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Dengue viruses cluster antigenically but not as discrete serotypes

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Abstract

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The four genetically divergent dengue virus (DENV) types are traditionally classified as serotypes. Antigenic and genetic differences among the DENV types influence disease outcome, vaccine-induced protection, epidemic magnitude, and viral evolution. We characterized antigenic diversity in the DENV types by antigenic maps constructed from neutralizing antibody titers obtained from African green monkeys and after human vaccination and natural infections. Genetically, geographically, and temporally, diverse DENV isolates clustered loosely by type, but we found many are as similar antigenically to a virus of a different type as to some viruses of the same type. Primary infection antisera did not neutralize all viruses of the same DENV type any better than other types did up to two years after infection and did not show improved neutralization to homologous type isolates. That the canonical DENV types are not antigenically homogenous has implications for vaccination and research on the dynamics of immunity, disease, and the evolution of DENV.

Dengue virus (DENV) infects up to 390 million people each year, and of the 96 million individuals who develop an acute systemic illness, approximately 500,000 experience potentially life-threatening complications, including hemorrhage and shock (1, 2). The four genetic DENV types have long been thought to exist as four serotypes, and the antigenic differences between the types are believed to have a key role in the severity of disease, epidemic magnitude, viral evolution, and design of vaccines (3–5).

The description of DENV types as serotypes originated with the observation that the human immune response following primary DENV infection fully protected against challenge with viruses of the homologous type but only partially, and transiently, protected against challenge by viruses of a heterologous type (6). This finding was supported by *in vitro* neutralization experiments in which each DENV type was on average better neutralized by homologous than heterologous DENV infection antisera (7). The immune response immediately after a primary DENV infection varied from individual to individual, but generally was characterized by high levels of neutralizing antibody titers to multiple DENV types. The neutralizing response was observed to become more DENV type-specific over time (8). It was later shown that antibodies to a heterologous DENV type could enhance infection *in vivo* and were associated with increased risk of severe disease in nature (9, 10). Although antigenic variability was observed within DENV types from the earliest studies, this variation is generally considered to be substantially less than the differences between types, and not thought to modify type-specific protection (11, 12). Together, the DENV types clearly form an antigenic subgroup within the *Flaviviridae* (13, 14). Analyses of envelope (E) proteins, and later full genomes, showed that the four types are as genetically divergent among themselves as sequences assigned to different viruses within the genus *Flavivirus* (15). These deep evolutionary divergences between DENV types were evident in the phylogenetic tree of the genetically diverse E-gene sequences of the viruses we investigated here (Fig. 1A; fig. S1; and table S1) (16). Similarly, a map of amino acid differences between the E proteins revealed four compact, segregated types (Fig. 1B and fig. S2), as the number of amino acid substitutions between heterologous types far exceeded the maximum difference within a type.

However, investigations that rely on the classification of DENV into serotypes do not fully explain clinical and epidemiological phenomena. Despite this, antigenic properties are still thought to play a critical role in the biology of DENV infections. One hypothesis is that antigenic differences are critical, but that categorization by serotype alone is too coarse a measure. For example, differences in epidemic magnitude might be determined not only by the serotype but also by the antigenic differences between the particular infecting viruses that populations experience during sequential epidemics. Antigenic variation within and among the DENV types has also been hypothesized, in addition to intrinsic viral fitness and other factors, to explain phenomena including extinction and replacement of previously successful lineages and variation in disease outcome caused by genetically similar viruses (17–19). Here, we empirically test the antigenic relationships among a panel of diverse DENV isolates and re-examine the serotype concept.

Antigenic differences among viruses are caused by amino acid differences that lead to structural changes on viral proteins that modify antibody binding. The structural effect of such amino acid substitutions is difficult to predict from genetic sequences alone. In some instances substitutions have no antigenic effect, sometimes single substitutions cause substantial antigenic change, and other times it takes multiple substitutions (20, 21). Thus today, antigenic differences must be determined by phenotype, including by an antibody neutralization assay (13). Most often, viruses are measured against multiple sera to form a table of neutralization data from which antigenic relationships are inferred (22). However, such inferences are notoriously difficult to make, and this has hindered the reliable systematic antigenic characterization of DENV. The difficulties are caused by random error, the use of diverse methods among laboratories, and the intrinsic variability among immune sera due to differences in hosts and infection histories (23, 24). Moreover, neutralization data often contain apparent contradictions that are difficult to interpret, such as higher-than-homologous titers and sera that similarly neutralize multiple DENV types.

