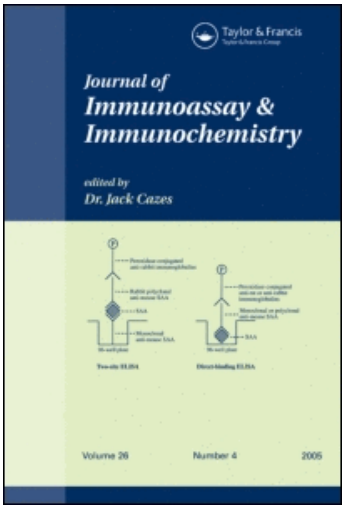

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Screening of Carbohydrate-Specific Phage Antibodies Against Recombinant Human Erythropoietin (rHuEPO) using a Phage Display Antibody Library: Preliminary Study

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Abstract: This paper is a preliminary report on development of a screening method for carbohydrate-specific phage antibodies against recombinant human erythropoietin (rHuEPO), using a phage display antibody library. rHuEPO was oxidized with sodium periodate or treated with 1,4-dithiothreitol and guanidine hydrochloride for detecting the specificity of obtained phage antibodies. Of 100 phage clones, three initially showed higher carbohydrate-related specificity. One of them (No. 62) bound specifically to the carbohydrate chains of rHuEPO, while the other two (Nos. 63 and 83) might recognize the steric conformation related to both the carbohydrate and the polypeptide chain of rHuEPO. These phage antibodies may serve as useful capture ligands for future development of efficient analytical methods for rHuEPO.

Keywords: Recombinant human erythropoietin (rHuEPO), Carbohydrate-specific, Phage display, Subtraction panning

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INTRODUCTION

As an endogenous growth factor, the glycoprotein erythropoietin (EPO) plays an important role in regulating the continuous formation of new red blood cells.[1] Recombinant human erythropoietin (rHuEPO) was introduced about twenty years ago by cloning the EPO gene into Chinese hamster ovarian (CHO) cells.[2,3] Soon, it was widely used in clinical treatment of anemia, especially anemia caused by chronic renal failure,[4–6] because of its similar biological activity as native EPO.[7] However, rHuEPO was also abused by endurance athletes as an illegal doping agent to improve the oxygen-transport capacity of the blood.[8,9]

EPO consists of a 165-amino acid polypeptide chain and four carbohydrate chains (3 N-linked chains and 1 O-linked chain). The sugar chains contribute to about 40% of the total molecular weight of EPO and act as the essential domain for keeping the active conformation[10] and in vivo biological activity of EPO.[1,10,11] rHuEPO has the same amino acid sequence as EPO, but its carbohydrate content and structures are different, since there are no proper sugar-transferring enzymes in these CHO cells.[11] These minor differences offer a slight chance for the analyst to distinguish rHuEPO from native EPO in clinical diagnosis[12] and detection of mis-usage of rHuEPO in sports. According to these differences, Lasne et al.[13–15] developed a test based on isoelectric focusing (IEF), followed by immunochemical detection of the resolved isoforms with a double blotting technique, which was accepted as the urine EPO test by International Olympic Committee (IOC) and World Anti-Doping Agency (WADA). The method can successfully distinguish the main isoforms of rHuEPO and native EPO, based on the net electric charge; however, it is expensive, time-consuming, and requires trained technicians and a well-equipped laboratory and can, thus, only be performed in a few specialized laboratories. Furthermore, IEF couldn't distinguish the isoforms of EPO which resulted from the microheterogeneity of EPO carbohydrate structures, including monosaccharide compositions, sequences, branching sites, and linkages.[12,16] Information concerning these isoforms is also an important dissimilarity between native EPO and rHuEPO. So, some improvements of IEF were suggested by WADA.[17] One of these improvements involved development of a more selective preconcentration step, which was essential to achieve the sensitivity of the current method and avoid the interference of many unrelated proteins in samples. Another was to use antibodies with specificity and capacity to distinguish rHuEPO and EPO, which can also recognize the various forms of EPO. Both of the improvements required appropriate reagents (antibodies or other materials with similar function). Recently, lectins were used to purify EPO and recognize isoforms after their separation by IEF, since they can combine to the glycosylation profiles of glycoproteins. But, they didn't possess specificity[18] as high as an antibody towards its antigen, so that other coexisting sugar groups might also be recognized by the lectins and interfere with the subsequent

detection. Therefore, it is necessary to produce antibodies directed at the carbohydrate structures and steric conformation of rHuEPO to discriminate rHuEPO from endogenous EPO and other glycoproteins in body fluids. It would be helpful to diagnose the diseases related to the changes of the glycosylation pattern of EPO.

