JOURNAL OF VIROLOGY, Oct. 1990, p. 5156–5159
0022-538X/90/105156-04$02.00/0

Vol. 64, No. 10

NOTES

A Single Amino Acid Substitution in Envelope Protein E of Tick-Borne Encephalitis Virus Leads to Attenuation in the Mouse Model

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Received 3 April 1990/Accepted 11 June 1990

We have determined the virulence characteristics of seven monoclonal antibody escape mutants of tick-borne encephalitis virus in the mouse model. One of the mutants with an amino acid substitution from tyrosine to histidine at residue 384 revealed strongly reduced pathogenicity after peripheral inoculation of adult mice but retained its capacity to replicate in the mice and to induce a high-titered antibody response. Infection with the attenuated mutant resulted in resistance to challenge with virulent virus. Assessment of nonconservative amino acid substitutions in other attenuated flaviviruses suggests that a structural element including residue 384 may represent an important determinant of flavivirus virulence in general.

Tick-borne encephalitis (TBE) virus is a human pathogenic flavivirus that is endemic in many parts of Europe (European subtype) and Asia (Far Eastern subtype). Like other flaviviruses, TBE virus contains a positive-stranded RNA genome (14, 15, 18, 19) and three structural proteins: the capsid protein C, the membrane protein M, and the envelope protein E (23). The flavivirus protein E contains about 500 amino acids and is usually glycosylated. It represents the viral hemagglutinin, induces a protective immune response, and plays a central role in the biology of flaviviruses. Like other viral envelope glycoproteins, protein E is believed to mediate important functions, such as receptor binding and the fusion of the virus membrane with cellular membranes after low-pH-induced conformational changes (4, 12; F. Guirakhoo et al., submitted for publication). Recently, a structural model of protein E has been established that contains information on the folding of the polypeptide chain and the locations and characteristics of monoclonal antibody (MAb)-defined epitopes (13). Most of these epitopes cluster in three antigenic domains (A, B, and C), whereas three isolated epitopes (i1, i2, and i3) do not overlap with any of the three domains. The model thus allows the correlation of the three antigenic domains to distinct protein domains.

By growing TBE virus in the presence of E protein-specific neutralizing MAbs, seven neutralization escape mutants were selected (designated VA3a, VA3b, VA4, VA5, Vi2, VB1, and VB4, corresponding to the selecting MAb), which revealed either reduced or no binding with the respective selecting MAb (10). Comparative sequence analyses demonstrated that each of the mutants differed from the wild type by only a single amino acid substitution in the E protein (13). The availability of these variants allowed us to assess the effect of single amino acid exchanges in different domains of the molecule on the virulence of TBE virus. This investigation thus aims at an understanding of the molecular basis of virulence and attenuation of flaviviruses.

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Comparative infectivity titrations of the seven mutants with the TBE wild type (strain Neudoerfl) were performed by intracerebral inoculation of Swiss albino suckling mice, by subcutaneous inoculation of 15-g mice, and in chicken embryo cells. Mouse titrations were scored by mortality, and titers were calculated according to Reed and Muench (20). Virus replication in chicken embryo cells was monitored by a four-layer enzyme-linked immunosorbent assay (ELISA) procedure as described previously (9). Most variants revealed titers similar to those of the wild-type virus in suckling mice, 15-g mice, and chicken embryo cells (Table 1). Variant VB4, however, showed a strongly reduced pathogenicity for 15-g mice after peripheral inoculation with a titer of less than $10^{2.0}$. The ratio of the titer in suckling mice to that in adult mice therefore exceeded $10^{6.2}$, whereas it was $10^{1.5}$ for wild-type strain Neudoerfl.

To further assess the virulence characteristics of the MAb escape mutants, we analyzed the induction of specific antibodies in the mice surviving infection. On day 28 postinfection, sera were taken from the survivors and screened for seroconversion by ELISA (7). The infectivity titers as measured by antibody induction in addition to mortality were calculated and related to those based on mortality only (Table 1). Most of the mice infected with either wild-type Neudoerfl or variants VA3a, VA3b, VA4, and Vi2 died from encephalitis. Only a few of the mice survived infection, as revealed by the induction of specific antibodies. The titers calculated from mortality and antibody induction were therefore only slightly higher than those based on mortality alone (ratio of $10^{0.4}$ to $10^{1.2}$). This was in contrast to the results obtained with variant VB4, which revealed a striking difference between virus replication indicated by antibody induction (titer of $10^{5.2}$) and mortality (titer of $<10^{2.0}$). Apparently, this variant has lost most of its potential to cause neurological disease and causes primarily subclinical infections.

