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Panning of a Phage VH Library Using Nitrocellulose Membranes: Application to Selection of a Human VH Library[1]

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We have established a method for selecting binding phages from a phage immunoglobulin heavy chain variable region (VH) library by panning with nitrocellulose membranes (membrane panning). To evaluate the concentrating ability of membrane panning for binding phages, a phage VH library containing clones that bind to hen egg white lysozyme (HEL) was used for panning against HEL. The efficiency of our method was as high as that of panning with magnetic beads. In addition, we performed membrane panning against target proteins and isolated the binding phages. The human VH genes of these phages were cloned and expressed as VH-bacterial alkaline phosphatase (PhoA) conjugates (VH-PhoA) in *Escherichia coli*. The dose-dependent binding of VH-PhoA to target proteins was confirmed by dot blotting. When applied to disease-associated antibodies, these methods will likely benefit clinical research. In addition, these techniques may be applicable to systematic analysis in proteome studies.

Key words: alkaline phosphatase, antibody, nitrocellulose membrane, phage display library, selection efficiency, variable region.

Phage display of antibody variable regions contitutes a powerful method for identifying the antibody fragments that bind to a target molecule (*1–4*). Immobilization of the target molecules on supportive materials is fundamentally important for the selection of phages. Traditionally, immunotubes have been used for the immobilization of target proteins. Recently, magnetic beads were introduced and offer some advantages (*5–7*). In the present study, we examined a new method for selecting phages involving nitrocellulose membranes as a support for the target proteins.

We first performed membrane panning against hen egg white lysozyme (HEL) using a phage library containing clones that bind to HEL (*8*), and confirmed the concentrating ability of this method by comparing it with that of panning with magnetic beads. Streptavidin magnetic beads (Promega, Madison, WI) were washed and blocked with SuperBlock (Pierce, CA) for 1 h. The phage heavy chain variable region (VH) library, which includes phages displaying the VH of the anti-HEL monoclonal antibody HyHEL10 (*8*), was blocked, and then mixed with biotinylated HEL (1 μg) and the light chain variable region (VL) of HyHEL10 (0.1 μg; *8*, *9*) for 1 h.

For control panning, the phage library solution including clones that bind to HEL was mixed with magnetic beads and incubated for 1 h. For nitrocellulose-based panning, a small amount of HEL (1 μg) was blotted directly onto a piece of nitrocellulose membrane (Amersham-Pharmacia, Tokyo) and air-dried; then the membrane was washed and blocked. The solution containing phages displaying the VH of HyHEL10 was blocked, mixed with the VL of HyHEL10 (0.1 μg) for 1 h, and then incubated with the membrane for 1 h. The phage solution was discarded, and the magnetic beads and the membrane were washed 5 times [ALF1]with phosphate-buffered saline (PBS) containing 0.5% Tween-20 and 5 times with PBS. Early log-phase *Escheirchia coli* JM109 cells were infected with the selected phages by adding the magnetic beads or the membrane to the cell suspension, which was then incubated at 37°C for 30 min and plated on LB plates containing 100 μg/ml ampicillin. The selected clones were amplified, and the binding ability of the phage library was assessed by ELISA as described previously (*8*).

Both methods markedly concentrated the binding phage (Fig. 1). The efficiency of the membrane panning was almost equal to that in the case of magnetic beads. In a comparative study on panning with polystyrene plates and magnetic beads, the magnetic beads more effective than were the polystyrene plates for isolating binding phages (*7*). In the present study, we used nitrocellulose membranes as the immobilization support and found that they could con-

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Abbreviations: VH, heavy chain variable region; VL, light chain variable region; HEL, hen egg white lysozyme; GST, glutathione *S*-transferase; PhoA, bacterial alkaline phosphatase; VH-PhoA, VH-bacterial alkaline phosphatase conjugates; cfu, colony-forming unit; PBS, phosphate-buffered saline; IPTG, isopropyl β-D-thiogalactopyranoside.

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centrate binding phages as effectively as magnetic beads.

By membrane panning, we isolated phages that bound to glutathione *S*-transferase (GST) using a human VH phage display library; we then expressed the selected clones in bacteria as VH-bacterial alkaline phosphatase (PhoA) conjugates (VH/GST-PhoA). We directly blotted 50 to 200 μg of GST onto pieces of nitrocellulose membrane and then air-dried them. To evaluate the nonspecific binding of phages to the membrane, non-GST-blotted membranes were prepared. All membranes were washed in PBS and then blocked with PBS containing 2% skim milk (MPBS). The selection of GST-binding phages from the phage VH library (2×10^9 cfu) was accomplished by means of 4 rounds of panning and GST-blotted membranes. After blocking, aliquots of the phage solution were incubated with the GST-blotted and non-GST-blotted membranes for 1 h. The phage solution was discarded, and the membranes were washed 10 to 20 times with PBS containing 0.5% Tween-20 followed by 10 to 20 times with PBS. Early log-phase *E. coli* JM109 cells were infected with the selected phages by adding each membrane to the cell solution, followed by incubation at 37°C for 30 min and then plating of the cells on LB plates containing 100 μg/ml ampicillin. The next day, the bacteria were recovered by scraping the plates, and the selected phages were amplified as described previously (*8*).

