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Enhanced antibody affinity to Japanese encephalitis virus E protein by phage display

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Abstract

Obtaining antibodies with high affinity and specificity against antigens are required for the development of therapeutic and diagnostic antibodies. In this study, the contributions to binding affinity in the CDR2 and CDR3 regions of two monoclonal antibodies E3.3 and 2H2 were investigated by random mutagenesis in a phage-display synthetic oligonucleotide library. One high-affinity clone (CDR3-30) was obtained with a 3-fold increase of the dissociation constant, resulting from the changes in amino acids at residues 95, 97, and 98 in the CDRH3 region. Analysis of the predicted structure by modeling suggested that the contributions of mutated residues in the CDR3 region to the binding affinity involved not only complementarity between antigen and CDR3, but also interaction between heavy and light chains. The information gained from this study may benefit the design of vaccines and therapeutic antibodies against Japanese encephalitis virus infection.

Keywords: Phage; Fab; Antibody; Japanese encephalitis virus

Antibody molecules are roughly Y-shaped and comprise two heavy (H) and two light (L) chains; the fragment antigen binding (Fab) fragment contains the variable region (V_H) and the first constant region (C_H1) of the H chain, which are paired with the variable region (V_L) and the constant region (C_L) of the L chain. Recently, recombinant Fab (rFab) fragments have been effectively generated by molecular cloning and gene manipulation in *Escherichia coli*. One of the most notable systems is the pComb3H phage vector, in which the pIII gene in the M13 filamentous phage was genetically modified to display the antibody fragments [1]. Since antibody-mediated virus neutralization is very important to vaccine design and anti-viral therapies, numerous neutralizing rFab antibodies have been successfully generated using the pComb3H vector, including some against dengue virus [2], hepatitis C virus [3], and West Nile virus [4].

In this study, the contributions to binding affinity of residues in the CDR2 and CDR3 regions were investigated by random mutagenesis in a phage-display library. Two recombinant Fab fragments, CDR2-26 and CDR3-30, were obtained to bind Japanese encephalitis virus (JEV) *in vitro,* as the high affinity of their parental murine monoclonal antibodies (mAbs) E3.3 and 2H2. The nucleotide sequences that encode the CDR in these two high-affinity Fabs were determined and the chimeric antibodies were constructed and expressed in CHO cells. The specificity to domain III of JEV envelope protein was characterized using antigen-based enzyme-linked immunosorbent assay (ELISA) and the dissociation constant was determined by the Friguet method [5]. Analysis of the predicted structure of Fab by modeling raised the possibility that the contributions of mutated residues in the CDR3 region to the binding affinity involved not only complementarity between antigen and CDR3, but also interaction between heavy and light chains. The information obtained in this study may support the design of vaccines and therapeutic antibodies against JEV infection.

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Materials and methods

Cloning and sequencing of two recombinant Fab fragments from hybridoma cells. Two hybridoma cell lines producing neutralizing mAbs E3.3 (IgG_{2a}/κ) and 2H2 (IgG_3/κ) [6] were grown in IMDM (Invitrogen) with 10% FBS and 100 g/ml P/S. The genes of the heavy- and light-chain variable region in mAbs E3.3 and 2H2 were amplified by RT-PCR using a set of primers, as described previously [7,8], and then cloned into the phagemid expression vector pComb3H [1]. The nucleotide sequences of the variable regions of E3.3 and 2H2 Fab fragments were sequenced and the complementarity-determining regions (CDRs), according to the Kabat definition (http://www.bioinf.org.uk/abs) [9], were analyzed.

CDR-based mutagenesis and DNA amplification. The five amino acids of the CDR2 were randomized and the four amino acids of the CDR3 in the heavy-chain variable region were introduced by the PCR. The sequences of the primers for the mutagenesis of CDRH2 and CDRH3 regions are shown in Table 1. The usage of a restricted number of nucleotides at the third codon position avoids stop codons TGA and TAA.

Primer-induced mutagenesis was carried out by a two-step PCR. The first PCR was carried out with 2H2-f and CDR2-r or CDR3-r, and with primers 2H2-r and CDR2-f or CDR3-f. The second PCR product with two fragments was cloned into phagemid expression vector pComb3H to construct CDR-based shuffled libraries.