Despite the high degree of VB4 attenuation, some mice died from encephalitis after high-titered infection with VB4. This result could be due to (i) contamination of the mutant

TABLE 1. Evaluation of MAb escape mutants by comparative infectivity titrations in suckling mice, 15-g mice, and chicken embryo cells

Host system and ratios of titers[a]	Wild-type Neudoerfl	MAb escape mutant						
		VA3a	VA3b	VA4	VA5	Vi2	VB1	VB4
Host systems								
A. Suckling mice i.c.	8.6	7.7	8.4	7.6	8.0	7.7	7.5	8.2
B. 15-g mice s.c.[b]	7.1	6.6	6.3	6.3	6.4	4.8	5.3	<2.0
C. 15-g mice s.c.[c]	7.6	7.2	6.9	6.7	ND[d]	5.5	6.5	5.2
D. Chicken embryo cells	8.0	9.0	9.0	9.0	8.0	8.0	7.0	8.0
Ratios of titers								
A/B	1.5	1.1	2.1	1.3	1.6	2.9	2.2	>6.2
D/B	0.9	2.4	2.7	2.7	1.6	3.2	1.7	>6.0
C/B	0.5	0.6	0.6	0.4	ND	0.7	1.2	>3.2

[a] i.c., Intracerebrally; s.c., subcutaneously.
[b] Titers were scored by mortality only.
[c] Titers were scored by mortality plus antibody induction.
[d] ND, Not done.

virus stock with wild-type virus, (ii) the generation of revertants, or (iii) residual encephalitogenic potential of the mutant itself. We therefore analyzed the antigenic phenotype of the virus in brain homogenates of deceased mice in a four-layer ELISA (9), using a panel of protein E-specific MAbs including that used for mutant selection (4). All mouse brain suspensions tested revealed the same reactivity pattern as VB4 (reduced binding of MAbs B1 and B4). We can therefore assume that most of the virus which replicated in the brain was the mutant itself, although a minor population of mouse-virulent revertant viruses could have gone undetected by this procedure.

We further investigated whether infection with the attenuated mutant VB4 resulted in a solid protection against virulent virus. For this experiment, we used as a challenge virus the highly mouse-pathogenic human TBE virus isolate strain Hypr, which inevitably kills mice after peripheral inoculation. Mice were immunized with increasing doses of VB4 (16, 160, and 1,600 50% infectious doses) and challenged on day 28 postinfection with either 100 or 1,000 50%

