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Short Communication

Dengue-Immune Humans Have Higher Levels of Complement-Independent Enhancing Antibody than Complement-Dependent Neutralizing Antibody

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SUMMARY: Dengue is the most important arboviral disease worldwide. We previously reported that most inhabitants of dengue-endemic countries who are naturally immune to the disease have infection-enhancing antibodies whose in vitro activity does not decrease in the presence of complement (complement-independent enhancing antibodies, or CiEAb). Here, we compared levels of CiEAb and complement-dependent neutralizing antibodies (CdNAb) in dengue-immune humans. A typical antibody dose-response pattern obtained in our assay system to measure the balance between neutralizing and enhancing antibodies showed both neutralizing and enhancing activities depending on serum dilution factor. The addition of complement to the assay system increased the activity of neutralizing antibodies at lower dilutions, indicating the presence of CdNAb. In contrast, similar dose-response curves were obtained with and without complement at higher dilutions, indicating higher levels of CiEAb than CdNAb. For experimental support for the higher CiEAb levels, a cocktail of mouse monoclonal antibodies against dengue virus type 1 was prepared. The antibody dose-response curves obtained in this assay, with or without complement, were similar to those obtained with human serum samples when a high proportion of D1-V-3H12 (an antibody exhibiting only enhancing activity and thus a model for CiEAb) was used in the cocktail. This study revealed higher-level induction of CiEAb than CdNAb in humans naturally infected with dengue viruses.

Dengue fever and dengue hemorrhagic fever are globally important mosquito-transmitted viral diseases, with an estimated 390 million infections annually (1). The causative agents are dengue viruses (DENV-1 to DENV-4) of the genus *Flavivirus*, which are distributed throughout tropical regions (2,3). The only available licensed vaccine against dengue is not highly protective (4–6). Infection-enhancing antibodies are hypothesized to be involved in disease severity (7,8), while neutralizing antibodies are thought to be protective (9). Complement in the circulation may reduce the activity of enhancing antibodies (10,11). We previously reported that most dengue-immune inhabitants in endemic countries induce complement-independent enhancing antibodies (CiEAb; 12). Here, we compared levels of CiEAb and complement-dependent neutralizing antibodies (CdNAb) in dengue-immune humans.

In this study, we used human serum samples that were collected from general patients in Indonesia between 1999 and 2000 (13). The use of human serum samples was approved by the Ethical Committees of Kobe University Graduate School of Medicine and the Faculty of Tropical Medicine, Mahidol University. Before the antibody assays, the sera were heat-inactivated at 56°C for 30 min. Mouse monoclonal antibodies (MAbs) against DENV-1 (D1-IV-7F4, D1-V-3H12) have been reported (14). Another MAb against DENV-1 (D1-IV-1C8) was obtained when the MAbs described above were generated. The balance between the enhancing and neutralizing activities was measured in semi-adherent K562 cells, as previously described (15). Briefly, serial dilutions of antibody specimens were mixed with DENV-1 (Mochizuki strain) in the absence or presence of 5% rabbit complement, and then incubated at 37°C for 2 h. K562 cells were then added and incubated at 37°C for 2 days. After fixation and immunostaining, the numbers of infected cells were counted. The cut-off values for the enhancing and neutralizing activities were calculated as the means ± 3SD of 8 negative controls, adjusted for approximately 100 infected cells.

The leftmost lower panel of Fig. 1A shows the typical dose-dependent neutralizing and enhancing antibody activity patterns observed in dengue-immune human serum (I#230) in our assay system, with and without complement. This sample displayed neutralizing, enhancing, and no activities at low, medium, and high serum dilutions, respectively. Addition of complement to the assay system reduced the infected cell counts at dilutions of 1:10 to 1:2,560, but not at 1:10,240 to 1:163,840, indicating that the activity level of the complement-independent antibody was higher than that of complement-dependent antibody.