Previous antigenic analyses of DENV have addressed such challenges by using monoclonal antibodies, averaging responses of many individuals, or excluding sera with unusual patterns of reactivity. Despite careful work, these approaches have not produced a unified framework for understanding patterns across large neutralization data sets. Antigenic cartography is a method that positions viruses and antisera as points in a map, such that the distance between each virus and antiserum is derived from the corresponding neutralization titer in the tabular data. This method exploits variation in host responses to better triangulate the map, reduces the effect of some measurement errors because each virus is measured against multiple antisera (and vice versa), and has been shown to accurately interpret apparent contradictions in the data (25).

We formed the Dengue Antigenic Cartography Consortium, an open collaboration of international research laboratories, to establish empirically how DENV types relate to one another antigenically. Thirty-six African green monkeys (*Chlorocebus sabaeus*, hereafter NHP) were experimentally inoculated with diverse DENV isolates, and their sera were tested for neutralizing antibody potency against the genetically (all known genotypes), temporally (1944–2012), and geographically (20 countries) diverse panel of DENV isolates shown in Fig. 1 (table S1). Serum samples were taken three months post-inoculation, and

titrations were conducted using an immunofocus reduction neutralization test on mosquito cells (C6/36, *Aedes albopictus*) (tables S2-S7 and fig. S3) (16, 26). A conventional interpretation of the raw antibody neutralization titers was consistent with previous observations, both for DENV and for other flaviviruses: antisera were generally able to neutralize viruses of the infecting type better than heterologous types.

The cartographic analyses fit these data with low error and were internally consistent (figs. S4, S6, and S7). Only 1% of map distances differed by more than four-fold from the measured titer (table S8). The positions of viruses and antisera were robust to different methods of calculating neutralization titers and to the exclusion of outliers (figs. S5, S8-S12 and table S10). Maps made with random subsets of the data set could predict excluded titers within two-fold error ($r=0.90$ for the relation between all measured and predicted titers) (table S9).

Our analyses showed that the DENV isolates in our panel did group according to current serotype classification (Fig. 2), and the majority of viruses neighboring any given virus are of the same DENV type. However, many of the viruses were positioned as close to a virus of another DENV type as to some viruses of their own type, and the distance within and between types was comparable. Similarly, while neutralizing antisera responses clustered closely to viruses of the homologous type, almost all were at least as close to a heterologous-type isolate (table S11, table S12).

To examine these findings in detail, we evaluated whether the observed antigenic diversity of the virus types was also observed with human antisera and over time, and whether the neutralizing responses of individual antisera became increasingly type-specific over time.

We titrated human antisera derived from vaccination with a live-attenuated chimeric DENV vaccine against the genetically diverse DENV panel. Individuals lacking detectable neutralizing antibodies against DENV or other flaviviruses were each inoculated with one monovalent component of the National Institutes of Health DENV vaccine ($n=40$ in total, 10 per DENV type). Antisera drawn 42 days post-injection were titrated against the DENV panel ($n=36$) using the neutralization test on mosquito cells. The resulting antigenic map is consistent with the NHP map in that the distance between DENV types was equivalent to the spread within type, and the overall orientation of DENV1-4 was the same (Fig. 3A).

We measured the antigenic relationships among the DENV panel as recognized by antisera drawn from naturally-infected individuals, who had neutralizing responses representative of the cohort study from which they were selected. Serum samples drawn from 20 Nicaraguan children in the year following their first DENV infection were titrated, using the neutralization test on mosquito cells, against 14 viruses that captured the breadth of variation seen in the DENV panel in Fig. 2. Again, the antigenic distances among the DENV types were similar to those observed with NHP and human vaccine antisera, although the DENV4 cluster was positioned adjacent to DENV1 and DENV2 (Fig. 3B).

