Association of Tonate Virus (Subtype IIIB of the Venezuelan Equine Encephalitis Complex) with Encephalitis in a Human

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Tonate virus, subtype IIIB of the Venezuelan equine encephalitis (VEE) complex, was first isolated in 1973 in French Guiana, South America. However, very little is known about its pathogenicity; it was considered to be responsible for only mild dengue-like syndromes. In 1998, a 2-month-old boy living along the Oyapock river in French Guiana was hospitalized for fever and generalized status myoclonus, and despite treatment the patient died 72 h after admission. Testing showed the presence of IgM specific for viruses of the VEE complex. A sensitive seminested polymerase chain reaction derived from a previous study was developed to detect viruses from the VEE complex, since no virus could be recovered from clinical specimens cultured on mosquito cells or from intracerebral inoculation into newborn mice. The genome of a virus from the VEE complex was detected in postmortem brain biopsies, and Tonate virus was identified by direct sequencing. This is the first reported case of human encephalitis due to Tonate virus.

The Venezuelan equine encephalitis (VEE) complex is an antigenically related group of arboviruses in the family Togaviridae, genus *Alphavirus.* The viruses of the VEE antigenic complex are currently classified into 6 antigenic subtypes by cross-neutralization and hemagglutination-inhibition tests [1]. Subtypes IAB and IC are highly virulent for horses and responsible for equine epizootics and epidemic human disease throughout South and Central America [2]. Clinical symptoms in humans may vary from undifferentiated fever to serious encephalitis [2].

Subtype IIIB (Tonate virus) was first isolated in French Guiana, an overseas French department located between Brazil and Surinam, from *Psarocolius decumanus,* a bird captured in 1973 [3]. Thereafter, it was repeatedly isolated from various mosquitoes, especially *Culex portesi,* in French Guiana and Surinam [4, 5]. The virus has previously been isolated from humans presenting with mild febrile illnesses [3], and antibodies to this virus are frequently detected in individuals living in French Guiana. However, little is known about its pathogenicity. Tonate virus was considered to be responsible for a mild dengue-like syndrome in humans, and there are no previously obtained data concerning its neurovirulence. We report here the first case of encephalitis due to Tonate virus in a human.

Case Report

In June 1998 a boy aged 2 months, who lived along the Oyapock River in French Guiana, was hospitalized 4 days after onset of fever in the intensive care unit of the Cayenne General Hospital, French Guiana, for fever and generalized status myoclonus. Specific treatment was given (diazepam, 0.5 mg/kg; and phenobarbital, 20 mg/kg), but myoclonic seizures persisted. He was given general anesthesia (pentothal), underwent tracheal intubation, and received ventilation support for cerebral protection.

At the time of admission the laboratory values were as follows: hemoglobin, 7.3 g/dL; total WBCs, 17,300/mm^3; platelets, 302,000/mm^3; glucose, 6.2 mmol/L; sodium, 135 mmol/L; potassium, 4.7 mmol/L; urea, 7.3 mmol/L; and prothrombin time ratio, 1.51 (normal range, 1.0–1.2). CSF findings included the presence of 100 WBCs/mm^3 (neutrophils/lymphocytes, 40/60 per mm^3); glucose and protein concentrations were normal. Blood smears for diagnosis of malaria were negative. Neither bacterial infection nor herpes simplex virus could be detected in CSF. Titers of antibodies to different viruses, especially adenoviruses, enteroviruses, myxoviruses, measles virus, and herpetoviridae, were not elevated in CF tests.

Despite treatment, the patient's neurological condition continued to deteriorate, and he died 72 h after admission. A CT scan showed generalized cerebral edema without focal lesions.

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Postmortem liver and brain biopsies were performed only after informed consent was obtained from the parents of each patient.

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Materials and Methods

Virological studies. All virological tests for arboviruses were performed at the Institut Pasteur de la Guyane, National Reference Center for Arboviruses.

Serological tests. The serum of the patient was tested for IgM antibodies to 3 flaviviruses (yellow fever, dengue, and St. Louis encephalitis) and to alphaviruses (in the VEE complex and Mayaro) with use of an IgM capture enzyme immunoassay (MAC-ELISA) modified from that previously published [6]. The antigens were prepared by extraction of the brains of suckling mice with sucrose-acetone [7]. To detect IgM antibodies to viruses of the VEE complex, of which cross-reactions are important [1], antigens prepared with a Tonate virus were used.

Cell culture analysis and inoculation into newborn mice. Samples of serum, homogenized liver (since many arboviruses have a tropism for liver), and the brain of the patient were diluted 10-fold in Leibowitz medium containing 3% fetal calf serum, and dilutions were used to inoculate subconfluent AP 61 and Vero cell cultures [8]. After 7 days of culture, cells were harvested and tested by an indirect immunofluorescence assay for the presence of yellow fever, St. Louis encephalitis, and dengue 1, 2, 3, and 4 viruses, with use of monoclonal antibodies obtained from the Centers for Disease Control and Prevention (Fort Collins, CO, branch); and for the presence of Mucambo, Pixuna, Tonate, Venezuelan equine encephalitis, Ilheus, Oriboca, Murutucu, Caraparu, Guama, Catu, Wyeomyia, Oropouche, Chagres, and Punta Toro viruses, with use of mouse ascitic fluids obtained from the University of Texas, Medical Branch (Galveston, TX). Clinical specimens were also inoculated intracerebrally into 2-day-old suckling mice.

