Monoclonal Antibodies Capable of Distinguishing Epizootic from Enzootic Varieties of Subtype 1 Venezuelan Equine Encephalitis Viruses in a Rapid Indirect Immunofluorescence Assay

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We used previously characterized murine monoclonal antibodies to develop a panel useful in subtyping Venezuelan equine encephalitis (VEE) viruses by an indirect fluorescent antibody assay. This panel worked well with either prototype VEE viruses or a series of more recent VEE virus isolates. The panel is particularly useful for rapidly differentiating VEE viruses with epidemic-epizootic potential from other endemic varieties of this virus. Using this panel, we identified an antigenic variant of prototype VEE subtype 1E virus currently present in Mexico. This antigenic change in the E2 glycoprotein was confirmed by enzyme-linked immunosorbent assay. Because VEE virus virulence has been associated in part with the E2 glycoprotein, this observed antigenic change in the 1E virus E2 glycoprotein may explain the apparent equine virulence of this unusual VEE 1E virus.

The Venezuelan equine encephalitis (VEE) virus serocomplex is currently composed of six subtypes (subtypes 1 to 6), with subtype 1 including five varieties (1AB, 1C, 1D, 1E, and 1F) and subtype 3 including three varieties (3A, 3B, and 3C). The important epidemiological correlation of this subtyping scheme is that only members of varieties 1AB and 1C have been associated with large human epidemics and equine epizootics (7). The other VEE viruses have only been associated with enzootic transmission.

Evaluation of the genomic sequences of various VEE virus isolates has suggested that the last major VEE virus epizootic, occurring from 1969 to 1972 by viruses belonging to the variety 1AB, may have been caused by poorly inactivated VEE virus vaccines (4). This hypothesis is supported by the observation that 1AB viruses have not been isolated from nature since this epidemic. Similarly, epizootic viruses of the 1C variety have not been isolated in interepidemic years. Recent studies in this laboratory and others suggest that the epizootic 1C variety may evolve from the enzootic 1D variety, as demonstrated by their strong genome sequence homologies. However, these sequence data are only an indirect measurement of genetic relatedness and are not conclusive evidence for direct evolution (5, 7, 14, 16).

In the last few years, we have experienced a reemergence of VEE. In 1993, an equine outbreak of variety 1E virus occurred in Mexico (5a). Interestingly, this virus killed horses, a characteristic not normally associated with 1E VEE viruses. In 1994, VEE virus variety 1D human disease was identified in Peru (15). In the fall of 1995, a large VEE epidemic-epizootic caused by variety 1C virus occurred in the La Guajira peninsula of Venezuela and Colombia. Current estimates of human disease in this variety 1C virus epidemic stand at 90,000 cases (14a). This was the first major VEE epidemic-epizootic since the epidemic of 1969 to 1972. The reoccurrence of epizootic VEE and the association of this severe form of disease with VEE virus variety 1C have redirected the need for a rapid, cheap, and standardizable laboratory test to differentiate VEE virus subtypes and varieties, particularly variety 1C viruses from variety 1D, 1E, and 1F viruses.

Over the last 15 years, we have characterized the antigenic structure of alphaviruses, focusing on the VEE viruses (1a, 2, 6, 9–12). We have previously prepared and used murine monoclonal antibodies (MAbs) to locate and define the function of epitopes on the surface glycoproteins of these viruses (9, 10, 12). Recently we identified a MAb prepared against a synthetic peptide derived from the E2 glycoprotein amino terminus and used this MAb in an enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody assay (IFA) to identify its serological activity with a wide variety of VEE viruses and other alphaviruses (9). This MAb can be used to differentiate most wild-type VEE viruses from the live attenuated VEE vaccine strain, TC-83.

