

Generation of high affinity human single-chain antibody against PreS1 of hepatitis B virus from immune phage-display antibody library

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BACKGROUND: A single-chain antibody (ScFv) phage display library was created by cloning antigen-binding regions of V_H (variable domain) and V_L gene repertoires as fusion proteins with a minor coat protein of filamentous phage, from which high affinity completely humanized ScFv against PreS1 of hepatitis B virus could be screened and characterized.

METHODS: A combinatorial library of phage-display human ScFv genes, which were derived from peripheral blood lymphocytes immunized by peptide PreS1 in vitro, was constructed. The library contained 7×10^8 clones.

RESULTS: After 3 rounds panning, a high affinity ($K = 10^{-7}$ - 10^{-8} mol/L) ScFv specific to PreS1 was obtained. Sequence analysis showed that the V_H belonged to the V_{H4} family and V_L to V_{L4} .

CONCLUSIONS: The described ScFv may provide a more satisfactory therapy. This application further illustrates that the method of in vitro antigen stimulation is expeditious for the source of human immune antibody library.

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KEY WORDS: hepatitis B virus; PreS1; single-chain antibody; immune antibody library; panning

Introduction

Antibodies play a crucial role in protecting the human body from viral infection. But there is doubt of the therapeutic potential of rodent antibodies in humans owing to the immune response they elicit.^[1,2] Fortunately, recent advances in using antibody phage-display have made it possible to get completely humanized antibodies, among which the single-chain antibody (ScFv) is particularly useful.^[3,4]

The major humoral immune response to hepatitis B virus (HBV) is mounted against HBsAg, which is composed of 3 related membrane-bound proteins:^[5-7] S (226aa), PreS2 (55aa) and PreS1 (108aa). The results of clinical research have shown that the antibody specific to PreS1 may neutralize HBV and disturb its replication.^[8]

Keeping these in mind, we initiated this project to get completely humanized ScFv against PreS1, which would provide a more satisfactory therapy of hepatitis B. It is more likely that the high affinity ScFv can be obtained from the immune library other than from naive one^[9-13] because the irrelevant antibodies may be removed by immune response, followed by the improvement of the V-gene before cloning.^[14] Hence, we used the method of in vitro antigen stimulation to obtain immune B cells of an immune source. The result proved that it is powerful to isolate human ScFv of interest.

Methods

Immunization and lymphocyte RNA preparation

Each of 6 healthy volunteers donated 5 ml of PBLs, from which 1×10^8 lymphocytes were obtained after density-gradient separation. The cells were cultured in the presence of 100 μ g/ml BSA-PreS1 (gift of Mr. R. Vinas, Pasteur Merieux, USA) covering amino acids P20-47 of HBV for 5 days in a RPMI 1640 medium with 10% human AB serum and 1% PHA. Then RNA was isolated by Trizol reagent (Gibico-BRL), dried

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and stored at $-70\text{ }^{\circ}\text{C}$ till use for reverse transcription.

Amplification and assembling of the V_H and V_L genes

cDNA synthesis was performed by RT-PCR kit (Takara, Dalian, China). PCR amplification was carried out using Pyrobest DNA polymerase with GC buffer kit in $50\text{ }\mu\text{l}$ reaction mixture (Takara, Dalian, China). Primers used for PCR amplification of human heavy and light chain V-genes are shown in Table 1. To maintain a maximal diversity, half-nest PCR was employed.^[15] IgM-derived heavy chain genes ($V_H + C_H1$) were amplified by a primary PCR with an IgM constant region primer (forward primer) and separate 5 V_H primers (reverse primers). Then the products were reamplified with J_H primer (forward primer) annealing to 3' end of V_H and the V_H primers described above. All the primers were designed according to V-BASE.^[16] The PCR condition was adjusted at $94\text{ }^{\circ}\text{C}$ 1 min ($98\text{ }^{\circ}\text{C}$

