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Localization of four antigenic sites involved in Venezuelan equine encephalomyelitis virus protection

Brief Report

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Summary. Stable neutralization and protection escape variants of a virulent strain (Trinidad Donkey) of the VEE virus were selected by monoclonal antibodies (MAbs). Determination of nucleotide sequences of nine variants revealed a clustering of single mutations in four regions of the E1 and E2 glycoproteins. Involvement of amino acid residues 206 (site E1-1), 57 and 59 (site E2-2), 180, 182, 213, 214 and 216 (site E2-6) and 232 (site E2-3) in protective epitopes was demonstrated.

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Venezuelan equine encephalomyelitis (VEE) virus is an arthropod-borne member of the *Alphavirus* genus within the *Togaviridae* family. The VEE virus is composed of an icosahedral nucleocapsid containing a positive-sense, single-stranded RNA genome. The host derived lipid bilayer surrounding the nucleocapsid contains the glycoproteins E1 and E2 [6, 7]. Based on extensive studies, the antigenic structure of the E1 and E2 surface proteins of the TC-83 vaccine strains of the VEE virus has been determined. With the use of MAbs by competitive binding assay (CBA), mapping of the spatial arrangement of four epitopes on the E1 and 8 on the E2 proteins was achieved [21, 22]. A major neutralization (N) domain formed by clustering of six of the epitopes (five on the E2 and one on the E1) was revealed by the CBA. Variants of the TC-83 strain of the VEE virus have been selected using four MAbs recognizing epitopes of the N domain [11]. Determination of the nucleotide sequence

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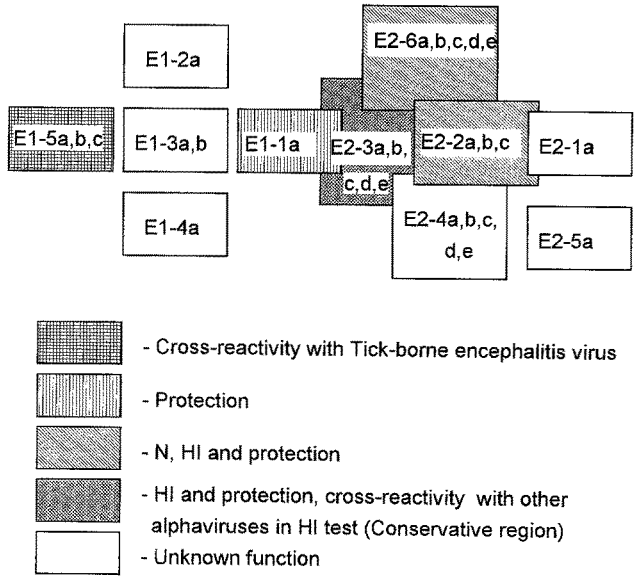

Fig. 1. Map of a spike of the VEE virus glycoprotein. The E1 or E2 sites are boxed; *a*–*f* are MAb epitopes. MAb competition is indicated by overlapping of the sites. The shaded area represents the MAb function

of the E1 and E2 genes of four variants revealed a clustering of single mutations in a domain spanning positions E2-182 to E2-207.

We have prepared a panel of MAbs against the VEE virus and with its use we have determined the topology of antigenic determinants of the E1 and E2 proteins of the Trinidad Donkey (Trd) virulent strain of the VEE virus [15–19]. There are 5 non-overlappings sites on the E1 glycoprotein including 8 epitopes of MAb binding, and 6 sites including 21 epitopes on the E2 glycoprotein (Fig.1). Roehrig's TC-83 epitope model [11, 21, 22] and our map of Trd virus have a number of common (structural and serological heterogeneity of epitopes, the presence of several neutralizing and hemagglutinating epitopes), as well as different (epitope number ranging from 5–13 protection (P) epitopes) features. Because each MAb panel used for epitope mapping is unique, differences in epitope maps were expected. These observations yield additional information about the antigenic structure of the E1 and E2 heterodimer.

It has been demonstrated that MAbs to three sites of E2 and one site of E1 can possess P activity [18, 19]. The monoclonal antibodies against these sites protect outbred albino mice from lethal infections caused by the virulent strain Trd of the VEE virus. Of 13 identified epitopes responsible for protective activity, three alterations in the TC-83 vaccine strain, and six epitopes are referred to two sites in vaccine strain 230 of the VEE virus [19].

