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Affinity Maturation of an Anti-Hepatitis B Virus PreS1 Humanized Antibody by Phage Display

Gi-Hyeok Yang, Sun Ok Yoon, Myung Hee Jang, and Hyo Jeong Hong*

Therapeutic Antibody Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejon 305-333, Republic of Korea

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In a previous study we generated an anti-Hepatitis B Virus (HBV) preS1 humanized antibody (HzKR127) that showed *in vivo* HBV-neutralizing activity in chimpanzees. However, the antigen-binding affinity of the humanized antibody may not be sufficient for clinical use and thus affinity maturation is required for better therapeutic efficacy. In this study, phage display technique was employed to increase the affinity of HzKR127. All six amino acid residues (Glu95-Tyr96-Asp97-Glu98-Ala99-Tyr100) in the heavy (H) chain complementary-determining region 3 (HCDR3) of HzKR127 were randomized and phage-displayed single chain Fv (scFv) library was constructed. After three rounds of panning, 12 different clones exhibiting higher antigen-binding activity than the wild type ScFv were selected and their antigen-binding specificity for the preS1 confirmed. Subsequently, five ScFv clones were converted to whole IgG and subjected to affinity determination. The results showed that two clones (B3 and A19) exhibited an approximately 6 fold higher affinities than that of HzKR127. The affinity-matured humanized antibodies may be useful in anti-HBV immunotherapy.

Keywords: humanized antibody, phage display, random mutagenesis, HCDR3, affinity maturation, hepatitis B virus, preS1

Despite the availability of efficient vaccines, human hepatitis B virus (HBV) is a major causative agent for liver diseases and hepatocarcinoma in humans. HBV is a small-enveloped DNA virus containing three related surface glycoproteins called the large (L), middle (M), and small (S) proteins. These proteins are the product of a single open reading frame, which is divided into the preS1, preS2, and S regions (Heermann *et al.*, 1984). The S protein (P24) and its glycosylated form (GP27) are encoded by the S region. The M protein (GP33/GP36) contains preS2 (55 amino acids, aa) and S regions, while the L protein (P39/GP42) contains preS1 (108 or 119 aa, depending on the antigenic subtype), preS2, and S regions. In addition to the infectious viral particles, cells infected by HBV produce large quantities of non-infectious subviral particles. The S protein is found in large quantities on both particle types, whereas the L protein is preferentially localized on infectious viral particles (Stibbe and Gerlich, 1983; Heermann *et al.*, 1984). Thus, the L protein has been speculated to have a pivotal function in viral infectivity and assembly (Neurath *et al.*, 1986; Persing *et al.*, 1986). In addition, aa 21-47 of the preS1 was proposed to contain a specific binding site for human hepatocyte receptor (Pontisso *et al.*, 1989).

The envelope proteins carry epitopes involved in immune defense mechanisms (Heermann *et al.*, 1987; Milich, 1987). The preS1, preS2, and S antigens have been shown to elicit virus neutralizing and protective antibodies (Bhatnager *et al.*, 1982; Dreesman *et al.*, 1982; Itoh *et al.*, 1986; Heermann *et al.*, 1987; Milich, 1987; Neurath *et al.*, 1989). However, since the preS1 antigen is preferentially localized on infectious viral particles, anti-preS1 antibody will be beneficial in antibody-based HBV therapy. Encouragingly, antiserum to peptide (aa 21-47) of preS1 has been reported to neutralize HBV infection in chimpanzees (Neurath *et al.*, 1989). In previous studies, we generated an anti-preS1 murine monoclonal antibody KR127, which specifically recognizes aa 37-45 of the preS1, that showed HBV-neutralizing activity in an *in vitro* neutalization assay using *in vitro* infection of adult human hepatocyte culture by HBV (Maeng *et al.*, 2000; Ryu *et al.*, 2000).