We now report the validation of a panel of these previously prepared murine MAbs capable of differentiating the important varieties of subtype 1 VEE viruses. While the serological specificities of these MAbs have been defined by an indirect ELISA, we have now used IFA as a rapid and easy test for antigen identification. We have validated these reactivities with prototype VEE viruses and a number of more recent VEE virus isolates. These MAbs were isolated by standard techniques; some of their previously defined characteristics are included in Table 1. The protocol for this IFA has also been previously published (8, 9). Briefly, virus-infected 75-cm² tissue culture flasks of Vero cells were harvested at 20 h postinfection by trypsinization. The prototype viruses used in this analysis were Trinidad Donkey (TRD), P676, 3880, Mena 2, 78V-3531, Everglades (Fe3-7c), Mucambo (BeAn 8), Pixuna (BeAr 35645), Cabassou (CaAr 508), Ag80-663, eastern equine encephalitis (EEE) (82V-2137), western equine encephalitis (WEE) (McMillan), and St. Louis encephalitis (SLE) (MSI-7).
viruses. Cells were spotted and air dried on 12-well slides. Dried slides were fixed for 30 min in ice-cold acetone. The MAb titrations were begun at 1:1,000 and continued to the end point. The MAbs were incubated for 30 min at 37°C. Bound MAbs were detected with a 1:100 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (Jackson Laboratories, West Grove, Pa.) incubated for 30 min at 37°C. The IFA reactions were scored from 1+ to 4+, with 4+ indicating maximum intensity. End point titration was necessary to clearly define antibody cross-reactivities in the IFA. The end points were defined by dilutions that demonstrated fluorescence with a score of ≥2+. The IFA cross-reactivities of these MAbs with the prototype VEE viruses were nearly identical to their indirect ELISA reactivities (Table 1). The observed MAb reactivity patterns were 1A3A-5 (epizootic varieties 1AB and 1C and enzootic subtype 2), 1A4D-1 (epizootic varieties 1AB and 1C and enzootic variety 1D), 1A3A-9 (all subtype 1 viruses), 1A1B-9 (enzootic subtype 1 varieties 1D, 1E, and 1F), and 1A3B-7 (all VEE viruses). The control antibody reactivities were 2A2C-3 (all alphaviruses), 2A6C-7 (all WEE viruses), 1B1C-4 (all EEE viruses), and 6B6C-1 (all flaviviruses). Two differences noted were the failure of 1A2B-10 to react with Pixuna virus, and the failure of 1A3A-5 to react with 3880 virus. The failure of 1A2B-10 to react with Pixuna virus in the IFA, while reacting in the ELISA, was most likely due to the lower sensitivity of IFA compared to that of ELISA. This MAb was at least 40-fold less reactive with Pixuna virus in the ELISA than with the other VEE viruses (9). The inability of 1A3A-5 to react with 3880 virus was most likely due to the altered expression of the epitope defined by this MAb on 3880 compared with variety 1AB or 1C viruses. Similar differential reactivities of this MAb have been observed previously (12). While the ELISA titer of 1A3A-5 with variety 1AB, 1C, and 1D viruses is within fourfold, the MAb reactivity is 1C specific in both plaque reduction neutralization and hemagglutination-inhibition assays. Based on these IFA results, we developed the following scheme for differentiating variety 1AB and 1C VEE viruses from variety 1D, 1E, and 1F viruses, using all MAbs at a single screening dilution of 1:400 (Fig. 1). A positive reaction with MAb 2A2C-3 identifies an isolate as an alphavirus. A positive reaction with MAb 1A3B-7 identifies an isolate as a VEE virus. A positive reaction with MAb 1A3A-9 identifies an isolate as a subtype 1 VEE virus. A positive reaction with MAb 1A1B-9 identifies an isolate as an enzootic variety 1D, 1E, or 1F virus (subtype 5 viruses are previously excluded, based on 1A3A-9 reactivity). A positive reaction with MAb 1A4D-1 combined with a negative reaction with MAb 1A3A-5 confirms an isolate as variety 1D virus (and further excludes variety 1E and 1F and subtype 2 viruses). A positive reaction with MAb 1A3A-5 confirms an isolate as an epizootic variety 1AB or 1C (subtype 2 viruses are previously excluded based on 1A3A-9 reactivity). This algorithm was then applied to a number of more recent variety 1C, 1D, and 1E viruses, including isolates from the recent epidemic-epizootic in Venezuela and Colombia in 1995 (Table 2). Virus identification by IFA was as predicted, with subtype 1, variety 1E, and 1F viruses (end point titers are reported as log10 values).
titers of ≤3.0). The genetic reason for this reactivity will require further analysis. The presence of an epizootic VEE virus epitope on this enzootic 1E subtype virus might be consistent with its unusual ability to kill horses. The location of the 1A4D-1 (E2f) epitope has been previously identified within the VEE virus critical neutralization domain (E2 amino acids 180 to 220), and changes in the E2 glycoprotein have been shown to have substantial effects on virus virulence (3). Recently (in 1996), a similar outbreak of equine virulent 1E VEE occurred in the neighboring state of Oaxaca, Mexico (1). It will be of interest to subtype this new VEE virus with these MAbs to determine whether this area of Mexico has an emergent enzootic focus of this unusual variety 1E VEE virus.