Three MAbs recognizing the N sites E2-2 and E2-6 were used to select escape variants of the TC-83 and 230 strains of the VEE virus [1]. The nucleotide sequence of the E2 gene of four variants revealed single mutations at positions 57, 60 (site E2-2) and 213 (site E2-6) of the E2 glycoprotein of the VEE virus.

To identify P epitopes for inclusion in the alphavirus vaccine design, we selected variants of the virulent strain of the VEE virus that escaped N and P by each of their selective MAbs.

Table 1. Characterization of the VEE virus neutralization escape variants

Epitope	Immunogen	MAbs	Assays	Viruses						
				Trd	TC-83	230	AV. 2D4[a] (TC-83)	AV. 2D4 (Trd)	AV/1. 5A12 (Trd)	AV/2. 5A12 (Trd)
E2-6b	Trd	2D4	ELISA	730000	730000	<1000	<1000	<1000	730000	730000
			HI	1280	1280	<10	<10	<10	1280	1280
			N	5120	5120	<10	<10	<10	5120	5120
E2-6d	Trd	5A12	ELISA	145000	<100	<100	<100	145000	<1000	<1000
			HI	40	nd	nd	nd	40	<10	<10
			N	800	nd	nd	nd	800	<10	<10

AV. 2D4 (Trd) Antigenic variant of the VEE virus (strain Trd) was obtained with MAb 2D4

[a] From Agapov et al. [1]

nd Not determined

The VEE virus strains Trinidad Donkey, TC-83, and 230 were used in the study. They were received from the National Collection of Virus Strains at the Ivanovsky Institute of Virology of the Academy of Medical Sciences of Russia. Alphaviruses were grown on Vero cells and purified in a sucrose density gradient as described by Enzmann and Welland [4].

The collection of rat and murine hybridomas to the VEE virus used has been described elsewhere by Razumov et al. [15–17]. Ascitic fluids containing rat MAbs were produced by intraperitoneal injection of 1–2 $\times 10^6$ cells of the corresponding hybridomas into female LOU rats. Ascitic fluids were collected after 7–10 days. Murine MAbs were produced using syngenic BALB/c mice.

Neutralization reaction with alphaviruses was done by microtechnique on Vero cell cultures [2]. Hemagglutination inhibition (HI) tests were performed using goose erythrocytes and eight hemagglutinating units of VEE virus antigen [3]. Protective MAb activity was assayed on outbred albino mice weighing 8–10 g [17, 18]. An enzyme linked immunoabsorbent assay (ELISA) was carried out with the use of antispecies antibodies conjugated to horseradish peroxidase [20].

Antigenic variants (AVs) resistant to the protective effect of MAbs were selected as follows. Albino mice were passively immunized with 0.2 ml of MAbs (ascites) and, after 24 h, they were injected with various dilutions of the VEE virus, strain Trd. The AVs were isolated from affected mice infected with the minimum dose of virus. After three cycles of cloning by the method of limiting dilutions, the AVs were amplified on Vero cells or culture and used for further analyses. The AVs resistant to the neutralizing effect of MAbs were selected from Trd in cell culture as described by Agapov et al. [1]. The AVs produced in cell culture were used to isolate genomic RNA. cDNA produced with specific primers and corresponding to the genes of the

Table 2. Characterization of the VEE protection escape variants

MAbs	Immunogen	Epitope	Virus	Titer[a] logLD50/ml MAbs (-)	Titer[a] logLD50/ml MAbs(+)	Index protection $\log_{10}$	Titer[b] ELISA	HI
8D2	TC-83	E1-1a	Trd	10.2 ± 0.4	4.0 ± 0.7	6.2 ± 0.8	270000	<10
			AV.8D2	7.2 ± 0.4	7.3 ± 0.5	0.1 ± 0.6	270000	<10
B12	TC-83	E2-2a	Trd	9.9 ± 0.4	3.1 ± 0.5	6.8 ± 0.6	540000	640
			AV.B12	8.0 ± 0.5	8.0 ± 0.5	0 ± 0.7	<500	<10
7D1	TC-83	E2-2c	Trd	10.2 ± 0.4	5.1 ± 0.4	5.1 ± 0.6	540000	40
			AV.7D1	8.5 ± 0.3	8.7 ± 0.3	0.2 ± 0.4	<500	<10
7A6	TC-83	E2-3d	Trd	10.2 ± 0.4	8.1 ± 0.6	2.1 ± 0.7	180000	160
			AV.7A6	10.4 ± 0.4	10.2 ± 0.4	0.2 ± 0.6	180000	160
3E11	TC-83	E2-6a	Trd	10.4 ± 0.3	2.0 ± 0.4	8.4 ± 0.5	220000	320
			AV.3E11	9.5 ± 0.3	9.3 ± 0.4	0.2 ± 0.5	<500	<10
2D4	Trd	E2-6b	Trd	10.4 ± 0.3	4.0 ± 0.5	6.4 ± 0.6	730000	1280
			AV.2D4	10.5 ± 0.3	10.2 ± 0.4	0.3 ± 0.5	<500	<10

