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Antibody germline characterization of cross-neutralizing human IgGs against 4 serotypes of dengue virus

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ABSTRACT

Dengue virus (DENV), a re-emerging virus, constitutes the largest vector-borne disease virus, with 50–100 million cases reported every year. Although DENV infection induces lifelong immunity against viruses of the same serotypes, the subsequent infection with the heterologous serotypes can cause more severe form of the disease, such as Dengue Haemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS). However, there is neither approved vaccine nor specific drugs available to treat this disease. In this study, previously developed 19 human monoclonal antibodies (HuMAbs) showing strong to moderate cross neutralizing activity were selected. Most of them (13/19) were targeted to domain II of envelop glycoprotein. To understand and clarify the recognition properties, the maturation mechanisms comprising Variable/Diversity/Joining (VDJ) recombination, Variable Heavy (VH)/Variable Light (VL) chain pairing, variability at junctional site, and somatic hypermutation (SHM) of those antibodies were studied and compared with their predecessor germline sequences. IMGT/V-QUEST database was applied to analyze the isolated VH and VL sequences. To confirm the correction of isolated VH/VL, 3 HuMAbs (1A10H7, 1B3B9, 1G7C2) was transiently expressed in HEK293T cell. All three clones of the expressed recombinant IgG (rIgG) showed the same binding and neutralizing activity as same as those from hybridomas. The data obtained in this study will elucidate the properties of those HuMAbs for further genetic modification, and its binding epitopes.

1. Introduction

Dengue virus (DENV) is transmitted to humans by infected *Aedes* mosquitoes in tropical and subtropical areas. It is a member of the *Flavivirus* genus of the *Flaviviridae* family. It is the enveloped icosahedral, positive-stranded RNA viruses. The approximately 11-kb genome is translated as a single polyprotein, which is cleaved into three structural proteins (capsid [C], premembrane/membrane [prM/M], and envelop [E]), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by host and viral protease [1]. They comprise four distinct serotypes, DENV1 through DENV4. This virus is the major cause of morbidity in tropical and subtropical regions, and have been estimated that there are 50–100 millions of infected cases annually around the world. However, nowadays, there is neither proven vaccine nor specific anti-viral drug to all serotypes available. Infection with DENV can cause a spectrum of clinical symptoms ranging from flu-like illness, dengue fever (DF), to a severe fatal disease known as DHF or DSS. These fatal forms are often attributed to re-infection by heterologous serotypes [2]. Primary infection with any of the four serotypes can produce lifelong immunity to that serotype, but only temporary immunity to the others; moreover, the sequential infections in the presence of heterologous dengue antibodies often lead to a more severe secondary infection of DHF/DSS [3]. These is attributed to antibody-dependent enhancement (ADE) when non-neutralizing antibodies form a complex with the virus and infect phagocytes via Fc receptors, causing more viral load and enhance infection involving DHF/DSS [3].

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Passive antibody therapies, based on targeted-specific monoclonal antibodies (MAb) have been widely studied in several infectious diseases including viral diseases, such as cytomegalovirus (CMV), respiratory syncytial virus (RSV), hepatitis B virus (HBV), hepatitis C virus (HCV), rabies, vaccinia, varicella-zoster virus (VZV), and West Nile virus (WNV), which some of them have been approved to use both in prophylaxis or treatment in the US market [4]. However, therapeutic antibodies to treat dengue virus are still limited.

Several studies on both mouse and human antibodies to DENV have implied that antibodies targeted to envelope protein of DENV (ED) are the principal determinants of virus neutralization [5]. For ED itself, it is composed of 3 domains (DI, DII, and DIII). DII is located at one end of the molecule covering the fusion peptide and responsible for host cell-viral fusion. At the other end is located DIII which is involved in host cell binding [6]. Both DI/II and DIII have been studied to be targeted by several mouse and human antibodies.

Even though mouse MAbs can normally show strong neutralizing activity with serotype specific to dengue virus that targeted to EDIII [7–8], human MAbs (HuMAbs) usually show more diversity of antibody response; serotype- or cross reactive with weak to strong neutralizing activity with several target epitope (DI/II or DIII) of E protein [9–10]. Similar with our 20 anti-DENV HuMAbs (19 IgGs and 1 IgA) that have been previously generated, using hybridoma technique [11], all of them showed strong to moderate cross-neutralizing activity to 4 serotypes of dengue virus tested *in vitro*. They were targeted to envelop protein of DENV. Moreover, thirteen of them were further characterized and found to target to domain II of E protein using truncated E protein [12].

