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Isolation of Human Antibodies Against Hepatitis E Virus From Phage Display Library by Immobilized Metal Affinity Chromatography[1]

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Objective To isolate human antibodies against hepatitis E virus from phage display library by a new method of panning phage antibody library based on immobilized metal affinity chromatography (IMAC). **Methods** Phage antibody library was allowed to mix with hex-His tagged expressed HEV specific antigen, NE2, in solution for adequate binding before affinity resin for hex-His was added. The non-specific phage antibodies were removed by extensive washing and the specific bound phage antibodies could then be eluted to infect TG1 or repeat the binding process for subsequent rounds of purification. The specificity of the selected human antibodies were tested by antigen competitive ELISA, human sera blocking ELISA, scFv expression, and sequence analysis. **Results** His-NE2 specific recombinant phages were successfully enriched after panning procedure. Two individual phage clones, 126 and 138, showed 50% inhibition in NE2 antigen competition ELISA and obvious blocking effect by HEV positive serum in blocking ELISA. Soluble scFv of 126, 138 bound to NE2 specifically. **Conclusion** Two specific human phage antibodies against hepatitis E virus (HEV) from phage display library were isolated by immobilized metal affinity chromatography. The immobilized metal affinity chromatography applied to phage antibody selection was a helpful supplement to the selection in solution.

Key words: HEV antibodies; Phage antibody library; Affinity chromatography; Panning

INTRODUCTION

In the last decade, an increasing number of antibodies have made their way from research bench tops into clinical practice, and many more are currently under clinical trial. Among monoclonal antibody-producing techniques, phage-display is undoubtedly the most effective and versatile. It has allowed the isolation of numerous proteins and peptides of therapeutic value, and provides access to a vast untapped pool of human monoclonal antibodies with anti-tumor and antiviral activities. It is making a significant difference for patients with cancer, autoimmune diseases and infectious diseases.

Theoretically, human antibodies binding to any target antigen of interest can be selected from a large naive phage display library. Currently, many selection methods have been developed, including pure or impure antigen selection, functional selection and 'selectively infective phage' (SIP) method[1]. The impure antigen selection can be performed on cell surface[2], tissue sections[3], and in living animals[4]. In addition, biological effects derived from the reaction of antigens and antibodies are introduced into selection, such as antibody enzyme[5], fluorescence-activated cell sorting (FACS)[6] and magnetic-activated cell sorting (MACS)[7]. Generally, the antibodies binding to the target antigen could be selected after 2-4 rounds of selection.

There are two traditional selection methods with purified antigen as target. One is to bio-panning on immobilized antigen coated on solid supports, such as enzyme-linked immunosorbent assay (ELISA) plates[8], immunotubes[9], BIAcore sensorchips[10] or columns[11]. After incubating with antigen, the unbinding phage antibodies are washed away from the bound phage antibodies and ready to be eluted for the next round purification. It should be taken into consideration that antigen immobilization may cause conformation change or loss of activity. Some phage

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antibodies selected against an adsorbed antigen may not be capable of recognizing the native form of the antigen or lose the high affinity ones[12]. Another method is to select with biotinylated antigen which reacts with phage antibody library in the solution. The complexes of antigen and phage antibody are captured by streptavidin-coated magnetic beads. The specific phage antibodies are selected subsequently[13]. This technique allows solution binding of antigens and antibodies. However, antigen biotinylation is likely to disrupt the protein structure or inactivate the binding site(s).

In this paper, we introduce an immobilized metal affinity chromatography (IMAC) method for selecting phage antibodies. This is an improvement by taking advantage of keeping target antigen in solution to preserve its native conformation while unbound antibodies can easily be washed away. In the present study, recombinant peptide NE2 of HEV[14] open reading frame 2 (ORF 2) was tagged with 6×His and used as target antigen. Specific human antibodies against the antigen were selected from a naïve phage antibody library by the affinity chromatography method.