The binding of phages to unblotted membrane became negligible after 3 rounds of panning (Table 1). These findings suggest that the phages binding to target proteins were concentrated and that nonspecific binding decreased as panning progressed. After 4 rounds of panning, individual phages were tested by means of an ELISA in which the plate was coated with GST (50 μg/ml), and 12 of the 48 phages tested bound to GST. The gene encoding VH was amplified from GST-binding phages by means of *Taq* polymerase-based PCR amplification, and oligonucleotide primers 5′-ATGAAATACCTATTGCCTACG-3′ (sense primer) and 5′-CTAGTCTAGACTAGCTTGATATTCACAAACGAATGGAGA-3′ (antisense primer). The DNA sequences of the 12 selected clones were determined, and the CDR3 region of all clones had sequences corresponding to HGQDCSDASCSGWFDP (data not shown).

The PCR products were inserted into pGEM-T vectors (Promega, Tokyo), and then excised using *Sfi*I and *Xba*I. The resulting fragments were inserted into pEL-DBAP vectors (*10*) from which the D10 scFv fragment was removed by digestion with the same enzymes, yielding pEL-VH/GST-PhoA constructs. *E. coli* BL21(DE3)pLysS cells were transformed with the pEL-VH/GST-PhoA constructs, and the transformants were grown at 37°C in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. When each culture reached the early stationary phase, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a

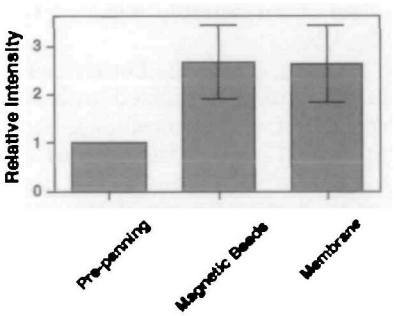

Fig. 1. **Comparison of panning with a nitrocellulose membrane and magnetic beads.** Both methods were performed with a phage library (2×10^9 cfu) containing clones (6×10^6 cfu) that bind to hen egg white lysozyme (HEL); the target protein used was HEL. The binding activity of each phage library before and after one round of selection (9×10^6 cfu) was evaluated by means of ELISA. Both methods markedly concentrated the binding phages compared to the results before panning, demonstrating that the efficiency of the membrane panning was almost equal to that in the case of magnetic beads. Each value is expressed as the ratio of the absorbance at 415 nm to that obtained prior to panning, and is the mean ± standard deviation for 3 separate experiments.

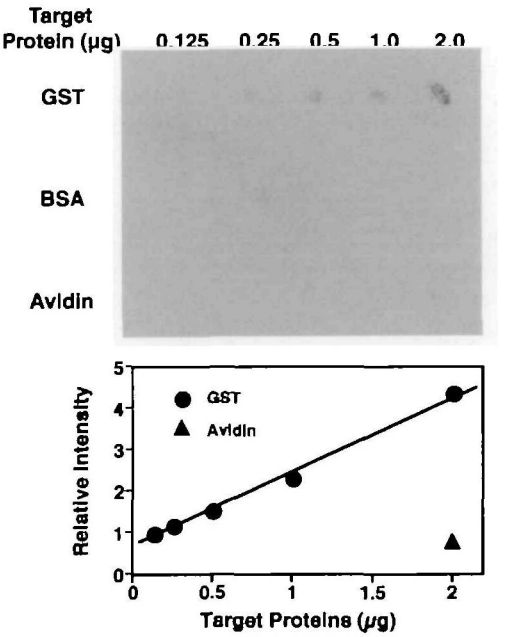

Fig. 2. **Dot blotting using VH-PhoA.** Top, the results of dot blotting analysis. Various amounts of glutathione *S*-transferase (GST), bovine serum albumin (BSA), and avidin were bound to a poly(vinyl difluoride) membrane, and then visualized using VH-bacterial alkaline phosphatase (PhoA) conjugates (VH/GST-PhoA) and a chemiluminescent substrate. Bottom, a plot of the relative intensity of each spot on the membrane. Each value is the ratio of each density to that of 0.125 μg GST. The results demonstrate that the chemiluminescence intensity depends on the amount of GST, while no signal was observed for BSA and only a slight one for avidin. Symbols used: Solid circles, GST; solid triangle, avidin.

