

Enzyme-Linked Immunosorbent Assay Detection of Immunoglobulins G and M to Venezuelan Equine Encephalomyelitis Virus in Vaccinated and Naturally Infected Humans

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Enzyme-linked immunosorbent assays (ELISAs) were developed for detection of immunoglobulin G (IgG) and IgM antibodies to Venezuelan equine encephalomyelitis (VEE) virus in vaccinated and naturally infected humans. A total of 441 sera found negative for VEE antibodies by plaque reduction neutralization were examined by IgG ELISA and gave a 1.0% false-positive rate; no false-positives were found in the IgM ELISA. Sera with neutralizing antibody to western or eastern equine encephalomyelitis virus did not react with VEE antigen in the IgG ELISA. Sensitivity of the IgG ELISA was determined by testing 100 coded pre- and postvaccination human sera. Sixty-two were positive by ELISA; 58 of these 62 were also positive by neutralization tests, and 38 were negative by both tests. No neutralization-positive, ELISA-negative sera were found. Comparison of titers obtained by ELISA and neutralization tests indicated that 88% varied randomly by a fourfold dilution factor or less, while 61% were identical or varied only twofold. In sera obtained sequentially from 10 vaccinees and 5 naturally infected patients, both IgG and IgM antibodies appeared between 2 and 3 weeks after vaccination or onset of symptoms. The IgG and IgM antibody ELISAs described are rapid, specific, and sensitive indicators of VEE antibody status in vaccinated and naturally infected individuals.

Enzyme-linked immunosorbent assays (ELISAs) are widely available for detection of both immunoglobulin G (IgG) and IgM antibodies to a variety of common viruses; however, this technique has been applied to relatively few arboviruses (2, 6, 9, 10, 12, 14). Venezuelan equine encephalomyelitis (VEE) virus, an alphavirus of the family *Togaviridae*, is a veterinary and human public health problem in the Americas, including the southern United States (3, 7). Many strains of VEE virus infect horses (17) and have been associated with equine epizootics as well as outbreaks of disease in humans. Both epizootic and enzootic VEE strains have high potentials to infect humans (5, 7, 8, 15). We report here the development of sensitive and specific ELISAs for the detection of IgM and IgG antibody responses to VEE virus in humans after natural infection or immunization with live, attenuated vaccine. It is our contention that this assay provides a rapid, specific, and sensitive method for diagnosis of this disease.

MATERIALS AND METHODS

ELISA procedures. (i) **IgG antibody test.** The procedure used is essentially that of Yolken and Leister (18, 19). Polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with VEE TC-83 antigen diluted 1:200 in pH 9.6 coating buffer (16), incubated overnight at 4°C, and washed before the addition of test sera. Sera for screening were diluted 1:20 in phosphate-buffered saline (PBS) containing 0.5% gelatin (19). Samples for titration were tested in duplicate with serial two- or fourfold dilutions from 1:20 to 1:20,480. All reagents were added in

0.1-ml volumes per well and incubated for 1 h at 37°C, and the plates were washed five times after each incubation with PBS containing 0.05% Tween 20 (PBST), pH 7.4. Alkaline phosphatase-labeled rabbit anti-human IgG (M. A. Bioproducts, Walkersville, Md.) diluted 1:200 in PBST with 4% bovine serum albumin was used as the conjugate. *p*-Nitrophenyl phosphate substrate (1.0 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in diethanolamine buffer (pH 9.8) was added to each well and incubated for 30 to 60 min. The reaction was stopped by the addition of 3 M NaOH.

(ii) **IgM antibody test.** The IgM capture assay of Roggen-dorf et al. (14) was used with the addition of a blocking step. Plates were coated overnight at 4°C with goat anti-human IgM (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:500 in coating buffer. PBST with 5% bovine serum albumin was added to each well as a nonspecific blocking agent and incubated for 30 min at 37°C, and the plates were washed five times after this and each subsequent 37°C incubation. Test sera dilutions in PBST containing 2% sheep serum and 3% Tween 20 were then incubated for 2 h. VEE TC-83 antigen (1:200 in PBS with 2% sheep serum) was added and incubated for 1 h, followed by a 1-h incubation with rabbit anti-VEE antiserum (1:100 in PBS-2% sheep serum diluent). Alkaline phosphatase-labeled goat anti-rabbit IgG conjugate diluted 1:200 was added. After incubation, *p*-nitrophenyl phosphate substrate was added and the reaction was stopped with 3 M NaOH after a 30- to 60-min incubation. Positive and negative serum controls were included in each test. Optical densities were read at 405 nm in a Dynatech MR 580 automatic microELISA spectrophotometer interfaced with an Apple II computer programmed to report values as positive which were equal to or greater than the mean of the negative serum control \times 2.1.

