Neutralizing and enhancing antibody responses to five genotypes of dengue virus type 1 (DENV-1) in DENV-1 patients

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Abstract

Dengue virus (DENV) has four distinct serotypes, DENV-1–4, with four to six genotypes in each serotype. The World Health Organization recommends tetravalent formulations including one genotype of each serotype as safe and effective dengue vaccines. Here, we investigated the impact of genotype on the neutralizing antibody responses to DENV-1 in humans. Convalescent sera collected from patients with primary infection of DENV-1 were examined for neutralizing antibody against single-round infectious particles of the five DENV-1 genotypes (GI–GV). In both GI- and GIV-infected patients, their neutralizing antibody titres against the five genotypes were similar, differing ≤4-fold from the homogenotypic responses. The enhancing activities against the five genotypes were also similar in these sera. Thus, the genotype strains of DENV-1 showed no significant antigenic differences in these patients, suggesting that GI- or GIV-derived vaccine antigens should induce equivalent levels of neutralizing antibodies against all DENV-1 genotypes.

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Abbreviations: ADE, antibody-dependent enhancement; DENV, dengue virus; SRIP, single-round infectious particle.

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The GenBank/EMBL/DDBJ/PIR accession numbers for the dengue virus type 1 sequence derived from the patient code 1 to 10 are LC148022, LC148029, LC148031, LC148025, LC148030, LC148023, LC148024, LC148028, LC148026 and LC148027, in order.

One supplementary figure and three supplementary tables are available with the online Supplementary Material.

Dengue diseases are the most important mosquito-borne viral diseases in the world. Dengue fever and its severe form, dengue haemorrhagic fever, are caused by each of the four dengue virus (DENV) serotypes (DENV-1 to DENV-4), which are distributed throughout tropical and subtropical areas [1, 2]. Approximately 3.9 billion inhabitants of these areas are at risk of infection, and 390 million dengue infections and 100 million cases of disease are estimated to occur annually [3, 4]. The total abolition of the global dengue burden is afforded the highest priority because such large numbers of patients are affected across a wide geographic distribution. Therefore, efforts have been made to develop a dengue vaccine, and a chimeric dengue vaccine has been licensed very recently in some countries [5, 6].

The World Health Organization recommends that a tetravalent dengue vaccine be developed, to induce specific antibodies against all four DENV serotypes [7] because a monovalent vaccine composed of a single serotype antigen may increase the potential risk of vaccination-enhanced dengue disease after infection with any of the other serotypes [8]. Antibody-dependent enhancement (ADE) of infection is one of the most important hypothetical mechanisms explaining the increased disease severity of dengue haemorrhagic fever [9]. ADE is caused by serotype-cross-reactive subneutralizing antibodies (enhancing antibodies), which are thought to increase the efficiency of infection in Fc-gamma-receptor-bearing cells, such as monocytes/macrophages, and to thus increase viraemia levels and disease severity [10]. This is in contrast to neutralizing antibodies, which effectively reduce viraemia levels and are therefore believed to play a protective role [11]. Consequently, most dengue vaccines already licensed or still under development have been designed to induce neutralizing antibodies specific for each dengue serotype.

Each DENV serotype has four to six genotypes, based on the nucleotide sequence of the gene encoding the envelope (E) protein, the major surface protein of the DENV virion [12]. Therefore, to develop a tetravalent formulation, only one genotype is used as the vaccine antigen to represent each serotype. The amino acid sequence similarities among
the E proteins of the four serotypes are approximately 60–70%, whereas those among the genotypes of each serotype are at least 90–96% [13]. Although this greater amino acid similarity underlies their greater antigenic cross-reactivity, whether vaccination with one genotype strain can protect the host from infection with the other genotype strains within a particular serotype is an important issue for dengue vaccine development. A study using a mouse model demonstrated that monoclonal antibodies induced by one genotype antigen show various neutralizing activities against other genotype antigens, and low or undetectable neutralizing activity was demonstrated against at least one other genotype [14–16]. In addition to the potential failure to cover all other genotypes, the induction of insufficient amounts of antibodies against a certain genotype may increase disease severity via the mechanism of ADE. Although the details of these mechanisms have not been determined, it has been reported that the introduction of a new genotype or clade strain to a certain area increased the number of cases of severe disease and/or the total number of patients [17–20].