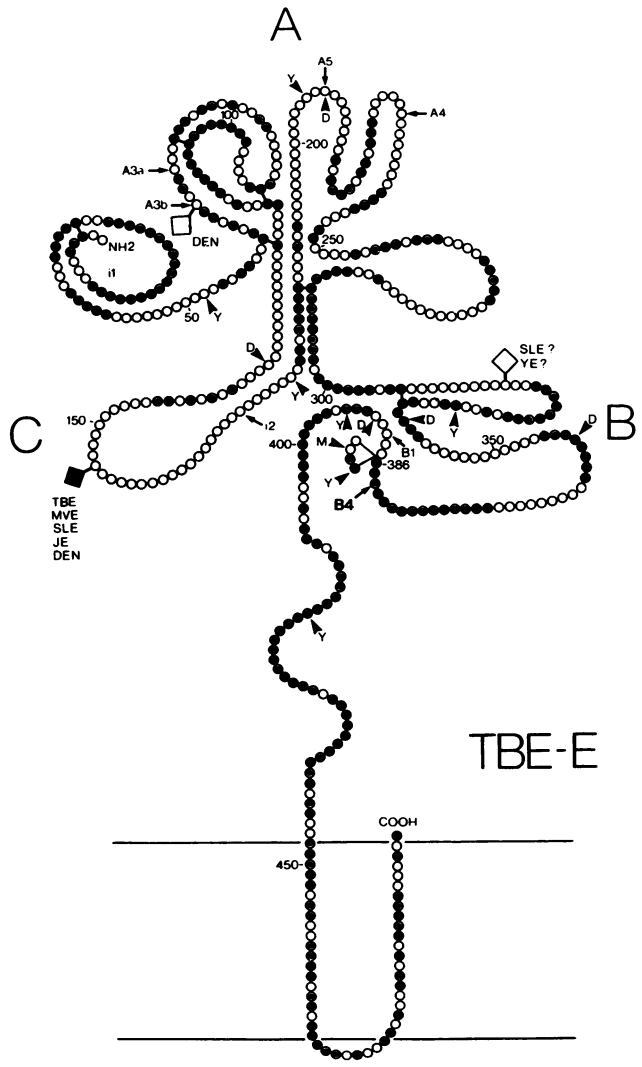

FIG. 1. Model of the TBE virus protein E (13) modified to show conserved and variable amino acid sequences. The amino acid sequence of the E protein of TBE virus strain Neudoerfl (14) was aligned with the corresponding sequences for MVE (1), JE (25), West Nile (WN) (28), St. Louis encephalitis (SLE) (26), YF (22), DEN-2 (2), DEN-1 (16), and DEN-4 (30) viruses. For the definition of conserved and variable positions in the E protein, only nonconservative amino acid substitutions were considered (conservative amino acid exchanges: Arg = Lys; Ser = Thr; Asp = Glu; Gln = Asn; Val = Leu = Ile = Met; Ala = Gly; Ala = Val; Tyr = Phe). Symbols: ●, conserved residues (no nonconservative amino acid substitutions in any of the flaviviruses compared); ◉, partially conserved residues (identity of amino acids between all viruses of the DEN and JE virus serocomplex but not TBE and YF viruses or conservation between all viruses investigated except one); ○, variable amino acid residues. Position numbers are indicated every 50 amino acids. Cysteine residues forming disulfide bridges (17) are connected by solid lines. Two solid lines represent the lipid membrane that is spanned by two transmembrane regions of protein E. The polypeptide chain is folded to indicate the MAb-defined antigenic domains A, B, and C, which are designated by capital letters. Arrows together with the names of neutralizing MAbs mark the locations of the mutations identified in the respective antigenic variants of TBE virus by sequence analysis. Arrowheads indicate positions of nonconservative amino acid substitutions in the YF 17D vaccine strain (5) (Y), the attenuated DEN-2 S1 strain (6) (D), and an attenuated MVE virus passage variant (12a) (M). The four-amino-acid sequence element after position 386 lacks in the TBE virus E protein but is present in all other flaviviruses sequenced so far. Diamonds mark potential N-glycosylation sites for TBE virus and other flaviviruses.

TABLE 2. Challenge of mice immunized with attenuated variant VB4[a]

Immunization dose (ID_{50})	No. of surviving mice/no. tested at challenge dose of:	
	100 LD_{50}	1,000 LD_{50}
16	6/9	6/10
160	8/9	9/9
1,600	ND	8/8

[a] ID_{50}, 50% infectious dose; LD_{50}, 50% lethal dose of strain Hypr; ND, not done.

lethal doses of strain Hypr (Table 2). In accordance with the induction rate of specific antibodies by increasing doses of the attenuated mutant, mice were protected against lethal challenge after infection with VB4.

On the basis of these data, one can assume that the amino acid substitution at position 384 affects a functionally important structural element that is a major determinant of neurovirulence. It is characteristic of functionally active sites to exhibit a high degree of conservation among homologous proteins. The alignment of the E protein sequences from different flaviviruses reveals a number of conserved sequences alternating with variable sequences along the polypeptide chain (Fig. 1). Antigenic domain B, which includes the attenuation mutation at position 384 and corresponds to an independently folding protein domain (8, 29), also contains several stretches of conserved amino acids. Most strikingly, of the seven MAb-resistant mutants, only the attenuated variant VB4 has an amino acid substitution within a conserved sequence (Fig. 1).

