Production of High Affinity Human Single-chain Antibody Against PreS1 of Hepatitis B Virus: Comparison of Large Naïve and *In vitro* Immune Phage Displayed Antibody Library

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Abstract A large naïve phage displayed human single-chain variable fragments antibody (scFv) library and an *in vitro* immune library were constructed in parallel conditions, based on the PBLs from healthy and sero-negative blood donors, part of which were *in vitro* immunized by peptide PreS1 conjugated to BSA. After 3 rounds of panning against PreS1, measurement of antibody-antigen reaction revealed: a scFv specific to PreS1 from the immune library was obtained, which affinity ($k = 10^{-7} \sim 10^{-8}$ M) was higher than that from the naïve one ($k = 10^{-6} \sim 10^{-7}$ M). Sequencing of the two scFv showed they were human antibodies, which may be of interest in therapy of Hepatitis B. This investigation also illustrated that the method of *in vitro* immunization results in antibody library more satisfied even than the large naïve one.

Key words *in vitro* immunization, single-chain antibody, PreS1, phage display

Hepatitis B is a viral disease caused by hepatitis B virus (HBV). Because the disorder of immune system, the active immunization often does not work well to produce antibody against HBV. So it is necessary to investigate passive immunization especially in therapy of Hepatitis B. As to passive immune, the lately research showed: immunogenicity is fulfilled through epitope instead of the whole protein molecule[1], while antibodies elicited by peptide encoded by epitope can neutralize the parent protein[2]. Consequently, the research on passive immunization would be diverted from nature antigen molecule to its epitope.

PreS1 is one of the most important epitope of HBsAg. Anti-preS1 antibody may neutralize HBV[3]. With these considerations in mind, we started this project to get high affinity humanized scFv against PreS1, which may provide a new field in therapy of hepatitis B. We constructed both naïve and immune library and accomplished isolating a sequence encode higher affinity single-chain variable fragments antibody (scFv) from the immune one.

1 Materials and methods

1.1 Materials

1.1.1 PBLs: Every one of 60 healthy and sero-negative volunteers donated 5 ml of his PBLs, which resulted in 1×10^9 lymphocytes after density-gradient separation.

1.1.2 Reagents: PreS1 peptide conjugated to BSA (BSA-PreS1) was from Shanghai Biochemical Institute. PreS1 peptide, covering amino acids P20~47 of HBV, was synthesized by solid-phase techniques and analysed by standard reverse-phase high performance liquid chromatography (HPLC), the purity was > 90%[4]. TRIZOL was purchased from GIBICO, L-leucine methylester hydrobromide was purchased from Shanghai HuaMei Biocompany. pCANTAB5E phagemid vector and *E. coli* HB2151, *E. coli* TG1 were from Phamarcia. scFv detection module was from Phamarcia. PEG600 and IPTG were from Sigma. Restriction endonuclease were from TAKARA Co. Ltd(DaLian, China). Immuno tube was from NUNC.

1.2 Methods

1.2.1 *In vitro* Immunization: 5×10^8 PBLs each were taken for two parallel experiments. one was nonimmunized, the other was *in vitro* antigen stimulation. For the former one, RNA was isolated immediately by TRIZOL reagent; and for the other group, the cells were treated with L-leucine methylester hydrobromide (LeuLeuOMe), as previously described[5]. Then they were cultured in the presence of BSA-PreS1 (containing 100 mg/L PreS1) in RPMI 1640 medium with 10% human AB serum and 1% PHA for 5 days when the PBLs proliferated twice confirmed by cell counting. RNA was isolated as described above.

1.2.2 Library construction: Two phage-displayed libraries were constructed as described in our former research[6]. Briefly, half-nest PCRs were employed

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in amplifying V_H, V_κ and V_λ. All primers were designed according to V-BASE[7], covering all of the 7 subgroups of V_H, 4 subgroups of V_κ, and 7 subgroups of V_λ. scFv genes were assembled though $(G_4S)_3$ linker bridge between V_H and V_L (V_κ and V_λ). After ligation with pCANTAB5E phagemid vector and electroparition into *E. coli* TG1, libraries were serially diluted to titer the size.

1.2.3 Library panning for antigen binding: Phagemid particles were rescued from the two libraries as described[8]. Briefly, the two libraries were inoculated into 2YT-AG medium (containing 100 g/L ampicillin and 2% glucose) separately. Then M13K07 were added (Moi= 10) to rescue phage. After overnight culture in 2YT-AK medium (containing 100 g/L ampicillin and 25 g/L kanamycin), it was centrifuged and the phage antibodies in supernatant were precipitated by PEG and resuspended in PBS.