Construction of recombinant phage libraries and biopanning. Ligation products were mixed and electroporated into electro-competent cells, and then recovered in SOC medium to generate a large and highly diverse library of fused antibody fragments. Following the infection of VCS M13 helper phage (10^{12}–10^{13} pfu/ml), the culture was pelleted and resuspended with SB for the expression of recombinant Fab fragments. The titer of the prepared recombinant phage pool was measured by infecting ER2738 and plating it on ampicillin plates. The phage libraries were subjected to four-round biopanning for screening and enriching clones. The neutralized elute was then utilized to infect growing ER2738 to amplify selected phage and the amplified phage was used directly in subsequent panning.

SDS–PAGE, Western blotting. Purified proteins were separated by electrophoresis on an SDS–PAGE gel and stained with Coomassie brilliant blue. The antibody fragments expressed in CHO cells were further electrotransferred onto nitrocellulose membranes (Millipore) for Western blotting. The membrane was blocked and probed with horseradish peroxidase (HRP)-conjugated anti-mouse IgG-Fab or anti-human IgG-Fc antibodies. The membranes were then washed three times and immune complexes were visualized with ECL (Pierce).

Expression and purification of the chimeric full-length IgG in CHO cells. The expression vector pFab CMV was used to express chimeric full-length IgG in CHO cells. After 4-day transfection with *BsiWI*-treated linear vectors in the presence of Exgen 500 (Fermentas), the medium was harvested and applied to a column with protein A–Sepharose (GE). The column was then washed and eluted with an elution buffer gradient.

Determination of dissociation constant of antibodies. The dissociation constant, K_D, of chimeric antibodies and domain III of JEV E protein was determined in solution according to the ELISA method that was described by Friguet et al. [5]. The formula $A_0/(A_0 - A) = K_D/[Ag] + 1$ was utilized to determine the KD of chimeric antibodies.

Results

Determination of sequence of E3.3 and 2H2 JEV-neutralizing mAbs

The Fabs of two JEV-neutralizing mAbs E3.3 [7] and 2H2 [8] were cloned into the pCom3H vector and their encoding sequences were determined using Kabat approach [9]. The number of differences between the amino acid sequences of E3.3 and 2H2 were five in CDRH2, eight in FRH3, four in CDRH3, one in FRL1, one in CDRL1, one in FRL2, one in CDRL2, one in FRL3, and one in FRL3 (data not shown). We have previously utilized site-directed mutagenesis [7] or knowledge-based homologous modeling [8] to demonstrate the combining sites in the CDRH2 and CDRH3 regions. This work further investigated the contributions to the binding affinity of residues 53 (Y for 2H2; S for E3.3), 56 (G for 2H2; H for E3.3), 57 (N for 2H2, K for E3.3), 61 (A for 2H2, E for E3.3), and 62 (K for 2H2, M for E3.3) in the CDRH2 region and residues 95 (W for 2H2, L for E3.3), 96 (G for 2H2, S for E3.3), 97 (N for 2H2, Y for E3.3), and 98 (L for 2H2, Y for E3.3) in the CDRH3 region.

Selection of high-affinity Fab clones using random mutagenesis with phage-display synthetic oligonucleotide libraries

Phage-display Fab libraries were generated by introducing synthetic amino-acid diversity at the specified five positions in CDRH2 and the specified three positions in CDRH3. Random mutagenesis of phage-display synthetic oligonucleotides was performed using the primers designed with the triplet NNS at the specified residues (Table 1). Following two-step PCR, fragments with random residues were cloned into the pComb3H vector to generate phage-display Fab libraries. The sizes of the CDR2 and CDR3 libraries were around 2.51×10^8 and 2.05×10^8, respectively. Phage ELISA was applied to monitor each round of biopanning and thus confirm the augmentation of binding affinity of the recombinant phage libraries (data not shown).

Table 1
Primer sequences designed for heavy-chain CDR2 and CDR3 random mutagenesis in phage-display synthetic oligonucleotides libraries

Primers	Sequence (5′ → 3′)
2H2-f	GCTGCTCGAGTCTGGGACTGAA
CDR2-r	GGCCTTGCTCTTAAASNNSNNATTGTAGTTSNNSNNACCATTSNNAGGATTAATATTTCCAATCCACTC
CDR2-f	TTTAAGAGCAAGGCCACACTGA
CDR3-r	GCCCCAGACATCGAAGTACCASNNSNNSNNSNNTCTTGCACAATAATAGACCGAAG
CDR3-f	TACTTCGATGTCTGGGGCACA
2H2-r	GACTAGTATGATGATGATGATGCTTGGGTATTCTAGGCTCGATT

(S = G/C, N = A/T/C/G).

