type VH-displaying phage (WT) was also used. Signals without antigen (Ag(-)) or with peptide (BGP-C8) at specified concentration are shown. The signals with immobilized anti-myc antibody are also shown to show the relative amounts of VH-displaying phages. (B) OS Phage ELISA of the phages from the initial library or obtained by the rounds of OS biopanning (OS-R1 and OS-R2) and conventional biopanning (C-R1 and C-R2). Keys are the same as in (A).
Analysis of O2AG2

From the clones obtained by the OS biopanning, a clone (O2AG2) that showed the most enhanced signal towards BGP-C8 was selected and used for further analyses. Since the clone had four mutations, namely, N31S, V37I, S58T and H82R, we analyzed the effect of each mutation on the detection sensitivity by OS-ELISA. First, four single mutants that have one of four mutations were constructed and analyzed by OS-ELISA. As shown in Fig. 3C, V37I mutation at the V\textsubscript{H}/V\textsubscript{L} interface showed the most significant improvement in the detection limit in OS-ELISA, while other two of three mutations did show some positive effects. Since the response of V37I mutant was still inferior to O2AG2, next we constructed three double mutants that harbor V37I and another mutation, and analyzed them by OS-ELISA. As shown in Fig. 3D, all three mutants showed enhanced detection sensitivity compared with V37I mutant, which were nearly comparable to O2AG2. These results indicate the importance of V37I mutation in binding activity and cooperative effects by other three mutations for the fine-tuning.

Table II. Amino acid alterations found in the selected clones with improved sensitivity in OS ELISA

<table>
<thead>
<tr>
<th>Clone</th>
<th>Substitutions</th>
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<tbody>
<tr>
<td>O2AG2</td>
<td>CDR1 N31S FR2 V37I CDR2 S58T FR3 H82R</td>
</tr>
<tr>
<td>O2AH2</td>
<td>FR1 E10V FR3 T69I</td>
</tr>
<tr>
<td>O2BH4</td>
<td>FR1 V37I CDR2 S58T CDR3 H82R</td>
</tr>
<tr>
<td>O2BC6</td>
<td>FR1 E6Q V12A FR1 F29S</td>
</tr>
<tr>
<td>O2BG5</td>
<td>E6Q FR1 N30Y</td>
</tr>
<tr>
<td>O2BB6</td>
<td>E10G FR1 Q108P</td>
</tr>
<tr>
<td>O2BD6</td>
<td>E6Q FR1 T28A Q43H</td>
</tr>
<tr>
<td>O3AE7</td>
<td>E6Q FR1 Q62H CDR2 G66D CDR3 H82R</td>
</tr>
<tr>
<td>O3AB8</td>
<td>E6Q FR1 S7P FR2 P41S S88P</td>
</tr>
<tr>
<td>O3AF10</td>
<td>E6Q FR1 S99G</td>
</tr>
<tr>
<td>O3AD11</td>
<td>E10V FR1 Y27D FR2 P41T</td>
</tr>
</tbody>
</table>

Construction and selection of O2AG2 randomized V\textsubscript{H} second library

To further optimize the detection sensitivity of V\textsubscript{H} fragment, we designed a second V\textsubscript{H} library by introducing saturation mutagenesis to all the four mutation sites of O2AG2. V\textsubscript{H} gene was randomized by degenerate codons (NNK) with PCR mutagenesis and ligated with pIT2 vector. Resultant colonies were checked by the same way as described earlier, and we finally obtained $2.5 \times 10^8$ repertoires of V\textsubscript{H} displayed on phage. Since the theoretical library size was $1.0 \times 10^6$.
(32), the constructed library was thought to fully cover its theoretical diversity.