Specific phage-displayed scFv were affinity-selected by using protein antigen absorbed to immuno tube (Gibco). Firstly, two tubes were coated overnight by PreS1(100 mg/L) at 4℃. Next day they were washed with PBS then blocked with 3% BSA-PBS. To deplete the libraries of casein antibodies, phagemid populations were preincubate with 3% BSA then poured into the prepared tube. After incubation for 1 h at room temperature, the tubes were emptied and washed with 0.05% PBST (containing 0.05% Tween20) for 20 times then PBS for another 20 times before log phase *E. coli* TG1 was added and incubated 1 h at 37℃ to allow reinfection. The output library was titered. The circle was repeated for 3 times.

1.2.4 Polyclonal ELISA: To determine the concentration of phage antibodies against PreS1 in each round of panning, an indirect ELISA was performed as follows: 100 μl(10 mg/L) PreS1 was coated in 96-well plates overnight at 4℃. Then 10 μl phage of each round was added after washing plates with PBS and blocking with 2% MPBS(containing 2% (*v*/*v*) non-fat milk). Incubated for another 2 h at room temperature, 100 μl HRP-antiM13 antibody was added in each well. After incubation for 1 h, the plates were washed with 0.05% PBST for 5 times then ABTS were added. 30 min incubation at room temperature was needed before measuring the absorbance of each well at 410 nm with ELISA reader.

1.2.5 Monoclonal ELISA: After the third panning, 94 clones of each library were inoculated into a 96-well plate with 100 μl 2YT-AG each well. Phage population was rescued from every clone as above. The supernatant containing phage antibodies was taken for ELISA as described above. The positive clones were examined by crossing reaction with BSA.

1.2.6 Fingerprinting assay: Positive clones were picked up to be assayed by restriction enzyme *Afa* I and *Hha* I [9].

1.2.7 Preparation of soluble scFv: Two clones, one enriched in naïve library and the other in immune one, were inoculated into 400 μl log phase *E. coli* HB2151 to transform the scFv genes into proper expression host. Under induction of IPTG, the supernatant containing soluble scFv was obtained, which was demonstrated by Western blot analysis[10].

1.2.8 Competition ELISA: To determine the affinity of the two clones, competition ELISA was performed to measure the binding constants of scFv[11]. Briefly, The 96-well plates were coated and blocked as above. The supernatant containing scFv were incubated with serially diluted buffer containing free PreS1 overnight at 4℃ to allow equilibration. Then ELISA was performed as above.

1.2.9 Sequencing: Plasmids of the specific phage antibody described above were prepared for sequencing in TAKARA Co. Ltd.

2 Results

2.1 Library construction

During construction of naïve antibody library, 80 electroporations resulted in 1×10^{10} pfu (plauhe forming units) library. 1: 10^9 diluted library plated on 2YT-AG plate resulted in 13 clones, while 1: 10^7 resulted in 1 005 clones. The immune library was 1×10^8 after 2 electroporations. PCR assay showed the full length insertion clones were about 90%.

2.2 Library panning

The specific phage antibodies were enriched as evidence by the increasing of the number of clones from the 1st panning to the 3rd panning, both in naïve library and immune one. But the result of every round panning of immune library was 4 ~ 50 folds higher than that of naïve one (Table 1). Polyclonal ELISA showed target phage antibodies were generated from both the two libraries. The immune

Table 1 panning of naïve and immune library

	Beginning (pfu)	1st panning (pfu)	2nd panning (pfu)	3rd panning (pfu)
Immune	1×10^8	6×10^4	1.4×10^6	1×10^7
Naïve	1×10^{10}	1.5×10^4	4×10^4	2.1×10^5

one was also better than naïve one (Table 2).

Table 2 polyclonal ELISA of every panning

	1st painning(A_{410})	2nd panning(A_{410})	3rd panning(A_{410})
Immune	0.23	0.56	0.88
Naïve	0.21	0.58	0.72

The phage population rescued from the 3rd panning was subjected to ELISA with PreS1. The result showed: for the immune library, frequency of positive clone was 52/94, with A_{410} about 1.0; for the naïve one, it was 53/94 with A_{410} about 0.6. While the bacterial supernatant containing negative clone as judged by the phage screening assay showed no reactivity with PreS1 in ELISA. Finger print assay of these positive clones showed, there were 44/52 and 40/53 clones with full-length insertion (about 700 bp) and the same fingerprinting respectively in immune and naïve library. It was showed that each of the two libraries enriched one target phage antibody. No cross reaction toward BSA was found in these two phage antibodies. They were donated as scFv ZG1 (immune) and scFv ZG2 (naïve).