Three high-affinity clones (CDR2-26, CDR2-109, and CDR2-314) from CDR2 libraries and two high-affinity clones (CDR3-30 and CDR3-313) from CDR3 libraries were obtained in a screen of 772 clones after biopanning (data not shown). These five clones were subjected to sequence analysis, and only clone CDR2-26 and clone CDR3-30 had different amino acid sequences from that of the wild-type 2H2 Fab (Table 2). Clone CDR2-26 exhibited changes in amino acids at residues 53 (Y → L), 56 (G → H), 57 (N → V), 61 (A → P), and 62 (K → R) in the CDRH2 region. Clone CDR3-30 exhibited changes in amino acids at residues 95 (W → S), 97 (N → C), and 98 (L → P) in the CDRH3 region.

Production of full-length IgG antibodies in CHO cells

Two high-affinity variants (CDR2-26 and CDR3-30) and parental 2H2 were constructed into an expression vector pFab CMV [2]. These three vectors were transiently transfected into CHO-K1 cells and the secretary antibodies were harvested 4-day post-transfection. The expression of the monomer (heavy chain or light chain) and the full-length chimeric mouse–human IgG was investigated on SDS–PAGE under reducing and non-reducing conditions (Fig. 1A). Bands that are consistent with a glycosylated heavy chain (50 kDa), light chain (25 kDa), and whole antibody (150 kDa) were observed from these three antibodies.

Dissociation constant of chimeric IgG

The dissociation constant, K_D, was determined by the Friguet method to estimate the affinity of antibody–antigen complexes in solution [5]. As calculated from the formula $A_0/(A_0 - A) = K_D/[Ag] + 1$, the slopes of the straight lines in Fig. 1B, which equal the K_D values of IgG CDR2-26, CDR3-30, and 2H2 in solution were 1177.9 pM ($R^2 = 0.996$), 41.5 pM ($R^2 = 0.945$), and 174.8 pM ($R^2 = 0.987$), respectively. This result demonstrated that antibody

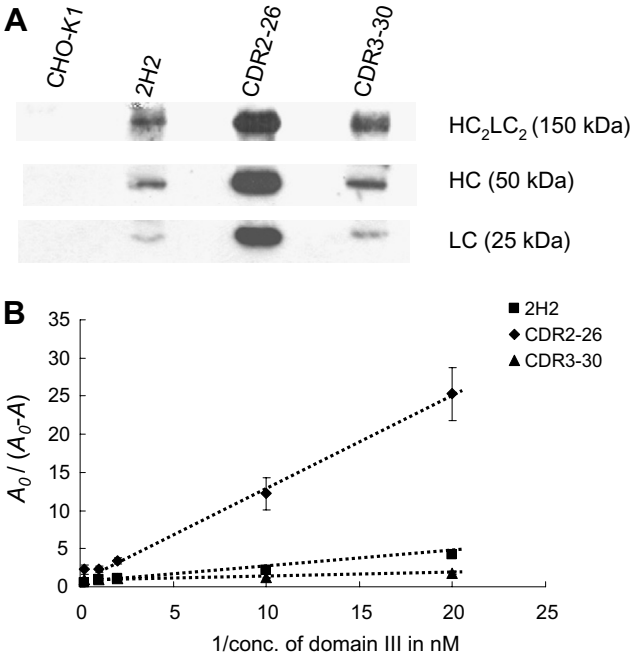

Fig. 1. Characterization of full-length chimeric antibodies expressed in CHO-K1 cells. (A) Western blot analysis of recombinant 2H2, CDR2-20, and CDR3-30 antibodies expressed in CHO-K1 cells. SDS–PAGE separation under non-reducing (full-length antibody, HC_2LC_2) and reducing (HC and LC) conditions of a whole antibody. (B) Determination of dissociation constant of JEV domain III-2H2 antibody, JEV domain III-CDR2-26 antibody and JEV domain III-CDR3-30 antibody complexes. Collected data were fitted using the formula $A_0/(A_0 - A) = K_D/[Ag] + 1$. A_0 is the absorbance when the antibody was incubated without an antigen; A is the absorbance that corresponds to free antibody after incubation with antigen, and [Ag] is the concentration of free antigen, which equals that of the antigen used in the experiment.

