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Monoclonal Antibodies That Bind to Domain III of Dengue Virus E Glycoprotein Are the Most Efficient Blockers of Virus Adsorption to Vero Cells

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The specific mechanisms by which antibodies neutralize flavivirus infectivity are not completely understood. To study these mechanisms in more detail, we analyzed the ability of a well-defined set of anti-dengue (DEN) virus E-glycoprotein-specific monoclonal antibodies (MAbs) to block virus adsorption to Vero cells. In contrast to previous studies, the binding sites of these MAbs were localized to one of three structural domains (I, II, and III) in the E glycoprotein. The results indicate that most MAbs that neutralize virus infectivity do so, at least in part, by the blocking of virus adsorption. However, MAbs specific for domain III were the strongest blockers of virus adsorption. These results extend our understanding of the structure-function relationships in the E glycoprotein of DEN virus and provide the first direct evidence that domain III encodes the primary flavivirus receptor-binding motif.

The flavivirus E glycoprotein is the primary antigen inducing protective immunity, is essential for membrane fusion, and mediates binding to cellular receptors. Therefore, this protein directly affects host range, cellular tropism, and, in part, the virulence of these viruses (17, 18). The crystal structure of the ectodomain of the tick-borne encephalitis (TBE) virus E-glycoprotein homodimer was recently solved at high resolution (16). Multiple lines of evidence indicate that this E-glycoprotein structure is strongly conserved across the *Flaviviridae* (16). This protein contains three structural domains. The central domain, domain I (DI), contains predominately type-specific nonneutralizing epitopes and is theorized to be the molecular hinge region involved in low-pH-triggered conformational changes (19). The dimerization domain, domain II (DII), makes important contacts with itself in the homodimer, is involved in virus-mediated membrane fusion, and contains many cross-reactive epitopes eliciting neutralizing and nonneutralizing monoclonal antibodies (MAbs) (16, 19). Domain III (DIII) is characterized by an immunoglobulin-like structure containing the most distal projecting loops from the virion surface. It contains multiple type- and subtype-specific epitopes eliciting only virus-neutralizing MAbs and has been hypothesized to contain the host cell-binding antireceptor (16, 18, 19). As part of our ongoing research to elucidate the structure-function relationships of the dengue (DEN) virus E glycoprotein, we have assessed the ability of a well-characterized panel of E-glycoprotein-specific MAbs to block virus adsorption to Vero cells. These results provide the first direct evidence that E glycoprotein DIII encodes a receptor-binding motif.

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DEN type 2 (DEN-2) virus strain 16681 was isolated in 1964 from the serum of a DEN hemorrhagic fever patient in Bangkok, Thailand. Virus seed was grown in *Aedes albopictus* C6/36 mosquito cells and contained 1.5×10^7 PFU/ml, as determined by plaque titration on Vero cells (19). Aliquots from the same seed were utilized for all assays. All MAbs utilized in this study have been described previously (19). The chemical and biological characteristics and the spatial arrangements and locations of the epitopes defined by these MAbs were determined previously (19).

To assess the effects of antibody-virus interaction on virus adsorption, a virus attachment curve was first established in Vero cell monolayers grown in six-well trays with minimal essential medium containing penicillin, streptomycin, and 5% fetal calf serum (20). We selected Vero cells because they are highly permissive to DEN virus infection and do not contain Fc receptors (2). They were therefore ideal for investigating DEN virus adsorption to mammalian cells without the confusing influence of potential virus-MAb-Fc receptor interactions. In addition, these cells were used in a previous investigation implicating the blocking of virus attachment as an important mechanism of neutralization for human DEN virus infection-immune serum (7). Attachment curves (50 to 100 PFU/assay) demonstrated that approximately 90% of virions had adsorbed to cells by 1 h at 4°C (data not shown).