Table 1. Oligonucleotide primers used for amplification of the V_H and V_L genes

A	Primary amplification
HZ5' I	GGGGCCCAGCCGGCCAGGTGCAGCTGGTGCAGTCTGG
HZ5' II	GGGGCCCAGCCGGCCAGGTGCAGCTGGTGCAGTCTGG
HZ5' III	GGGGCCCAGCCGGCCAGGTGCAGCTGGTGGAGTCTGG
HZ5' IV	GGGGCCCAGCCGGCCAGGTGCAGCTGCAGSAGTCGGG
HZ5' V	GGGGCCCAGCCGGCCAGGTGCAGCTGCAGSAGTCAGG
HZ3' M	GGACTAGTGGCAATCACTGGAAGAGGCAC
L_{κ} 5' I	GACATCCAGATGACCCAGTC
L_{κ} 5' II	GATATTGTGATGACTCAGTC
L_{κ} 5' III	GAAATTGTGTTGACGCAGTCTC
L_{κ} 3'	ACACTCTCCCCTGTTGAAGCTC
L_{λ} 5' I	TCTGTSBTGACKCAGCCRCC
L_{λ} 5' II	TMTGWGCTGACTCAGSMMCC
L_{λ} 5' III	GYTRTRCTGACTCARSMMCM
L_{λ} 3'	CGCCGTCTAGAACTATGAACATTCTGTAGG
B	Secondary amplification
J_H	TGARGAGACGGTGACCAKKG
$J_{\kappa}1$	GGGCGGCCGCACGTTTGATWTCCACYTTGGTCCC
$J_{\kappa}2$	GGGCGGCCGCACGTTTTRATCTCCAGYYKKGTC
$J_{\lambda}1$	GGGCGGCCGCACCTARRACGGTSAGCTRGGT

HZ5' I -HZ5' IV are 5' primers for V_H . HZ5' I is homologous to 1 and 7 subgroups of V_H , and HZ5' III, HZ5' IV, HZ5' V are homologous to 3-5 subgroups of V_H separately. HZ5' II is homologous to 2 and 6 subgroups of V_H . HZ3' M is homologous to the constant region of IgM heavy chain. L_{κ} 5' I, L_{κ} 5' II, L_{κ} 5' III are 5' primers for κ chain, corresponding to 1-4 subgroups of V_{κ} separately. So L_{λ} 5' I, L_{λ} 5' II, L_{λ} 5' III are corresponding to 1-7 subgroups of V_{λ} . L_{κ} 3' and L_{λ} 3' is homologous to the constant region of κ and λ chain separately. J_H , $J_{\kappa}1$, $J_{\kappa}2$, $J_{\lambda}1$ are the 3' primers homologous to the FW4 regions of V_H , V_{κ} and V_{λ} . Sites of Sfi I and Not I are underlined.

Table 2. Primers for linker

Primer	Sequence
LJH	CMMYGGWCACCGTCTCYTCATCGAGTGGTGGAGGCCG
$LV_{\kappa}1$	GACTGGGTGCATCTGATGTCCTACTACCGCCAGAGC
$LV_{\kappa}2$	GACTGAGTCATCACAATATCACTACCGCCAGAGC
$LV_{\kappa}3$	GACTGGGTCAACACAATTTCACTACCGCCAGAGC
$LV_{\lambda}1$	CTGMGTCAVSACAGAACTACCGCCAGAGC
$LV_{\lambda}2$	GACTCAGTCGWTMTACTACCGCCAGAGC
$LV_{\lambda}3$	YTGAGTCAGYAYARCACTACCGCCAGAGC

LJH is the 5' primer; $LV_{\kappa}1$, $LV_{\kappa}2$, and $LV_{\kappa}3$ are the 3' primers. $LV_{\lambda}1$, $LV_{\lambda}2$ and $LV_{\lambda}3$ are also the 3' primers. Underlined parts are homologous to the linker DNA, while the other parts are homologous to the V_H or V_L gene.

for 10 s, $52\text{ }^{\circ}\text{C}$ for 30 s, and $72\text{ }^{\circ}\text{C}$ for 30 s) \times 30 rounds. The PCR products were purified by a PCR fragment recovery kit (Takara, Dalian, China). The light chain V-genes of the κ and λ families were amplified by the PCR described above.