The other lower panels of Fig. 1A show the dose-dependent antibody activity patterns observed with 2 more

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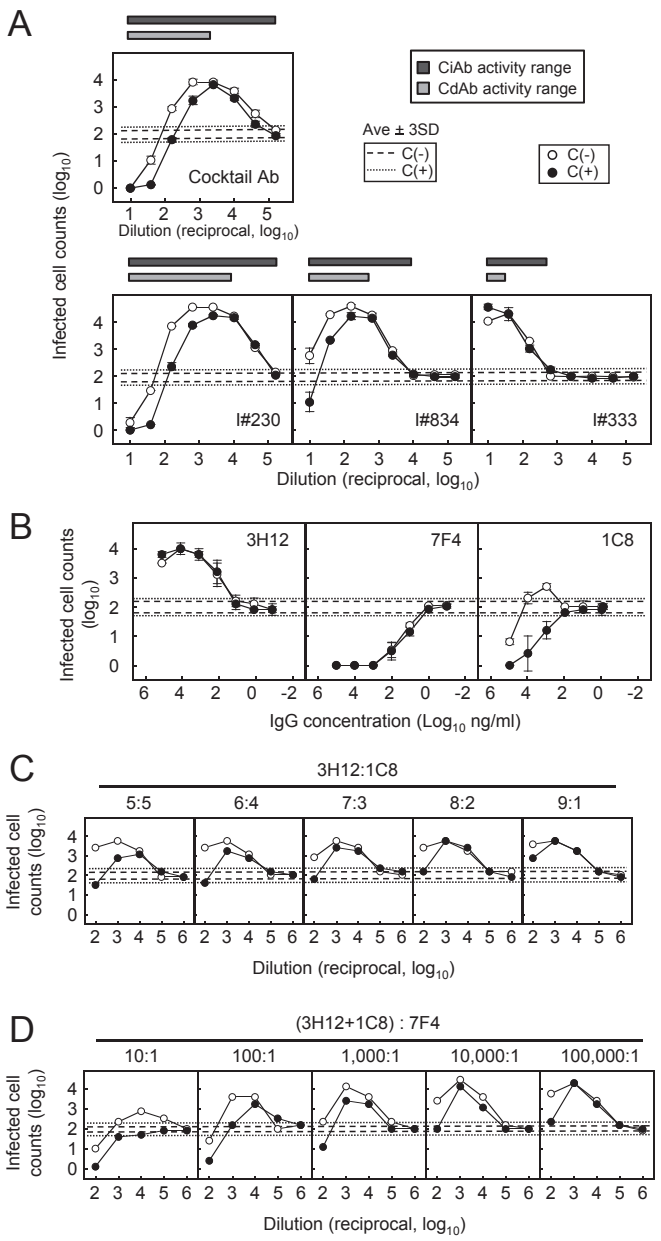

Fig. 1. Antibody cocktails composed of 3H12, 7F4, and 1C8 mimic.dengue-immune human sera. (A) Dose-dependent activity curves obtained with the antibody cocktail (upper panel) and human sera (lower panels) against DENV-1. Our antibody assay to measure the neutralizing/enhancing activity balance was carried out with DENV-1 in the presence (closed circles) or absence (open circles) of complement. The abscissa indicates dilutions of the serum sample, while the ordinates infected cell counts. Dark and light gray bars indicate the dilution range of complement-independent antibody (CiAb) and complement-dependent antibody (CdAb) displaying neutralizing/enhancing activities, respectively. The 3H12:7F4:1C8 ratio in the antibody cocktail was 8,000:1:2,000. (B) Dose-dependent activity curves obtained with DENV-1 MAbs used to prepare the antibody cocktail. (C, D) Preliminary experiments to decide the ratio of 3H12 and 1C8 (C) and the ratio of the 3H12–1C8 mixture and 7F4 (D) in the antibody cocktail. The IgG concentration of all MAbs was adjusted to 1 mg/ml before mixing. Dotted and broken lines indicate cut-off values used to differentiate neutralizing/enhancing activities from non-neutralizing/non-enhancing activities in the presence and absence of complement, respectively. In A and B, each datum represents the average result for 2 separate assays, with SD (error bars).

