Characterization of monoclonal antibody-escape mutants of tick-borne encephalitis virus with reduced neuroinvasiveness in mice

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Escape mutants of tick-borne encephalitis (TBE) virus were selected using neutralizing monoclonal antibodies (MAbs) that react with three different and previously unrecognized epitopes in the envelope protein E of TBE virus. Two of these variants (V-IC3 and V-IE3) exhibited a significantly reduced reactivity with their selecting MAbs, as determined by ELISA, whereas with one variant (V-IO3), reactivity was completely unchanged. Comparative sequence analyses demonstrated that each of the variants differed from the wild-type virus by a single amino acid substitution located at exposed positions within domains I, II and III of protein E. In the mouse model, all three mutants were still neuroviral but exhibited a significantly reduced neuroinvasiveness after subcutaneous inoculation. Virus replication, however, was sufficient to induce a specific antibody response. The observed alterations in virulence properties were not associated with reduced growth rates in vertebrate cell cultures, but one variant (V-IE3) exhibited a small plaque phenotype. The mutation of variant V-IO3 resulted in a temperature-sensitive phenotype and a significant elevation of the pH-threshold of the conformational change necessary for fusion activity.

Introduction

The genus Flavivirus, of the family Flaviviridae, comprises about 70 distinct viruses, most of which are transmitted to their mammalian hosts by mosquitoes or ticks (Monath & Heinz, 1996). Among these, yellow fever (YF) virus, the dengue (DEN 1–4) viruses, Japanese encephalitis (JE) virus and tick-borne encephalitis (TBE) virus have the highest worldwide impact as human pathogens, as indicated by the disease incidence in epidemic and endemic areas (reviewed by Monath & Heinz, 1996).

Considerable effort is currently being made towards a molecular understanding of flavivirus virulence and attenuation, which would form a rational basis for specific engineering of second generation vaccines by the use of infectious clones (Lai et al., 1992; Pletnev et al., 1993; Kawano et al., 1993; Cahour et al., 1995; Sumiyoshi et al., 1995; Chen et al., 1995). The single long open reading frame for these proteins is flanked by short 5’ and 3’ noncoding regions, which apparently contain important signals for RNA replication (Rice, 1996). The information on the molecular basis of flavivirus virulence accumulated so far reflects its multifactorial nature. Mutations in structural proteins, nonstructural proteins and also noncoding regions have all been shown to be capable of affecting virulence (reviewed by Monath & Heinz, 1996).

There is, however, general agreement that the viral envelope protein E represents an especially important determinant of virulence because of its dual function for cell penetration, i.e. receptor-binding and acid-pH-dependent fusion activity. Recently, the atomic structure of protein E from TBE virus was elucidated by X-ray crystallography (Rey et al., 1995). The basic subunit represents an elongated dimer that is oriented parallel to the membrane and gently curved to accommodate the surface of a virion with a diameter of approximately 50 nm. Each of the monomers is composed of three structural domains, designated domain I, II and III (Rey et al., 1995). It is likely that all flavivirus E proteins exhibit a very similar structure due to an amino acid sequence identity of at least 40% (Monath & Heinz, 1996) and conservation of all 12 cysteines which form 6 disulphide bridges (Nowak & Wengler,
1987). Mapping of mutations in the three-dimensional structure of protein E that were shown to affect virulence in studies with different flaviviruses (Mandl et al., 1989; Lobigs et al., 1990; Holzmann et al., 1990; Cecilia & Gould, 1991; Hasegawa et al., 1992; Guirakhoo et al., 1993; Jiang et al., 1993; Gao et al., 1994; McMinn et al., 1995; Sumiyoshi et al., 1995; Pletnev et al., 1993; Kawano et al., 1993) revealed clusters in three distinct regions: (a) domain III, especially its lateral surface, (b) the base of domain II and (c) the interface between domain I and III of opposite subunits including the carbohydrate side chain and the putative internal fusion peptide (Rey et al., 1995). These clusters are believed to indicate sites involved in receptor-binding (a) and fusion activity (b and c).