Other than epitope mapping studies for vaccine design, the genetic analysis of variable region sequences of anti-viral Abs have recently shown its potential for vaccine development. This germline characterization has recently been widely studied in several anti-viral MAb including HIV-1 virus [13–14], Influenza virus [15], and SARS coronavirus, Hendra virus, and Nipah virus [16] using phage display and high-throughput sequencing. One study of anti-influenza antibodies have shown that mutation at only 7 amino acids in the Heavy chain Complementary Determining Region 2 (HCDR2) and Framework Region 3 (FR3) are evolved for the full activity of neutralization, which can be applied for the rational design of epitope-specific vaccine inducing broadly-neutralizing antibody [15]. For anti-DENV MAbs characterization, there is one study characterizing mouse MAb 4E11 that targeted to EDIII which has been raised against serotype 1 dengue virus, but can neutralize all 4 serotypes with different efficacy. It was found that mutation at only some hotspot can greatly affect the binding activity of that MAb [17]. However, germline study of anti-DENV HuMAbs is still limited. For this reason, further characterization of genetic sequences of our 19 hybridoma IgGs – all targeted to E, mostly targeted to EDII and show strong to moderate neutralizing activity – is needed. These should be a good model of antibody's genetic study that will enlighten dengue vaccine development through the induction of cross-neutralizing activity.

2. Materials and methods

2.1. Hybridoma sample

Previously generated 19 hybridomas secreting HuMAb IgGs with cross-neutralizing activity were selected. These 19 neutralizing HuMAbs (NHuMAbs) were produced from 3 acute dengue patients of secondarily infection with dengue virus serotype 2.

2.2. Isolation of VH and VL from hybridoma cell

Ribonucleic acids (RNAs) were isolated from hybriboma cells using Trizol reagent (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from the isolated RNA, and then used as the template for amplification of Heavy chain (HC) and Light chain (LC) using 26 pairs of primer. Twelve reactions for HC, 10 reactions for LC kappa, and 4 reactions for LC lambda were performed (Table 1). Specific products of both HC and LC were purified and cloned to pGemT easy vector (Promega, USA) for sequencing.

2.3. Antibody gene analysis with its germline

The isolated HC/LC antibody gene covering the entire antibody variable domain with 3 CDRs and 4 FRs characterized by IMGT unique numbering system were entered to the immunogenetic IMGT/V-QUEST database [18,19]. The localization of CDR and FR, including % variation and mutation from its original germline sequence were analyzed.

2.4. Subcloning of VH and VL to mammalian expression vector

The VH and VL fragments were amplified from pGemT-easy vector with primers containing extended restriction site. The amplified VH and VL as well as the human antibody constant region-containing pQCX plasmid were then digested with *Not*I and *Xho*I restriction enzymes. The VH fragments were cloned to pQCXIP-CH containing HC constant region. Similarly, VL fragments were cloned to either pQCXIH-CKappa or CLambda, containing constant kappa or lambda LC, respectively. The recombinant DNA was transformed to DH5α competent cell. Plasmids were amplified from positive clones using Purelink plasmid midiprep kit (Invitrogen, USA.)

2.5. Transient expression of recombinant IgG (rIgG) in mammalian cells

Human embryonated kidney (HEK) 293T cells were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) without sodium pyruvate (Gibco, USA). The cells were seeded to 10 cm cell culture dish at 5.5×10^5 cell/ml, and incubated at 37 °C under 5% CO_2 overnight. On the next day, twelve micrograms

Table 1
Primers used to amplify Variable Heavy chain, and Variable Light chain (kappa and lambda).

Name	Sequence	*N*
Variable heavy chain		
hIGHV1/7-	ATGGACTGGACCTGGAGGATCCTC	24
hIGHV2	ATGGACATACTTTGTTCCACGCTCCT	26
hIGHV4	ATGAAACACCTGTGGTTCTTCCTCCT	26
hIGHV6	ATGTCTGTCTCCTTCCTCATCTTCCT	26
hIGHV3	ATGGAGTTTGGGCTGAGCTGGGTT	24
hIGHV5	ATGGGGTCAACCGCCATCCTCGC	23
37, hIgG1234(−)_PCR Center	CTCCCGCGGCTTTGTCTTGGCATTA	25
17,hIgG1234(−)_nPCR	CCTTGGTGTTGCTGGGCTTGTGAT	24
Variable kappa light chain		
IGKV1–5′L	ATGGACATGAGGGTCCCCGCTCAG	24
IGKV2–5′L	ATGAGGCTCCCTGCTCAGCTCCTG	24
IGKV3–5′L	ATGGAARCCCCAGCGCAGCTTCTC	24
IGKV4–5′L	ATGGTGTTGCAGACCCAGGTCTTCAT	26
IGKV5–5′L	ATGGGGTCCCAGGTTCACCTCCTC	24
18, hIgK_PCR(−)	GTGACACTCTCCTGGGAGTTACCC	24
11, Human specific 3′ primer for nested PCR	GAGTTACCCGATTGGAGGGCGTTAT	25
Variable lambda light chain		
IGLV_1/3/5/7–5′L	ATGGCCTGGWYYCCTCTCYTYCTS	24
IGLV_8–5′L	ATGGCCTGGATGATGCTTCTCCTCG	25
IGLV_2/9/10–5′L	ATGSCCTGGGCTCYKCTSCTCCTS	24
IGLV_4–5′L	ATGGCCTGGRYCYCMYTCYWCCTM	24
19, hIgL_nPCR(−)	TGGCAGCTGTAGCTTCTGTGGGACT	25