MATERIALS AND METHODS

Enzymes and Chemicals

The Recombinant Phage Antibody System (RPAS) was purchased from Pharmacia (Piscataway, USA). The restriction enzyme was purchased from TaKaRa (Dalian, China). BD TALON™ metal affinity chromatography resin was purchased from BD (Franklin Lakes, USA). The primers synthesis and sequence analysis were performed in Bioasia (Shanghai, China). The neutralizing mouse anti-HEV monoclonal antibody (McAb), designated as 8C11, was obtained and characterized in our laboratory. The antigen protein of NE2, HIV, HBcAg, and HCV were expressed and purified in our laboratory.

Construction and Purification of His-NE2

His-NE2 expression and analysis The pHis-NE2 plasmid was constructed according to the standard molecular biological procedure[15]. The NE2 fragment was digested with NdeI and *Eco*RI, and ligated with the vector pTO-T7[16] treated with the same restriction enzyme.

The pHis-NE2 was transformed into *E.coli* ER2566. An overnight culture of the transformant was transferred to 500 mL LB medium with 0.1 mg/mL kanamycin and grew at 37℃ until it reached an optical density of 0.8 at 600 nm. IPTG was added to a final concentration of 0.2 mmol/L, and the cultures were further incubated at 37℃ for 4 h. Bacterial cells harvested from the culture were lysed by sonication. It was found that all the recombinant proteins formed inclusion bodies in host cells. The inclusion bodies were treated with 2% Triton X-100 at 37℃ for 30 min and then dissolved in 4 mol/L urea. The proteins were renatured by dialysis against phosphate-buffered saline, pH 7.45, at room temperature. The fusion protein had a final purity of 95% and concentration of 1.7 mg/mL. The result of Western blot showed that the His-NE2 maintained the binding specificity and could be used for screening.

Determination of Imidazole Concentrations for Washing and Eluting

700 μL of fusion protein His-NE2 (0.6 mg/mL) were mixed with 1 mL of affinity resin, and shook at room temperature for 1 h. The protein was eluted by increasing concentration of imidazole at 5 mmol/L, 10mmol/L, 20 mmol/L, 40 mmol/L, 60 mmol/L, 80 mmol/L, 100 mmol/L, and 150 mmol/L. The eluant was subjected to SDS-PAGE.

Affinity Selection

A M13 phage-displayed human library composed of 1.2×10^8 different clones was used in selection experiments. 2 mL of phage library with approximate $2.0\text{-}3.5\times10^{10}$ cfu were blocked by incubation in 4% skim milk in PBS (MPBS) at room temperature for 30 min. The His-NE2 was diluted in PBS to 0.3 mg/mL, then the 10^{10} phage particles in 2 mL of 4% MPBS were added and allowed to bind to His-NE2 at room temperature for 2 h (for 1.5 h in the second and 1 h in the third round). Then the affinity resin was added to bind His-NE2-phage complex for 1 h by gently rotating continuously. All mixtures were added to a column. For the first round of selection, the column was washed 5 times with PBS containing 0.5% Tween-20 (PBST), 5 times with PBS and once with 5 mmol/L imidazole. The column was washed 10 times with PBST, 5 times with PBS and twice with 5 mmol/L imidazole in the second round, and 10 times with PBST, 10 times with PBS and three times with 5 mmol/L imidazole in the third round. The bound His-NE2-phage complex was then eluted from the resin by washing three times with total 2 mL of 150 mmol/L imidazole. The eluted phages then infected with *E coli.*TG1 and were incubated with shaking at 37℃ for 1 h. 10 μL was applied to titration and the rest were harvested by centrifugation, plated on 50 mL 2×YT-AG (0.1 mg/mL ampicillin，2% glucose) and incubated overnight at 30℃. The

colonies were then scraped and transferred to 2× YT-AG liquid medium with shaking at 37℃ to OD_{600} 0.4. Then 1×10^{12} pfu of M13KO7 was added and incubated at 37℃ for 1h. The infected cells were centrifuged and resuspended in 2×YT-AK (0.1 mg/mL ampicillin, 0.1 mg/mL kanamycin) with shaking overnight at 25℃. The amplified phages were purified by precipitating in 2.5 mol/L NaCl/20% PEG-8000. Serial dilutions were plated after the third round to obtain single colonies.