TABLE I. **Membrane panning against GST.**

	Rounds of panning			
	1	2	3	4
Input (cfu)	1.8×10^9	8.0×10^8	1.0×10^9	5.0×10^8
Output (cfu)				
Blotted membrane	1.3×10^5	1.2×10^5	1.2×10^4	1.5×10^5
Non-blotted membrane	1.1×10^5	1.3×10^4	<500	—

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final concentration of 1 mM and then incubation was performed for an additional 9 h. The cells were pelleted, resuspended in PBS containing 1% Triton X-100, and then sonicated with a Branson sonifier. The cell lysate was centrifuged at 10,000 ×*g* for 10 min, and the supernatant was collected as the VH/GST-PhoA solution.

The binding of the VH/GST-PhoA to GST was analyzed by dot blotting. Various amounts of GST were blotted onto a poly(vinyl difluoride) (PVDF) membrane (Amersham-Pharmacia, Tokyo) according to the manufacturer's recommendations. The membrane was washed once in PBS, blocked with MPBS, and then incubated with VH/GST-PhoA overnight at 4°C. After being washed with PBS containing 0.1% Tween-20, the blots were developed using a chemiluminescent substrate and then analyzed with an LAS-1000 luminescent image analyzer. The VH/GST-PhoA detected the GST on the membrane, and the chemiluminescence intensity depended upon the amount of GST (Fig. 2). The addition of soluble GST to the VH/GST-PhoA clearly decreased the intensity in a dose-dependent manner (data not shown), indicating the specific binding of the clone to GST. The VH/GST-PhoA exhibited no cross-reactivity with bovine serum albumin, although slight cross-reactivity with avidin was observed. Functional improvements of the VH/GST-PhoA by means of site-directed mutagenesis are now in progress.

Membrane-panning methods have many practical and theoretical advantages for the panning of phage libraries. First, membranes have large capacities for binding proteins. Typically, the binding capacity of a nitrocellulose membrane for proteins is 100 to 120 μg/cm^2. Second, membranes bind target proteins easily and quickly without any loss; therefore the amount of target proteins required is minimized. In contrast, for panning involving immunotubes, the binding of target proteins to the tube relies on passive adsorption, and the efficiency depends on the buffer, pH conditions, and incubation time. Therefore, much time, labor, and reagents are consumed to determine the optimal conditions for the immunotube system. Third, modifications of target proteins including biotinylation are generally not necessary for membrane panning, and the target proteins are bound to membranes in their native conditions. Fourth, the handling of membranes, including their washing and transfer, is straightforward. Fifth, the volume and concentration of the phage library solution are controllable. Increasing the concentration of phages in the solution can cause an increase in the nonspecific binding due to phage aggregation (*11*). In contrast, our method accommodates a low-concentration phage solution; therefore, nonspecific binding can be decreased. Sixth, using control membranes such as nonblotted ones we can monitor the progression of membrane panning. The decrease in phage binding to control membranes mirrors the progress of the panning. Seventh, our membrane-panning method is very economical because it requires only small pieces of membrane.

In the present study, we used a VH phage display library derived from a human gene and succeeded in isolating binding phages. In a previous study (*12*), the investigators introduced artificial mutations into a mouse VH, isolated the binding VH phages, and made stable and specific VH proteins without any VL interface. Our results further indicate that the selection of functional VH from a natural human VH phage library is promising. In addition, gene deletions were limited in our library; therefore, the phage library displaying the human VH was very stable. It should be noted that high-affinity antibodies cannot be obtained without somatic changes of the gene *in vivo*; thus, site-specific and/or random mutagenesis of the selected gene would be required for practical use (*13*).

We demonstrated that our novel method involving nitrocellulose membranes is quite effective and useful for panning. The phage-displayed and selected single-chain Fv specific for CD30 has been detected by using nitrocellulose filters, and the possibility of detecting the target antigen of a preselected scFv displayed on a filamentous phage in λ phage cDNA expression libraries has been demonstrated (*14*). Our results clearly show that the selection of target-specific clones (*i.e.*, panning) can be performed using a membrane. Further, we also used target proteins other than GST for membrane panning, and clones that bound the targets were obtained from the phage library displaying human VH (data not shown). We are now performing research and developing clinical applications of our membrane-panning technique, including the selection of autoantibodies and tumor-targeting antibodies. In addition, cell-surface membrane proteins would be appropriate targets for membrane panning. Finally, this method would benefit proteome research in that human VH fragments specific for novel proteins on membranes could be obtained and used for systematic analyses.

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