In general, immunization with carbohydrates leads only to a primary IgM response and, in some instances, no response at all, because most of the carbohydrates are self-antigens.[19] These facts indicated that the protein moiety of the glycoproteins is more efficient than the carbohydrate chains in stimulating an immune response. This explains why a number of antibodies[20–25] against EPO (or rHuEPO), obtained via conventional routes such as hybridoma cell lines or polyclonal sera were against the peptide chains. Until now, the only reported antibodies that show specificity to the carbohydrate chains of rHuEPO was reported by Pazur,[26] who used an agar diffusion method to prove that two sets of antibodies against carbohydrates of rHuEPO existed in the polyclonal sera. However, no further isolation or demonstration of results were proposed.

The advances in the field of recombinant antibody technology made it possible to produce large amounts of antibodies with desirable affinity and specificity by screening of human synthetic phage display libraries. The aim of this study is to screen phage antibodies (single chain antibody fragment of variable region, scFv) against the carbohydrates or the conformation related to carbohydrates of rHuEPO utilizing the technique of phage display. Three phage clones were isolated by performing a subtraction strategy of panning. Preliminary results of the binding specificity tests showed that the selected phage clones Nos. 63 and 83 tended to recognize the carbohydrate-related conformation of rHuEPO, while No. 62 had the specificity towards the carbohydrates of rHuEPO.

EXPERIMENTAL

Materials

Recombinant human erythropoietin (rHuEPO) was purchased from Beijing Bio-EPO Biotechnology Co. Ltd. Each sample vial contained about 1.5 mg rHuEPO. The phage display antibody library (Human synthetic VH+VL scFv Library), *Escherichia Coli* (*E.coli*) DH5α F′ and M13-K07 helper phage were kind gifts of the Life Centre of the College of the Life Sciences (Peking University). The antibody library was created in pDAN5 and the diversity was estimated to be 3×10^{11}.[27] Tryptone and yeast extract were obtained from Oxoid Ltd (England). Horseradish peroxidase (HRP) labeled anti-M13 phage antibody was purchased from Amersham Biosciences (Piscatsway, NJ, USA). 3,3′,5,5′-tetramethylbenzidine (TMB), sodium meta periodate ($NaIO_4$), 1,4-dithiothreitol (DTT), guanidine HCl (Gu-HCl), ampicillin, and kanamycin were all obtained from Sigma (St. Louis, MO, U.S.A.).

Super-clean bench (Beijing Changping Great Wall Equipment & Engineering Company for Air Purification Manager), water interlining thermostatic culturing cabinet (PYX-DHS, Shanghai Yuejing Medical Instrument Works), water-bath thermostatic vibrator (DSHZ-300, Jiangshu Taichang laboratory instrument works), thermostatic bath (Beijing medical instrument works) and Beckman Coulter Avanti J-25 centrifuge (Beckman Coulter, Fullerton, CA USA) were used in panning, enrichment, and infection of the anti-rHuEPO phage particles. All the data of ELISA were recorded with a Tecan Genios Microplate Reader (Austria).

Modification of rHuEPO

Sodium periodate was used to oxidize the carbohydrate of rHuEPO and keep the polypeptide chain intact. rHuEPO, 50 μg, was added to 50 μL of 0.05 mol/L $NaIO_4$ at pH 6.4. The reaction was allowed to proceed for 8 h at 25°C in darkness. The product was stored at 4°C for a short time or used immediately for the following experiments.

The reduction of rHuEPO was achieved by using DTT and Gu-HCl. 0.75 mg of rHuEPO and 0.53 g of Gu-HCl were mixed in 1 mL of 0.05 mol/L, pH 8.5, Tris containing 0.04 mol/L DTT. After reaction for1 h at 25°C, the mixture was stored at 4°C or used immediately for the following experiments.