The second $V_H$ library was selected by OS biopanning. While the selection procedure was the same as in the first OS biopanning, according to the results of titration (Table III) and polyclonal phage OS-ELISA (Fig. 4A), the specific binders were gradually concentrated up to the fourth round. This was probably because of more diverse mutations introduced by saturation mutagenesis compared with those introduced by error-prone PCR. The clones recovered from the phages from third and fourth rounds were isolated, and were screened for higher signal by monoclonal phage OS-ELISA (Supplementary, Fig. S6). The top eight clones that showed higher binding activity towards BGP-C8 were picked-up, and analyzed for their primary sequence (Fig. 4B). The residues of these clones represented at each position were apparently biased, in which N31P, V37I, S58V and H82T were selectively concentrated. Among them, the residue at position 37 was highly selective, where only Val (wild-type) or Ile (O2AG2-type) were observed. Then OS-ELISA at the lower BGP-C8 concentration range was conducted to investigate their detection limits. As a result, most of the clones showed either equal or lower detection limit compared with the clone O2AG2 (data not shown). As shown in Fig. 4C, the best clone R4A10 could detect 1 pg/ml of BGP-C8 (>100-fold improvement compared with the wild-type). Interestingly, the clone R4A10 retained all the four mutations as described earlier (N31P, V37I, S58V and H82T).

**Antigen binding affinity of soluble $V_H$ and $V_L$ fragments**

To investigate whether the increased sensitivity of the mutants was originated from the increased antigen binding affinity of the assembled variable domains, soluble proteins for His$_6$-tagged $V_H$/V$_L$ and MBP-$V_L$ without His$_6$-tag were prepared to perform normal ELISA with immobilized tethered antigen, as well as OS-ELISA with unmodified antigen. As shown in Fig. 5A, 1 μg/ml of $V_H$ mutants O2AG2 and R4A10 each mixed with the same amount of MBP-$V_L$ showed higher antigen-binding signal than the wild-type $V_H$, and especially clone R4A10 showed the highest signal. Also, as shown in Fig. 5B, the signals in OS-ELISA with immobilized tag-less MBP-$V_L$ showed the same trend, in accord with the results of phage OS ELISA. The kinetic analysis of these $V_H$'s mixed with equimolar $V_L$ toward immobilized biotinylated peptide was performed with an SPR biosensor Biacore 2000. As shown in Fig. 5C and Table IV, while O2AG2 showed 3.5-fold higher association rate than the wild-type, it also showed some compromise in dissociation rate, which resulted in slightly lower (0.65-fold) equilibrium dissociation constant $K_d$. On the other hand, R4A10 showed increased association and decreased dissociation rates (5.3-fold and 0.66-fold, respectively), which resulted in 8-fold reduction in $K_d$ than that of the wild-type Fv. These results clearly indicate the improved antigen-binding affinity of the mutants isolated from OS-selection, proving its utility as an affinity maturation method.

**Table III.** Titers of the phage recovered from each round of the second OS biopanning in the presence of BGP-C8 at indicated concentration

<table>
<thead>
<tr>
<th>(BGP C8 conc.)</th>
<th>(0 ng/ml)</th>
<th>(1 ng/ml)</th>
<th>(100 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered phage (cfu)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round 1</td>
<td>$4 \times 10^4$</td>
<td>$2 \times 10^4$</td>
<td>$6 \times 10^4$</td>
</tr>
<tr>
<td>Round 2</td>
<td>$1 \times 10^5$</td>
<td>$4 \times 10^4$</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>Round 3</td>
<td>$4 \times 10^4$</td>
<td>$3 \times 10^4$</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>Round 4</td>
<td>$8 \times 10^4$</td>
<td>$1 \times 10^6$</td>
<td>$7 \times 10^7$</td>
</tr>
</tbody>
</table>

![Fig. 4.](image-url)
Table IV. The calculated kinetic parameters of the Fv fragments (wild-type and mutants) against immobilized Bio-C11 peptide determined by Biacore 2000

<table>
<thead>
<tr>
<th>Clone</th>
<th>$k_a \times 10^3 M^{-1}s^{-1}$</th>
<th>$k_d \times 10^{-6} s^{-1}$</th>
<th>$K_D \times 10^{-9} M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.778 ± 0.061</td>
<td>107 ± 58</td>
<td>137 ± 74.6</td>
</tr>
<tr>
<td>O2AG2</td>
<td>2.73 ± 0.012</td>
<td>245 ± 256</td>
<td>89.6 ± 93.8</td>
</tr>
<tr>
<td>R4A10</td>
<td>4.1 ± 0.011</td>
<td>70.6 ± 9.54</td>
<td>17.2 ± 2.39</td>
</tr>
</tbody>
</table>