In the present study we have made use of three neutralizing monoclonal antibodies (MAbs) that react with previously unrecognized epitopes in protein E of TBE virus (Holzmann et al., 1995) to select neutralization-resistant mutants. All three mutants exhibited an attenuated phenotype in mice, and their E proteins differed from the wild-type by a single amino acid substitution, each occurring in a different structural domain. These data contribute to the fine mapping of key structural elements within protein E that control its function and thereby influence the virulence of the virus.

**Methods**

**Cell lines.** Primary chick embryo cells (CEC) were maintained in medium 199 with Hanks' salts buffered with 15 mM-HEPES, 15 mM-HEPPS pH 7.6 containing 1% neomycin and 0.1% BSA. Porcine kidney (PS) cells were grown in L-15 (Leibovitz) medium containing 3% fetal calf serum (FCS) and 1% penicillin–streptomycin. Baby hamster kidney (BHK-21) cells were grown in MEM–Earle's salts with 5% FCS and 1% neomycin.

**Selection and purification of antigenic variants.** Antigenic variants were selected by growing TBE virus strain Neudoerfl in primary CEC in the presence of neutralizing MAbs (Holzmann et al., 1995) as described previously (Holzmann et al., 1989). Briefly, serial 10-fold dilutions of the virus were incubated with MAbs at a concentration of either 0.25 or 0.5 µg/ml prior to infection of CEC monolayers with the virus–MAB mixtures. After infection, the inoculum was removed and incubation was continued with 25% of the initial MAB concentration. Samples of the supernatants were taken on days 3 and 6 post-infection (p.i.) and screened for the presence of viral antigen by ELISA (Heinz et al., 1986). Selection cycles were repeated with virus-containing supernatants until virus yields in the presence of the MAB were equal to those of the control lacking antibody. The variants were then purified by three cycles of end-point dilutions using CEC, before virus stocks in the form of infected suckling mouse brain suspensions were prepared.

**Antigenic analysis of the E protein variants.** For antigenic analysis, the viruses were grown in CEC, concentrated by ultracentrifugation and purified by two cycles of sucrose density gradient centrifugation as described previously (Heinz & Kunz, 1981).

The reactivity of these preparations with 19 protein-E-specific MAbs (Heinz et al., 1983; Guirakhoo et al., 1989; Holzmann et al., 1993), in addition to the three MAbs used for selection (Holzmann et al., 1995), was determined in a three-layer ELISA as described previously (Heinz et al., 1983).

For this purpose, purified virus preparations were coated onto the solid phase at a concentration of 1 µg/ml and titration curves were established with each MAB in order to quantify differences of reactivity in comparison with the parent virus.

**Sequence analysis.** Genomic RNA was isolated from purified virus suspensions by phenol–chloroform extractions and ethanol precipitation as described previously (Mandl et al., 1988). Approximately 1 µg of this RNA was used for cDNA synthesis and subsequent amplification of fragments corresponding to the protein-E coding region using primers derived from the sequence of TBE virus strain Neudoerfl (Mandl et al., 1988; GenBank accession no. U27495). PCR-derived fragments were used directly for sequence analysis and sequenced in both directions using TBE virus-specific oligonucleotide primers. Sequencing was performed with an automated ABI 370A DNA sequencer using Taq polymerase and fluorescent-dye-labelled dideoxy terminators following protocols provided by the manufacturers (Perkin Elmer, ABI). Sequences were compared using the Microgenie software package, version 4.0 (Beckman).

**Virulence assay.** Virulence assays were performed with 5-week-old outbred Swiss-albino mice-GP with bodyweights of about 20 g. Groups of 10 mice were inoculated either subcutaneously (s.c.) with 1000, 100 or 10 p.f.u., or intracerebrally (i.c.) with 100 or 10 p.f.u. of variant or wild-type virus, and scored by mortality for a period of 28 days. On day 28 p.i., blood samples were taken from the survivors and screened for TBE-specific antibodies by ELISA (Heinz et al., 1983).