To connect the V_H and V_L genes, an oligonucleotide coding for the $(G_4S)_3$ linker was used. Firstly, the template of linker was synthesized (GGTGGAGGCCGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCCGGAAGT). It was amplified subsequently by 6 groups of primers annealing to 3' end of V_H and 5' end of V_L (V_{κ} and V_{λ}) separately (Table 2). After PCR, 6 linker fragments which are hybrids to 3' end of V_H and 5' end of 6 V_L genes separately were obtained. PCR products were purified.

To join the V_H , V_L and linker fragment, splicing overlap extension PCR was performed. The equimolar of 3 fragments was mixed, cycled 7 rounds ($98\text{ }^{\circ}\text{C}$ for 10 s, $58\text{ }^{\circ}\text{C}$ for 30 s, and $72\text{ }^{\circ}\text{C}$ for 30 s) to join the fragments before amplification for 30 cycles ($98\text{ }^{\circ}\text{C}$ for 10 s, $55\text{ }^{\circ}\text{C}$ for 30 s, and $72\text{ }^{\circ}\text{C}$ for 30 s) after the addition of outer primers. V_H -linker- V_L (ScFv) gene fragments were purified as mentioned above.

Library construction

After the ScFv genes were digested with Sfi I and Not I restriction enzyme, they were cloned into pCANTAB5E phagemid vector (Pharmacia) digested with the same restriction enzyme. Following 10 electroporations into *E. coli* TG1, the library was titered as described previously^[14] and stored at $-70\text{ }^{\circ}\text{C}$.

Library panning

Phagemid population was rescued from the library as reported.^[17] Specific phage-displayed ScFv was affinity-selected via protein absorbed to an immuno tube (Gibco). Firstly the tube was coated overnight by PreS1 ($100\text{ }\mu\text{g/ml}$) at $4\text{ }^{\circ}\text{C}$ and washed next day with phosphate buffered saline (PBS) and blocked with 3% BSA-

PBS (contain 3% bovine serum persulfate). To deplete the libraries of casein antibodies, rescued phagemid population was preincubated with 3% BSA-PBS before being poured into a prepared tube. After incubation for 1 hour at room temperature, the tube was emptied and washed with 0.05% PBST (contain 0.05% tween-20) for 20 times, then with PBS for another 20 times before Log-phase TG1 cells were added and incubated at 37 °C for 1 hour to allow reinfection. The output phage titer was quantitated. The panning was repeated thrice.

Monoclonal ELISA

After the third panning, 96 clones of the library were inoculated into a 96-well plate. Phage population was rescued from every clone.^[17] 100 μ l supernatant containing phage was added into another 96-well plate coated with 100 μ l (10 μ g/ml) PreS1 overnight at 4 °C and blocked with 2% MPBS (2% no fat milk BSA). Incubated at room temperature for 2 hours, then 100 μ l HRP-antiM13 antibody was added into each well. After incubation for 1 hour, the plate was washed with 0.05% PBST for 5 times, then ABTS was added. Thirty minutes incubation at room temperature was needed before the measurement of the absorbance of each well at 410 nm with an enzyme-linked immunosorbent assay reader. Positive clones were tested using cross reaction with BSA.

Fingerprinting assay

The positive clones were assayed by restriction enzyme Afa I and Hha I.^[18]

Preparation of soluble ScFv

The positive clones identified by ELISA were inoculated into 400 μ l log phase *E. coli* HB2151. After shaking at 37 °C for 30 minutes, the culture was plated on a SOBAG-N medium. Next day, the individual colonies were inoculated into 5 ml 2YT-AG for overnight shaking at 30 °C. After centrifugation at 1800 g for 10 minutes, the pellet was resuspended in 50 ml 2YT-AI containing 1 mmol IPTG and shaken for 6 hours at 30 °C. After centrifugation at 2000 g for 20 minutes, the pellet was resuspended in TEX buffer (0.5 mmol sucrose, 0.5 mmol EDTA) on ice for 60 minutes. Periplasmic fractions containing soluble ScFv were prepared by the above centrifugation.