of each pQC plasmid containing HC or LC were co-transfected to the cell using lipofectamine 2000 (Invitrogen, USA.). Briefly, 60 μl of lipofectamine 2000 was diluted in 1.5 ml of Opti-MEM I reduced serum (Gibco, USA), and then incubated at room temperature for 5 min. At the same time, 12 μg each of pQCXIP-CH-VH, and pQCXIH-CL-Vkappa or -VLambda was diluted in Opti-MEM I reduced serum (Gibco, USA). Then, 1.5 ml of diluted lipofectamine was added to the tube containing diluted DNA. They were gently mixed and the complex was incubated at room temperature for 30–40 min. Then, 3 ml of lipofectamine-DNA complex was added to the cell, and incubated at 37 °C under 5% CO_2 for 4–6 h. After incubation, the medium was replaced with fresh 10 ml of culture medium, and incubated at 37 °C under 5% CO_2 for 3 days. The presence of IgG and binding activity were checked by immunofluorescense assay (IFA) using transfected culture medium.

The collected culture fluid from transfected cell was filtered before purification using Hitrap protein A affinity column (GE bioscience, Sweden). The concentration of purified IgGs was then measured using BCA protein assay kit (Pierce Thermo Scientific, USA).

2.6. Indirect immunofluorescence assay (IFA)

Vero cells monolayer in 96-well microplate were mock-infected or infected with DENV serotype 1–4 separately. After incubation for 2–3 days, the cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS), and permeabilized with 0.1% Triton X-100 in PBS. The cells were stained with hybridoma HuMAbs or rIgG at 4 °C overnight using anti-flavivirus HuMAbs as positive control. The bound antibodies were reacted with an AlexaFluor 488-conjugated anti-human IgG (1:1000; Invitrogen), and observed under fluorescence microscope.

2.7. Neutralization test using Focus Reduction Neutralization Test (FRNT)

The neutralization test was performed using Vero cells. The focus number was firstly adjusted to obtain approximately 100–150 focuses/well. Vero cells were seeded to 96 well plate at 2.0×10^4 cells/well. Twofold serial dilution of antibodies were prepared on 96 well round bottom plate, then added with the adjusted focus number of dengue virus serotype 1–4. The Ab-virus mixtures were then incubated at 37 °C for 30 min. After that, 50 μl of the mixtures were added to Vero cells, and incubated for 2 h with gently tapping every 30 min. One hundred μl of overlaid medium (2X MEM, 2% CMC, 3% FBS) were then added to the well and incubated for 3 days for Dengue virus serotypes 1, 2, and 3, and 2 days for serotype 4. The assays were performed in triplicate. After 2 or 3 days incubation, the cells were fixed with 3.7% formaldehyde, followed by Triton X-100. The focus number was immunostained by incubated with anti-DENV MAb, as for IF assay. The number of focus was counted and compared with that of no antibody control to calculate NT_{50}.

3. Results and discussions

In general, to generate mature B cell for antibody production, 4 main mechanisms including the rearrangement of V, D, and J segments, the pairing of different VH and VL germline, the addition or deletion at the junctional site, encoded for CDR3 loop are involved. More diversity is further increased once the B cells have been stimulated with target antigen, inducing SHM at some hotspot to increase the affinity toward the foreign antigen. These four genetic processes have built a high diversity of variable region of antibodies [20]).

To study the genetic basis of our anti-DENV HuMAbs, the variable sequences of 19 IgGs isolated from acute dengue patients was studied. All of them were derived from blood sample of 3 patients of secondarily infected dengue virus serotype 2, within one week after the onset of fever [10]. HC and LC of all 19 IgGs were sequenced starting from human IgG leader peptide until constant region of IgG HC and LC. Only the variable regions were submitted to IMGT/V-QUEST to localize the FR and CDR. All sequences were productive IG rearranged sequences with no stop codon and in-frame junction, except 1 clone (23-5G8E3) have 5 aa insertion at CDR2. Variable region of heavy and light chain of 17 clones of them were submitted to database of DNA Data Bank of Japan (DDBJ) [12].

After comparing those sequences with germline database, 10 germline HCs and 11 germline LCs was used (Table 2). IGHV1–69 (5/19) and IGHV3–23 (5/19) are the major germline HC whereas IGLV7–43 is the major germline LC (Fig. 1A and B). As IGHV1–69 was the highest HC germline used in our anti-DENV HuMAbs, interestingly, the highest percentage of IGHV1–69 HC germline was also

Table 2
Analysis of anti-DENV HuMAbs including its germline sequences, and immunogenetic data.