Table 2
Amino acid sequences of CDR regions in five clones obtained in four rounds of biopannings

	FR1	CDR1	FR2	CDR2
2H2	K V Q L L E S G T E L V K P G A S V K L S C K A S G Y T F T	N Y W M H	W V K Q R P G Q G L E W I G	N I N P Y N G G N N Y N A K F K S
E3.3	- -	- - - - -	- - - - - - - - - - - - - - -	- - - - S - - H K - - - E M - - -
CDR2-26*	- -	- - - - -	- - - - - - - - - - - - - - -	- - - - L - - H V - - - P R - - -
CDR2-109	- -	- - - - -	- - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
CDR2-314	- -	- - - - -	- - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
CDR3-30*	- -	- - - - -	- - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
CDR3-312	- -	- - - - -	- - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -

	FR3	CDR3	FR4
2H2	K A T L T V D T S S S T A F M L L S S L T S E D S S V Y Y C A	R W G N L W Y F D V	W G T G T T V T V S S
E3.3	Q - - - - - - K - - N - - Y - Q - - - - - - - - - A - - F - V	- L S Y Y - - - - -	- - - - - - - - - - -
CDR2-26*	- -	- - - - - - - - - -	- - - - - - - - - - -
CDR2-109	- -	- - - - - - - - - -	- - - - - - - - - - -
CDR2-314	- -	- - - - - - - - - -	- - - - - - - - - - -
CDR3-30*	- -	- S - C P - - - - -	- - - - - - - - - - -
CDR3-312	- -	- - - - - - - - - -	- - - - - - - - - - -

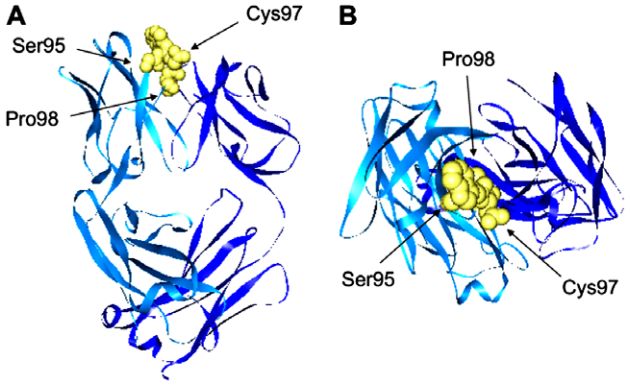

Fig. 2. Structural analysis of CDR3-30 Fab region by homology modeling. (A) View perpendicular to the 2-fold rotation axis between the heavy- and the light-chain heterodimer interface of CDR3-30. (B) View down the interface along the 2-fold rotation axis between the heavy- and the light-chain heterodimer. The locations of mutated residues Ser 95, Cys 97, and Pro 98 are presented in light gray in both figures.

CDR3-30 has a higher binding affinity than the parental 2H2 antibody.

Molecular modeling of Fab 2H2 structure

Computer modeling of the Fab was performed using the amino acid sequences of CDR3-30 to investigate the importance of the mutated residues (Ser 95, Cys 97, and Pro 98) in the variable region of IgG CDR3-30. The three-dimensional structure of Fab CDR3-30 was constructed based on the crystal structures of a Fab fragment of mAb 8f5 (PDB code, 1A3R). Fig. 2 shows the relative positions of the mutated Ser 95, Cys 97, and Pro 98 residues in the interface of the Fab dimer (heavy and light chains). Based on our previous docking model [8], residues 95 and 97 at CDR3 in the heavy chain were in the majority of the combining sites, in close contact with loop DxE and loop BCx of JEV domain III. The distinctive cyclic structure of the side chain of proline at residue 98 may change the relative position of the Fab dimer and indirectly influence the interface surfaces between the antigen and CDR3 of the heavy chain.

Discussion

Obtaining antibodies with high affinity and specificity against antigens are required for the development of therapeutic and diagnostic antibodies. In this study, the contributions to binding affinity of various residues in two high-affinity antibodies, 2H2 and E3.3, were investigated by random mutagenesis. Five high-affinity clones obtained in a phage-displayed library were analyzed and the same residues as those of parental 2H2 were found in three clones (CDR2-109, CDR2-314, and CDR3-312), illuminating the maturation of the binding affinity of antibody 2H2. Two variants, CDR2-26 and CDR3-30, with different amino acid sequences were further analyzed.

V_H and C_H1 regions of CDR2-26, CDR3-30, and parental 2H2 were constructed with entire human hinge C_H2 and C_H3 sequences to characterize these two high-affinity variants. Antibody CDR3-30 was confirmed to have a higher binding affinity than the parental 2H2 antibody (with a dissociation constant of 41.5 pM). Although high affinity does not always guarantee *in vivo* viral neutralization, this feature of recombinant antibody is the basic requirement of a neutralizing antibody. Since the occupancy of binding sites on the virion surface, and the inhibition of virus attachment, are directly correlated with neutralization capacity, high affinity to antigen in the physiological microenvironment is crucial for a neutralizing antibody. Therefore, the high-affinity antibody CDR3-30 is a potential molecule for therapeutic applications.