We also analyzed neutralization data from other studies that had used antisera from monovalent vaccine recipients and naturally infected human travelers, as well as different neutralization assays (22, 27, 28). Again, the antisera from these studies also recognized the

antigenic relationships among the DENV isolates similarly to the three-month NHP antisera (figs. S23-S25).

The early antibody response is assumed to broadly neutralize all DENV types, but over time cross-type neutralization is thought to be lost so that the antibody response remaining in the months to years after infection only potently neutralizes isolates of the infecting type (8, 29, 30). We compared how antisera taken at various time points after infection recognize antigenic relationships among the DENV panel. The human antisera used to make the antigenic maps described above were taken at various times following infection, ranging from 42 days for the monovalent vaccine antisera to more than one year for the natural infection antisera. We also made an antigenic map of a published neutralization data set of 44 DENV isolates titrated with one-year post-inoculation monkey antisera and found a similar range of antigenic variants among the four DENV types (fig. S26) (12). Thus, in maps made with early (one month) as well as late convalescent (three months to one year) antisera, the antigenic relationships among diverse DENV isolates were similar to those observed with three-month NHP antisera.

We tested if the patterns of antigenic recognition of the antisera from serially sampled individuals changed with time. We titrated antisera from the experimentally inoculated NHPs one month (n=36) and five months (n=16) post-infection against the DENV panel. As expected, the magnitude of the neutralizing titers generally dropped between one, three, and five months (table S14). However, viruses on the one and five-month antigenic maps showed the same orientation of types as the three-month antisera. At one month after infection, 55%, and at five months after infection, 41% of the viruses, respectively, clustered as closely to a virus in a heterologous type as to some viruses of the same type (Fig. 4A and B; table S11; table S13; and table S15). The antigenic relationships among isolates were conserved across time-points (fig. S13). We thus found that the antigenic relationships among the isolates in the DENV panel were recognized similarly by early and late convalescent antisera from the same individuals.

We measured changes in neutralizing type-specificity for each NHP by comparing the antiserum positions in the one, three, and five-month antigenic maps. The antiserum positions shifted (on average, greater than four-fold) between one month and three months, consistent with the period of somatic hypermutation and selection for affinity matured B cells (Fig. 4A and fig. S14). However, few antisera showed improved neutralization of the infecting DENV type relative to heterologous types between one and three months. The antiserum positions changed minimally between three months and five months, despite a significant decline in the magnitude of titers over that period, in some cases below the assay limit of detection (Fig. 4B and table S14). Thus, we did not observe a systematic shift toward increasing neutralizing specificity to viruses of the infecting type nor decreasing specificity toward heterotypic viruses (fig. S15 and fig. S21).

Published studies of neutralizing responses in the first year after experimental inoculation also reported stability of neutralization specificity. In one study, the ratio between homologous and heterologous neutralizing titers for 16 Rhesus monkeys between 4-13 months after experimental inoculation was remarkably consistent. NHPs that were initially

type-specific remained so, while those that exhibited early cross-type titers maintained titers to those types to the end of the study period (fig. S28) (31). A second study following the neutralizing responses of *Aotus nancymae* monkeys for 1-4 months to DENV1 and DENV2 isolates showed similarly stable neutralization specificity to the infecting type and heterologous types (fig. S29) (32).

We further analyzed the neutralizing responses in the natural human infection data set to look at the type-specificity of antisera obtained during the first two years after infection. The antisera in the map in Fig. 3B ranged in neutralizing type-specificity, with 55% of antisera responses clustering as closely to a heterologous isolate as some homologous isolates. For each individual, the serum position in Fig. 3B, made with titrations conducted on mosquito cells, closely corresponded to the serum position in the map made with titrations using human cells expressing the DENV attachment factor, DC-SIGN (Fig. 3B and fig. S16). The position of the DENV4 cluster was between DENV1 and DENV2 on both maps (Fig. 3B and fig. S16). We compared the antibody titrations after one and two years for each individual, and found that all maintained the pattern of neutralization, including cross-neutralization, observed in the first year after infection (fig. S17 and S18). Thus, neutralizing antibody responses in natural human DENV infections did not show a trend toward increasing type-specificity even two years after infection.