In this study, we compared the serum neutralizing or enhancing antibody levels against five DENV-1 genotypes (genotypes I–V; abbreviated D1-GI to D1-GV) in dengue fever patients in Japan. In 2004–2012, 36 human serum samples were collected from 18 dengue patients in the acute and convalescent phases of infection who had imported the disease into Japan. These patients were residents of Japan, who had recently returned from a dengue-endemic country, mainly in Southeast Asia. Acute sera were collected upon hospital admission and convalescent sera upon discharge. Their demographic data and laboratory data obtained from sera collected at hospital admission are listed in Table S1 (available in the online Supplementary Material). The study protocol was reviewed and approved by the Ethical Committees of the National Institute of Infectious Diseases and the Faculty of Tropical Medicine, Mahidol University.

All the patients were infected with DENV-1, determined with a real-time reverse transcription PCR (Table S1). The nucleotide sequences of the envelope protein region of DENV-1 were determined in 10 of these patients (designated Group I), and the genotypes infecting half (5) of them were identified as D1-GI and those infecting the other half were identified as D1-GIV. The DENV-1 genotypes infecting the remaining eight patients (designated Group II), which were not determined with a sequencing analysis, were estimated from the countries that the patients had visited (see below). The NS1 antigen was detected in all the patients tested for NS1 antigen. Eight patients were positive for IgM antibodies, constituting nearly half of the whole patient sample (n=18).

A phylogeny was constructed using the nucleotide sequences of the whole envelope protein region of all the DENV-1 strains registered in GenBank as of 29 March 2016. The DENV-1 strains that infected the patients in this study (Table S1) and those used for our neutralization test and enhancing antibody assay (Table S2) were included in the phylogeny. During a process to simplify the phylogenetic tree, most strains that did not significantly affect the tree topology were deleted, leaving only the strains in the clade containing the isolate(s) from the patients and closely related isolate(s) from the countries the patients had visited (Fig. 1). Consistent with a study that reported that each country usually has its own circulating genotype [21], the DENV-1 strains isolated in Thailand, the Maldives, Laos and Cambodia belonged almost exclusively to GI, whereas all the isolates from Yap Island, Samoa, East Timor and the Philippines belonged to GIV. All the strains that infected our patients were closely related to those isolated from the countries that the patients had visited. Therefore, the infecting genotype could be estimated from the country that the patient had visited. Eight patients confirmed this assumption, including four patients estimated to be infected with GI and another four with GIV (Table S1).

Conventional Vero cell plaque reduction neutralization tests were performed with DENV-1–4 or their single-round infectious particles (SRIPs), essentially as described previously [22]. The SRIPs are a reliable recombinant antigenic alternative to the live virus [23, 24]. The antibody dilution producing a 75% reduction in the number of infectious foci was determined as PRNT75 [25]. Table 1 shows the PRNT75 results of the Group I patients (see Table S3 for PRNT50 data). The acute sera from all the patients contained undetectable levels (<1:10) of neutralizing antibodies against DENV-1–4, confirming a primary infection. In the convalescent phase, these DENV-1-infected patients induced neutralizing antibody responses to the five DENV-1 genotypes. The countries and years of viral isolation and the GenBank accession numbers of these five genotype strains are listed in Table S2. As expected, the cross-neutralizing antibody titres against the heterotypes (DENV-2, DENV-3 and DENV-4) were lower than the titres against the DENV-1 genotypes, with differences of usually >4 fold (Table 1). In contrast, the differences among the five DENV-1 genotypes were <4 fold in all patients. These results indicate that similar neutralizing antibodies were induced against the five genotypes by a single infection with DENV-1 GI or GIV.