Positive Panning for Sub-Library EPO3′

First, a sub-library EPO3′ was established by three rounds of positive panning against the intact rHuEPO molecules using the original human synthetic phage display antibody library. In each round, 30 μg of intact rHuEPO was coated in the immunotube and the phage library (original or obtained from the previous round) was added. After combination, the supernatant was discarded and the phage antibodies bound to rHuEPO were eluted by 3 mL of a solution of fresh *E.coli* DH5α F′. The obtained phages were amplified and purified for the next round of panning.

Subtraction Panning for Carbohydrate-Specific Phage Antibodies

Based on the above sub-library EPO3′, a subtraction panning strategy was employed to produce antibodies with specificity for the carbohydrates of rHuEPO. Figure 1 shows the whole scheme of the subtraction panning used in this study. At the beginning of the procedure, the immunotube was coated with 2.5 mL of 12 μg/mL $NaIO_4$-oxidized rHuEPO in 0.1 mol/L bicarbonate buffer, pH 9.6, at 4°C, overnight. Then, 0.01 mol/L phosphate buffered

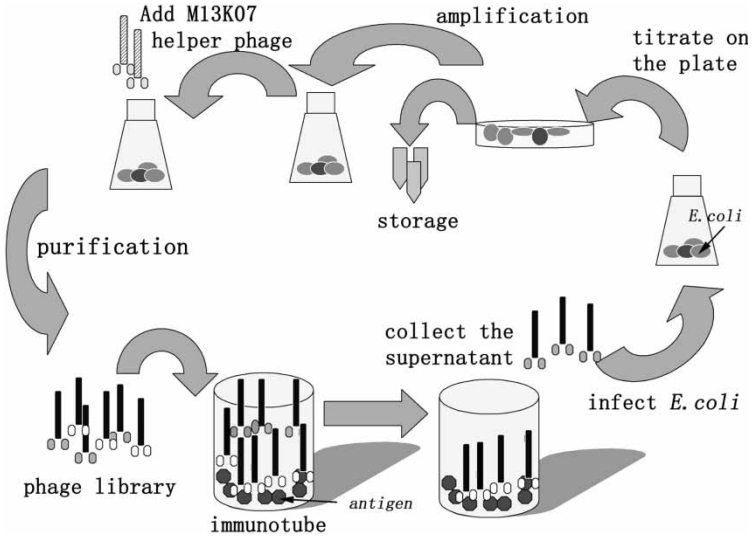

Figure 1. Flow chart of subtraction panning.

saline (PBS) containing 2% (w/v) gelatine was used to block the possibly remaining non-specific adsorption sites in the immunotube for 1 h at 37°C. After 3 cycles washing of the immunotube with PBST (PBS containing 0.05% (v/v) Tween 20) and PBS, sequentially, 20 μL of EPO3′ containing 10^6 colony forming units (CFU), diluted with 2.5 mL of PBS, was added to the tube and allowed to incubate for 2 h at 37°C. The phages collected from the supernatant were incubated with *E.coli* DH5α F' for 1 h at 37°C. Then, the infected bacteria were centrifuged at 4,000 rpm for 10 min at room temperature and the supernatant was discarded. The cell pellets were resuspended in 300 μL of 2 × TY medium and later titrated on 2 × TYAG plate (2 × TY medium containing 100 μg/mL ampicillin and 2% glucose) and allowed to grow overnight at 30°C. The next day, 4 mL of 2 × TYAG medium was used to wash the plate and the colonies were innoculated into 20 mL of 2 × TYAG medium, followed by incubation at 37°C with shaking at 250 rpm until the $A_{600\,nm}$ reached 0.4–0.8. Helper phage, M13-K07, 100 μL, was then added to the culture at a multiplicity of infection (MOI) of 20. After incubation at 37°C for 1 h with shaking at 100 rpm, the cells were pelleted by centrifugation at 5,000 rpm for 10 min at room temperature and then were resuspended in 20 mL of TYAK medium (2 × TY medium containing 100 μg/mL ampicillin and 50 μg/mL kanamycin). The culture obtained above was incubated at 30°C with shaking at 250 rpm overnight. After centrifugation at 5,000 rpm for 20 min at room temperature, the supernatant was collected and mixed with 4 mL of PEG/NaCl (20% (w/v) PEG8000 solution

containing 2.5 mol/L NaCl). After this step, the phage was allowed to gently precipitate for 1 h on an ice bath and pelleted by centrifugation at 12,000 rpm for 20 min at 4°C. In the final step, the obtained precipitate was resuspended in 500 μL of PBS, followed by centrifugation at 14,000 rpm for 20 min at 4°C to collect the supernatant. The phage in the supernatant was stored at −70°C until used for the next round of panning.