Antigen. VEE virus (TC-83 vaccine strain) was prepared as described by Jahrling and Gorelkin (11). Confluent BHK-

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TABLE 1. Lack of cross-reaction of eastern and western equine encephalitis virus N-test-positive sera with VEE ELISA antigen

Serum	N test ^a			ELISA (VEE)
	Eastern equine encephalitis virus	Western equine encephalitis virus	VEE	
A	160	10	<10	<20
B	10	40	<10	<20
C	640	20	<10	<20
D	320	160	<10	<20
E	160	40	<10	<20
F	20	40	<10	<20
G ^b	<10	<10	40	320

^a Reciprocal of serum dilution.

^b N-test-positive VEE serum control.

21 cells grown in 800-cm² roller bottles were infected at a multiplicity of 1.0 and incubated at 37°C for 2 days. Culture fluids were centrifuged (400 × *g*) to remove cellular debris and then centrifuged 60,000 × *g* for 90 min, and the pellet was extracted with Genitron 113 (Allied Chemical, Morristown, N.J.). After purification by rate zonal centrifugation (90,137 × *g*, 120 min) in a 10 to 30% (wt/wt) linear sucrose gradient, the opalescent virus band was collected and stored at -70°C in 0.2-ml aliquots. Virus titers ranged from 10⁹ to 10¹¹ PFU/ml.

N test. The plaque reduction neutralization test (N test) was similar to that described by Burke et al. (4). Human test sera were heated (56°C for 30 min), and a 1:10 dilution was prepared in Hanks balanced salt solution containing 100 units of penicillin and 100 µg of streptomycin per ml. Serial twofold dilutions were made in Hanks balanced salt solution containing 2% fetal bovine serum. VEE virus (Trinidad donkey strain, prepared in Vero cells) was diluted in Hanks balanced salt solution with 5% fetal bovine serum and antibiotics to yield 100 PFU/0.1 ml. Virus suspension (0.1 ml) was added to an equal volume of each serum dilution, and the mixture was incubated at 4°C overnight. Confluent monolayers of Vero cells in 12-well (4.5-cm²) plastic plates (Linbro Chemical Co., New Haven, Conn.) were inoculated in duplicate with each serum-virus mixture, absorbed for 1 h at 36°C, and then overlaid with 1.5 ml of 1% agarose containing Eagle basal medium with Earle salts with 4% fetal bovine serum and antibiotics. Plates were incubated for 24 h at 36°C in a humidified atmosphere of 5% CO₂ and then overlaid with an additional 0.75 ml of agarose overlay containing 0.017% neutral red. Plates were reincubated for 24 h, and plaques were counted. Neutralization titers were expressed as the highest dilution of serum resulting in an 80% reduction in the plaque counts.

Anti-VEE antibody. Rabbits were inoculated intramuscularly with 7.0 × 10⁹ PFU of VEE TC-83 virus (11) diluted with PBS and mixed with an equal volume of a liposomal preparation (L-1-dipalmitoyl-phosphatidylcholine, cholesterol [5-cholesten-3-ol, 99% pure], and L-dipalmitoyl phosphatidic acid [Sigma]) in a molar ratio of 2.0:1.5:2.2, respectively (1). Three boosters consisting of virus only (2.8 × 10⁹ PFU) were given at weekly intervals. Rabbits were bled from ear veins 2 weeks after the last injection, and the serum was separated, aliquoted, and stored at -20°C.

Human sera. Test sera were obtained from patients naturally infected in Panama, from United States Army Medical Research Institute of Infectious Diseases (USAMRIID) personnel undergoing immunization, and from the USAMRIID serum bank.

RESULTS

To determine the specificity of the IgG assay, we tested sera from 100 male, 18- to 35-year-old Special Forces troops lacking in neutralizing antibody to VEE at a 1:20 dilution in the VEE IgG assay. Two weakly positive reactions were seen. When retested, two different sera gave weakly positive results, indicating a false-positive rate of approximately 2%. Additional testing of other VEE N-test-negative sera (unpublished data) suggested that the total overall IgG false-positive rate was approximately 1.0% (4 of 441). In the IgM antibody capture assay, no false-positives occurred among 321 neutralizing antibody-negative sera tested.