**Plaque test and assay for temperature sensitivity.** Plaque assays were carried out with PS cells using 24-well clusterplates (Nunc). For this purpose, 200 µl of serial 10-fold dilutions of a suckling mouse brain suspension of the variants or wild-type virus (diluted in L-15 medium supplemented as above) was mixed with 300 µl of a PS cell suspension containing 10⁶ cells and incubated for 4 h at 37 or 40 °C before 400 µl of an overlay (15% carboxymethylcellulose in L-15 medium) was added. Incubation was then continued for 5 days and then the monolayers were stained with a 0.1% Naphthol blue black solution (Sigma) containing 1% acetic acid and 0.1% sodium acetate. Plaques were counted and infectivity titres were expressed as p.f.u./ml.

**Viruses growth in cell culture.** For examination of growth kinetics, BHK-21 cell and CEC monolayers in 24-well clusterplates were infected with 1000 p.f.u. of variant or wild-type virus (m.o.i. approx. 0.001) by adsorption of variant or wild-type virus to cells for 1 h at 37 °C. Cells were then washed once before fresh medium was added.

At 20, 25, 30 and 45 h.p.i. samples were collected from the supernatants, stored at −80 °C and titrated by plaque assay on PS cells for quantification of infectious virus.

**Low pH incubation.** Purified variant or wild-type viruses at a concentration of 50 µg/ml in TAN buffer (0.05 mM-triethanolamine and 0.1 mM-NaCl pH 8.0) were acidified by diluting the samples 1:10 in a buffer consisting of 0.05 mM-MES, 0.1 mM-NaCl and 0.1% BSA, which had been pretitrated to yield the desired final pH of between 5.5 and 6.9. For the pH 7.0 and 8.0 controls, samples were treated with buffers containing 0.05 mM-HEPES or triethanolamine, respectively, instead of MES. The viruses were incubated for 10 min at 37 °C at the low pH and then adjusted to pH 7.0 by a further 10-fold dilution in medium 199 buffered with 15 mM-HEPES, 15 mM-HEPPS containing 0.1% BSA. Samples were allowed to stand for 15 min at 37 °C before they were stored at −80 °C. The acid-pH-induced conformational change was monitored in a four-layer ELISA using MAB 12, which is specific for a low-pH-sensitive conformational epitope (Heinz et al., 1994).
Results

Selection and antigenic characterization of MAb-escape mutants

Neutralization-resistant mutants of TBE virus strain Neuendoerfl were selected in CEC using the protein-E-specific MAbs IC3, IE3 and IO3 as detailed in Methods. The escape mutants generated by this procedure were designated V-IC3, V-IE3 and V-IO3, according to the MAbs used for selection. The variants were analysed in a three-layer ELISA (see Methods) for their reactivity with the selecting antibody, as well as an additional panel of 19 MAbs which were previously shown to recognize different epitopes in each of the three structural domains of protein E (Guirakhoo et al., 1989; Mandl et al., 1989; Holzmann et al., 1993, 1995; Rey et al., 1995). The results of these analyses are presented in Table 1.

V-IC3 and V-IE3 exhibited a significantly reduced reactivity with their selecting MAbs, as expected. In contrast, despite the loss of neutralization, variant V-IO3 exhibited no change in its binding characteristic with its selecting MAb. The reactivity of the mutants with all of the other MAbs was identical to the wild-type, indicating that each of the three selecting MAbs defines a different, and previously unrecognized, epitope. The detailed results of the analyses are presented in Table 1.

Table 1. Comparative reactivities of the variants with MAbs (three-layer ELISA)

<table>
<thead>
<tr>
<th>Variant</th>
<th>V-IC3</th>
<th>V-IE3</th>
<th>V-IO3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC3</td>
<td>X*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IE3</td>
<td>0</td>
<td>X</td>
<td>0</td>
</tr>
<tr>
<td>IO3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
* More than 1000-fold reduced reactivity compared to parent strain Neuendoerfl.
† Same reactivity as with parent strain Neuendoerfl.