Phage Antibody ELISA

Indirect ELISA For polyclonal phage ELISA, rescued (recovered) phage particles obtained after each round of panning were tested on microtiterplate coated with recombinant NE2 protein to determine whether enrichment had taken place with each successive selection step. For monoclonal phage ELISA, single colonies were picked into 400 μL per well of 2×YT-AG in a deep 96-well plate and incubated at 37℃ for 5 h. The M13K07 was added. The infected cells were centrifuged and resuspended in 2×YT-AK with shaking overnight at 25℃. The amplified phage antibodies were separated from host cells by centrifugation and the resulting phage supernatants, blocked by MPBS, were tested by ELISA plates coated by NE2, or unrelated protein (HCV antigen) or no antigen coating as control. Antibodies that recognized NE2 with an ELISA signal at least threefold higher than that observed on the control antigens were deemed to be specific for NE2, with McAb 8C11 as positive control and McAb MA18/7 as negative control.

Phage antibody specificity test by antigen competition ELISA Bacterial supernatant containing phage antibodies was diluted with an equal volume of blocking buffer containing competitor NE2 antigen (10 μg/mL). After allowing to bind to the competitor for 0.5 h at room temperature, the mixed solution was added to the microplate coated with NE2 and tested by the usual ELISA procedure. Colonies that produced phage antibody showing a 50% reduction of ELISA reading in the antigen competition assay were considered as specific.

Antibody blocking ELISA HEV positive serum was diluted 1:100 in MPBS and added to NE2-coated microtiter plate (100 μL/well) and incubated at 37℃ for 30 min. The serum was removed and discarded; and then the phage antibody was added to be tested by the standard ELISA procedure.

Detection of Soluble Single Chain Fv (scFv)

The plasmid of selected clone was transformed into *E. coli* HB2151, which was incubated on 2× YT-A plate overnight. The colony was transferred to 2×YT-A liquid medium with shaking at 37℃ to OD 1.0. Then the culture was induced with IPTG at a final concentration of 1 mmol/L. The soluble scFv was expressed after incubation for 10-12 h at 25℃.

ELISA Crude bacterial pellets from 4 mL culture of soluble scFv-produing HB2151 clones were resuspended in PBS and extracted by three cycles of freezing and thawing. They were then centrifuged to eliminate bacterial debris and applied directly to ELISA testing.

Western blot The bacterium expressed scFv were harvested and made protein samples which were run on SDS-PAGE (10%) and electrobolted on nitrocellulose membranes. The testing was performed by standard technology with HRP/Anti-E tag conjugate.

RESULTS

Imidazole Concentrations for Washing and Eluting

Lower concentration of imidazole will eliminate the unspecific bound phage antibodies, and higher concentration of imidazole will elute the captured antigen from resin. To determine the appropriate concentration for washing and eluting, His-NE2 captured by affinity resin was eluted with imidazole in an increasing concentration. Different eluents were collected and underwent SDS-PAGE. The results showed that the His-NE2 began to be eluted when the imidazole concentration rose to 20 mmol/L. 5 mmol/L of imidazole was used to wash unspecific phage antibodies, and a higher concentration of 150 mmol/L was chosen to elute His-NE2 sufficiently.

Determination of Washing Condition During Panning

Washing condition is very important during panning. The specific His-NE2-phage scFv complexes were captured by affinity resin and unbound phage antibodies were then removed in washing steps. We integrated the washing in phage library panning with that in IMAC. PBST, PBS and imidazole solution were used for washing during each round. To analyze the effect of washing intensity, the cfus of eluent were tested (Fig. 1).

The results showed that many unspecific phage anbodies were removed with different washing solution, and with increasing times of the same washing solution, and the cfu decreased. The fact that the cfu increased with the changing washing solution suggested different effects of different washing solutions. The washing conditions were confirmed as follows: washing 5 times with PBST and PBS respectively (10 times with PBST, 5 times with PBS for