There are several lines of evidence that the stretches of conserved amino acids within domain B are of relevance for the virulence and attenuation of flaviviruses in general. This idea is supported by the location of nonconservative amino acid exchanges in the yellow fever (YF) 17D vaccine strain (5) and an attenuated dengue virus type 2 (DEN-2) S1 strain (6) compared with the wild-type viruses (Fig. 1). In YF 17D, four of the seven nonconservative amino acid substitutions affect conserved amino acids. Three of them are located in domain B, and one forms part of the strongly conserved stem structure leading to the membrane. The residual three substitutions are found in variable sequences in the rest of the molecule. The DEN-2 S1 strain reveals five nonconservative amino acid substitutions in comparison with the Jamaica strain. The only two exchanges that affect conserved sequence elements and one at a variable position are located within domain B. In contrast, sequence variations in natural strains are predominantly located at variable sequence positions throughout the molecule (6). This also holds true for nonconservative amino acid differences between the European and the Far Eastern subtypes of TBE virus (14, 18).

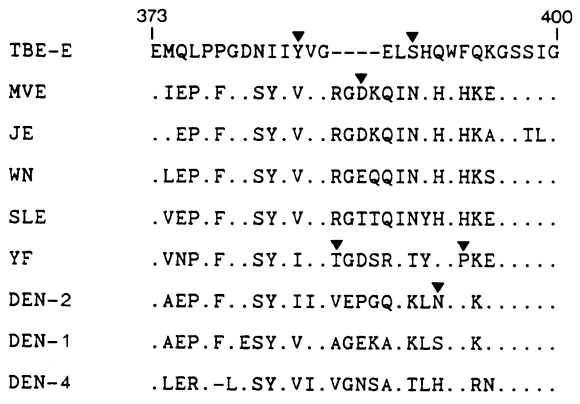

FIG. 2. Alignment of the TBE virus E (TBE-E) protein sequence (14, 18) from positions 373 to 400 with the corresponding sequences of other flaviviruses (1, 2, 5, 16, 25, 26, 28, 30). Dots indicate identity of amino acids with TBE; arrowheads mark the positions of sequence differences of attenuated virus strains in comparison with the parental viruses (YF 17D, DEN-2 S1, MVE host range mutant, and TBE MAb escape mutants) (5, 6, 12a, 13, 22). WN, West Nile virus; SLE, St. Louis encephalitis virus.

The importance of domain B is further substantiated by a Murray Valley encephalitis (MVE) virus passage variant altered at Asp-390 which was shown to be attenuated in 21-day-old mice after intraperitoneal inoculation (12a). Residue 390 in MVE virus is part of a four-amino-acid sequence (388-RGDK-391) that is deleted in the TBE virus E protein but immediately follows the last amino acid of the conserved sequence which includes the B4 mutation (Fig. 1 and 2). The RGD sequence motif is involved in cell attachment with different extracellular proteins such as fibronectin (11, 24) as well as foot-and-mouth disease virus (3). Although an RGD sequence is also found at the homologous position in Japanese encephalitis (JE) and YF 17D viruses, it is either modified (West Nile, Kunjin, and St. Louis encephalitis viruses) or not present (DEN-1, -2, and -4 and TBE viruses) (12a) in other flaviviruses (Fig. 2). Its significance for cell attachment therefore remains to be elucidated. Nevertheless it is tempting to speculate that sequence elements within domain B are involved in the putative receptor binding site. Subtle changes of receptor specificity or other aspects of early virus-cell interactions may affect the tissue tropism of the virus and thus lead to attenuation.

We are fully aware that mutations in domain B are involved in only one of several specific mechanisms that may determine the virulence of flaviviruses. Extensive studies with several different viruses have revealed that virulence is multifactorial and depends on a variety of viral and host factors (for a review, see reference 27). The YF 17D vaccine strain, for instance, contains a total of 68 nucleotide exchanges, several of which may contribute to attenuation involving either the E protein, other proteins, or even noncoding sequences (5). The data presented, however, add an additional piece of information toward our understanding of the molecular basis of virulence of flaviviruses. This knowledge may be helpful for the generation of new attenuated vaccine strains derived from full-length cDNAs (21).

We gratefully acknowledge the expert technical assistance of Angela Dohnal and Walter Holzer and thank Susanne Pfauser for typing the manuscript. We thank Lynn Dalgarno and Ron Weir for helpful discussions and the communication of their data on MVE virus variants.

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