HuMAbs	IGHV gene	IGKV/IGLV gene	No. of aa mutations /Identity(% variation)		HCDR3 aa sequence (length)
			VH	VL	
1A10H7	HV1–69*01F	KV1-NL1*01 F	12 /85(12.4)	9/85 (9.6)	ARSRYYYDSDASNYGMDV (18)
1G7C2	HV1–69*01F	LV2–23*01 F	17/80(19.5)	11/85 (11.5)	ATLIAVAGSEGAGSFDI (17)
1E7B8	HV1–69*01F	KV3–11*01 F	17 /81(17.3)	4/90 (4.2)	ARHRAVAGGDSDHDENNWFGP (21)
2D1G5	HV1–69*01F	LV2–11*01 F	14 /84(14.3)	11/86 (11.3)	ARAGPIAATGVQYEMDV (17)
1H5A11	HV3–23*01F	LV1–47*02 F	12 /84(12.5)	5/93 (5.1)	ATGSQWPGDY (10)
3A10G12	HV3–23*01F	LV1–47*02 F	10 /87(10.3)	5/93 (5.1)	AAGSQWPGDY (10)
4A6F9	HV3–23*01F	KV1–12*01 F	6 /91(6.2)	4/90 (4.2)	ANTLWTVGSKGGFDY (15)
4D10E9	HV3–23*01F	KV1–12*01 F	21 /76(21.6)	12/82 (12.8)	TKIDWSIRGTFDN (13)
4F5E1	HV3–23*01F	KV4–1*01 F	6 /92(6.1)	5/96 (5)	ARVTGGWSDY(10)
1B3B9	HV7–4*02F	LV7–43*01 F	14 /83(14.4)	13/83 (13.5)	TTLSGYSADWPEDY (14)
4H12C8	HV7–4*02F	LV7–43*01 F	5 /92(5.1)	3/93 (3.1)	TTLSGYSADWPEDY (14)
5E6B1	HV7–4*02F	LV7–43*01 F	13 /84(13.4)	13/83 (13.5)	TTLSGYSADWPEDY (14)
1C2D2	HV4–59*01F	KV1–33*01 F	13 /84(13.4)	10/84 (10.6)	ARVAKLFGSATYGMDV (16)
5G8E3	HV3–30*04F	LV1–40*01 F	12 /86(12.2)	12/87 (12.1)	AVYYCARRGDYSSSAENFQH (20)
1C1G4	HV1–18*01F	LV1–47*02 F	9 /89(9.2)	6/92 (6.1)	ARGPDYESSDSPWFDY (16)
3A1E2	HV3–11*01F	LV1–40*01 F	17 /80(19.5)	9/90 (9.1)	ARGMTGFTTSNTESFDL (17)
3B6C7	HV4–39*01F	LV7–43*01 F	15 /83(15.3)	8/89 (8.2)	ASPGGLISDEAMAGYFDY (18)
2H8G1	HV3–30*03F	KV1–39*01 F	13 /85(13.3)	12/83 (12.6)	ATGGGRFSGSGNYYYYGMDV (20)
5G2D2	HV1–69*06F	KV1-NL1*01 F	14 /83(14.4)	6/88 (11.7)	ARSTYYYDGSDLTYGMDV (18)

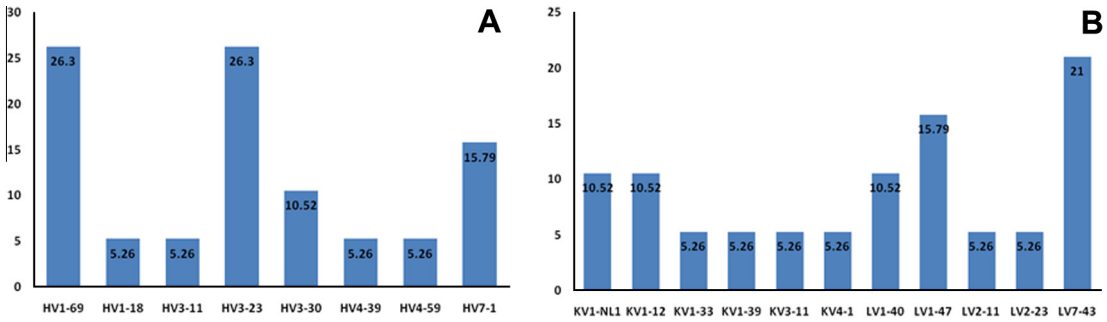

Fig. 1. Germline usage frequencies. (A) The heavy chain variable (HV) germline sequences. (B) The light chain variable (kV for kappa and LV for lambda) germline sequences related to 19 cross-neutralizing anti-DENV HuMAbs derived from acute secondarily infected dengue patients. The *Y* axis represents the usage percentage of each germline sequences, as also indicated at the top of bar graph.

Table 3
The amino acid sequences of heavy chain from 3 major groups, classified by type of heavy chain germline, of 13 HuMAbs in alignment of their predecessors germline, including type of its germline light chain and NT_{50}. The CDRs and FR are located according to the immunogenetic annotation. The somatic mutations in the VH of each HuMAb are highlighted in gray color.