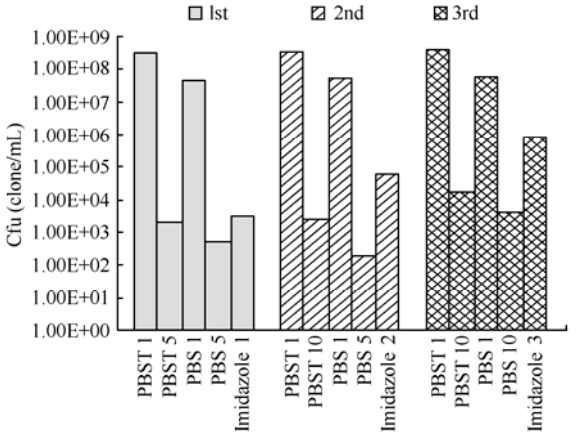

FIG. 1. The cfu under different washing conditions. PBST 1: washed once by PBST; PBST 5 or 10: washed 5 or 10 times by PBST; PBS 1: washed once by PBS; PBS 5 or 10: washed 5 or 10 times by PBS; Imidazole 1, 2, or 3: washed one, two, or 3 times by imidazole.

the second round; 10 times with PBST, 10 times with PBS for the third round), then washing once with 5 mol/L imidazole for the first round (twice for the second round, three times for the third round).

Affinity Selection

The naive human scFv display library was directed for the selection against recombinant His-NE2 protein by panning. Total 10^{10} recombinant phages were incubated with His-NE2 in the solution, and bound phages were eluted by trypsin treatment. The eluted phages were amplified by infecting freshly grown TG1 cells with M13 phage super-infection, and used in the subsequent panning. Panning was repeated three times. Titer of each input and output phage during panning was determined by colony forming unit (CFU), and it was found that the yield of antigen-bound phages was increased about 100-fold after the third round of panning, indicating the enrichment of recombinant phages through panning (Table 1).

TABLE 1

Enrichment of Phage Antibodies by Affinity Chromatograph Panning With His-NE2

Round of Panning	Input Phage (cfu)	Output Phage (cfu)	Output/Input (%)
1st	3.5×10^{10}	5×10^{4}	1.4×10^{-4}
2nd	2.0×10^{10}	6.0×10^{5}	3.0×10^{-3}
3rd	3.0×10^{10}	8.0×10^{6}	2.7×10^{-2}

Note. Output/Input(%)＝amount of output phage×100/amount of input phage.

In order to confirm the successful enrichment of His-NE2 specific recombinant phages after panning, polyclonal phage ELISA was performed using amplified phages from each eluate of the first, the second and the third round of panning (Fig. 2). The phage ELISA showed a substantial increase of His-NE2 binding phages.

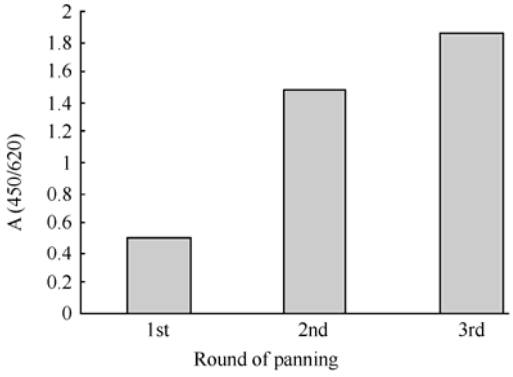

FIG. 2. Enrichment of antigen specific antibodies by affinity chromatography. After each round of panning by affinity chromatography, 100 μL polyclonal phage antibodies mixture was tested by the standard HEV ELISA. The end-point titers are shown as isolation of specific binding phage antibodies.

After three rounds of panning, 200 of individual phage clones were detected for specific binding by phage ELISA (Fig. 3). Ten different clones specifically recognized NE2 were selected for competitive ELISA and serum blocking ELISA. Among them, 126 and 138, showed 50% inhibition in NE2 antigen competition ELISA and obvious blocking effect by HEV positive serum in blocking ELISA (Fig. 4).

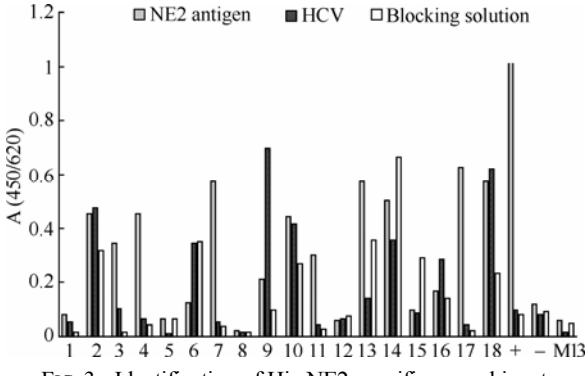

FIG. 3. Identification of His-NE2-specific recombinant phage clones by ELISA. Microtiter plates coated with either HEV or HCV antigen or blank with over-coating solution only were charged with 100 μL supernatants from phage antibody producing monoclones, and then standard ELISA was performed. +: Positive control; -: Negative control; M13: Wild-type phage.