The most problematic MAb in this panel is 2A2C-3. It defines an alphavirus group-reactive epitope located on the E1 glycoprotein that is conformationally dependent and is cryptic on the native virion. This means that the level of reactivity of this MAb is somewhat variable in each antigen, depending on

![Image](http://jcm.asm.org.org/download/)

**FIG. 1.** Identification algorithm for VEE viruses. The MAbs used are shown beside the vertical arrows on the left side of the figure. + and −, positive and negative reactions, respectively.

**TABLE 2.** IFA reactivities on contemporary strains of VEE virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Variety or subtype</th>
<th>Yr isolated</th>
<th>Location</th>
<th>Source</th>
<th>2A2C-3</th>
<th>1A3B-7</th>
<th>1A3A-9</th>
<th>1A4D-1</th>
<th>1A3A-5</th>
<th>1A1B-9</th>
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<td>1992</td>
<td>VE</td>
<td>Horse</td>
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<td>VE</td>
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<td>SH5</td>
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<td>VE</td>
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<td>1961</td>
<td>PN</td>
<td>Human</td>
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<td>4+</td>
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<td>4+</td>
<td>0</td>
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<td>1969</td>
<td>CO</td>
<td>Hamster</td>
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<tr>
<td>93-42124</td>
<td>1E</td>
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<td>MX</td>
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<td>US</td>
<td>Mosquito</td>
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</table>

*All MAb ascitic fluids were used at a 1:400 dilution, except 1A3A-9, which was used at 1:100. All viruses were negative with a 1:400 dilution of SLE 6B6C-1.

Abbreviations: VE, Venezuela; CO, Colombia; EC, Ecuador; PE, Peru; PN, Panama; MX, Mexico; US, United States (Florida).
the amount of denaturation occurring during preparation. Consequently, low reactivity with 2A2C-3 should be confirmed by 1A3B-7 staining. The 1A3B-7 MAb defines a more stable epitope on the E2 glycoprotein and readily identifies all members of the VEE virus serocomplex.

While this MAb panel fails to differentiate enzootic viruses of subtypes 2, 3, 4, 5, and 6, these subtypes of viruses are rarely isolated and have never been associated with major VEE outbreaks or epidemics. It is also important to emphasize that because of the potency of these MAb reagents, they must be used at suitably high dilutions (e.g., 1:400) in this IFA. Using these MAbs at low dilutions (which is customary for polyclonal antisera) magnifies low-level cross-reactivities, which confuse this identification scheme. For example, MAb 1A2B-10, which has an IFA end point of 1:32,000 with most wild-type VEE viruses, will react with TC-83 virus with a 1:40 end point (9). Using this reagent at a low dilution, therefore, negates its usefulness in discriminating wild-type VEE viruses from the live attenuated vaccine strain. With the reemergence of epidemic-epizootic VEE activity, we believe that this MAb panel will serve to rapidly assess the epidemic potential of a new VEE isolate and allow for worldwide standardization of laboratory reagents used for this purpose. We are currently preparing a kit from these reagents for distribution to reference laboratories used for this purpose. We are currently preparing a kit from these reagents for distribution to reference and diagnostic laboratories involved in VEE virus isolation and identification.

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REFERENCES
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