HC	FRW1	CDR1	FRW2	CDR2	FRW3	CDR3-IMGT	VL germline used	NT50 (μg/ml)
IGHV1-69	QVQLVQSGAEVKKPGSSVKVSCKAS	GGTFSSYA	ISWVRQAPGQGLEWMGG	IIPIFGTA	NYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYC			
1A10H7	QVQLVQSGAEVKKPGSSVRVSCKAS	GGTFRNYA	ISWVRQAPGQGLEWMGG	VIPTLHTT	NYVERFQGRITITADESTSTAYMELSSLRSEDTAVYYC	ARSRYYYDSDASNYGMDV	IGKV1-NL1*01 F	1.6
1G7C2	QVQLVQSGAEVKKPASSVKVSCKAS	GGTFGTYA	ISWVRQAPGKGLEWMGG	IIPLYKQS	TYAQKFRGRVTITADESTNTAYMELNGLSLEDTAVFYC	ATLIAVAGSEGAGSFDIW	IGLV2-23*01 F	1.1
1E7B8	QVQLVQSGAEVKKPGSSVRVSCRAS	GGALANYA	ISWVRQAPGQGLEWMGA	IIPMSRTT	DYAQKFRGRVTISADESRSTAYMELNSLRSDDTAVYYC	ARHRAVAGGDSDHDENNWFGPW	IGKV3-11*01 F	1.5
2D1G5	QVQLMQSGPEVKKPGSSMKVSCKAS	GGTLRSYS	ISWVRQAPGQGLEWMGG	TIPFFGRA	SYAQKFQGRVTITADEATSTAFMELSSLSSEDTAVYFC	ARAGPIAATGVQYEMDVW	IGLV2-11*01 F	13
5G2D2	QVQLVQSGAEVEKPGSSVKVSCKAS	GDTFSKYA	INWVRQAPGQGLDWMGG	IIPILTTT	TYAQRFQGRVTITADKSTNTAYMELRSLRFDDTAVYYC	CARSTYYYDGSDLTYGMDVW		
IGHV3-23	EVQLLESGGGLVQPGGSLRLSCAAS	GFTFSSYA	MSWVRQAPGKGLEWVSA	ISGSGGST	YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC			
1H5A11	EVQLLESGGDLVQPGGSLRLSCAAS	GFTFSTYA	MSWVRQAPGKGLEWVSG	IGDSGHSI	YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC	CATGSQWPGDYW	IGLV1-47*02 F	1.6
3A10G12	EVQLLESGGGLVQPGGSLRLSCTAS	GFTFSNYA	MTWVRQAPRKGLEWVSA	IGGSGHSI	YYGDSVKGRFTISRDNSNNTLYLQMNTLRAEDTAVYYC	CAAGSQWPGDYW	IGLV1-47*02 F	1.4
4A6F9	EVQLLESGGGLVQPGGSLRLSCAAS	GFTFSSNA	MSWVRQAPGKGLEWVSG	VSNSGGDT	YYADSVKGRFAISRDNSKNTLYLQMNSLRAEDTAVYYC	CANTLWTVGSKGGFDYW	IGKV1-12*01 F	2.3
4D10E9	EVQLLESGGGLAQPGGSLRLSCAAS	GFSFSTQA	MAWVRQAPGKGLEWVSA	IGGRDEGS	YYAESLKGRLTISRDNSKNTVYLQMDRLRADDTALYFC	CTKIDWSIRGTFDNW	IGKV1-12*01 F	
4F5E1	EVQLLESGGGLVQPGGSLRLSCAAS	GFSFSNSA	MSWVRQAQGKGLEWVSA	FSGSGGST	YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC	CARVTGGWSDYW	IGKV4-1*01 F	4.6
IGHV7-4	QVQLVQSGSELKKPGASVKVSCKAS	GYTPTSYA	MNWVRQAPGQGLEWMGW	INTNTGNP	TYAQGFTGRFVFSLDTSVSTAYLQISSLKAEDTAVYYC			
1B3B9	QVHLVQSESEVKKPGASVKVSCQAS	GYPPTRYI	MNWVRQAPGQGLEWMGW	IDTKTGNP	TYAQAFTGRFVFSLDTSVSTAYLQINNLKAEDTAVYFC	CTTLSGYSADWPEDYW	IGLV7-43*01 F	3.1
4H12C8	QVQLVQSGSELKKPGASVKVSCKAS	GYTPTTYP	MNWVRQAPGQGLEWMGW	IDTNTGNP	TYAQAFTGRFVFSLDTSVSTAYLQISSLKAEDTAVYYC	CTTLSGYSADWPEDYW	IGLV7-43*01 F	5.5
5E6B1	QVHLVQSGSEVKKPGASVKVSCQAS	GYPPTRYI	MNWVRQAPGQGLEWMGW	IDTKTGNP	TYAQAFTGRFVFSLDTSVSTAYLQINRLKAEDTAVYFC	CTTLSGYSADWPEDYW	IGLV7-43*01 F	3.1

previously reported from other anti-viral MAbs, such as influenza virus [14,19], HIV, SARS CoV, Hendra, and Nipah virus [16].