To differentiate MAbs that neutralized virus by blocking virus adsorption from MAbs that neutralized virus postadsorption, we performed pre- and postadsorption assays (11). For the preadsorption assay, 0.5 ml of a virus dilution containing 2.5×10^2 PFU/ml (50 to 100 PFU/well, final virus concentration) was mixed with 0.5 ml of 10-fold MAb dilutions, and the mixture was incubated for 1 h at 4°C. The virus plus MAb mixture was then added to cells (80 to 90% confluent), and incubation continued for an additional hour at 4°C, a temperature that allows only virus adsorption to occur. Negative controls received 0.5 ml of phosphate-buffered saline (PBS) in-

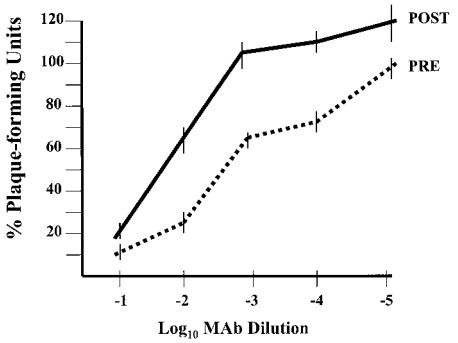

FIG. 1. Representative dose-dependent blocking of adsorption by a DIII MAb, 9D12, in the pre- and postadsorption assays.

stead of MAb. Cell sheets were washed three times with 2 ml of PBS at 4°C, the liquid was aspirated from the cells, and cells were overlaid with 4 ml of a 1% agarose–medium mixture (12). After 5 days of incubation at 37°C, the plates were again overlaid with 1% agarose–medium containing 0.01% neutral red, and plaques were counted over the next 30 to 50 h. In this assay, MAbs were present prior to, during, and just after virus adsorption to cells. The preadsorption assay, therefore, measured potential neutralization by any mechanism early in the infection cycle, including the direct blocking of adsorption.

For the postadsorption assay, 0.5 ml of the virus seed dilution from the preadsorption assay was mixed with 0.5 ml of PBS and added directly to cells, and the mixture was incubated for 1 h at 4°C. Unadsorbed virus was removed by three washes with PBS at 4°C. The 10-fold MAb dilutions were then added directly to washed cells containing adsorbed virus, followed by incubation for 1 h at 4°C. Negative controls received 0.5 ml of PBS at 4°C instead of MAb dilutions during this incubation. Following MAb binding, cells were washed three times with PBS at 4°C, overlaid with agarose, and incubated, and PFU were counted as in the preadsorption assay. In this assay, MAb is present only after virus has adsorbed to cells. Therefore, the ability of a MAb to block adsorption is represented as the difference in its abilities to neutralize virus infectivity in the post- and preadsorption assays. This value was calculated using the following formula:

$$\%\ \text{blocking} = 100 \times [(\text{PFU upon MAb treatment in the postadsorption assay/PFU upon negative control treatment in the postadsorption assay}) - (\text{PFU upon MAb treatment in the preadsorption assay/PFU upon negative control treatment in the preadsorption assay})]$$

An example of the pre- and postadsorption neutralizing activities of a representative blocking MAb, 9D12, are shown in Fig. 1.

In comparing the abilities of MAbs to block adsorption, we found internally consistent patterns for epitopes within a structural domain and distinct patterns of blocking between domains (Table 1). Representative blocking profiles for DI-, DII-, and DIII-specific MAbs and appropriate positive and negative control antibodies are shown in Fig. 2. All MAbs recognizing epitopes in DIII strongly blocked virus adsorption (36 to 49% blocking) (Table 1; Fig. 2C). All DIII-specific MAbs blocked adsorption to maximal levels greater than or equal to the maximal blocking ability (34%) of a polyclonal murine anti-DEN-2 virus hyperimmune ascitic fluid (HIAF) (Table 1; Fig. 2C and E). Moreover, all DIII-specific MAbs significantly blocked adsorption (i.e., the percent blocking minus the 95% confidence interval [CI] was greater than zero) across a wide range of MAb dilutions (Table 1). The apparent low levels of blocking shown in Fig. 2C and E suggest a possible prozone at high MAb concentrations. However, this phenomenon is actually due to high levels of virus neutralization obscuring the difference between the post- and preadsorption