Discussion

Compared with the corresponding conventional biopanning with the same amount of biotinylated antigen peptide, OS-biopanning showed higher efficiency in concentrating better binders in OS-ELISA (Fig. 2B). This can be partly explained by the lower titer of recovered phages in the each step of OS-biopanning (Table I), which in turn indicates more stringent selection condition in biopanning than in conventional biopanning. Generally, OS phage ELISA needs larger amount of phage to obtain similar signal than in conventional ELISA. The somewhat lower efficiency of ternary complex formation in OS-biopanning might also have resulted in its higher selection pressure.

In this study, we could not observe significant effect of ‘negative selection’ to remove antigen-independent $\gamma_L$ binders prior to OS-selection. This was probably because of the relatively small amount ($\sim 10^3$ cfu) of $\gamma_H$-phages in the library that bound to MBP-$\gamma_L$ in the absence of antigen, which was significantly less than that bound in the presence of 1 ng/ml BGP-C8 ($\sim 10^4$ cfu). This was probably because we used a clone of antibody that has a good aptitude for OS-IA as a starting point of randomization. However, if the antibody with stronger $V_H-V_L$ interaction is used as a source of the diversification, or the selection of the libraries from more diverse sources is attempted, such negative pre-selection might show some greater merits.

In our first three rounds of selection of the randomized library, no obvious convergence of the obtained mutated amino acid residues was observed. However, it is worth noticing that there was a tendency that a glutamate in FR1 was changed to a non-charged residue such as glutamine, valine or alanine, which might indicate the importance of the charge of the $V_H$ N-terminal regions in the specific recognition of antigen peptide (Table II). Especially, the frequently found E6Q mutation was in good agreement with the previous report showing the importance of glutamine in this position for $V_H$ folding (de Haard et al., 1998). The fact that observed mutations exist in not only CDR but also FR sequences are in good agreement with previous affinity-maturation experiments of anti-hapten antibodies (Gram et al., 1992; Miyazaki et al., 1999). Probably, it is the result of overall optimization including that of antigen binding site, $V_H-V_L$ interaction, and stability of the whole variable region including antigen itself. While we focused on one clone for further optimization, it will be also interesting to shuffle the $V_H$ gene of this clone O2AG2 with those of other higher-sensitivity clones such as O2BB6, O3BC6 and O3BE7 to get more sensitive clones.

Besides these possibilities, the mutation V37I at the $V_H/V_L$ interface was found to be the most effective mutation among the four found in O2AG2. In addition, most of the selected clones after saturation mutagenesis at the second screening possessed this mutation, which also indicates its importance. The residue is known as one of the invariant residues in $V_H$, and dominated by Val (9962/13 535) and Ile (3236/13 535) in mouse according to Abysis database (http://www.bioinf.org.uk/abysis/). The residue has been also recognized as a key residue buried in $V_H$-$V_L$ interface (Chothia et al., 1985; Chatellier et al., 1996), and found to be substituted by Phe in camelid $V_{HH}$, which does not associate with $V_L$ (Hamers-Casterman et al., 1993). In our case, the added methyl group at the side chain might have increased the hydrophobicity of the cavity between the domain interface, thus driven the $V_H$-$V_L$ association and also Fv-antigen association rates. It is worth noting that the V37I and E6Q mutations were both observed for the affinity-matured variants of anti-botulinum neurotoxin antibodies (Garcia-Rodriguez et al., 2007). Since the site is not in the vicinity of antigen-binding site, the V37I mutation can be a general way to increase the $V_H$-$V_L$ association, affinity to antigen, and also the sensitivity of OS-IA.

The utility of OS selection will be best demonstrated when an unconjugated hapten molecule is used as an antigen. In conjunction with appropriate negative selections, the OS selection will have a range of applications to improve the affinity and specificity of anti-hapten antibodies, and also the sensitivity and selectivity of corresponding OS-IAs.
**Supplementary data**

Supplementary data are available at *PEDS* online.

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**References**