Murine monoclonal antibodies (mAbs) are easy to produce, but their therapeutic use in humans is limited because of human anti-mouse antibody response during treatment (Shawler *et al.*, 1985). We developed a humanized version (HzKR127) of KR127 whose antigen-binding affinity (K_D, 2×10^{-8} M) was similar to that of KR127 and demonstrated that the humanized antibody showed *in vivo* HBV-neutralizing activity in chimpanzees (Hong *et al.*, 2004). However, the antigen-binding affinity of the humanized antibody is not sufficient for clinical use and therefore affinity maturation is necessary before positive therapeutic efficacy can be expected.

Phage display of antibody libraries is a powerful tool for the isolation of mAbs against specific antigens (Burton *et al.*, 1991; Marks *et al.*, 1991; Winter *et al.*, 1994). Large repertoires of antibodies can be displayed on the surface of filamentous phage particles, and antibodies with the desired specificity can be isolated by panning against the antigen of interest. ScFv (single-chain Fv antibody) consist of the variable (V) domains of immunoglobulin heavy (H) and light (L) chains connected by flexible peptide linker, and can be

* To whom correspondence should be addressed.
(Tel) 82-42-869-4122; (Fax) 82-42-860-4597
(E-mail) hjhong@kribb.re.kr

used for phage display to substitute for large intact immunoglobulin molecules (Griffiths *et al.*, 1994).

Within each V domain are three regions that are hypervariable in sequence and that form loops at the ends of rigid β-sheets. The hypervariable loops are primarily responsible for antigen recognition and are referred to as complementarity determining regions (CDRs), while the remaining V region amino acids act as a scaffold to support the loops and are referred to as framework residues (FR). Among the CDRs, the heavy chain CDR3 (HCDR3) are the most variable as a result of somatic mutations and the recombination of the variable (V), diversity (D) and joining (J) segments, while concomitantly essential in determining the specificity and affinity of an antibody. Therefore, the HCDR3 seems to be an excellent target for mutagenesis for affinity maturation of antibody.

In the present study, we obtained and characterized affinity-matured variants of HzKR127 by phage display of HCDR3-randomized ScFv library.

Materials and Methods

Bacterial strain

The *Escherichia coli strain* TG1 [Δ(*lac-pro*), *supE, thi, hsdD5/F, traD36, proAB, LacI^q, lac ZΔM15*] was used as the bacterial host for the preparation of phagemids and as the host for bacteriophage M13KO7. *E. coli* HB2151 [Δ(*lac-pro*), *ara, nal^r, thi/F proAB, LacI^q, lacZΔM15*] was used for expression of soluble scFv. *E. coli* DH5α (*supE44, Δ lacU169, hsdR17, recA1, endA1, gyrA96, thi-1, relA1*) was used for general subcloning procedure.

Construction of HzKR127 ScFv

In order to construct HzKR127 ScFv, the VH and VL genes were amplified by PCR from pCMV-HKR127(I) and pKC-dhfr-HKR127 plasmids, respectively (Hong *et al.*, 2004). The HF primer; 5'-TTTGGCCCAGCCGGCCATGGCCCA GGTCCAGCTGGTGC-3'; *Sfi*I site is underlined and HR primer; 5'-AGAGCCACCTCCGCCTGAACCGCCTCCACC TGAAGAGACAGTG-3'; linker site is underlined, were used to amplify VH gene. The LR primer; 5'-CCTGCGGCCGC CCGTTTGATTTCC-3'; *Not*I site is underlined, and LF primer; 5'-GGCGGAGGTGGCTCTGGCGGTGGCGGATCG GATATCGTGATG-3'; linker site is indicated by bold type and overlapping site is underlined, were used to amplify VL gene. The ScFv was assembled by splice overlap extension PCR (Horton *et al.*, 1989). The resulting PCR products were introduced into pCANTAB5E vector (Amersham Pharmacia Biotech, Sweden) by using *Sfi*I and *Not*I sites to give pCANTAB5E-HzKR127 (Fig. 1).

