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Comparisons of Venezuelan Encephalitis Virus Strains by Hemagglutination-Inhibition Tests with Chicken Antibodies

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Twenty strains of Venezuelan encephalitis (VE) virus inoculated intravenously in large doses into roosters produced hemagglutination-inhibition (HI) antibodies detectable in plasmas within 7 to 10 days. No signs of illness occurred, and there was no evidence of viral growth in tissues since blood concentrations of infectious virus steadily decreased after inoculation. HI antibodies in early plasmas were specific for VE virus and did not cross-react significantly with two other North American alphaviruses, eastern and western encephalitis viruses. VE virus strains could be distinguished by virus-dilution, short-incubation HI, but not by plasma-dilution neutralization tests, by using early rooster antibodies. The distinctions by HI test were similar with some strains to, but different with other strains from, those described by Young and Johnson with the spiny rat antisera used to establish their subtype classifications of VE virus (14, 28). Nevertheless, results of HI tests with rooster antibodies correlated with equine virulence, as did results with spiny rat antibodies, and distinguished the new strains of virus that appeared in Middle America during the VE outbreak of 1969 from preexisting strains.

A system for subtyping Venezuelan encephalitis (VE) virus strains was reported by Young and Johnson based on a "kinetic" or short-incubation, virus-dilution, hemagglutination-inhibition (HI) test (28). Goose erythrocyte agglutinins were made in cultures of the Vero line of African green monkey kidney cells, and antibodies were produced in spiny rats (*Proechimys semispinosus*). Unlike spiny rat antiserum, mouse, rabbit, marmoset, and human antisera did not satisfactorily differentiate strains of VE virus from two regions of Panama. The reasons for the specificities of spiny rat immune sera were not clear, although they seemed to be unrelated to the single injection of live virus required to engender antibody formation in spiny rats, since marmoset and human antisera were also obtained after one inoculation, but were not specific. The authors concluded that "constituent antigens exist which vary in their capacity to elicit antibody responses in different hosts."

For laboratories not located in tropical America, spiny rats have been difficult to obtain. Therefore a more convenient laboratory animal has been sought for production of antisera that will distinguish strains of VE virus. Many types of laboratory animals are killed by infections with some strains of VE virus, and thus immunizations must begin with inactivated virus. Mice and rabbits are in this category, and they did not produce satisfactory antisera for Young and Johnson. White rats (*Rattus rattus*) and cotton rats (*Sigmodon hispidus*) died from infections with some strains of VE virus (8, 29), and, therefore, we considered them to be unlikely improvements over mice and rabbits as sources of strain-specific antibodies. Adult squirrel monkeys survived infections with three strains of VE virus from epizootic and three from enzootic habitats, but they produced only low titers of HI antibody in serum (8). Adult rhesus monkeys also survived infection with three epizootic and two enzootic strains, but the abilities of sera taken 12 or 47 days after inoculation were "somewhat limited" to distinguish among VE subtypes by HI or serum-dilution neutralization (N) tests (17). Since adult birds of several species survived infections with VE viruses (4, 10), the possibility arose that adult chickens might also survive. This possibility was supported by the observation that resistance of chickens to VE viruses increased as they matured from in ovo to newly hatched chicks (9). Moreover, young chickens were shown to make antibodies that distinguished North and South American strains of another alphavirus, eastern encephalitis (EE) virus (2). Therefore roosters, which can be easily utilized in most laboratories and which provide large volumes of plasma, were

examined for susceptibility to VE virus and as sources of antibodies to distinguish VE strains by HI or N tests.

MATERIALS AND METHODS

VE virus strains. Sources, HI subtypes according to the Young and Johnson classification (14, 28), and passage histories of the 25 strains of VE virus studied by HI tests with rooster antibodies are given in Table 1. Viruses for inoculations of roosters were grown in primary chicken embryonic cell cultures (CEC) by methods essentially similar to those already described and in maintenance solution without serum (19, 24). Supernatant culture fluids were harvested when 95 to

TABLE 1. *Sources, HI subtypes, and passage histories of the 25 strains of VE virus studied by HI tests with rooster antibodies*