Table 2. Antigenic variants of TBE virus

<table>
<thead>
<tr>
<th>Variant</th>
<th>Nucleotide change</th>
<th>Position</th>
<th>Amino acid exchange</th>
<th>Position</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-IC3</td>
<td>G → U</td>
<td>1513</td>
<td>Asp → Tyr</td>
<td>181</td>
<td>I</td>
</tr>
<tr>
<td>V-IE3</td>
<td>G → A</td>
<td>1339</td>
<td>Ala → Lys</td>
<td>123</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>C → A</td>
<td>1340</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-IO3</td>
<td>G → A</td>
<td>2074</td>
<td>Gly → Arg</td>
<td>368</td>
<td>III</td>
</tr>
</tbody>
</table>
* Position numbers are counted from the first nucleotide of the protein-E-coding region and the first amino acid of protein E, respectively.
† According to Rey et al. (1995).

Sequence analysis of the mutants

The sequences of the protein-E-coding regions were determined by direct sequencing of PCR-amplified cDNA. The sequences of the mutants V-IC3 and V-IO3 differed from that of the wild-type by one nucleotide, and that of V-IE3 differed by two nucleotides, leading in each case to single amino acid substitutions in the corresponding E proteins. The V-IC3, V-IE3 and V-IO3 mutations mapped to domain I, II and III, respectively. The details are given in Table 2.

Mouse virulence

The virulence properties of the mutants were investigated in mice by comparing the neuroinvasiveness (s.c. inoculation) and neurovirulence (i.c. inoculation) of the three variants with that of the wild-type.

As can be seen in Fig. 1(a), all three mutants were significantly attenuated in their neuroinvasiveness, whereas only minor differences were observed in their neurovirulence, as indicated by a slight increase of the mean survival times of mice infected with V-IO3 and V-IE3 (Fig. 1b).

To assess whether the mutants had replicated in the mice surviving s.c. infection with 1000 p.f.u. virus (Fig. 1a), blood samples were collected on day 28 p.i. and tested for the presence of TBE-specific antibodies by ELISA. With the exception of one individual mouse in the case of variant V-IE3, all of the mice had seroconverted (Table 3), which strongly suggests that virus replication had taken place in the surviving mice.

Growth properties, plaque phenotype and temperature sensitivity

Since an attenuated phenotype is also frequently reflected by a reduction in replication efficiency, plaque size or temperature sensitivity, we examined these properties for each of the three MAb-escape mutants (Table 4). V-IO3 was temperature sensitive, but exhibited a normal plaque size, whereas V-IE3 produced significantly smaller plaques than the other two mutants and the wild-type, but was not temperature sensitive. V-IC3 did not differ from the wild-type in either of these properties.
Fig. 1. Mouse-neuroinvasiveness and -neurovirulence of TBE virus and its antigenic variants. Groups of 10 adult mice were infected subcutaneously with 1000 p.f.u. (a), or intracerebrally with 10 p.f.u. (b) of TBE virus strain Neudoerfl (△) or the variants V-IC3 (○), V-IE3 (□) or V-IO3 (△), respectively. Survival was monitored for 28 days p.i.

Table 3. Detection of TBE-specific antibodies in mice surviving s.c. infection

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0</td>
</tr>
<tr>
<td>V-IC3</td>
<td>6</td>
</tr>
<tr>
<td>V-IE3</td>
<td>7</td>
</tr>
<tr>
<td>V-IO3</td>
<td>5</td>
</tr>
</tbody>
</table>

Interestingly, the observed alterations in virulence properties were not associated with reduced growth rates of the variants compared to the parent virus, either in BHK-21 cells or in CEC (data not shown).

Sensitivity to acidic pH

Protein E undergoes an irreversible conformational rearrangement at pH 6.5 or below which is necessary for fusion activity. To assess the possible effect of the mutations in protein E on this functional property, we compared the pH-threshold for acid-induced conformational changes of the three mutants to that of the wild-type. This analysis was carried out by ELISA using a conformation-specific MAb (i2), which recognizes a highly acid-pH-sensitive epitope (Heinz et al., 1994; Allison et al., 1995). As shown in Fig. 2, all three mutants were more sensitive to acidic pH than the wild-type, and this effect on conformational stability was most pronounced with V-IO3.
Discussion