TABLE 1. Comparison of biological activities of anti-DEN-2 virus MAbs

Epitope (MAb)	E-glycoprotein domain	90% PRNT[a]	Fusion Blocking	ELISA result[b]		Maximum % blocking[c]	Log_{10} MAb dilution exhibiting blocking[f]
				Bound	Captured		
A1 (6B6C-1)	II	±	+	5.2	≥5.3	18 (11)	1
A1 (4G2)	II	+	ND[d]	5.3	≥5.3	32 (9)	1–2
A2 (4E5)	II	+	+	4.2	2.6	12 (12)	5
A3 (1A5D-1)	II	−	−	5.3	3.5	—	NA[e]
A5 (1B7)	II	+	+	5.3	≥5.3	23 (6)	1–3
A/C (10A1D-2)	I/II	−	−	4.4	2.9	—	NA
C1 (1B4C-2)	I	−	−	6.2	≥5.3	46 (15)	2, 6
C2 (4A5C-8)	I	−	−	3.2	2.0	ND	ND
C3 (2B3A-1)	I	−	−	5.3	2.0	ND	ND
C4 (9A4D-1)	I	−	−	4.4	2.9	ND	ND
B1 (3H5)	III	+	ND	5.3	≥5.3	36 (9)	3–5
B2 (9A3D-8)	III	+	−	5.8	≥5.3	42 (6)	2–3
B3 (10A4D-2)	III	+	−	5.3	≥5.3	44 (7)	2–5
B4 (1A1D-2)	III	+	−	5.3	≥5.3	49 (9)	2–5
B4 (9D12)	III	+	−	ND	ND	41 (7)	1–5
DEN-2 virus HIAF	NA	+	+	+	+	34 (18)	1–5
DEN-1 virus (1F1)	NA	−	−	≤1.0	≤1.0	10 (12)	None

[a] Plaque reduction neutralizing activity (PRNT) at a 1:100 dilution of ascitic fluid (19).
[b] Values are reciprocal end-point titers (log_{10}) determined by plate-bound (indirect) or antibody-captured virus (four-layer) ELISA (19).
[c] Values are average maximum percent blocking based on six replicates (values in parentheses are 95% CIs). —, antibodies enhanced virus binding.
[d] ND, not done.
[e] NA, not applicable.
[f] We considered a MAb to significantly block adsorption only if the percent blocking minus the 95% CI was greater than zero.