Type-specific and cross-reactive neutralizing antibodies are thought to target distinct viral structures, and thus potentially may produce different antigenic maps (33). We therefore tested whether cross-reactive neutralizing antisera recognized different antigenic relationships among the DENV panel than type-specific neutralizing responses, using the serum positions of the monovalent vaccine map (Fig. 3A). Despite the fact that all ten individuals for each DENV type were inoculated with the same vaccine component, the antisera responses to the isolates varied. Collectively, the antisera provided a coherent description of antigenic patterns among the isolates (fig. S19). The relationships among the DENV panel changed minimally between maps made with only the most central, cross-reactive 20 antisera or only the most peripheral, type-specific 20 antisera (fig. S20 and fig. S22). Thus, the DENV type-specific and cross-reactive neutralizing responses recognized the same antigenic relationships among the DENV panel.

The antigenic characterization of any pathogen relies on the biological relevance of the assay used to generate the data. Both recent and historical studies have found significant associations between pre-infection neutralization titers and DENV viremia or infection outcome (34-37); however, other studies have been inconclusive (38, 39). Thus, the identification of immune correlates of protection including, but not exclusively, potentially neutralizing antibodies, is an active area of research for DENV (40-42). Notably, the antigenic patterns in our data are similar to those in antigenic maps we made of DENV antibody neutralization data from other published studies using different cell lines, virus preparations, methods for detecting infected cells, and plaque or immunofocus reduction end-points (figs. S23-S27) (12, 19, 22, 27, 28). We also found that the human antisera from natural infections titrated on mosquito cells showed similar neutralization profiles to those titrated on human cells (fig. S16 and S18). The antigenic variation we observed is thus not limited to the assay or samples that we used.

While overall, prior immunity to a heterologous DENV type still remains the strongest risk factor for disease, there is evidence that neutralizing responses to the particular DENV lineages circulating in a population modifies the magnitude and severity of epidemics caused by subsequent infecting lineages (17, 18). In one study, cross-type neutralization provided by prior DENV1 immunity correlated with a mild epidemic caused by one lineage of DENV2, but showed no neutralization of other DENV2 lineages that in immunologically similar populations caused severe epidemics (fig. S27) (19). These, and our, studies highlight the importance of studying the specific relationship between antigenic distances as measured with neutralizing antibody titers and protection. The approach described here, in combination with global surveillance of the genetic, antigenic, and clinical features of DENVs as well as further detailed studies of natural infection and vaccination-derived protection, has the potential to inform whether vaccination protects against circulating isolates as well as recognize gaps in vaccine-induced protection should they emerge over time.