The specificity of the VEE IgG ELISA was further demonstrated by testing eight sera from vaccinees previously immunized with multiple viral agents with the exception of VEE. All sera contained neutralizing antibody to eastern or western equine encephalomyelitis viruses, but were negative for VEE (Table 1).

The sensitivity of the VEE IgG assay was compared with that of the N test with a second set of 100 coded pre- and postimmunization human sera from laboratory personnel receiving bunyavirus, flavivirus, and alphavirus vaccines. Sixty-two VEE-positive and 38 VEE-negative sera were found by ELISA. Of the 62 VEE-positive sera, 58 (94%) were also positive by the N test. No N-test-positive, ELISA-negative sera were found. Immunization histories of persons providing the four ELISA-positive, N-test-negative sera indicated that two of the four had been immunized with VEE vaccine more than 8 years earlier. The other two had no known exposure to VEE virus. Therefore, 2 of 62 were designated as ELISA false-positive in this series.

Of the 58 ELISA-positive sera, the titers of 43 were determined by both IgG ELISA and the N test (15 lacked sufficient volume for further testing). The degree of correlation of titers ($p = 0.78$) is shown in Fig. 1. For 38 of 43 sera (88%), ELISA and neutralization titers differed by fourfold or less, and for 26 of these 43 sera (61%), titers were identical or differed by only a twofold dilution. In the remaining five (12%) sera, ELISA values were sixfold greater than those of

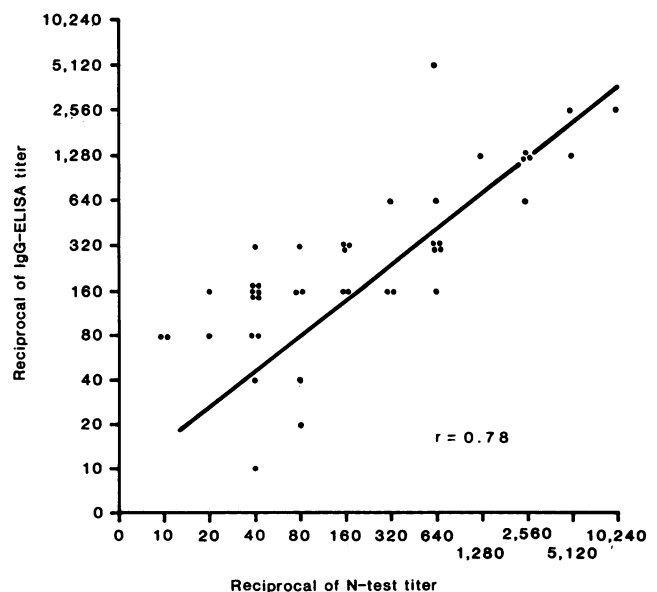


FIG. 1. Correlation of VEE IgG antibodies in vaccinees by ELISA and N test.

the N test. Overall, of the 38 ELISA- and N-test-positive samples, six were identical; ELISA titers were greater than those of the N test for 21; and the N-test titers were greater than those of the ELISA for the remaining 16. Positive and negative values coincided in 96 of the 100 sera tested.

To determine the appearance and persistence of IgG and IgM antibody, 13 VEE TC-83 vaccinees were bled weekly for 4 weeks and intermittently thereafter (Table 2). Three individuals failed to produce detectable IgG or IgM antibodies by either ELISA or N test and were excluded from further studies. IgM antibody was detected at week 2 post-immunization in 8 of 10 vaccinees and at week 3 in the ninth. The remaining vaccinee did not have blood drawn until week 4 postimmunization and was found to be IgM positive. Overall, peak titers of IgM occurred by weeks 2 to 3 and ranged from 1:80 to 1:5,120. Specific IgM antibody was detected in eight of the nine sera tested at 7 to 12 weeks, in three of the eight sera tested at 15 to 19 weeks, and in only one of the three sera tested at 24 weeks.