The neutralizing antibody levels against all four DENV serotypes were also undetectable in the acute sera from the Group II patients (data not shown). Similar to the results obtained for the Group I patient sera, those of Group II showed equivalent neutralizing antibody titres against all five DENV-1 genotypes, with differences of <4 fold. The neutralizing antibody titres against the five DENV-1 genotypes are expressed as ratios relative to those obtained against the homologous genotype that infected individual patients (Fig. 2a). In the total population of Group I and II patients infected or estimated to be infected with D1-GI, similar average relative ratios (P>0.05 by Student’s t-test) of antibody titres were obtained against D1–GII (1.17), D1–GIII (1.04), D1–GIV (1.12) and D1–GV (0.98). Similarly, the total population of Group I and II patients infected or estimated to be infected with D1-GIV showed equivalent average relative ratios (P>0.05) of antibody
titres against D1-GI (0.93), D1-GII (0.91), D1-GIII (0.94) and D1-GV (0.94).

The infection-enhancing antibody activities against the five DENV-1 genotypes were compared in each of the Group I and II patients. Enhancing activity was measured with semi-adherent K562 cells, essentially as described previously [26]. The serum dilutions used in our enhancing antibody assay started from 1 : 160 because enhancing activity usually appears at subneutralizing doses and the amount of serum in the samples was limited. Similar dose-dependent antibody activity patterns were obtained against the five genotypes, regardless of whether the patients were infected with D1-GI or D1-GIV; examples are shown in Fig. 2(b), and other patient data in Fig. S1. To further analyse the enhancing activities, we used two indicators (see Fig. 2b; patient code 6): (i) the fold enhancement, calculated from the largest number of infected cells recorded on the dose-dependent antibody response curves and expressed as the increase in the infected cell count relative to the control level, and (ii) the enhancement titre, expressed as the highest antibody dilution showing enhancing activity. To compare the enhancing activities against the five genotypes in patients infected or estimated to be infected with D1-GI or D1-GIV, the two indicators obtained against the five genotypes were expressed as ratios relative to those obtained against the infecting genotype.

Fig. 1. Nucleotide sequence phylogeny of the complete E gene (1485 nt) of DENV-1, which was used as the serological assay antigen and was present in the patient sera. The tree was reconstructed essentially according to a previous study [20]. The GenBank accession numbers are given, followed by the countries and years in which the strains were isolated. The DENV-1 detected in the patients with imported infections and the strains used as serological assay antigens are indicated in blue and red, respectively. Pink rectangles show closely related isolate(s) from the country the patient had visited. The horizontal axis is drawn to scale and shows the estimated year of divergence, with the time at the branch tip indicating the sampling date (year). Posterior probability values ≥0.9, indicating statistical support for clades, are shown above the nodes. The tree was automatically rooted under the assumption of a molecular clock. Because the nucleotide sequence of the complete E gene was not obtained from patient code 3, it was excluded from this analysis.
Individual data and their averages calculated from the total population of Group I and II patients are shown in Fig. 2(c) for fold enhancement and Fig. 2(d) for enhancement titre. The average relative ratios for both fold enhancement and enhancement titre against the heterologous genotypes did not differ significantly from those against the homologous genotype in patients infected either with D1-GI or D1-GIV (P>0.05). These results indicate that similar enhancing antibody responses were induced against the five DENV-1 genotypes when patients were infected with either D1-GI or D1-GIV.

In this patient population, infection with D1-GI or D1-GIV induced equivalent neutralizing antibody responses against the five DENV-1 genotypes. An advantage of our study was the use of infections imported by residents of a non-DENV-endemic country. Humans with only a single DENV infection must be used for an exact comparison of the antibody responses to multiple genotypes within a single serotype. In the present study, we used Japanese patients who had imported DENV infections from elsewhere, who had undetectable levels of neutralizing antibodies, and half of whom had IgM/IgG antibody ratios indicative of primary infection. Therefore, it is highly probable that the induced antibody responses of these patients truly involved only one genotype. We focused on DENV-1-infected patients because, at this time, many travellers visit (Southeast) Asia, where DENV-1 is predominant [21, 27, 28].