The antigenic analyses shown here using one, three, and five-month NHP antisera, human monovalent vaccine antisera, late-convalescent human natural infection antisera, and published neutralization data show that the DENV types do not fall into order as distinct serotypes. We have found that while DENV isolates are usually located closer to other viruses of the same type, some viruses, both modern and historical, have greater antigenic resemblance to viruses of a different type than to some viruses of the same type. We find that primary infection neutralizing antibody titers, although they drop in magnitude, do not systematically become more type-specific in the year after primary infection. As expected, individuals infected with the same or different antigens have variable patterns of neutralization, but cross-neutralizing responses consistently recognize the same antigenic relationships within the DENV panel as do the neutralizing responses that are most type-specific. These findings shift our understanding of the antigenic properties of DENV, enable more detailed study of the antigenic determinants of clinical severity, epidemic magnitude, and DENV evolution, and provide additional methods for the selection of future vaccine strains and global surveillance of the antigenic dynamics of dengue viruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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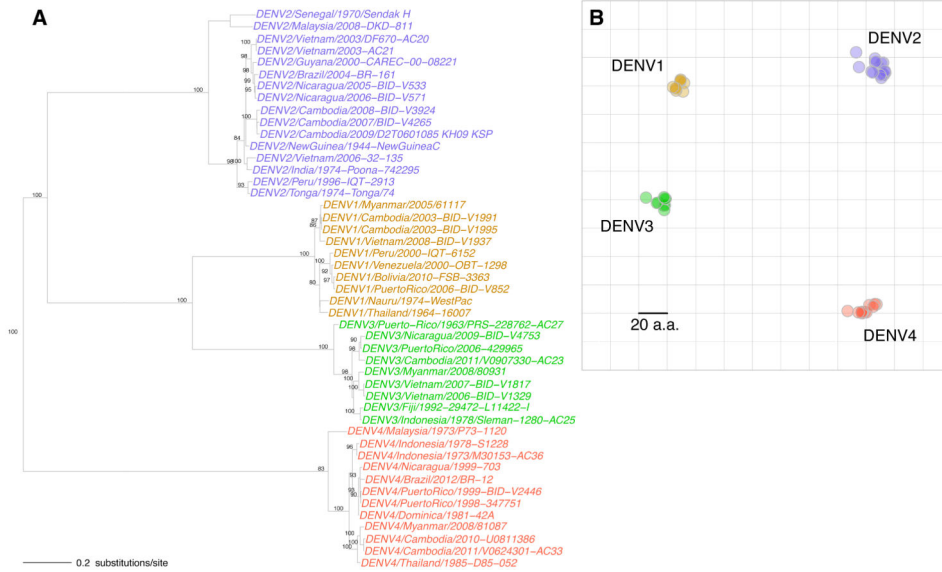


Fig. 1. Genetic analyses of the DENV panel (n=47). **(A)** Phylogenetic tree showing the evolutionary relationships of DENV E gene sequences. Sequences were aligned with MAFFT, and a maximum likelihood tree (ML) was estimated using a general time reversible model, accounting for both among site rate variation and invariant sites (GTR+G₄+I). Bootstrap support values of at least 75% are shown. **(B)** Amino acid map of dengue E protein sequences (493-495 amino acids in length). The total amino acid differences between pairs of E sequences correspond to distances between points on the geometric display.

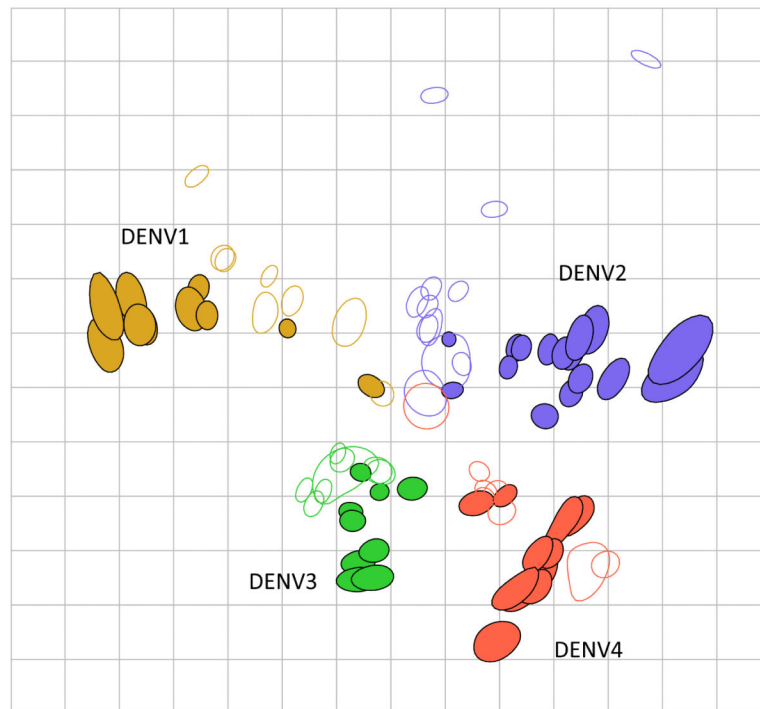


Fig. 2. Antigenic map of the DENV panel (n=46) titrated against three-month post-infection African green monkey antisera (n=36). Each unit of antigenic distance (length of one grid-square side, measured in any direction) is equivalent to a two-fold dilution in the neutralization assay. Each antiserum (open shape) and virus (closed shape) is colored according to the infecting genetic type (*16*). The size and shape of each point is the confidence area of its position.