To rule out the possibility of false-positive reactions in the IgM assay, we tested all sera from week 2, plus randomly selected samples from other periods, for rheumatoid factor using the Rheumaton slide agglutination test (Wampole Laboratories, Div. Carter-Wallace, Inc., Cranbury, N.J.) and found them to be uniformly negative. Adsorption and subsequent elution of IgM-positive sera from IgM separation columns (Isolabs Inc., Akron, Ohio) did not affect specific IgM titers. Additionally, all IgM ELISA-reactive antibody was removed from IgM-positive, IgG-negative sera by adsorption with goat anti-human IgM antiserum. Rheumatoid-positive sera processed on the IgM separation columns did not interfere with the assay, i.e., they did not give a false-positive reaction.

IgG antibody was detected in low titer in six of the nine samples tested at the week 2 postimmunization and in all 10 samples by week 4 (Table 2). IgG titers remained elevated beyond 19 weeks in all cases. Both IgG and IgM appeared concurrently in six of nine vaccinees tested at week 2 and in all sera tested at week 4. In only two cases (C and I tested at week 2) did IgM clearly precede IgG. Figure 2 illustrates the onset and duration of both IgG and IgM antibodies in vaccinees. Both antibody levels rose between weeks 2 and 3; IgM peaked and began to decline by week 4, while IgG remained relatively constant.

To determine the ability of the ELISAs to detect VEE antibodies in naturally infected individuals, we examined 220 sera from soldiers stationed in Panama, some of whom had experienced febrile illness compatible with VEE infection, by both ELISA and N tests (Table 3). Twelve sera from five patients were positive by N test; 11 of these 12 sera were also positive by IgG ELISA. In four of five patients (A through D), fourfold rises in neutralization titers were demonstrated in sequential samples. The N-test-positive, ELISA IgG-negative specimen (E) was ELISA positive on repeat assay, but never had titers of more than 1:20. Subsequent serum samples never reached more than a 1:80 IgG titer. The IgM ELISA titer was 1:80 on the sample from week 2. Although a weak responder, this patient had an unequivocal 1:10 neutralization titer at week 5 and a fourfold rise in IgG ELISA titer.

IgM responses are also shown (Table 3) and are similar to previous observations in TC-83 recipients; early IgM rise concomitant with IgG in all sera tested, followed by a subsequent decline in IgM titer. Peak IgM titers were 4- to 12-fold greater than either the IgG ELISA or N-test titers.

TABLE 2. Detection of IgG and IgM antibodies by ELISA in 10 VEE (TC-83) vaccinees

Vaccinee	Antibody	Reciprocal of serum dilution at the following time of serum collection post immunization (wk) ^a :									
		Pre	1	2	3	4	5	7-9	11-12	15-19	24
A	IgM	<20	<20	<20	1,280	1,280		1,280	320		320
	IgG	<20	<20	<20	80	320		320	320		1,280
B	IgM	<20		1,280	1,280	1,280		320	<20	<20	
	IgG	<20		20	80	80		80	80	80	
C	IgM	<20	<20	80	1,280	1,280	320			<20	
	IgG	<20	<20	<20	20	80	20			20	
D	IgM	<20				1,280		320	320	20	
	IgG	<20				80		80	80	80	
E	IgM	<20	<20	1,280	5,120	1,280		1,280	<20	<20	
	IgG	<20	<20	20	80	80		80	80	80	
F	IgM	<20	<20	5,120	5,120	5,120		<20		<20	
	IgG	<20	<20	20	80	80		80		80	
G	IgM	<20	<20	1,280	5,120	5,120		5,120		1,280	
	IgG	<20	<20	20	80	80		80		320	
H	IgM	<20	<20	5,120	5,120	5,120		1,280	320		<20
	IgG	<20	<20	80	80	320		320	320		320
I	IgM	<20	<20	320	5,120	5,120		320		320	<20
	IgG	<20	<20	<20	320	320		320		320	320
J	IgM	<20	<20	1,280	1,280	1,280	1,280	320		<20	
	IgG	<20	<20	320	1,280	1,280	1,280	1,280	1,280	320	

^a Blank spaces, Serum not available.