The limited availability of antibody assay antigens can be overcome with a genetic engineering technology that generates viral or subviral assay antigens equivalent to those of the authentic virus, if the corresponding nucleotide sequence information is available. In this study, it was difficult to obtain five DENV-1 genotype strains because the transport of live viral materials across national borders is strictly regulated by governmental security export control policies [29] and the restrictions imposed on access and benefit sharing by the Convention on Biological Diversity [30]. Therefore, we used our previously established system to generate flavivirus SRIPs [23]. DENV-1 SRIP has been shown to be a valid alternative antigen to authentic DENV-1 in the neutralization test and enhancing antibody assay [24]. For the present study, we synthesized DNA fragments corresponding to the prM and E gene regions of the five DENV-1 genotype strains based on nucleotide sequence information available in GenBank. We used these to prepare the assay antigens with which we compared the antibody responses to the five DENV-1 genotypes induced in DENV-1-infected patients.

Although some individual variations were observed, almost equivalent levels of neutralizing antibodies against the five DENV-1 genotypes were induced, with <4 fold differences, regardless of whether the infecting genotype was D1-GI or D1-GIV (Table 1). These results suggest that when genotype I or IV is used as a vaccine strain, similar neutralizing antibody levels will be produced against all five genotypes. Similarly, there were no significant differences in the levels of enhancing activity (average relative ratios) against the five DENV-1 genotypes in the D1-GI- and D1-GIV-infected patients (Fig. 2). D1-GI or D1-GIV strains have been used as the antigens for at least four of the six currently licensed vaccines or in the clinical stages of vaccine development: an attenuated chimeric vaccine (CYD-TDV [31, 32], TetraVax-DV [33]), an inactivated virus vaccine (TDENV-PIV [34]) and a DNA vaccine (TVDV [35]).

### Table 1. Neutralizing antibody titres in Group I patients

The antibody dilution producing a 75 % reduction in the number of infectious foci was determined with the FORECAST function in Microsoft Excel and expressed as PRNT75 (reciprocal).

<table>
<thead>
<tr>
<th>Infecting genotype</th>
<th>Patient code*</th>
<th>Acute phase</th>
<th>Convalescent phase</th>
</tr>
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<tbody>
<tr>
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</table>

*These codes correspond to those in Table S1.
†DENV-1 (Mochizuki strain), DENV-2 (New Guinea C strain), DENV-3 (H87 strain) and DENV-4 (H241 strain) were used.
‡ Not tested because too little serum was available.

NT, Not tested.
Although genotypic differences were shown to have a large effect in a mouse model [14–16], the antibody response of humans may differ from that of mice. In fact, although mouse antibodies that target domain III of the envelope protein and thus efficiently interrupt viral attachment have strong neutralizing activity, human antibodies that target domain III do not play an important role in neutralization [36, 37], and studies with a human monoclonal antibody showed that domains I and II or their hinge region contain strong neutralizing epitopes [38].
In conclusion, in patients singly infected with D1-GI or D1-GIV, equivalent neutralizing antibody responses were induced against all five DENV-1 genotypes. Although we only used a small number (n=18) of patients in this study, similar genotype-cross-reactive neutralizing antibody levels were induced in all the patients. We are currently comparing the neutralizing antibody responses against genotypes of DENV-2–4 in Japanese patients with imported infections of a single genotype strain of the corresponding serotype. Such a comparison of genotypes may help vaccine developers to select vaccine strains.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References
33. Kirkpatrick BD, Durbin AP, Pierce KK, Carmolli MP, Tibery CM et al. Robust and balanced immune responses to all 4 dengue


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