VE virus HI subtype[a]/strain	Isolation			Reference	Passage history before inoculation of roosters[b]
	Country	Year	Host		
I-A					
Kubes (Beck-Wycoff)	Venezuela	1938	Horse	16	M8, C2
TC83	Human vaccine			1	—[c]
I-B					
69Z1	Guatemala	1969	Human	25	M2, C2
52/73	Peru	1973	Burro	—[d]	M3, C1
69T1597[e]	Guatemala	1969	Burro	25	M3
69Z1126[e]	Guatemala	1969	Human	25	M3
I-C					
CBSI-9 (P676)	Venezuela	1963	*A. triannulatus*	28, 29	M1, V1, C2
I-D					
V209A	Colombia	1960	Sentinel mouse	28, 29	M2, V2, C2
I-E					
63A216	Mexico	1963	*Culex* sp.	22	M1, C1
63U2[e]	Mexico	1963	Sentinel hamster	29	M6, C2, M1
63Z1[e]	Mexico	1963	Human	29	M3
65U64[e]	Mexico	1965	Sentinel hamster	29	M3
68U200	Guatemala	1968	Sentinel hamster	21, 23	M1, C1
68U201	Guatemala	1968	Sentinel hamster	21, 23	M1, C1 (cloned)[f]
71U338	Guatemala	1971	Sentinel hamster	21	M1, C1, V5, C1
II					
Fe3-7c	U.S.A. (Florida)	1963	*Culex* sp.	5	M6, C1
Fe5-47et	U.S.A. (Florida)	1965	*Aedes taeniorhynchus*	6, 28	M2, C1
III (Mucambo)					
BeAn8	Brazil	1954	*Cebus apella*	27	M9, C1
52049	Trinidad	1963	*Zygodontomys brevicauda*	15, 28	V1, C3[g], M1, C1
IV (Pixuna)					
BeAr35645	Brazil	1961	*Anopheles nimbus*	27	M4, C1
BeAr40403	Brazil	1962	*Trichoprosopon digitatum*	27	M5, C1
Uncertain					
?I-D					
70U1129	Peru	1971	Sentinel hamster	20	M1, C1
71D1249	Peru	1971	Mosquitoes	20	M1, C1
71D1316	Peru	1971	Mosquitoes	20	M1, C1
?V					
71D1252	Peru	1971	Mosquitoes	20	M1, C4

[a] By Young and Johnson classification (14, 18).

[b] M, Suckling, 1- to 4-day-old white mice. C or V, Primary chicken embryonic or Vero African green monkey kidney cells in culture.

[c] A total of 83 passages in cultures of embryonic guinea pig heart or embryonic chicken cells of Trinidad strain isolated from burro brain in 1943.

[d] J. Madalengoitia. Personal communication.

[e] Used only as hemagglutinins and not inoculated into roosters.

[f] Cloned strain 68U201 was M1, C2 (clonings), M1, C1 and was used to inoculate roosters.

[g] Three clonings with single plaques picked each time.

100% of the cells were destroyed; after freezing and thawing, they were centrifuged at 1,000 × *g* for 30 min at 0°C and stored at −60°C. They contained $10^{6.9}$ to 10^{10} CEC plaque-forming units (PFU) per ml. VE strains V209A and Fe5-47et were purified by the following sequential centrifugations at 5°C: (i) CEC fluid harvest (10,000 × *g*, 1 h); (ii) supernatant fluid of the CEC fluid harvest to pellet virus (83,000 × *g*, 4 h); (iii) virus into a 10 to 30% sucrose gradient (83,000 × *g*, 2 h); (iv) virus into a 30 to 60% sucrose gradient (83,000 × *g*, 14 h); and (v) the visible band of the sucrose gradient to pellet virus (83,000 × *g*, 5 h). Suspensions of purified virus contained $10^{8.8}$ and $10^{9.2}$ CEC PFU per ml of strains V209A and Fe5-47et, respectively.

Antibody production in roosters. Adult roosters (1 to 2 years of age) were caged separately and inoculated with $10^{7.6}$ to $10^{10.7}$ PFU per 5 ml of virus suspension intravenously (iv) into the jugular vein; a few roosters received similar doses of virus intraperitoneally (i.p.). Roosters were bled from jugular or wing veins, and blood contained 2 to 4 U of heparin per ml. Whole blood assayed for virus content was stored at −60°C, and plasmas for antibody tests were stored at −20°C. Preinoculation plasmas from 23 roosters were tested for VE, EE, and western encephalitis (WE) antibodies. EE and WE were included because they are alphaviruses that exist in New Jersey, the state where the roosters were raised (13). No VE, EE, or WE HI antibodies were found at 1:10 dilutions of plasmas. VE N antibodies were absent in six preinoculation plasmas ($<\log_{10}$ neutralization index 1.3 with plasmas diluted 1:2), and there were no detectable EE N antibodies in 23 preinoculation plasmas ($<\log_{10}$ neutralization index 1.6 at 1:2 dilution).