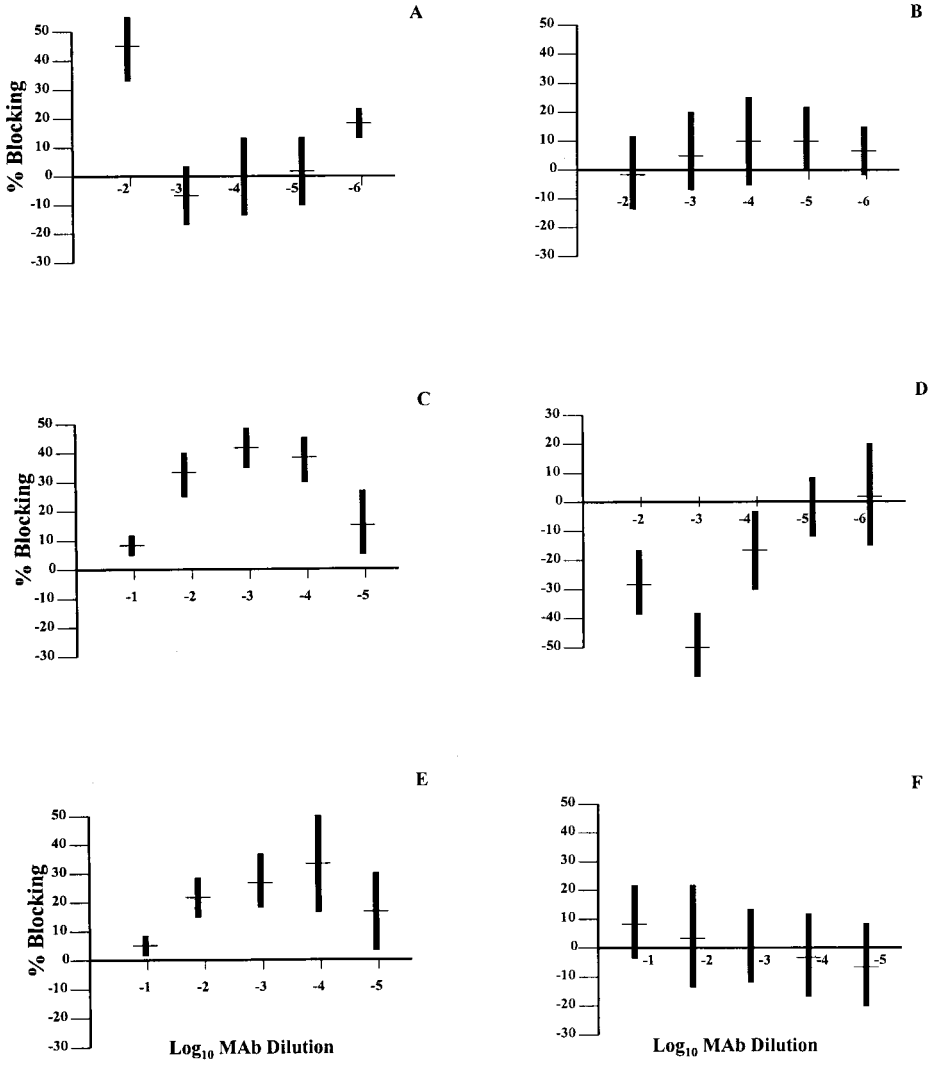

FIG. 2. Comparison of percent blocking of adsorption (±95% CI) by representative MAbs for the three distinct E-glycoprotein domains. (A) DI MAb 1B4C-2; (B) DII MAb 4E5; (C) DIII MAb 9D12; (D) DII MAb 1A5D-1; (E) DEN-2 virus polyclonal murine HIAF; (F) DEN-1-specific negative-control MAb 1F1. Panel D illustrates enhancement of virus adsorption.

assays and is not due to excess MAb (Fig. 1). DII MAbs fell into two distinct patterns of adsorption blocking (Table 1; Fig. 2B and D). The DII-specific MAbs that neutralized virus blocked adsorption but not as strongly as (12 to 32%) and less significantly than did those specific for DIII. Blocking by DII-specific MAbs was more similar to the statistically insignificant blocking (10%) of a DEN-1-specific negative control MAb, 1F1 (Table 1; Fig. 2B and F). Moreover, DII-specific neutralizing MAbs blocked adsorption only at relatively high concentrations (Table 1). Two nonneutralizing DII-specific MAbs actually enhanced virus adsorption at high MAb concentrations (Table 1; Fig. 2D). The single nonneutralizing DI-specific MAb that had been shown previously to recognize native virus strongly blocked adsorption (46%) but only at the 1:100 dilution of this highest-titer MAb (Table 1; Fig. 2A). Our MAbs specific for DI epitopes C2, C3, and C4 were not used in this analysis because of their poor ability to recognize native virus (Table 1). They are, therefore, presumably unable to block virus adsorption.

These results indicate that most anti-DEN virus MAbs neutralize, at least in part, by disrupting the virus's ability to bind mammalian cellular receptors. As with neutralization, the ability of a MAb to block adsorption also correlates with its ability to recognize and attach to native virus as measured in an enzyme-linked immunosorbent assay (ELISA) with captured virus (Table 1). Furthermore, by using a well-defined panel of MAbs, we have implicated DIII as the most likely region of the E glycoprotein to interact with the cell membrane receptors on Vero cells. This conclusion is supported by the consistently higher levels of blocking across multiple MAb dilutions observed with all DIII-specific MAbs and by their greater adsorption-blocking abilities, compared to polyclonal anti-DEN-2 virus HIAF. These results are consistent with those of others indicating that the blocking of adsorption is a common mech-