Production of phage-displayed ScFv and soluble ScFv

To produce phage-displayed ScFv, TG1 cells harboring phagemid were grown to an $O.D._{600}$ of 0.5 in 2YT containing 100 μg/ml ampicillin and 2% glucose (2YTAG) at 37°C. Following, 1×10^{10} plaque forming units of helper phage M13KO7 was added to the culture. After incubation for 30 min at 37°C without agitation and further 30 min at 37°C with agitation, cells were centrifuged for 10 min at 3,000×g, and the cells were resuspended in the same volume of 2YT

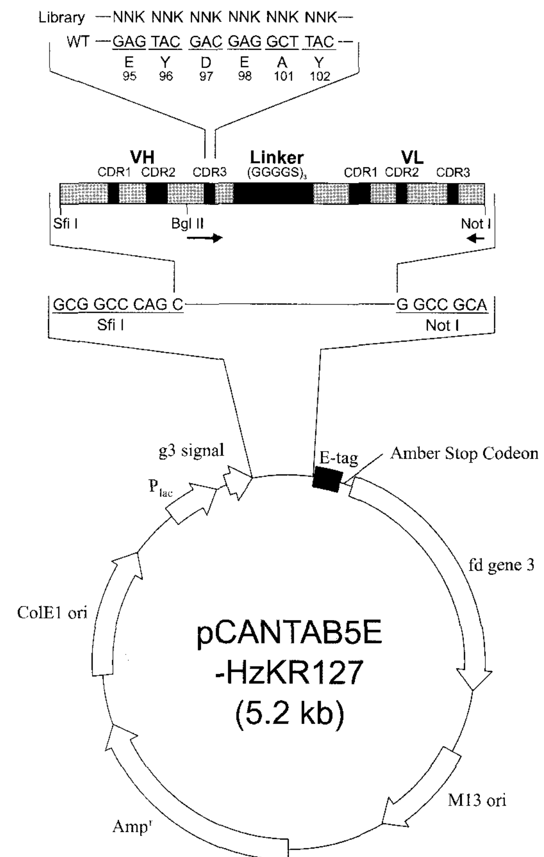


Fig. 1. Construction of HzKR127 scFv expression vector and schematic representation of HCDR3 randomized library. Two arrows indicate the primers (BglII primer and LR primer) to construct HCDR3-randomized library.

containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. The culture was incubated at 30°C overnight with agitation.

The expression vector pCANTAB5E contains a *lac* promoter and a gene 3 leader sequence upstream of the VH-$(GGGGS)_3$-VL insert, which is then followed by E-tags, an amber stop codon and the gene 3 encoding the pIII phage coat protein. Thus, in a suitable non-suppressor strain (HB2151), addition of isopropyl-thio-β-D-galactoside (IPTG) induces only ScFv and not ScFv-pIII fusion expression. In contrast, in suppressor strain (TG1), addition of isopropyl-thio-β-D-galactoside (IPTG) induces not only ScFv and but-also ScFv-pIII fusion expression. ScFvs are then directed to the periplasm while some of them diffuse out into the supernatant. For soluble ScFv production, TG1 or HB2151 cells harboring the phagimid pCANTAB5E-HzKR127 were grown to an $O.D._{600}$ of 0.5 in 2YTAG at 37°C and induced with 1 mM IPTG. The induced cells were grown at 30°C for 4 h or overnight. The cells were harvested by centrifugation and the pellet was resuspended in ice-cold 1× periplasmic extraction buffer (0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 mM sucrose, pH 8.0). Subsequently, 1.5 volumes of 1/5 diluted periplasmic extraction buffer was added to the mixture and incubated on ice for 30 min. After centrifugation, the resulting supernatants containing the soluble ScFv were store at -20°C until used.

Construction of phage-displayed ScFv carrying HCDR3 mutants

PCR was performed with pCANTAB5E-HzKR127 as the template and with LR primer and *Bgl*II primer; 5'-CTGA **GATCT**GAGGACACGGCGGTCTATTTCTGTGCAAGAN (G/A/T/C)NK(G/T)NNKNNKNNKNNKNNKTGGGGCCA AGGGACTCTGGTC-3' randomized HCDR3 nucleotide sequence was underlined and *Bgl*II site was indicated by bold type which includes a part of FR3 sequence, HCDR3 and a part of FR4 sequence in the VH region. The resulting PCR product was inserted into pCANTAB5E-HzKR127 by using *Bgl*II and *Not*I sites (Fig. 1). Three electroporations allowed us to obtain a library of 3.0×10^8 clones which is larger than the calculated library size (6.4×10^7).