[a] Titers were assayed on outbred albino mice, each weighing 8–10 g

[b] Reciprocal titers

Table 3. Selection and mutations observed in the VEE variants

MAbs used for selection	Virus	Variants	Observed mutations in E1 or E2 mutation	change	position	Site epitope
			Variants selected in Vero cells			
2D4	Trd	AV.2D4	AAG→AAC	Lys→Asn	213	E2-6b
5A12	Trd	AV/1.5A12	TCC→TTC	Ser→Phe	180	E2-6d
		AV/2.5A12	AGC→AGA	Ser→Arg	182	E2-6d
			Variants selected in mice			
8D2	Trd	AV.8D2	GAA→AAA	Arg→Lys	206	E1-la
B12	Trd	AV.B12	TCC→TTC	Ser→Phe	57	E2-2a
7D1	Trd	AV.7D1	GGC→GAC	Gly→Asp	59	E2-2c
7A6	Trd	AV.7A6	CAG→GAG	Gln→Arg	232	E2-3d
3E11	Trd	AV.3E11	CAG→CCG	Gln→Pro	216	E2-6a
2D4	Trd	AV.2D4	ACA→CCA	Thr→Pro	214	E2-6b

E1 and E2 glycoproteins was cloned in E. coli cells as previously described [24]; nucleotide sequencing was done according to the procedure of Maxam and Gilbert [14].

The two MAbs recognizing the N site E2-6 were used to select variants of the virulent strains Trd of the VEE virus. Stable variant viruses were derived that escaped N by each of their selective MAbs (Table 1). All the three variants also showed reduced titers in ELISA, N and HI.

The six MAbs that we used to select P-resistant variants of the VEE virus define discrete sites in the E1–E2 heterodimer (E1-1, E2-2, E3-3 and E2-6). The MAbs to the VEE virus have been characterized in greater detail [15–19]. The MAbs to the E2-2 and E2-6 sites of the VEE virus had N, HI and P activities. Those to E2-3 had HI and P activities and the MAb to E1-1 possessed only P activity. Stable variant viruses were derived that escaped P by each of their selective MAbs (Table 2). Four variants demonstrated reduced titers in ELISA and HI. Antigenic variants produced with 7A6 and 8D2 MAb retained the ability to bind MAbs used for selection in ELISA.

The virulence of the variants was examined in outbred albino mice and rabbits by both intracerebral (only mouse) and subcutaneous inoculation. In mice, the virulence of all the variants was indistinguishable from that of Trd virus. None of the N and P escape variants was more virulent than its parent. In rabbits, all the variants (except AV 7D1) were attenuated when inoculated subcutaneously.

The Trd antigenic variants were analyzed by sequencing the genes encoding the E1 and E2 proteins and comparing these sequencing with those of the parent virus [5]. A single nucleotide change in the E1 or E2 genes was detected for each of the variant viruses. The predicted amino acid substitutions are shown in Table 3. The eight amino acid changes cluster in two regions of the E2 protein spanning positions 57 to 59 (site E2-2) and 180 to 216 (site E2-6). Two amino acid substitutions mapped at position 232 (epitope E2-3d) of E2 and 206 of E1 (site E1-1). Four of the

predicted amino acid substitutions (Gly→Asp, E2-59; Ser→Arg, E2-182; Lys→Asn, E2-213 and Gln→ Arg, E2-232) alter the charge of the E2 glycoprotein. Two mutations (Ser →Phe, E2-57 and Ser→Phe, E2-180) may result in perturbation of the surface topography of the E2 protein by insertion of a hydrophobic side chain. Another mutation (Thr→Pro, E2-214) altered the glycosylation site of the E2 glycoprotein.

We present serological and sequence evidence that the P sites of the VEE virus includes four regions from E2-182 to E2-216 (site E2-6), E2-57 to E2-59 (site E2-2), E2-232 (site E2-3) and E1-206 (site E1-1). Antigenic variants, which escaped the P or N effect of MAbs on E2-2 and E2-6 sites, escaped binding by selective MAbs. Mutations in the E1-1 and E2-3 sites of the VEE virus did not alter binding to homologous MAbs.