HA. We attempted to follow the method of Young and Johnson for making hemagglutinins (HA) in cultures of the Vero line of African green monkey kidney cells covered with serum-free Eagle minimum essential medium (28), but were unable to obtain high HA titers. For example, attempts to harvest virus before complete cytopathic effect developed yielded infectious virus titers in culture fluids of $10^{8.9 \text{ to } 9.3}$ CEC PFU per ml for strain 69Z1 and $10^{7.9 \text{ to } 8.2}$ for strain BeAn8, but HA titers were only 1:40 or less at optimal pH 6.2. Concentration of virus from Vero cell cultures using ammonium sulphate (60% final concentration) or polyethylene glycol (10 g/100 ml) failed to yield HA titers of >1:80 at optimal pH despite 2- to 80-fold increases in PFUs. Similar concentrations of virus from CEC produced maximal HA titers of 1:80 to 1:320 at optimal pH, and a few HA were made in CEC as follows. Cell sheets were washed three times with volumes of Hanks solution equal to the volume of growth medium to remove serum of the growth medium. Otherwise, the procedure was the same as for virus inoculated into roosters. However, unless noted otherwise, VE HA were prepared from infected suckling mouse brains by the sucrose-acetone method (7) since Young and Johnson reported that this type of HA was equally suitable to that from Vero cell cultures (28). Suckling mouse brain HA of EE virus strain NJ-1959 (3) represented the fourth mouse brain passage from mosquitoes (kindly supplied by J. Casals); WE virus strain 1985-60 was in the seventh mouse brain passage from *Culex tarsalis* (26).

HI tests. Plasma-dilution HI tests were done in small wells of plastic plates by methods similar to those of Clarke and Casals (7). Preinoculation rooster plasmas were tested with the same VE strain that was subsequently inoculated, except that strain 68U201 HA was used for roosters receiving strains 68U200 and 63A216. Virus-dilution HI tests were performed similarly to methods already described (2, 3, 28). Since our HA often had significantly higher titers at pH values lower than that used for HI tests by Young and Johnson (pH 6.3) (28), we used the optimal pH of hemagglutination for virus-dilution HI tests. These were usually either pH 6.0 or 6.2, except for some HA preparations of strain TC83, which were tested at pH 6.4. HA and acetone-extracted rooster plasmas were diluted with serological pipettes. The dilution of rooster plasma that reduced the homologous HA titer 16- to 32-fold (usually 1:80 to 1:640) was used to test heterologous HA. To minimize technical errors, this dilution of acetone-extracted rooster plasma in pH 9 buffer was determined in a preliminary 1-h "box" plasma- and virus-dilution HI test with homologous HA. The dilution was then remade in a 15- to 20-ml volume, retested, and frozen in working volumes at −20°C. A separate sample was thawed for each test during the next 7 to 10 days. Plasma-dilution HI tests were incubated for 14 to 18 h at 5°C, and virus-dilution HI tests were incubated for 1 h at 22 to 25°C unless noted otherwise. Antibody and HA end points per 0.025 ml of plasma or HA were based on complete HI.

N antibody tests. N antibody tests were done by plaque reduction in CEC by methods described previously and 80% or more plaque reduction as an end point (24). VE strains are mentioned in the text. EE virus was Guatemalan strain 68U230 (18).

RESULTS

Inoculation of roosters with strains of VE virus to produce antibodies. No signs of overt illness developed in 45 roosters inoculated with 20 strains of VE virus (Table 1). Concentrations of VE virus in blood decreased steadily following i.v. inoculations of each of four virus strains, and HI antibodies were present in plasmas 10 days later (Table 2).

Homologous HI titers were essentially equal at 7 and 10 days after inoculation (1:320 versus 1:160 and 1:80 versus 1:160 in two roosters inoculated i.v. with strain 68U200 or 1:80 versus 1:80 and 1:320 versus 1:160 with cloned strain 68U201). HI titers were slightly lower at 13 to 14 days than at 10 days with strain 68U201 (uncloned) (1:640 versus 1:160 and 1:320 versus 1:160 with two roosters inoculated i.v., 1:320 versus 1:160 and 1:640 versus 1:320 with two i.v. and i.p. roosters, and 1:40 versus 1:20 and 1:160 versus 1:80 with two i.p. roosters). These data also showed that homologous VE HI antibody titers were slightly higher when i.v. or i.v. and i.p. routes were used than when virus was inoculated only i.p. Plasmas used for subsequent HI

tests were collected on day 10 after a single i.v. inoculation.