Competitive ELISA

To determine the affinity, competition ELISA was performed to measure the binding constants of ScFv. Briefly, a 96-well plate was coated and blocked. Periplasmic fractions containing ScFv were incubated with serially diluted buffer containing free PreS1 overnight at 4 °C to allow equilibration. After the plate was washed, ScFv-PreS1 mixture was added, then analyzed by ELISA.

Sequencing

Plasmids of the specific phage antibody described above were prepared for sequencing in Takara Co., Ltd., Dalian, China.

Results

Immunization in vitro

1×10^8 lymphocytes were cultured in the presence of PreS1 (100 μ g/ml). After 5 days, the cells proliferated to 2×10^8 .

Library construction

After half-nest PCR, 5 IgM derived V_H , 6 V_K and 3 V_L fragments were obtained. Subsequently, 6 linkers bridged between V_H , V_K and V_L were also amplified, resulting in the accomplishment of 45 ScFv fragments. From these products, 10 electroporations showed in 7×10^8 immune phage-display library.

Library panning

The specific phage antibodies were enriched evidently by the increase of the number of clones from the first panning to the third panning (Table 2).

ELISA

The phage population rescued from the third panning was subjected to ELISA with PreS1. The results showed that the frequency of positive clone was 55/94, whereas the bacterial supernatant containing negative clones as judged by the phage screening assay showed no reactivity with PreS1 in ELISA. No cross reaction toward BSA was found. Finger print assay of these positive clones showed that there were 44/55 clones with full-length insertion (about 700bp) and that these clones had the same fingerprinting. We believe that one clone of

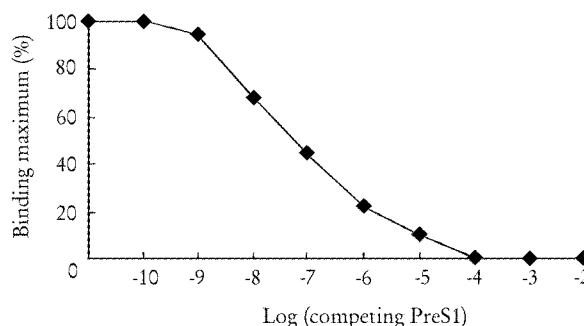


Fig. 1. Specificity of PreS1 binding as revealed by competitive ELISA. Experiments were carried out with the periplasmic fractions from the positive clone, induced with 1 mmol IPTG for 6 h at 30. Apparent affinity was determined as the reciprocal of the PreS1 concentration required to inhibit 50% of maximal binding in a competitive ELISA.

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CAGGTGCAGC TGCAGGACTC GGGCCCAAGG CTGGTGAAGC CTTCGGAGAC CCTGTCCCTC
Q V Q L Q E S G P G L V K P S E T L S L
ACCTGGCGTG TCTCTGGTGG CTCCATCAGC AGTAGTAACT GGTGGAGITG GGTCCGCCAG
T C A V S G G S I S S N W W S M Y R Q
CCCCAGGGA AGGGGCTGGA GTGGATTGGG GAAATCTATC ATACTGGGAG CACCAACTAC
P P G K G L E U I G E I Y H S G S T N Y
AACCCTGCC TCAAGACTCG AETCACCATA TCACTAGACA AGTCCAGCAA CAGITCCGCC
N P S L K S R V T I S V D K S K N Q F P
CTGAAGCTGA GCTCTGTGAC CGCGGCGGAC ACGGCGGTGT ATTACTGTGC AAGACATTCT
L K L S S V T A A D T A V Y Y C A R H S
GGTCAGCTGT GGCCTGTTG GGGCCAAAGT ACCCTGCTCA CCGCTCGAG TGGTGGAGGC
G Q L M P C M G Q G T L V T V S S G G E
GGTTCAGGCG GAGGTGGCTC TGGTGGAGGC GGTAGTGCAC TTCTGCCTGT GCTGACTCAG
G S G E G G S G G G S A L L P V L T Q
GAGCAGCACT CCTACACCAT CGAATGGTAT CAACAGAGAC CAGGGAGTCC CCCCAGTAT
E H S T Y T I E W Y Q Q R P G R S P Q Y
ATAATGAAGG TTAAGACTGA TGGCAGCCAC AGCAAGGGGG ACGGCACTCC CGATCGCTTC
I M K V K S D G S H S K G D G I P D R F
ATGGGCTCCA GTTCTGGGCG TGACCGCTAC CTCACCTTCT CCAAGCTCCA GTCTGACGAT
M G S S S G A D R Y L T F S H L Q S D D
GAGGCTGAGT ATCACTGTGG AGAGAGCCAC ACGATTGATG GCCAAGTCTG A
E A E Y H C G E S H T I D G Q V V
    