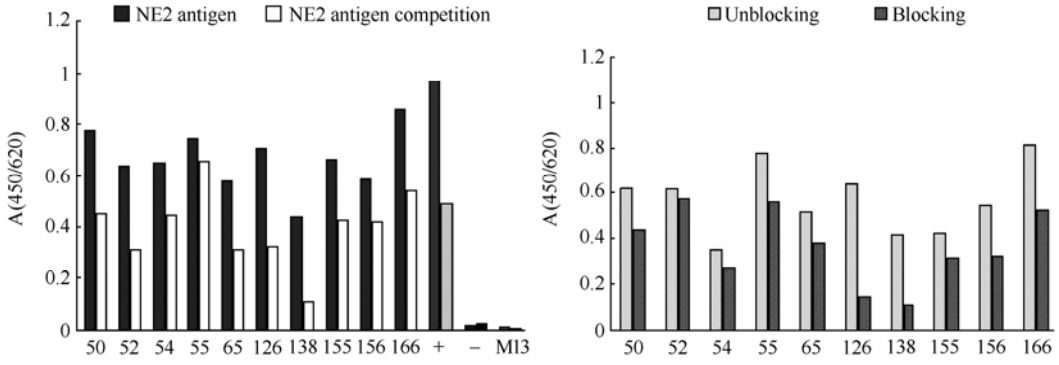

FIG. 4. Specificity test for selected monoclonal phage antibodies by competitive and blocking ELISA. A. Competitive ELISA of phage antibodies by NE2 antigen; supernatant of monoclonal phage was incubated with 10 μg of NE2 antigen before standard ELISA. B. Blocking monoclonal phage antibodies by anti-HEV positive serum. +: Positive control; -: Negative control; M13: Wild-type phage.

Detection of Binding Specificity of Soluble scFv by ELISA and Western Bloting

Crude bacterial pellets from 4 mL culture of soluble scFv-produing HB2151 clones of 126 and 138 were frozen and thawed to apply to ELISA. Figure 5 shows that the soluble scFv of 126, and 138 bound to NE2 specifically and did not cross-react with the unrelated protein. Total protein of scFv-produing HB2151 bacteria was tested by SDS-PAGE (Fig. 6A). The result of Western blotting (Fig. 6B) confirmed the expression of scFv with the clear band at approximate 35kD.

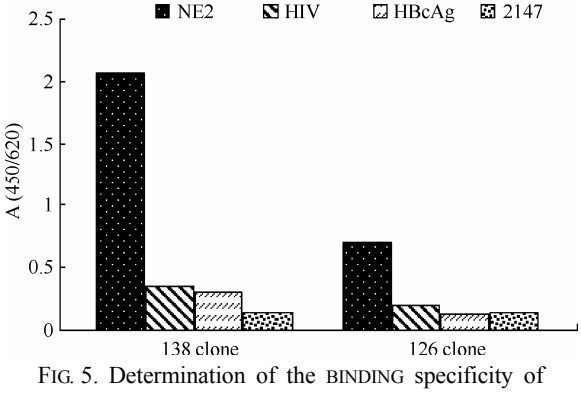

FIG. 5. Determination of the BINDING specificity of soluble scFv antibodies by ELISA. One hundred μL of supernatants from 4 ml of soluble scFv producing bacterial culture were tested by standard ELISA with microtiterplates coated with different antigens.