Panning of phage-displayed ScFv against preS1 antigen

Streptavidin-coated Dynabead (Dynal, Norway) was incubated with MPBS (2% skim milk in PBS, pH 7.4) for 2 h at 25°C with agitation. Freshly prepared phage-ScFv were also pre-incubated with MPBS for 15 min at 25°C with agitation. The phage-ScFv was then incubated with biotinylated GST-preS1 (1 μg, 0.1 μg, and 0.01 μg for the first, second, and third panning, respectively) for 1 h at 25°C with agitation and mixed with the Streptavidin-coated Dynabead for 15 min at 25°C. The beads were washed 7 times with PBST (PBS with 0.05 % Tween 20) then three times with PBS for the first panning and 17 and 27 times with PBST then three times with PBS for the second and third panning, respectively. Bound phages were eluted with 200 μl of 100 mM-triethylamine for 10 min, followed by neutralization with 50 μl of 1 M Tris-HCl (pH 7.4) and then used to infect TG1 cells. Colonies were obtained by plating on 2 YTAG. The output phages from each round were tested for antigen binding activity by an indirect ELISA using the GST-preS1 or preS1 peptide (aa 1-56) (Kim and Hong, 1995).

Initial characterization of mutants

After the third round of panning, 250 clones were randomly picked and cultured in 2YTA in microtiter plates overnight at 37°C. The clones were then subcultured (20 μl in 200 μl) and grown for 3 h at which point helper phage and kanamycin were added and the cultivation continued overnight. Cells were removed by centrifugation and the phages in the supernatants were added to the microtiter plates coated with 1 μg/ml of the preS1 peptide and blocked with MTBS. Bound phages were detected with a horseradish peroxidase-conjugated anti-M13 antibody (Amersham Pharmacia Biotech). To confirm binding activity, periplasmic extracts containing scFv were tested by an indirect ELISA, as described above. Clones showing the highest binding activity were sequenced. The binding activities of the mutants for GST-preS1 (1-56, *adr* subtype) and GST-preS1 (1-45, *ayw* type) (Maeng *et al.*, 2000) were also tested by Western blot analysis using anti-E tag antibody (Pharmacia).

Construction of mutant heavy chain expression plasmids

For convenient construction of mutant H chain expression plasmids, Apa I site in the pcDNA (Invitrogen) was destroyed to yield pcDdA. Then, the heavy chain gene of wild type HzKR127 was inserted into the EcoRI-NotI sites of pcDdA to yield pcDdA-HzKR127. To construct the mutant heavy chain expression plasmids, the VHs were amplified by splice overlap extension PCR (Horton *et al.*, 1989) using primers containing *Eco*RI or *Apa*I site, and after digestion were subcloned into the EcoRI-ApaI sites of pcDdA-HzKR127.

Expression of whole IgG in COS-7 cells

The resulting mutant heavy chain expression plasmid and the wild type humanized light chain expression plasmid pKC-dhfr-HKR127 were co-transfected into COS-7 cells using LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. The concentrations of the humanized whole IgG in the culture supernatants were determined by sandwich ELISA using goat anti-human IgG (Sigma) and horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fc-specific) (Sigma). Human IgG (Sigma) was used as the standard protein for quantification. For indirect ELISA, the antibody samples were added to 96 well microtiter plates that had been previously coated with GST-preS1 (1-56) at 37°C for 2 h and blocked with 2% bovine serum albumin (BSA) at 37°C for 1 h. After washing, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fc-specific) (Sigma) was added to each well and incubated at 37°C for 1 h.

Affinity determination

Affinities of the mutant humanized antibodies for GST-preS1 (aa 1-56) were determined by competition ELISA, as described previously (Hong *et al.*, 2004). Solutions containing 3 ng of the respective antibodies and various concentrations ($5\times10^{-12}\sim5\times10^{-7}$ M) of the preS1 antigen as a competing antigen were incubated at 37°C for 2 h and then added to separate wells that had been previously coated with 200 ng antigen. An indirect ELISA detected the bound antibody, while apparent affinity was determined using a Klotz plot (Oh *et al.*, 2003).