Amplification of the Selected scFv-Phage

After five rounds of panning, the obtained scFv-phages were 10-fold serially diluted. *E.coli* DH5α F′ cells were infected with the diluted scFv-phages and then titrated on 2 × TY plate containing 100 μg/mL ampicillin and 2% glucose. The plate was incubated at 30°C overnight and, on the next day, 100 individual separated clones on the plate were picked randomly and amplified in 1 mL of 2 × TYA medium. M13-K07 was added to the amplified solution at an MOI of 20 and the scFv-phages were released into the medium during the incubation in 2 × TYAK medium at 30°C with shaking at 250 rpm, overnight. The supernatant was collected after centrifugation at 5,000 rpm for 20 min at room temperature and used for the binding specificity test by enzyme linked immunosorbent assay (ELISA).

scFv-Phage ELISA

96-Well microtiter plates were coated with 100 μL/well of bicarbonate buffer containing serially diluted (no dilution in the primary positive determination) intact rHuEPO, or rHuEPO oxidized by $NaIO_4$, or treated with DTT and Gu-HCl at 4°C, overnight. After blocking with PBS containing 2% (w/v) skimmed milk at 37°C for 1 h, 100 μL of the supernatant obtained as described above was added and incubated at 37°C for 2 h. Then, the bound phages were detected by HRP conjugated anti-M13 phage antibody and TMB substrate. The color development was stopped after 25 min with 60 μL of 2 mol/L H_2SO_4 and the absorbance was read at 450 nm. A negative control was run, using the supernatant containing phages which were selected against irrespective protein instead of rHuEPO.

RESULTS

Optimization of the Operating Conditions in ELISA

The operating conditions of scFv-phage ELISA were optimized before the specificity test. Skimmed milk (5%, 2%) and gelatin (0.8%) were compared as blocking reagents to decrease the interference of irrespective proteins

and non-specific adsorption. The results revealed that skimmed milk was better than gelatin. Although the blank absorbance using 5% skimmed milk was lower than that of 2% skimmed milk, the former also had strong interference on the absorbance of targeted proteins.[28] So, 2% skimmed milk was chosen to block the wells in scFv-phage ELISA. The diluting buffer of the HRP conjugated anti-M13 phage antibody (secondary antibody) was also an important factor that might affect the combination of the second antibody with scFv-phage and the background. 2%, 1%, and 0.5% skimmed milk and PBS were used to dilute the secondary antibody, respectively, and the results were compared. Considering both the absorbance value and the repeatability of the scFv-phage ELISA comprehensively, 0.5% skimmed milk was found to be the optimal diluting buffer for the secondary antibody. Skimmed milk of higher concentrations (2% or 1%) showed too strong a blocking effect to observe satisfactory signal change when serially diluted antigens were coated; PBS didn't possess enough blocking ability and the non-specific adsorption of secondary antibody resulted in high background and bad repetition.

Panning

During the subtraction panning, since the immunotube was coated with the oxidized rHuEPO in which the carbohydrate structure has been destroyed, phage antibodies against the polypeptide chain are easier to be captured. As can be seen in Figure 2, the number of phages binding to the oxidized rHuEPO gradually decreases after 5 rounds of subtraction panning, indicating that the phage antibodies that can recognize the carbohydrate profiles of rHuEPO are enriched in the supernatant during each round of panning. 100 clones were randomly picked out from the supernatant of the last round for further evaluation of binding ability to the carbohydrates of rHuEPO. In the primary positive screening, as described above, 28 clones were selected since, in scFv-phage ELISA, their absorbance values with the intact coated rHuEPO were higher than those with rHuEPO oxidized by $NaIO_4$.