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Fig. 2. Nucleotide sequence and aminoacid sequence of the positive clone. CDRs and linker are indicated.

phage antibody specific to PreS1 was enriched.

Competitive ELISA

This clone was transformed into *E. coli* HB-2151 to produce soluble ScFv fragment. Then the specificity was confirmed by competitive ELISA. The result indicated apparent inhibition constant in the range of 10⁻⁷–10⁻⁸ mol/L (Fig. 1).

Sequence of the specific ScFv

DNAPLOT analysis showed that the sequence of heavy chain belongs to V_H4 subgroup and that of light chain belongs to V_L4 subgroup (Fig. 2). GeneBank number is AF422193.

Discussion

Hepatitis B is caused by HBV. Because the disturbance of the immune system, active immunization often does not work well to produce antibody against HBV. As to protective immune response, the late discovery is that immunogenicity is fulfilled through epitope instead of the whole molecule. The immune response^[19] elicited by the native antigen molecule can't satisfy the prevention or protection. Hence research into protective immunization would transfer to epitope.

PreS1 is one of the most important epitope of HBV.^[20-22] To obtain the high affinity ScFv against it, the construction immune antibody library is ideal. In other research, sensitized lymphocytes were obtained from normal persons injected with vaccine^[23] or from patients.^[24] In the former, however, it was difficult to immunize humans because it is rarely possible to ensure

Table 3. Enrichment of the ScFv library to PreS1

Library	Phage inpute	cfu	Yielding
Primary library		7 × 10 ⁸	
1st panning	1.25 × 10 ¹¹	6 × 10 ⁴	4.8 × 10 ⁻⁵ (c)
2nd panning	1.03 × 10 ¹¹	8 × 10 ⁵	7.7 × 10 ⁻⁴ (c)
3rd panning	1.10 × 10 ¹¹	5 × 10 ⁶	4.5 × 10 ⁻³ (c)

the presence of specific B cells at an acceptable frequency in the PBLs^[25] and the availability of vaccine also limits its application. In the latter, most of patients especially those infected with HBV were tolerant to the virus, which is meant that the titer of serum antibody is lower than the acceptable level.^[26] To overcome these difficulties, the method of in vitro antigen stimulation was employed in this study. Because the peptide PreS1 is too small to induce immune response, it was conjugated with BSA with a high concentration of 100 µg/ml. To delete cross reaction of BSA, the rescued phage antibodies were incubated with 3% BSA prior to panning against PreS1. During ELISA, plates were blocked by 2% MPBS to reduce background and false positive.

From the first to the third panning, increasing output clone forming unit (cfu) showed enrichment of specific phages (Table 3). A 100-fold increase in the yield of phage relevant to the first panning also demonstrated accomplishment of the panning. Finally, ScFv specific to PreS1 with affinity of 10⁻⁷–10⁻⁸ mol/L was confirmed by competitive ELISA. The yield of elute phage was higher than that of other research.^[27] This may be due to the in vitro stimulation of antigen. During the procedure, PBLs proliferated twice as compared with the original. It is reasonable to suggest that the genes of lymphocytes rearranged against PreS1.

During construction of the ScFv library, the half-nest PCR was employed because it would optimize the diversity of ScFv gene repertoire, which would also ensure the accomplishment of the panning.

In summary, a sequence encoding the human ScFv against PreS1 of HBV generated would provide a safe method for clinical treatment of patients with hepatitis B virus. Further investigation, however, should be continued including site-directed mutagenesis or mutator strain expression.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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