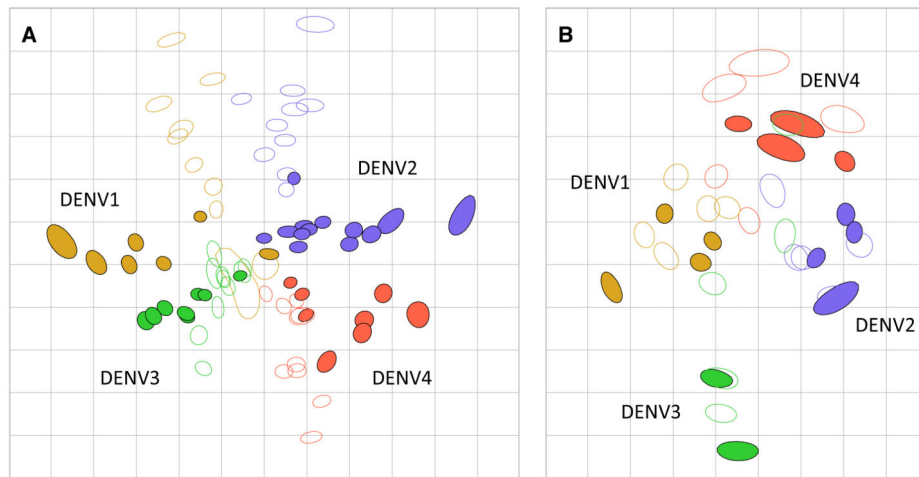


Fig. 3. Human primary infection antigenic maps. **(A)** Antisera from individuals inoculated with each monovalent component of the NIH live vaccine (10 per group) were drawn 42 days post-infection and titrated against 36 viruses in the DENV panel. **(B)** Antisera from 20 Nicaraguan children drawn in the year after their first DENV infections were titrated against an antigenically diverse subset of the DENV panel (n=14).

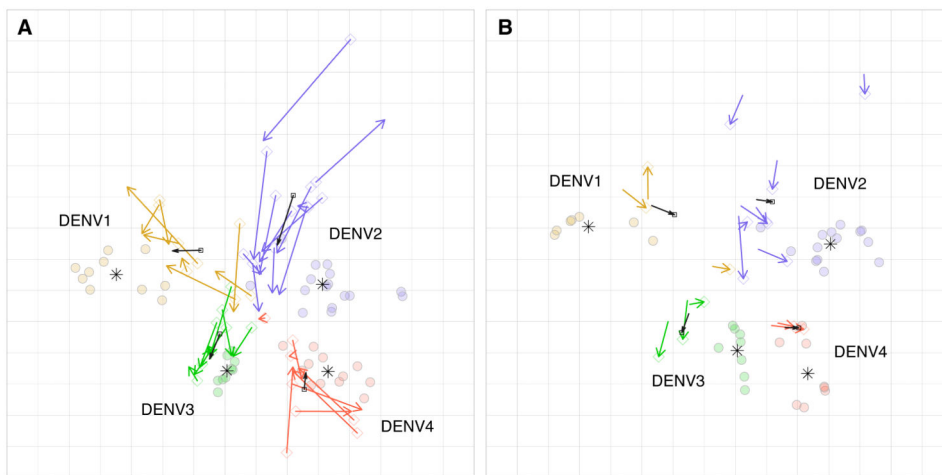


Fig. 4. Antigenic maps of the DENV panel made with antisera drawn from NHPs one and five months post-infection. **(A)** An antigenic map of 47 DENV isolates titrated against 36 NHP antisera drawn one month post-infection. Colored arrows (DENV1=yellow, DENV2=blue, DENV3=green, DENV4=red) show the change in antiserum positions between one and three months. The black arrows show the average shift in serum position for each DENV type. The star denotes the antigenic center for each DENV type. **(B)** An antigenic map of 37 DENV isolates titrated against 16 NHP antisera drawn five months post-infection. Arrows point from positions of antisera at three months to the corresponding five-month positions.