ScFv-phage ELISA

To further confirm the specificity of phage antibodies, scFv-phage ELISA was performed with serially diluted rHuEPO and rHuEPO treated with $NaIO_4$ or Gu-HCl/DTT coated onto the wells of microtiter plate. Generally, the periodate reagent (e.g., $NaIO_4$) can destroy the carbohydrate groups without affecting the protein moiety of a glycoprotein.[25] So, the conformation related to the protein moiety would most likely remain unaltered during the periodate oxidation. While Gu-HCl/DTT can break intermolecular or intramolecular hydrogen bonds and disulfide linkages to make the protein

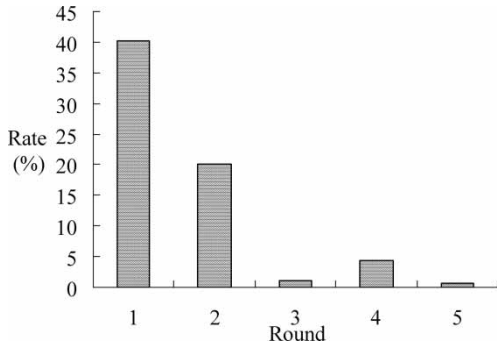

Figure 2. Rate of the phage combined with rHuEPO oxidized by $NaIO_4$ after each of 5 rounds of subtraction panning. The number of total phages put into the immunotube in each round was 1×10^6 CFU.

stretch, the carbohydrate structures would still exist. Based on these principles, two clones (No. 63 and No. 83) in our experiments showed the ability to recognize the conformation related to both the carbohydrate and protein moieties of rHuEPO (Fig. 3). In Fig. 3, there appeared little changes in the values of ELISA when different concentrations of denatured rHuEPO were coated. The results suggest that No.63 and No.83 did not recognize the protein moiety, including polypeptide chain and its steric conformations that were preserved during oxidation, nor did they recognize the structure and conformation of carbohydrate chains which were not destroyed by Gu-HCl/DTT. On the contrary, the values of absorbance in the scFv-phage ELISA with intact rHuEPO coated declined when the concentration of rHuEPO decreased, indicating these two phage antibodies may recognize the conformation constructed by the carbohydrate chains and protein moiety together. Another clone (No. 62) showed stronger binding ability to the intact rHuEPO or rHuEPO treated with Gu-HCl/DTT than rHuEPO oxidized by $NaIO_4$. Thus, clone No. 62 is more likely to bind with the carbohydrates of rHuEPO; its binding affinity may be independent of the senior structure of rHuEPO (Fig. 4).

Figure 5 shows the reaction of the phage antibody against irrespective protein (negative control) with different rHuEPOs coated. Little change was observed when the concentrations of antigens changed. During the panning, some scFv-phages with specificity to the protein moiety of rHuEPO were also obtained and their absorbance values in scFv-phage ELISA showed little difference between the intact rHuEPO and oxidized rHuEPO. Figure 6 shows the reaction of clone No. 33, which shows special binding tendency to the polypeptide chain. Comparing the results in Figs. 5 and 6 with those in Figs. 3 and 4, it can be basically concluded that No. 62, No. 63, and No. 83 had special binding tendencies towards the carbohydrates or carbohydrate-related conformation of rHuEPO.