In 2012, Prabakaran et al., used IgM derived B cell for naïve Fab antibody library construction and screened for several viral targets. They found that IGHV1–69 were the highest heavy chain germline gene used. Similar with the result from Chen et al. (2012a) who constructed 2 libraries from peripheral blood mononuclear cells (PBMCs) from HIV-1 patients from different time point after infection, one from 40 days, and the other from 8 months post infection. It was found that germline gene usages of both HC and LC were different among two libraries. HV1–46, HC3–33, and HV5–51 were most frequently found from library constructed from 40 days post infection PBMCs. In contrast, HV1–69 (26%) was the most frequently used HC germline from library constructed from 8 months PBMCs. Comparing with our study, that all 19 IgGs were generated from acute patient PBMCs from secondary dengue infection, it can be suggested that after primary infection, this IGHV1–69, which is the high frequency heavy chain germline presence in IgM repertoires [16], should be the major group of germline heavy chain responsible for antibody production. After recovery, the human immune response can stay long in the human blood circulation. Once re-infection has occurred, the memory B cells were stimulated; therefore, high possibility of antibody production from this kind of germline heavy chain was observed.

Other than the different combination of VH/VL to generate a high diversity of our anti-DENV HuMAbs, more variable were also found at the junctional site (length of CDR3). We found more variable occurred in HCDR3 of our HuMAbs, which ranged from 10 to 21 aa (Table 2), but not for LCDR3 which ranged from 9 to 11 aa (data not shown). This high diversity of HCDR3 was also observed from anti-HIV-1 antibody, which ranged from 4 to 27 aa, and considering as the determination of VH diversity of antibody repertoire [13].

Once the mature B cells have faced the dengue virus antigen, more diversity was enhanced by SHM. We observed these mutations throughout the variable regions with different degree, 5.1–21.6% variation for VH, with less variation for VL at 3.1–12.8%.

Among 19 IgGs, three major groups are classified by type of germline HC. These 3 kinds of HC germline, which combined with 8 kinds of germline LC shared 13 HuMAbs. The other 7 HuMAbs have unique HC germline as shown in Table 2. The alignment of HuMAbs HC from these 3 groups, along with their closest germline sequences, including their germline LC, as well as annotated FRs and CDRs are shown in Table 3. It was noticed that among 4 HuMAbs in Gr. 1, which used IGHV1–69 as HC germline, all of them used different germline LC (Table 3). However, there is 1 HuMAb that showed the highest NT_{50}, at 13 μg/ml, comparing with the other 3 which have NT_{50} ranged from 1.1 to 1.6 μg/ml. It is implied

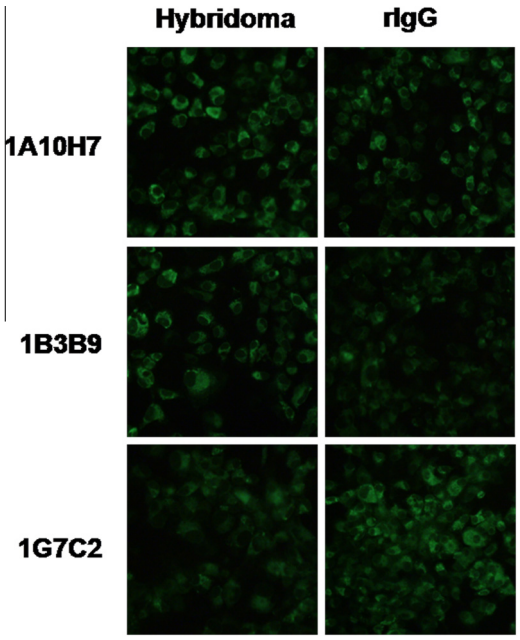

Fig. 2. Fluorescence immunostaining of three expressed rIgG to Dengue virus serotype 2 compared with IgG from hybridoma secretion.