dengue-immune human sera (I#834 and I#333). These samples showed dose-response curves equivalent to a portion of the curve obtained for I#230, at dilutions of 1:160 or higher for I#834, and 1:2,560 or higher for I#333. Because the waning of neutralizing antibodies is hypothesized to be a mechanism by which dengue viral infection is enhanced (8), the patterns of I#834 and I#333 may be attributable to antibody waning compared to the pattern of I#230. In our previous study using 94 dengue-immune humans (12), we found that neutralizing activity in humans was exhibited only by CdNAb, and not by complement-independent antibodies. Furthermore, although complement-dependent enhancing antibody activities were also observed (e.g., 1:40 dilution of I#834), they always appeared at higher dilutions than those at which complement-dependent neutralizing activities were observed (e.g., 1:10 dilution of I#834). Thus, we regarded complement-dependent antibody as CdNAb, while complement-independent antibody CiEAb. Because these 3 dose-response patterns were generally observed in sera from dengue-immune humans (12), our results suggest that CiEAb is induced at higher levels than CdNAb in these subjects.

Another method we used to determine the proportion of CiEAb in dengue-immune human sera involved the preparation of a MAb cocktail used to mimic the human antibody dose-dependent activity patterns. The cocktail was composed of 3H12 (an enhancing-only antibody and thus a model for CiEAb), 7F4 (a neutralizing-only antibody), and 1C8 (CdNAb; Fig. 1B). When these MAbs were adjusted to an IgG concentration of 1 mg/ml and mixed in a specific ratio (3H12:7F4:1C8 = 8,000:1:2,000), the dose-response curves obtained in the assay, with or without complement (Fig. 1A: upper panel), were similar to those obtained with human serum samples (Fig. 1A: lower panels). The first step in determining this mixing ratio was to mix various ratios of 1C8 to 3H12 (Fig. 1C). Because mouse MAbs with both neutralizing and enhancing activities usually do not show enhancing activity in the presence of complement, the addition of the enhancing-only antibody was required to reproduce the dose-response patterns characteristic of dengue-immune human sera, in which complement-independent enhancing activities were observed in the high-dilution range. We selected a 3H12:1C8 ratio of 8:2 and then combined this mixture with 7F4 in varying ratios to adjust the neutralizing activity at low dilutions. From the dose-response curves (Fig. 1D), we selected a 3H12 + 1C8:7F4 ratio of 10,000:1, because ratios of 10:1–1,000:1 did not maintain the dose-response patterns characteristic of complement-independent enhancing activity at high dilutions. The mixing ratio of the MAbs in the antibody cocktail that mimicked the dose-response patterns of dengue-immune human sera suggested that a 3H12-like antibody was predominant in the sera. This result supports the presence of a high proportion of CiEAb in dengue-immune human sera.

In conclusion, this study revealed that natural infection with DENVs in humans induces CiEAb more than CdNAb. Although the serotype that had infected the dengue-immune people in this study is unknown, dose-dependent neutralizing and enhancing antibody activity patterns obtained in our assay system were similar,

irrespective of the infecting serotype (12,16). Because of the difference between humans and mice and the presence of various antibodies in polyclonal status, further studies are needed using a sufficient number of human MAbs. Recently, we found an inhibitory effect of CiEAb on the neutralizing activity of CdNAb (17), suggesting that concomitant induction of CiEAb suppresses the protective effect of CdNAb in vivo. Thus, further studies are also needed examining this aspect. These findings have implications for dengue vaccine development, because the currently licensed dengue vaccines are created from antigens derived from viral strains isolated from nature.

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Conflict of interest None to declare.

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