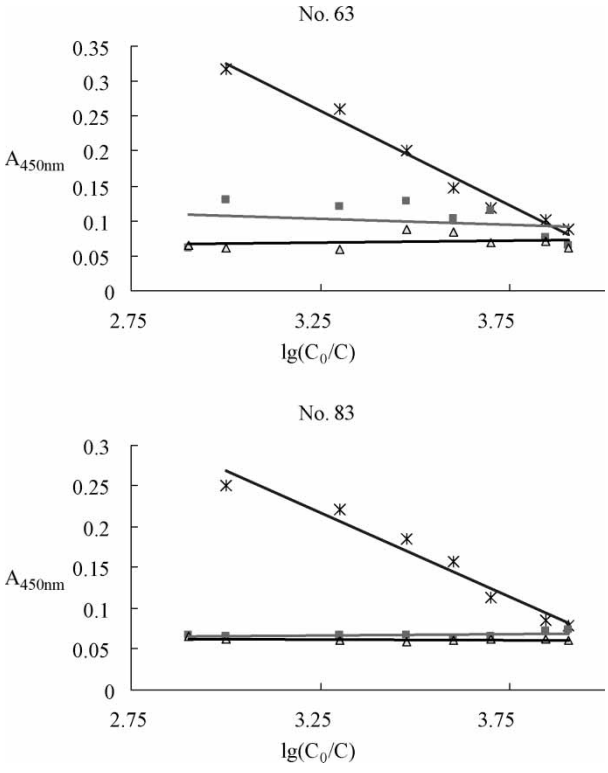

Figure 3. Results of scFv-phage ELISA of clones No. 63 and No. 83. The concentrations of the three types of rHuEPOs coated were 1.88, 1.50, 0.75, 0.50, 0.38, 0.30, 0.21, and 0.19 ng/mL. The primary concentration (C_0) of rHuEPO was 1.5 mg/mL. (✳), the intact rHuEPO; (■), rHuEPO oxidized by $NaIO_4$; (△), rHuEPO treated with Gu-HCl/DTT.

DISCUSSION

In general, carbohydrate-specific antibodies are difficult to produce by conventional techniques, for many carbohydrates are self-antigens. Phage display technique possesses the higher rate of selection and the ability of obtaining antibodies without immunization, so it is considered as a good method to isolate carbohydrate-specific antibodies. In fact, some research work has proven that carbohydrate-specific antibodies can be produced by phage display.[19,29] In this study, a phage display antibody library and subtraction panning strategy were used to obtain phage antibodies with specificity for the carbohydrates of rHuEPO. Subtraction panning greatly decreased the number of phages against the protein moiety of rHuEPO and, at the same time, increased the rate of phage recognizing the carbohydrates of rHuEPO. Compared with the common positive panning procedure, subtraction

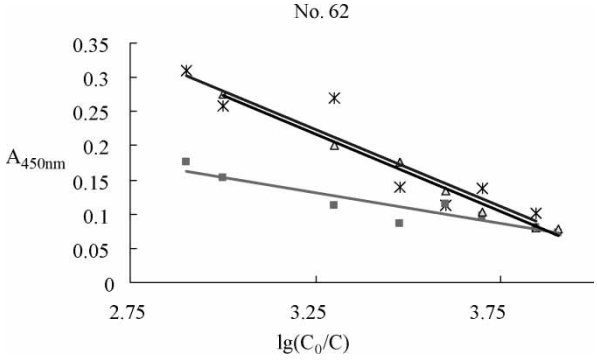

Figure 4. Results of scFv-phage ELISA of the clone (No. 62). The concentrations of the three types of rHuEPOs coated were 1.88, 1.50, 0.75, 0.50, 0.38, 0.30, 0.21, and 0.19 ng/mL. The primary concentration (C_0) of rHuEPO was 1.5 mg/mL. (✳), the intact rHuEPO; (■), rHuEPO oxidized by $NaIO_4$; (△), rHuEPO treated with Gu-HCl/DTT.

panning provides a more effective way for selection of antibody against rare or non-immunodominant antigens.[30,31] However, it is not able to remove all of those unwanted phages perfectly. As a matter of fact, it is a technique suited for the purpose of enrichment of targeted scFv-phages.[32] This could explain why only 28 of 100 clones seemed to be specific to the carbohydrates of rHuEPO after the fifth panning round.

During the subtraction panning, the input number of phage library and the maximum binding capacity of the coated antigens are two important factors that should be taken into account. In our study, we found that the maximum

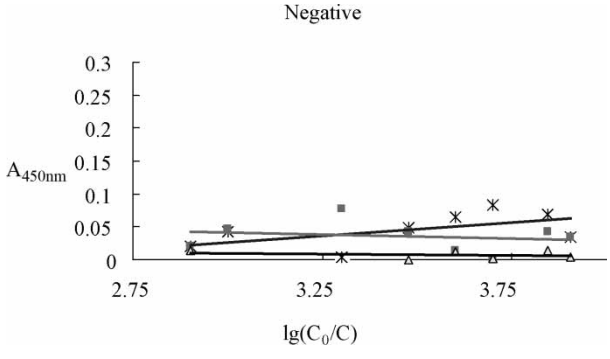

Figure 5. Results of scFv-phage ELISA of the phage antibody against irrespective protein (negative control). The concentrations of the three types of rHuEPOs coated were 1.88, 1.50, 0.75, 0.50, 0.38, 0.30, 0.21, and 0.19 ng/mL. The primary concentration (C_0) of rHuEPO was 1.5 mg/mL. (✳), the intact rHuEPO; (■), rHuEPO oxidized by $NaIO_4$; (△), rHuEPO treated with Gu-HCl/DTT.