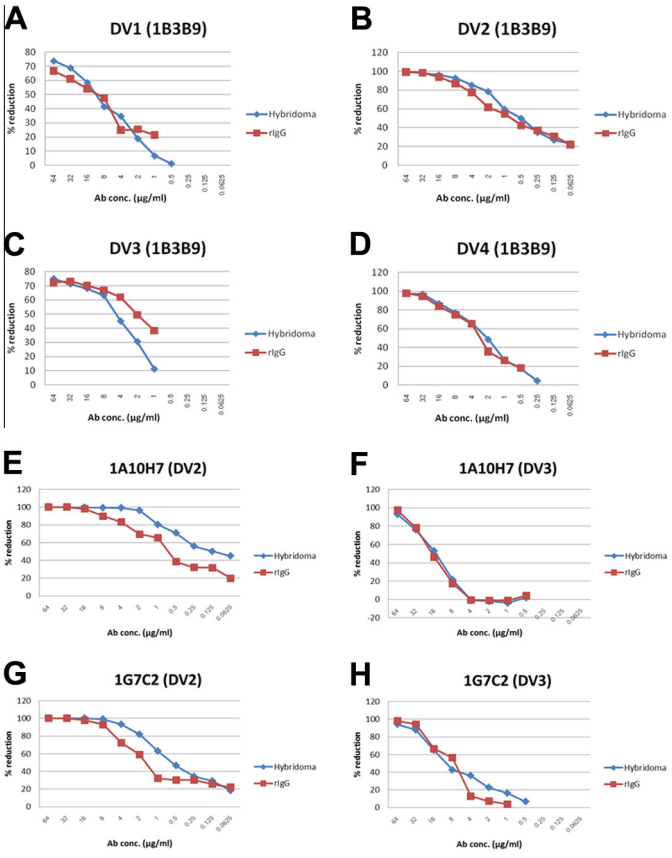

Fig. 3. Neutralizing activity (NT) of HuMAb 1B3B9 to 4 serotypes of dengue virus (A–D), and 1A10H7 (E and F), 1G7C2 (G and H) to dengue virus serotype 2 and 3, demonstrated by the comparison of rIgG and IgG from hybridoma secretion.

that the combination of different HC and LC can greatly affect the binding of our antibodies with the target antigen (IGHV1–69 paired with IGLV2–11 is the less matched). In contrary, Gr. 3 composing of 3 HuMAbs (HuMAb 1B3B9, 4H12C8, 5E6B1) used the same HC and LC germline with different level of mutations. We found that, using the same originated germline, hypermutation also affect their NT_{50} (Table 3). Among these 3 HuMAbs, there are 2 HuMAbs (1B3B9 and 5E6B1) that showed the same level of mutation (14.4% and 13.4% for VH, and the same 13.5% for VL, respectively). Both of them showed the same NT_{50}. In contrast, the other HuMAb (4H12C8) which have less variation comparing with its germline counterpart (5.1% for VH and 3.1% for VL) showed almost 2 times higher NT_{50} concentration (Table 3).

However, for the less variation of same germline sequences, it can be suggested that germline-like antibody with low level of SHM can showed the relatively high binding with target antigen. This phenomenon was also previously evidenced by Chen et al. (2012b). They supported that antibodies with minimally mutated can bind virus causing acute infection like SARS CoV, and Hendra virus with relatively high affinity, but not for chronic disease like HIV-1 [14].

Moreover, after detail characterization, 3 HuMAbs (1A10H7, 1B3B9, 1G7C2), which show the highest activity both in binding and neutralizing activities as previously described [12] was selected. It was noticed that these 3 clones did not showed the highest variation. Hence, it was suggested that the 4 mechanisms was generally play a role in increasing the binding affinity of one antibody toward the target antigen.

To study the function of isolated VH and VL, those 3 best HuMAbs were selected for production of rIgG, by subcloning of variable region of HC and LC to another 2 mammalian expression plasmids containing either constant HC or constant LC (kappa or lambda). The constructed plasmids were co-transfected to human embryonated kidney (HEK) 293T cell for rIgG expression, for testing of both binding and neutralizing activity. All three clones can bind to 4 serotypes of dengue virus infected Vero cell with the same manner as IgG secreted from hybridoma cell (Fig. 2). Moreover, three of them also show almost the same neutralizing activity as those from hybridomas (Fig. 3).

To confirm the right combination of VH/VL from each monoclonal antibody, using the same constructed plasmid, different combinations of VH and VL constructs of those 3 HuMAbs were tested in the total of 9 combinations including the original VH/VL pairing. It was found that only the correct combination of each three clones can show the optimal binding activity by IFA. It was implied that the CDR loop, which is the region much responsible for antigen binding of these three clones need the correct combination of VH and VL and conformation to form the antigen binding site.

For more understanding of affinity pathway, many more HuMAbs with various activity, for example, low, moderate, and high NT titer are needed in identification of the critical position (hotspot) that are most likely to have a major impact on antibody activity and affinity.

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References

[1] R.F. Qi, L. Zhang, C.W. Chi, Biological characteristics of dengue virus and potential targets for drug design, Acta Biochem. Biophys. Sin. 40 (2008) 91–101.
[2] K. Clyde, J.L. Kyle, E. Harris, Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis, J. Virol. 80 (2006) 11418–11431.
[3] S. Murrell, S.C. Wu, M. Butler, Review of dengue virus and the development of a vaccine, Biotechnol. Adv. 29 (2011) 239–247.