Results

Construction of HzKR127 ScFv expression vector

To construct phage-displayed HCDR3 mutant libraryScFv of wild type HzKR127 was constructed and subcloned into

Table 1. Enrichment of PreS1-Specific phages during three rounds of panning

Round	Capturing Ag (ug/ml)	Washing frequency	Input titre (cfu[a])	Output titre (cfu)	Ratio (Output/Input)
1	10	10×	3×10^{10}	5×10^4	1.7×10^{-6}
2	1	20×	2×10^9	5×10^3	2.5×10^{-6}
3	0.1	30×	2×10^{10}	1×10^5	5.0×10^{-6}

[a] cfu means colony forming unit.

pCANTAB5E vector to yield pCANTAB5E-HzKR127 (Fig. 1). Phage-displayed HzKR127 ScFv, which was produced in *E. coli* TG1 cells, was capable of binding to its target antigen, preS1(1-56) peptide (data not shown). Furthermore, soluble ScFv expressed in *E. coli* HB2151 or TG1 cells retained the antigen binding activity in indirect ELISA (data not shown).

Construction and panning of phage displayed-HCDR3 mutant library

Mutagenic primer was designed to randomize the six amino acid residues of HCDR3 and used to synthesize mutants by PCR. The PCR products were subcloned into the *Bgl*II-*Not*I sites of pCANTAB5E-HzKR127. The HCDR3-randomized ScFv library was transformed into TG1 by several electroporations. The constructed library had a size of 3×10^8 which is 4-5 times more than all possible amino acid combinations (6.4×10^7).

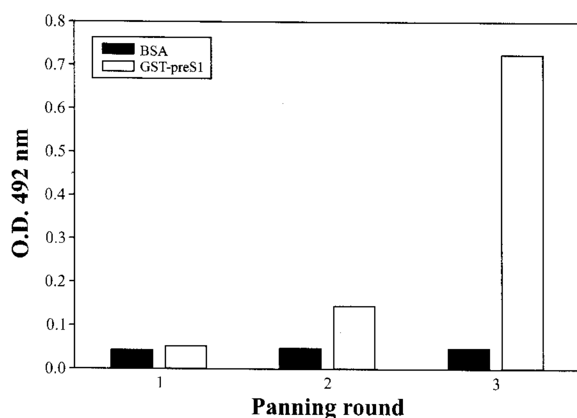


Fig. 2. Phage ELISA showing the enrichment of ScFv-phages specific for preS1 during the panning cycles. After each round of panning, the output phages (2×10^{10}) were added to microtiter wells coated with 200 ng BSA (■) or 100 ng preS1(1-56) (□). Bound phages were detected by horseradish peroxidase (HRP)-conjugated anti-M13 antibody.

To screen the phage-displayed ScFv library, liquid phase selection using the biotinylated preS1(1-56) as a capture antigen and Streptavidin-coated magnetic bead was performed. This was because the preS1 peptide attached to microtiter plate in solid phase selection may cause structural alteration. Before panning, it was confirmed that the antibody-binding activity of biotinylated preS1(1-56) was the same as that of unbiotinylated preS1(1-56) by indirect ELISA (data not shown). Three rounds of panning were performed by decreasing the concentration of antigen and increasing the number of washing (Table 1). Antigen-binding activity of the pooled phages from each round of the panning was analyzed by ELISA, which showed successful enrichment of preS1-specific binders during the panning cycles (Fig. 2).

After the third round of panning, 250 colonies were randomly picked, subjected to production of phage-ScFv and soluble ScFv, and tested for their antigen-binding activities by indirect ELISA using GST-preS1(1-56). Among them, 28 clones showed higher antigen-binding activity compared with wild type ScFv, while 12 were identified as different clones by DNA sequence analysis (Table 2). Sequence analysis of the different clones revealed that tyrosine at position 96 was mostly changed to glycine, while the other positions in the HCDR3 showed limited sequence variations.