The single amino acid substitutions in the three neutralization-resistant mutants described above (V-IC3, V-IE3 and V-IO3) map to different structural domains (I, II and III, respectively) of protein E. Previous investigations with MAb-escape mutants had already indicated that antibody-binding to each of the structural entities could lead to virus neutralization, presumably by blocking different protein-E-mediated functions, i.e. receptor-binding and/or fusion (Mandl et al., 1989; Holzmann et al., 1990; Rey et al., 1995). In the case of V-IE3 and V-IC3 it is likely that the mutated amino acids indeed represent part of the epitopes recognized by the selecting MABs because their reactivity was almost completely abolished by the amino acid substitutions. These mutations map to exposed areas on the surface of domain I (V-IC3) and domain II (V-IE3) and are adjacent to previously mapped epitopes (Mandl et al., 1989; Rey et al., 1995). Structurally, there seems to be little overlap since both variants exhibit an unchanged reactivity pattern with all of the other protein-E-specific MABs analysed so far (Table 1). Consistent with the observation that the low-pH-induced structural rearrangements necessary for fusion activity affect domain I as well as domain II (Guirakhoo et al., 1989; Heinz et al., 1994; Rey et al., 1995), MABs IC3 and IE3 lose their reactivity when incubated at acidic pH (Holzmann et al., 1995). However, it remains to be determined whether antibody-binding to these sites neutralizes the virus by preventing fusion after uptake through receptor-mediated endocytosis or by simple steric hinderance of virus-binding to the cell surface receptor.

Previous studies of flavivirus escape variants have shown that the loss of neutralization due to the loss of antibody-binding appears to be the most frequent mechanism leading to selection of mutants by MABs (Holzmann et al., 1989; Cecilia & Gould, 1991; Hasegawa et al., 1992; Sil et al., 1992; Jiang et al., 1993; Lin et al., 1994; McMinn et al., 1995). This is in concordance with observations made in other viral systems such as picorna and influenza viruses, in which the majority of mutations were found to be in very prominently exposed positions that allow direct interaction with the antibody (Hogle et al., 1985; Sherry et al., 1986; Page et al., 1988; Wiley et al., 1981; Colman et al., 1983; Tulip et al., 1991; Saito et al., 1994; Rossmann et al., 1989).

Variant V-IO3, however, reveals a different and apparently more complex mechanism of neutralization because, in this case, the ‘escape mutation’ does not affect antibody-binding. Similar observations have also been made with escape mutants of JE virus (Cecilia & Gould, 1991), coxsackie virus B4 (Prabhakar et al., 1987), New cauliflower disease virus (Iorio & Bratt, 1985), poliovirus (Diamond et al., 1985; Blindel et al., 1986; Page et al., 1988) and variants of YF virus (Buckley & Gould, 1985). These mutations apparently do not affect the epitope itself, but alter the effect of antibody-binding (i.e. virus neutralization), which probably involves additional structural elements located outside the antibody-binding site. The mutation in V-IO3 (Gly 368→Arg) is located within domain III in a loop that connects the Dx and Eβ sheets, rather than at the lateral surface which has been suggested to be involved in receptor-binding (Rey et al., 1995). The functional or structural importance of glycine-368 is suggested by the fact that it is part of a series of three glycines which are absolutely conserved among all tick-borne flaviviruses sequenced so far (Marin et al., 1995; Gritsun et al., 1995).

All three mutants exhibited a significantly reduced neuroinvasiveness in the mouse, even though the inoculated viruses had replicated in the animals, as indicated by the induction of specific antibodies. The underlying mechanism(s) of attenuation remain a matter of speculation since it is not clear, at present, which molecular properties were affected by the mutations in protein E, and whether the variants had undergone changes in other parts of the genome during the selection and growth procedures. In the case of V-IO3, attenuation may be related to the observed increased lability of protein E at acidic pH and its temperature sensitivity.

The data presented provide additional clues to the identification of structural elements in protein E that can influence neuroinvasiveness in the mouse. This information will facilitate the specific engineering of stably attenuated mutants of TBE virus using infectious cDNA clone technology.

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References


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