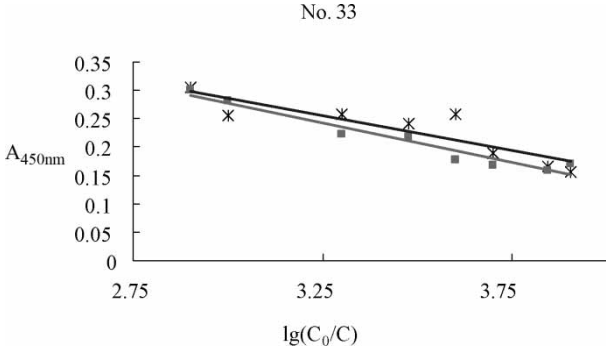

Figure 6. The results of scFv-phage ELISA of the clone (No. 33) against the polypeptide chain of rHuEPO. The concentrations of the three types of rHuEPOs coated were 1.88, 1.50, 0.75, 0.50, 0.38, 0.30, 0.21, and 0.19 ng/mL. The primary concentration (C_0) of rHuEPO was 1.5 mg/mL. (∗), the intact rHuEPO; (■), rHuEPO oxidized by $NaIO_4$.

number of binding phages was 10^6-10^7 when 30 μg of oxidized rHuEPO was coated in the immunotube. To perform the subtraction panning efficiently, the input number of phage library should be no more than 10^6-10^7. Otherwise, most of the unwanted phages would remain in the supernatant and lead to a lower panning efficiency. On the other hand, the phage library used in subtraction panning should not be diluted excessively because excessive dilution may lose the diversity of the phage library or even cause a failure in screening the targeted scFv-phages. In summary, the input number of phage library should be necessarily around the maximum binding capacity of the antigens coated in the immunotube.

Glycosylation of rHuEPO is a complex process that often results in several glycoforms of the same glycoprotein.[18] Although there has been certain evidence indicating that the epitopes of EPO consisted solely of peptide elements, the carbohydrates may play an important role in maintaining the conformation and reactivity of the protein moiety.[33] In order to illustrate the composition, structure, and function of carbohydrates of rHuEPO (or EPO), as well as the subtle relationship between the carbohydrates and protein moieties, it is quite necessary to develop more effective methods and better reagents. In recent studies focused on EPO, lectins have been utilized to analyze and enrich this glycoprotein.[12,13,34]. As specific reagents, lectins can bind to the individual carbohydrate structures and their steric conformations. However, they are not able to distinguish proteins that have similar carbohydrate structures. This means other glycoproteins in the sample (serum or urine) might affect, or even have a negative effect on, the analysis of rHuEPO. Delorme has proved that N-glycosylation chains can affect EPO conformation, i.e., the N-link carbohydrates may take part in the formation of EPO conformation.[35] If the antibody is produced against

these conformations, it shall have the ability to recognize the conformation related to both the carbohydrates and protein moieties. In our study, the phages No. 63 and No. 83 showed such characteristics. No. 62 seems to bind to the carbohydrate structure just as do lectins, while, since they were produced by panning against rHuEPO and its specificity to rHuEPO is surely better than lectins. Although the low intrinsic affinity of the phage antibodies may affect the range of their use, maturation of affinity in vitro make it possible to increase the affinities of phage antibodies. So, these phage antibodies with specificity for the carbohydrates of rHuEPO are potential tools to analyze the carbohydrate structure of rHuEPO. Furthermore, they might be useful in purification and enrichment of rHuEPO in serum or urine, for doping control, or for clinical applications.

CONCLUSIONS

This paper reports a new approach to obtain carbohydrate-specific phage antibodies of rHuEPO by phage display combined with subtraction panning. The unique specificity of these phage antibodies make them potential tools in future analytical studies related to rHuEPO.

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