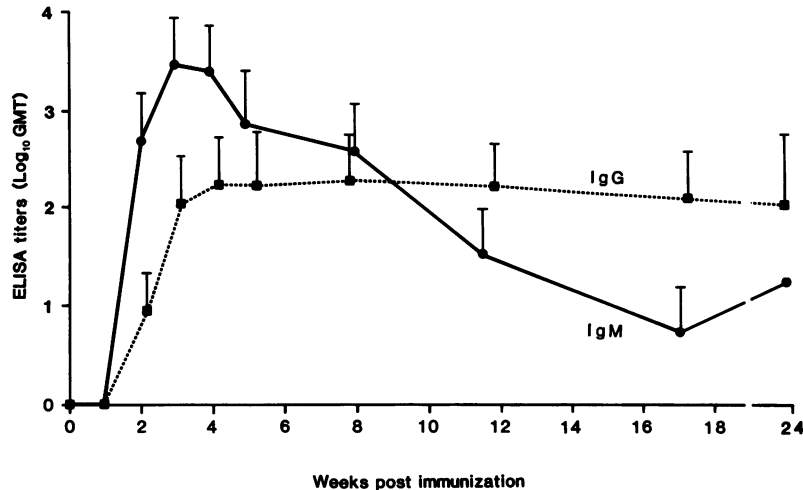


FIG. 2. Detection of IgM and IgG antibodies by ELISA in 10 subjects immunized with attenuated VEE (TC-83) vaccine. Standard error indicated by vertical bars. GMT, Geometric mean titer.

DISCUSSION

The VEE ELISAs described are specific, as sensitive as the N test for VEE antibodies, and have great potential as tools for use in rapid diagnosis of acute infections. IgM and, in most instances, IgG antibodies are present in both vaccinees and naturally infected patients 2 weeks postvaccination or after onset of symptoms. At 2 weeks, IgM antibody titers exceed IgG titers detected by IgG ELISA or N test by up to 12-fold.

In contrast to a previous report that specific IgM preceded IgG for a different flavivirus infection (Dengue [6]) our results suggest that for VEE, the shift from IgM to IgG production occurs early or perhaps their production occurs simultaneously. In 8 of 10 VEE vaccinees, high IgM and low

IgG antibody titers were detected concurrently 2 weeks after immunization; in only two instances did the IgM response clearly precede IgG. In addition, in four naturally infected individuals, sera obtained 8 to 10 days after onset of illness similarly contained both high IgM and low IgG antibody titers. These findings imply that the time to appearance of VEE IgM and IgG antibodies is relatively short. Yolken et al. (20) demonstrated that IgM could be detected in human reoviruslike infection as early as 5 days after onset of illness. Beaty et al. (2) reported the appearance of IgM antibodies to La Crosse virus by indirect fluorescence assay and IgM capture ELISA in sera obtained from the day of onset of clinical symptoms to day 12. Low levels of IgM were still present up to 7 years later in those patients showing recurrent seizures, possibly indicative of persistence of latent virus in the host rather than actual persistence of IgM antibody resulting from the primary infection.

In the majority of VEE vaccinees, IgM antibodies persist longer than in the naturally infected individuals. This apparent quantitative difference could be attributable to the use of VEE TC-83 vaccine as antigen in the assay, since the patients were most likely infected with a VEE serotype ID (strain 3880) which is endemic in Panama (5) and antigenically distinguishable from TC-83 (13, 21). This is similar to the report of Burke et al. (4) who found that after immunization with VEE TC-83, N-test titers to the homologous epizootic virus were much higher and persisted longer compared with those for heterologous enzootic VEE virus strains. Preliminary studies (unpublished data) on a limited number of individuals presumably naturally infected with strain 3880 indicate that the endemic strain 3880 might react with IgM antibodies not detected by the TC-83 antigen-containing ELISA, whereas TC-83 reacts later with developing IgG antibodies more readily than the 3880 strain.

The results presented here suggest that the described VEE IgG and IgM ELISAs are rapid, sensitive, and specific means of measuring the antibody status of vaccinated and naturally infected humans.

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TABLE 3. VEE antibody levels detected by ELISA and N test in patients with febrile illness

Patient	Antibody	Reciprocal of antibody titer at the following time (wk) after onset of illness ^a :						
		1	2	3	4	5	9	
A	IgM	<20		1,280		1,280	80	
	IgG	<20		320		320	320	
	NT ^b	<10		40		160	40	
B	IgM		1,280				<20	
	IgG		20				320	
	NT		10				160	
C	IgM		320		80			
	IgG		20		320			
	NT		10		40			
D	IgM		1,280		1,280	320		
	IgG		20		320	320		
	NT		10		20	40		
E	IgM		80				<20	
	IgG		20				80	
	NT		10				10	

^a Blank spaces, Serum not available.

^b NT, 80% Plaque reduction neutralization.

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