Three Fab clones were obtained in the CDR2 phage library through four rounds of biopannings. CDR2-109 and CDR2-314 have the same residue as their parental Fab 2H2; the five target residues in the CDRH2 region of the high-affinity clone CDR2-26 were altered but the binding affinity was lower than that of their parental antibody 2H2. This result is also supported by our previous finding that none of the residues 53, 56, 57, 61, and 62 were randomly changed in CDR2 phage library was located at the predicted combining sites in the docking model of Fab 2H2 and antigen E domain III [8]. On the contrary, Fab CDR3-30, with a higher affinity, included mutated residues 95, 97, and 98 in the CDR3 region, which were located at the combining sites in the docking model.

In this study, contributions to the binding affinity of residues were examined by random mutagenesis in phage-display synthetic oligonucleotide libraries. Two rFab fragments, CDR2-26 and CDR3-30, containing different amino acid sequences were obtained to bind antigens with increased affinities. Mouse–human chimeric antibodies CDR2-26 and CDR3-30 were expressed in CHO cells, and their specific binding affinity to domain III of JEV E protein was characterized using ELISA. One high-affinity clone (CDR3-30) was obtained with a 3-fold increase of the dissociation constant, resulting from the changes in amino acids at residues 95, 97, and 98 in the CDRH3 region. The structure of Fab CDR3-30 predicted by modeling and docking model provide a possible explanation of the contribution of mutated residues in the CDR3 region to the binding affinity involved not only complementarity between antigen and CDR3, but also interaction between heavy and light chains. The information gained from this study may benefit the design of vaccines and therapeutic antibodies against JEV infection.

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References

[1] C.F. Barbas 3rd, J.E. Crowe Jr., D. Cababa, T.M. Jones, S.L. Zebedee, B.R. Murphy, R.M. Chanock, D.R. Burton, Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus F glycoprotein and neutralize infectivity, Proc. Natl. Acad. Sci. USA 89 (1992) 10164–10168.

[2] R. Men, T. Yamashiro, A.P. Goncalvez, C. Wernly, D.J. Schofield, S.U. Emerson, R.H. Purcell, C.J. Lai, Identification of chimpanzee Fab fragments by repertoire cloning and production of a full-length humanized immunoglobulin G1 antibody that is highly efficient for neutralization of dengue type 4 virus, J. Virol. 78 (2004) 4665–4674.

[3] R. Burioni, N. Mancini, S. Carletti, M. Perotti, A. Grieco, F. Canducci, P.E. Varaldo, M. Clementi, Cross-reactive pseudovirus-neutralizing anti-envelope antibodies coexist with antibodies devoid of such activity in persistent hepatitis C virus infection, Virology 327 (2004) 242–248.

[4] G.E. Nybakken, T. Oliphant, S. Johnson, S. Burke, M.S. Diamond, D.H. Fremont, Structural basis of West Nile virus neutralization by a therapeutic antibody, Nature 437 (2005) 764–769.

[5] B. Friguet, A.F. Chaffotte, L. Djavadi-Ohaniance, M.E. Goldberg, Measurements of the true affinity constant in solution of antigen–antibody complexes by enzyme-linked immunosorbent assay, J. Immunol. Methods 77 (1985) 305–319.

[6] S.C. Wu, W.C. Lian, L.C. Hsu, M.Y. Liau, Japanese encephalitis virus antigenic variants with characteristic differences in neutralization resistance and mouse virulence, Virus Res. 51 (1997) 173–181.

[7] C.W. Lin, S.C. Wu, A functional epitope determinant on domain III of the Japanese encephalitis virus envelope protein interacted with neutralizing-antibody combining sites, J. Virol. 77 (2003) 2600–2606.

[8] S.C. Wu, Y.J. Lin, J.W. Chou, C.W. Lin, Construction and characterization of a Fab recombinant protein for Japanese encephalitis virus neutralization, Vaccine 23 (2004) 163–171.

[9] E.A. Kabat, T.T. Wu, H.M. Perry, K.S. Gottesman, C. Foeller, Sequences of proteins of immunological interest, publication no. 91-3242, Natioanl Institutes of Health, Maryland, 1991.