Characterization of selected ScFv clones

Western blot analysis was performed to characterize the antigen-binding activity and antigen-binding specificity of the selected 12 mutant ScFvs using *E. coli* cell lysates containing GST-preS1(1-56, *adr* subtype) and GST-preS1(1-45, *ayw* subtype). Figure 3 showed that there was only one specific bands corresponding to GST-preS1 fusion protein among uncountable

Table 2. HCDR3 mutants isolated from phage display of antibody library

Clone[a]	Selection frequency	Amino acid[b]						Binding reactivity[c]		Affinity of whole IgG
		95	96	97	98	101	102	Adr(1-56)	Ayw(1-45)	
WT	(1)	Glu	Tyr	Asp	Glu	Ala	Tyr	++	++	2.5×10^{-8}
B3	(5)	Ala	Gly	Leu	Gln	Gly	Gln	++	++	4.0×10^{-9}
B84	(1)	Asn	Gly	Gln	Lys	Glu	Val	++	++	
C20	(4)	Asn	Gly	Arg	Gln	Glu	Val	++	++	
C55	(2)	Asn	Gly	Ala	Gln	Glu	Val	++	++	
C75	(8)	Thr	Gly	Arg	Thr	Glu	Lys	++	++	1.6×10^{-7}
C78	(1)	Glu	Gly	Arg	Val	Glu	Arg	++	++	9.0×10^{-8}
A18	(1)	Gly	Gly	Leu	Gln	Gly	Lys	++	++	
A19	(1)	Asn	Ala	Leu	Ala	Gly	Gln	++	++	4.4×10^{-9}
A21	(1)	Ala	Gly	Leu	Arg	Gly	Gln	++	++	9.0×10^{-9}
A26	(1)	Gly	Gly	Leu	Met	Gly	Gln	++	++	
A33	(1)	Gly	Gly	Leu	Gln	Gly	Arg	++	++	
A57	(1)	Pro	Gly	Leu	Glu	Gly	Arg	++	++	

[a] HCDR3 mutants showing higher binding activity compared to ScFv
[b] Numbering is according to Kabat *et al.* (1991)
[c] Based on Western blot analysis using *E. coli* cell lysates containing GST-preS1(1-56, *adr* type) or GST-preS1(1-45, *ayw* type)

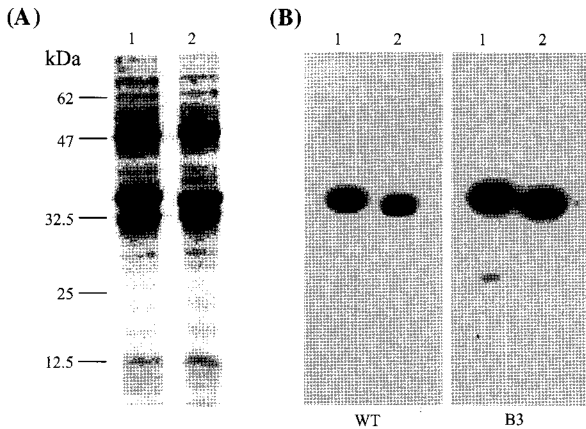


Fig. 3. Western blot analysis of soluble ScFv of HCDR3 mutant (B3) and wild type for antigen-binding specificity. Equal amount of *E. coli* cell lysates containing GST-preS1(1-56, *adr* type) (lane 1) or GST-preS1(1-45, *ayw* type) (lane 2) were subjected to SDS-PAGE (A) and Western blot analysis using anti-E tag antibody (B).

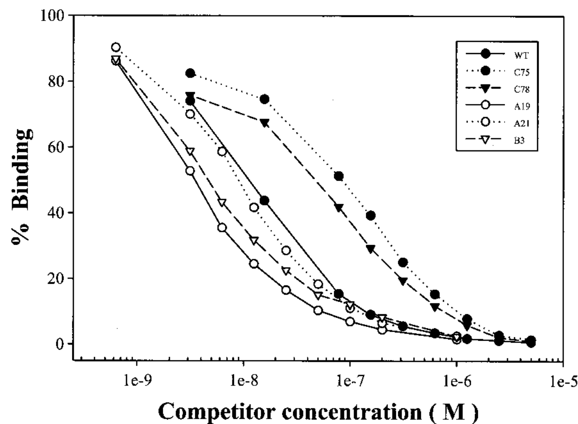


Fig. 4. Competition ELISA of whole IgG form of selected mutants to determine antigen-binding affinities for preS1.