2.3 Competition ELISA

ZG1 and ZG2 were transformed into *E. coli* HB2151 to produce scFv fragment. Western blot showed the desired band (about 30 ku), then the affinity was confirmed by competition ELISA. The result indicated affinity of ZG1 was in the range of 10^{-7}~ 10^{-8} M, while that of ZG2 was 10^{-6}~ 10^{-7} M (Figure 1).

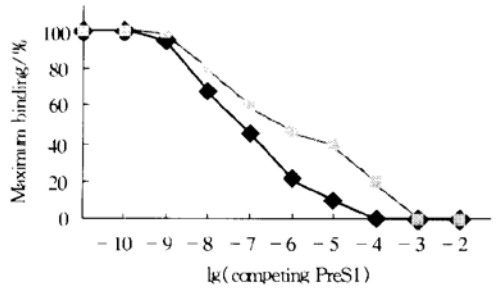

Fig. 1 Specificity of PreS1 binding as revealed by competition ELISA

Apparent affinity was determined as the reciprocal of the PreS1 concentration required to inhibit 50% of maximal binding in a competition ELISA. ◆——◆: ZG1; ☆——☆: ZG2.

2.4 Sequencing

Sequencing result showed both ZG1 and ZG2 were proper consistent of human antibody gene. DNAPLOT software analysis showed heavy chain of ZG1 belongs to V_H4 and light chain belongs to $V_\lambda4$. The heavy chain of ZG2 is V_H1 and light chain is $V_\lambda1$. GenBank numbers of them are AF422193 and AF427148 respectively.

3 Discussion

Phage display makes it possible to get completely humanized antibody, which is of interest in therapy of viral disease if the affinity is higher than 10^{-6} M[12]. To obtain high affinity antibodies, two ways are available. One is construction of large naïve phage antibody library, size of which is up to 10^{10}[13]; the other is immune library with only 10^7~ 10^8. Construction of immune library is much easier than the former one. But the material of immune library is something of trouble. In other investigations the immunized material was from human injected with vaccine[12], or from patients[14]. In the former case, however. it is difficult to immunize human to order because it is rarely possible to ensure the presence of specific B cells at acceptable frequency in the PBLs[15]; In the latter case, most of patients especially whom infected with HBV are tolerant to the virus. It means the titer of serum antibody is lower than acceptable level. To overcome these difficulties, the method of *in vitro* antigen stimulation was employed in this paper.

Because the peptide PreS1 is too small to induce immune response, it was conjugated with BSA and high concentration was employed. To delete the cross reaction of BSA, the rescued phage antibodies were incubated with 3% BSA prior to panning against PreS1. During ELISA, wells were blocked by 2% MPBS to the reduce background then false positive.

Although *in vitro* immunization of naïve B cells has been shown to result in the induction of antigen-specific of antibodies[16], some of the attempts have met with disappointment[12,17] due to target peptide antigen difference in conformation, and difficulties in creating an adequate micro-environment outside the human host. To investigate the way of obtaining high affinity genetic antibody, we constructed both large naïve and immune library in parallel conditions. Comparison of the result showed, although scFv specific against PreS1 was generated from both of the two libraries, the result of polyclonal ELISA and monoclonal ELISA demonstrated immune library is more satisfied than naïve one. Result of every round panning of immune library was 4~ 50 folds higher than that of naïve one, because of which we believe the concentration of target phage antibodies of immune library is higher than that of naïve one at the very beginning. It is reasonable to suggest that the

genes of lymphocytes were rearranged against the PreS1 during *in vitro* immunization. Finally, the result of competition ELISA confirmed the higher affinity scFv was from immune one.

In sum, a more satisfied scFv against PreS1 was generated. It would provide a more satisfied therapy of hepatitis B. Some further research, however, should be continued including site-directed mutagenesis, or mutator strain expression.

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高亲和力抗乙型肝炎病毒 PreS1 的人源单链抗体的获得：天然及免疫抗体库的对比研究

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摘要 以健康人的外周血淋巴细胞为来源，以偶联 BSA 的乙型肝炎病毒 PreS1 肽体外免疫．分别从免疫和未经免疫的淋巴细胞提取 RNA，扩增抗体基因，构建大容量天然单链抗体 (scFv) 噬菌体展示文库和体外免疫 scFv 抗体库．以 PreS1 肽进行 3 轮淘选后，抗原抗体反应结果显示，从免疫库中获得了亲和力 10^{-7}~ 10^{-8} M 的抗乙型肝炎病毒 PreS1 的单链抗体，高于天然库的结果 (10^{-6}~ 10^{-7} M)．测序结果表明两株抗体均为人抗体．为基因工程抗体用于临床治疗乙型肝炎奠定基础．同时证明淋巴细胞体外免疫方法构建的免疫抗体库优于大容量天然抗体库．

关键词 体外免疫，单链抗体，PreS1，噬菌体展示

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