[4] W.A. Marasco, J. Sui, The growth and potential of human antiviral monoclonal antibody therapeutics, Nat. Biotechnol. 25 (2007) 1421–1434.
[5] H.E. Lin, W.Y. Tsai, I.J. Liu, P.C. Li, M.Y. Liao, J.J. Tsai, Y.C. Wu, C.Y. Lai, C.H. Lu, J.H. Huang, G.J. Chang, H.C. Wu, W.K. Wang, Analysis of epitopes on dengue virus envelope protein recognized by monoclonal antibodies and polyclonal human sera by a high throughput assay, Plos Negl. Trop. Dis. 6 (2012) e1447, http://dx.doi.org/10.1371/journal.pntd.0001447.
[6] R.T. He, B.L. Innis, A. Nisalak, W. Usawattanakul, S. Wang, S. Kalayanarooj, R. Anderson, Antibodies that block virus attachment to Vero cells are a major component of the human neutralizing antibody response against dengue virus type 2, J. Med. Virol. 45 (1995) 451–461.
[7] W.D. Crill, J.T. Roehrig, Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells, J. Virol. 75 (2001) 7769–7773.
[8] S. Sukupolvi-Petty, S.K. Austin, M. Engle, J.D. Brien, K.A. Dowd, K.L. Williams, S. Johnson, R. Rico-Hesse, E. Harris, T.C. Pierson, D.H. Fremont, M.S. Diamond, Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2, J. Virol. 84 (2010) 9227–9239.
[9] M. Beltramello, K.L. Williams, C.P. Simmons, A. Macagno, L. Simonelli, N.T. Quyen, S. Sukupolvi-Petty, E. Navarro-Sanchez, P.R. Young, A.M. de Silva, F.A. Rey, L. Varani, S.S. Whitehead, M.S. Diamond, E. Harris, A. Lanzavecchia, F. Sallusto, The human immune response to dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity, Cell Host Microbe 8 (2010) 271–283.
[10] R. de Alwis, S.A. Smith, N.P. Olivarez, W.B. Messer, J.P. Huynh, W.M.P.B. Wahala, L.J. White, M.S. Diamond, R.S. Baric, J.E. Crowe, A.M. de Silva, Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions, PNAS 8 (2012) 7439–7444.
[11] C. Setthapramote, T. Sasaki, O. Puiprom, K. Limkittikul, P. Pitaksajjakul, P. Pipattanaboon, M. Sasayama, P. Leuangwutiwong, W. Phumratanaprapin, S. Chamnachanan, T. Kusolsuk, A. Jittmittraphap, A. Asai, J.F. Arias, I. Hirai, M. Kuhara, Y. Okuno, T. Kurosu, P. Ramasoota, K. Ikuta, Human monoclonal antibodies to neutralize all dengue-virus serotypes using lymphocytes from patients at acute phase of the secondary infection, Biochem. Biophys. Res. Commun. 423 (2012) 867–872.
[12] T. Sasaki, C. Setthapramote, T. Kurosu, M. Nishimura, A. Asai, M.D. Omokoko, C. Pipattanaboon, P. Pitaksajjakul, K. Limkittikul, A. Subchareon, P. Chaichana, T. Okabayashi, I. Hirai, P. Leaungwutiwong, R. Misaki, K. Fujiyama, K. Ono, Y. Okuno, P. Ramasoota, K. Ikuta, Dengue virus neutralization and antibody-dependent enhancement activities of human monoclonal antibodies derived from dengue patients at acute phase of secondary infection, Antiviral Res. 98 (2013) 423–431.
[13] W. Chen, P. Prabakaran, Z. Zhu, Y. Feng, E.D. Streaker, D.S. Dimitrov, Characterization of human IgG repertoires in an acute HIV-1 infection, Exp. Mol. Pathol. 93 (2012) 399–407.
[14] W. Chen, E.D. Streaker, D.E. Russ, Y. Feng, P. Prabakaran, D.S. Dimitrov, Characterization of germline antibody libraries from human umbilical cord blood and selection of monoclonal antibodies to viral envelope glycoproteins: implications for mechanisms of immune evasion and design of vaccine immunogens, Biochem. Biophys. Res. Commun. 27 (2012) 1164–1169.
[15] D. Lingwood, P.M. McTamney, H.M. Yassine, J.R.R. Whittle, X. Guo, J.C. Boyington, C.J. Wei, G.J. Nabel, Structural and genetic basis for development of broadly neutralizing influenza antibodies, Nature 489 (2012) 566–571.
[16] P. Prabakaran, Z. Zhu, W. Chen, R. Gong, Y. Feng, E. Streaker, et al., Origin, diversity, and maturation of human antiviral antibodies analysed by high-throughput sequencing, Front. Microbiol. 3 (2012) 1–7.
[17] H. Bedouelle, L. Belkadi, P. England, J.I. Guijarro, O. Lisova, A. Urvoas, et al., Diversity and junction residues as hotspots of binding energy in an antibody neutralizing dengue virus, FEBS J. 273 (2006) 34–46.
[18] X. Brochet, M.P. Lefranc, V. Giudicelli, IMGT/V-QUEST the highly customized and integrated system for IG and TR standardized V–J and V–D–J sequence analysis, Nucleic Acids Res. 36 (2008) W503–W508. Web Server Issue.
[19] V. Giudicelli, P. Duroux, C. Ginestoux, G. Folch, J. Jabado-Michalud, D. Chaume, et al., IMGT/LIGM-DB, the IMGT comprehensive database of immunoglobulin and T cell receptor nucleotide sequences, Nucleic Acids Res. 34 (2006) D781–D784.
[20] J. Sui, W.C. Hwang, S. Perez, G. Wei, D. Aird, L. Chen, et al., Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses, Nat. Struct. Mol. Biol. 16 (3) (2009) 265–273.