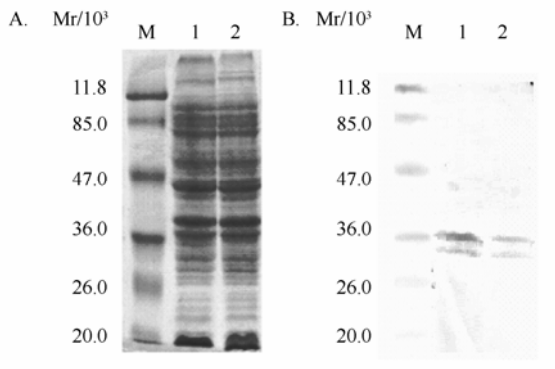

FIG. 6. Western blot analysis of expressed clone 138 and clone 126 scFv. A. Coomassie blue staining of SDS-PAGE (12%). B. HRP-Western blot detecting antibody was HRP/anti-E Tag at 1:5000 dilution. M: Protein marker. 1: 138 clone. 2: 126 clone.

Sequences of Specific scFvs

The two specific clones were sequenced and applied to IMGT/V-base database to analyze the variable region genes. The data in Table 2 verified that the sequences were human IgG, with different CDR region of VH and VL, belonging to different antibody family.

TABLE 2

Classification of the Selected Antibody Clones According to Their Amino Acid Sequences

Clone	CDR1	CDR2	CDR3	Closest V Family
126VH	......NGSLSGYY.....	NHSGGA.....	ARGQHGSGSYVGGFYYYDC.....	IGHV4
126VL	......QDIDDD.....	QEATT.....	LQHDNFPYTF.....	IGKV5
138VH	......GDSVSSTFAA.....	TYYSHWNY.....	ARGWEGNIWYTEAYDT.....	IGHV6
138VL	......QSVSSY.....	YDASN.....	QQRSNWP.....	IGKV2

Note. Sequences were compared with the human Ig set from the IMGT/V-base database.

DISCUSSION

Whether the antigen in solid phase can maintain its native conformation is a common problem in the selection of phage antibody by immobilized antigen. Some phage antibodies selected against an adsorbed antigen may not be able to recognize the native form of the antigen. This drawback can be somewhat eliminated by selection in solution. Most selection in solution adopts biotinylated antigen, which is most suitable for selection of antibodies for polypeptide which is not prone to immobilize on solid phase. Biolatinylation is carried out by combining biotin and amino acid side chain of antigen of interset, which may lead to the inactivation of binding site of antigen and antibody, or destruction of protein structure. Streptavidin in solid is also a likely source of the unspecific phage in the selection[17].

Immobilized metal affinity chromatography (IMAC) has been developed for purification of protein in recent years. Protein is tagged with polypeptide, e.g. 6×histidine (6×His), glutathione S-transferase (GST), and purified *via* this tag. In recent researches, affinity chromatography is widely used as an important method in downstream bioengineering technology and is usually applied to the purification of protein[18-19].

In this study, we introduced affinity chromatography to the phage library selection. To improve its efficiency, we examined the cfu under different washing intensity. The experiment demonstrated that increasing of washing times by the same solution was not enough for the elimination of unspecific antibodies, and different washing solution had different effects.

Compared with the construction of biotinylated protein, the novel selection method just generated a protein with an affinity tag of 6 amino acid. The procedure was simple and had little influence on the protein conformation. The fused protein with a small tag for purification conserved not only the binding activity of antigen and antibody, but also the affinity with resin. The selection completed in solution avoided the influence on the epitope of antigen fasten on solid phase, and was in favor of the sufficient reaction of antigen and antibody.

Furthermore, in conventional ways, the bound phage antibodies could be eluted by acid solution, or infected TG1 directly. In the way of biotinylation, the bound phage antibodies could be cleaved by proteinase. The former could not separate the antigen specific antibodies from those unspecifically bound to other members, while the latter was complicated because of the necessity of digestion by proteinase. Overall, the method with high concentration of imidazole to elute the antigen-antibody complex from resin in our research was simple and specific, and possessed both advantages of selection in solid phase and in solution. The new method was applied to select HEV NE2 specific phage antibody. The results of antigen competitive ELISA, sera-blocking ELISA, scFv expression and sequence analysis indicated that two specific human phage antibodies were generated.

To fully exploit a large naïve human phage antibody library, many different selection strategies should be employed for different antigens. Different method might result in quite different outcomes[20]. The affinity chromatography applied to phage antibody selection might be a helpful supplement to the selection in solution.

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