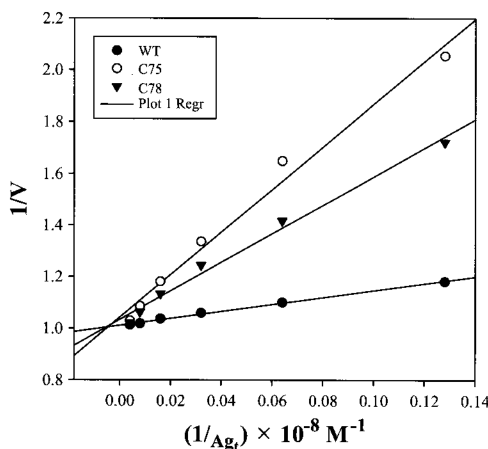
proteins from *E. coli*, suggesting that the selected mutant ScFvs exclusively recognized the preS1 epitope. All 12 mutants showed reactivity to the preS1 (Table 2) and did not exhibit cross reactivity with BSA or skim-milk proteins (data not shown).

Conversion of ScFv to whole IgG and affinity determination of selected clones

For precise characterization of the selected mutants, the ScFv was converted to whole IgG (γ1, κ) with human heavy and κ light chain constant regions. Five clones (C75, C78, A19, A21, and B3) were expressed in COS7 cells and antigen binding affinities of assembled whole humanized antibodies were determined by competition ELISA. As the results show, B3, A19, and A21 exhibited approximately 6.3-, 5.7-, and 2.8-fold higher antigen-binding affinities than wild type antibody, while C75 and C78 mutants showed lower affinities (Fig. 4). Apparent affinity was determined using a Klotz plot (Fig. 5) and summarized in Table 2.

Discussion

To date, the phage display technique has been used as a promising technique to mature antibody affinity through mimicking *in vivo* selection of antibodies in the human immune system. HCDR3 is far more variable than HCDR1 and HCDR2 in length, sequence, and structure, and plays a crucial role in antigen recognition. It has been shown that somatic mutation of HCDR3 could allow antibodies to achieve high affinity and antigen specificity (Xu and Davis, 2000). Affinity maturation by randomization of HCDR3 in combination with phage display has become a relatively straightforward procedure that has been employed by a number of research groups (Komissarov *et al.*, 1997; Krykbaev *et al.*, 2001). In this study, we constructed HCDR3-randomized library in scFv-phage display format (Fig. 1) while several mutants with higher affinity, compared to wild type, were selected through panning (Table 2). Conversion of the ScFv clones to whole IgG and affinity determination showed that two clones (B3 and A19) exhibited approximately 6.3- and



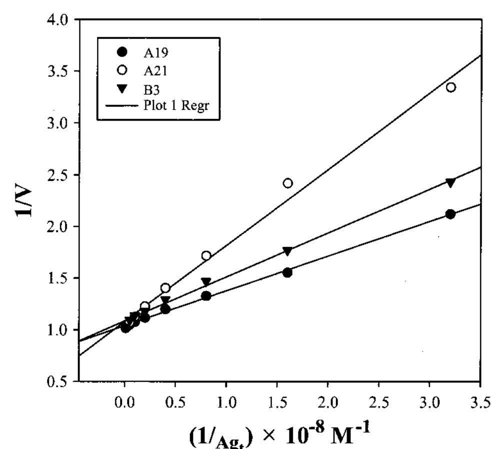


Fig. 5. Klotz plots to determine the affinity constants of selected mutants.

5.7-fold higher affinities compared with HzKR127, respectively (Table 2). The results demonstrate that the HCDR3 randomization and phage display of mutant antibody library is useful in affinity maturation of an antibody. The affinity-matured humanized antibody may neutralize HBV infection more effectively than HzKR127 and thus be useful in the prophylaxis of HBV infection.

Acknowledgements

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