Three MAbs recognizing sites E2-2 and E2-6 were used to select variants of the attenuated strains (TC-83 and 230) of the VEE virus [1]. The nucleotide sequence of the E2 gene of four variants revealed single mutations at positions 57, 60 (site E2-2) and 213 (site E2-6) of the E2 glycoprotein .

The data shown in Fig.1 allowed us to compare our maps of Trd virus with the epitope model of the TC-83 virus [11, 21, 22]. According to this model six epitopes of the E1 and E2 proteins form a single N domain. The immunodominant N domain of TC-83 virus was mapped to the region from E2-182 to E2-207 with MAbs with $E2^c$, $E2^f$, $E2^g$ and $E2^h$ epitopes [11]. In addition to the mutations localized at E2-182, -183, -199, and -207 two other relevant amino acid substitutions in this N domain were identified. E2-192 and E2-209 also affected the binding of N MAbs to the E2 and E2 epitopes [11, 13]. All the epitopes within this N domain, with the exception of $E2^a$, elicited MAbs that could protect mice from peripheral Trd virus challenge. Site E2-6 is continuous, it contains amino acid residues 182–216.These data show that site E2-6 corresponds to the N domain. We believe that our map is in good agreement with the TC-83 virus E1 and E2 protein epitope model developed by Roehrig [11, 21, 22]. The main difference is that we observed three additional P sites (E1-1, E2-2 and E2-3) which have no analogue in Roehrig's model.

Previous studies with 14 synthetic peptides representing the first 245 amino acids of the E2 glycoprotein demonstrated that these peptides elicited antiviral antibodies in peptide-immunized mice, and two peptides protected great numbers of outbred mice from challenge with the Trd strain of the VEE virus [9, 10, 12]. The protective peptides represent residues 1–25 of the deduced amino acid sequence of the E2 protein of the TC-83 strain or Trd strain of the VEE virus. The results of ELISAs designed to detect MAb binding to all peptides were ambiguous. Our data show that nine synthetic peptides representing the 30–75 and 202–250 amino acids do not react in ELISA with a panel of 17 MAbs to the VEE virus [23]. This suggested the possibility that protective MAbs may, perhaps, recognize discontinuous epitopes.

The presence of a highly conserved region (C-region) forming HA alphavirus-group-reactive antibodies was identified in the E2 protein of the VEE and EEE viruses [17]. Eight MAbs to the C-region inhibited hemagglutination activity of

Western equine encephalomyelitis, Semliki Forest, Sindbis, Getah, Aura, Chihungunya and Pixuna viruses. Two antigenic variants from Trd VEE virus that escaped the protective effect of MAb 7D1 (epitope E2-2c) and MAb 7A6 (epitope E2-3d) were produced for the C-region. Nucleotide sequencing of the E2 gene revealed non-synonymous point mutations at position 59 and 232 (Table 3). An antigenic variant of TC-83 VEE virus produced with 7D1 MAb in Vero cells also had a substitution at position 59 (Gln→Asp) [1]. This suggested that there may be a relation between the observed substitutions and the effect of MAbs used to obtain them.

The mutation in a 59 a.a. region induced by 7D1 MAb was localized close to mutations induced by B12 and E8 MAbs. These MAbs also recognize site E2-2 and induce mutations at positions 57 and 60 [1]. The results of the CBA demonstrated that E2-2c and E2-3d epitopes are located in very close proximity and that MAbs to these epitopes compete with each other [19].

Involvement of amino acids 59 and 232 in the formation of the C-region implies that this region is discontinuous. This is supported by the steric proximity of the sites E2-2 and E2-6 of the VEE virus, the conformational instability of a number of C-region epitopes to treatment with denaturation agents as well as by mapping of the E2-6 site at position 180–216 of the protein E2. Previous studies with synthetic peptides of the VEE virus also demonstrated that the amino terminal 25 amino acid of the VEE virus overlaps with the N domain composed of residues 180–210 [9, 10, 12].

The $E1^d$ epitope fulfills an analogous function and provides alphavirus-group-reactive antibodies in ELISA. This epitope was mapped previously to the E1 glycoprotein of the Western equine encephalomyelitis virus. MAbs to this epitope also possess HI activity to homologous virus [8]. The steric interrelations of the $E1^d$ epitope and the C-region remain unclear. However, their functional similarity suggests that the E1 epitope may be located immediately adjacent to the C-region of the E2 protein.

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