Molecular detection of Tonate virus. RNA extraction was performed with 130 μL of serum or brain or liver biopsy specimens by use of the QiaAmp Viral RNA Kit (QIAGEN S.A., Courtaboeuf, France) according to the recommendations of the manufacturer [9]. Reverse transcription (RT)–PCR were performed, as described elsewhere [10]. For RT, primer VEE 116 (5′-TACACCCAYTTRTCRTTCTG-3′) was used; and for the seminested PCR, primers VEE130 (5′-GAGAACTGCGAGCAATGGTCA-3′) and VEE 116, described by Oberste et al. [11], were used as outer primers; and VEE130 and TonnestA, a primer developed in our laboratory (5′-GCCATGTACGGGCGTGTTA-3′), were used as inner primers to amplify a portion of the PE2 glycoprotein gene. In preliminary assays this seminested PCR was able to amplify a 256-bp product for the different VEE complex viruses tested (i.e., subtypes IE, IIIA, IIIB, and V).

The PCR was performed in 3 different rooms by 3 different researchers; a negative control was included and the positive control was tested separately to avoid any contamination. The 256-bp PCR product was purified and directly sequenced with an automatic sequencing system (ACTgene; EuroSequence Gene Services, Evry, France). The sequence was aligned by use of Gene Works 2.5.1 software (IntelliGenetics, Mountain View, CA), with previously sequenced VEE strains of subtypes IAB, IC, ID, IE, IF, II, IIIA, IIIB, IIIC, IV, and V (Genbank numbers AF004436 to AF004438, AF004451 to AF004454, AF004463, AF004469, U55360, U88632, and U88650) [11, 12].

Results

MAC-ELISA showed the presence in serum of IgM antibodies specific for viruses of the VEE complex and the absence of IgM to dengue, St. Louis encephalitis, yellow fever, and Mayaro viruses.

Tonate virus could not be isolated by cell culture from blood, brain, or liver samples, and the mice (which were observed daily) remained healthy for 1 month. Tonate virus RNA was detected by RT-PCR after seminested PCR in the brain sample but not in the serum or liver specimens.

No Tonate virus–specific probes had been described, so the virus was identified by direct sequencing of the PCR product on both strands. The comparison of the sequence with sequences of all subtypes of the VEE complex obtained from Genbank showed that the virus was more closely related to Tonate virus reference strain CaAn410d, isolated in 1973 in French Guiana from the bird *P. decumanus* (1.3% nucleotide divergence), than to any other subtype (20%–55% nucleotide divergence from subtypes IIIA and II, respectively). This virus was therefore identified as a strain of Tonate virus. The sequence of this new Tonate virus strain has been deposited in Genbank (accession number AF135803).

Discussion

Although Tonate virus was first isolated >25 years ago, very little is known about its virulence. This first reported fatal case of encephalitis due to Tonate virus demonstrates that this virus may be neurovirulent and that it should not be considered, as it used to be [3], as being responsible for only mild disease. There are several possible reasons why this virus was not recovered from patients with encephalitis before 1998: a low rate of encephalitis with this virus, the small number of inhabitants in French Guiana (150,000), or the fact that surveys to determine the causes of encephalitis had not been carried out in French Guiana.

The case described here was confirmed by an RT-PCR test on samples only from the brain because, presumably, in cases of encephalitis the virus load is higher in the brain than in serum. Without molecular diagnosis this case would have been considered to be a probable case of encephalitis due to a virus of the VEE complex, but Tonate virus would not have been identified as the pathogenic agent. Indeed, IgM to viruses of the VEE complex can cross-react with Tonate virus, and other viruses of this complex (Cabassou virus, Mucambo virus) have been previously isolated in French Guiana from vertebrates and mosquito pools [4, 13].

Only a 1.3% nucleotide divergence was observed between the strains recovered from the brain and the Tonate virus reference strain CaAn410d. However, since all precautionary measures were taken, contamination is highly improbable. Moreover, sequences performed on both strands of the genome of the new strain had 100% homology, and when sequencing of the ref-

erence strain was performed to eliminate errors due to the PCR, the sequence was 100% identical to that previously published by Powers et al. [12].

This case is a further demonstration of the value of molecular methods to detect arboviruses in clinical specimens. The need for such methods has previously been described with regard to cases of yellow fever and in 1 case of dengue encephalitis [14, 15].

This case stresses the importance of compliance with the recommendations of the Subcommittee on Arbovirus Laboratory Safety for the handling of Tonate viruses in laboratories: obligatory safety level 3 containment and previous vaccination of workers against VEE virus [16].

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