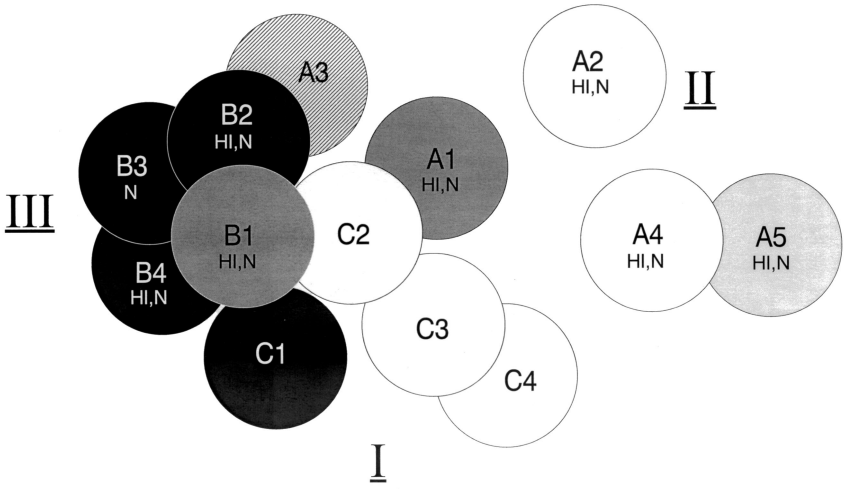

FIG. 3. Summary of biological activities and spatial arrangements of the DEN-2 virus E-glycoprotein epitopes listed in Table 1. The biological activities of MAbs elicited by these epitopes were hemagglutination inhibition (HI) and virus neutralization (N). The maximal values for the blocking of virus adsorption are indicated as follows: black, greater than 40%; dark gray, 30 to 40%; light gray, 20 to 30%; white, less than 20% (for A2) or not tested (for A4, C2, C3 and C4); cross-hatched, binding enhancement. Overlapping circles indicate spatially proximal epitopes. Epitope designations: A, DII; B, DIII; C, DI. The HA and N activities of these epitopes were reported previously (19).

anism of MAb neutralization for flaviviruses (7), rhinoviruses (5, 23), and influenza virus (25). We found previously that the majority of anti-Venezuelan equine encephalomyelitis virus MAbs also blocked virus adsorption to Vero cells and that one MAb enhanced virus adsorption (21).

The magnitude of MAb blocking of adsorption that we observed did not exceed 50%. Our observation that maximal blocking measured for MAbs is equal to or greater than that observed with a high-titer anti-DEN-2 virus polyclonal antibody suggests, however, that these results are representative of actual maximal blocking activity (Table 1; Fig. 2E). The use of isotopically labeled virus to quantify MAb blocking showed higher percent blocking activities than we observed (7, 21). However, consistent with our results, Hung et al. also observed maximal MAb blocking of DEN virus adsorption in the 20 to 60% range using an assay similar to ours (11). A major limitation of isotope-binding assays is that they measure only virus attachment and not the virus adsorption that ultimately leads to productive infection.

Some DI- and DII-reactive MAbs were able to block virus adsorption to Vero cells but not as strongly as did MAbs specific for DIII. Moreover, these MAbs blocked adsorption only at high concentrations of antibody. Although DI and DII are not, per se, believed to be part of the flavivirus antireceptor, a previous analysis of the spatial arrangement of these epitopes using competitive antibody-binding assays indicated that two of these epitopes that react with blocking MAbs (A1 and C1) and are accessible on the virion surface were proximal to DIII epitopes (19) (Fig. 3). Another explanation for the ability of DI- and DII-reactive MAbs to block virus adsorption may be the induction of distal conformational changes within DIII following MAb binding (8). In support of induced conformational changes following MAb binding, we found that two nonneutralizing DII-reactive MAbs enhanced virus adsorption. Heinz et al. (8) demonstrated that for TBE virus, the binding of one MAb was able to induce conformational changes in the E glycoprotein that enhanced the binding of a second MAb reactive with a distal epitope. A similar though not well-characterized MAb-induced enhancement of secondary MAb avidity has also been observed with DEN-2 virus (9). Finally, MAbs might interfere with normal homodimer contacts between DII and DIII, thereby indirectly interfering with virus adsorption (16). This might explain the blocking mediated by MAb 1B7, specific for epitope A5, which is not spatially close to DIII (Fig. 3). These hypotheses suggest that DII mediates neutralization by mechanisms other than the direct blocking of virus adsorption. It should be noted that MAbs specific for DII epitopes A1, A2, and A5 block virus-mediated cell membrane fusion (1, 19), previously demonstrated to be an important mechanism of flavivirus neutralization (6).

The finding that epitopes within DIII elicited the strongest MAb blockers of virus adsorption is consistent with previous studies suggesting that DIII is the location of the flavivirus antireceptor. These studies include the identification of the immunoglobulin-like structure of DIII in the TBE virus E glycoprotein, a structural motif common to many cellular adhesion proteins (16); the localization of mutations altering viral entry, cellular tropism, and virulence to DIII (10, 13–16, 24); the identification of putative GAG-binding motifs within DIII as possible receptors for heparan sulfate (4); the ability of soluble DIII from Langat virus to function as an antagonist for virus infectivity (3); and the use of MAbs recognizing defined epitopes within DIII to directly interfere with virus adsorption and entry processes (7, 11, 22). We are now producing mutations in DIII using an infectious cDNA clone of DEN-2 virus strain 16681. With this approach, we hope to more precisely identify the DIII structures involved in virus adsorption and MAb binding.

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