EE HI antibodies were not detected in day-10 plasmas of two roosters inoculated i.v. with VE strain BeAr35645, two with 68U201, two with 63A216, one with BeAn8, and one with Fe5-47et. EE HI titers were 1:10 in one of two day-10 plasmas from roosters inoculated with strain Kubes, one of two with 69Z1, one of two with CBSI-9, and two of two with V209A; the second rooster with strain CBSI-9 had a titer of 1:20 and the others had titers of <1:10. No WE HI antibodies were found in these day-10 plasmas from 16 roosters inoculated with VE strains.

Incubation time for virus-dilution HI tests. Specificities of two rooster plasmas made with Kubes strain VE virus were maximal at 0.5 and 1 h since cross-reactions occurred at these times only with closely related strain 69Z1 and not with six other strains (Table 3). Thereafter at 2, 4, and 18 h, more cross-reactions appeared. We therefore used 1 h for subsequent tests.

Comparisons of VE virus strains by HI tests with rooster antibodies. The Kubes (Beck-Wycoff) strain (subtype I), which represents the original discovery of VE virus in 1938, (16) and Mucambo and Pixuna viruses (subtypes III and IV), which were historically the next members of the VE virus complex to be distinguished from previously isolated strains (27), were used as points of reference in HI tests. There were no significant reactions between Kubes rooster plasmas and Mucambo (strain BeAn8) and Pixuna (strain BeAr35645) HA nor between Mucambo and Pixuna plasmas and Kubes HA (Table 4). Cross-reactions however did occur between Mucambo and Pixuna viruses and their antibodies. Plasmas made with either Mucambo strain BeAn8 or strain 52049 inhibited Pixuna HA, and a plasma of Pixuna strain BeAr40403 inhibited Mucambo HA (Table 4).

Against nine strains of subtype I and two strains of subtype II, Kubes plasmas reacted with HA of subtypes I-A, I-B, I-C, and I-D. However, significant reactions did not occur with four I-E strains except for a barely positive result with one of two Kubes plasmas and strain 68U201 (Table 4). Kubes plasmas also reacted with HA of one of two subtype II strains. Mucambo plasmas reacted with subtype I-D and I-

TABLE 2. *VE virus concentrations in blood of roosters after i.v. inoculation*

Strains inoculated/dose (log_{10} CEC PFU in 5 ml per rooster)	Rooster designation	Virus titers in blood (log_{10} CEC PFU/ml) by time after inoculation:						Reciprocal of VE HI antibody titers 10 days postinoculation[a]
		min		h				
		2-5	2	4-5	24	48-53	72-77	
63A216/9.1	a	6.0	5.6	5.6	2.7	+[b]	0	160
	b	6.0	5.4	4.2	0	0	0	160
71U338/9.5	a	6.0		3.0	<1.4	0	0	640
	b	5.9		4.8	<1.4	0	0	640
Fe3-7c/8.7	a	6.8		3.0	<1.4	0	0	2,560
	b	6.7		4.7	<1.4	0	0	1,280
Pixuna (BeAr 40403)/8.9	a	5.6	3.2	+	+	0	0	160
	b	6.2	6.0	4.8	0	0	0	40

[a] Rooster plasmas were tested by overnight incubation of serum and 4 to 8 U of HA at 4°C for 63A216 and for 1 h at 22°C for the others. HA were made from strains 68U201, 71U338, Fe3-7c, and BeAr35645, respectively.
[b] +, Of eight suckling mice, one to five died after inoculation of blood, 0.01 ml per mouse. 0, <0.4 log_{10} CEC PFU/ml of blood, and none of seven or eight suckling mice died.

TABLE 3. *Effects of reaction times on abilities of rooster antibodies to VE strain Kubes to inhibit HA of homologous and heterologous VE strains*

Reaction time (h)	Avg-fold reduction of HA titers of VE strains[a] by subtype:							
	I-A Kubes	I-B 69Z1	I-C CBSI-9	I-D V209A	I-E 68U201	II Fe5-47et	III BeAn8	IV BeAr35645
0.5	13, 20	10, 20	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
1	20, 18	20, 18	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
2	>32, >20	32, 39	10, 13	8, 10	0, 0	0, 13	0, 0	0, 0
4	>32, >32	32, >64	>8, >11	16, 11	0, 0	8, >8	0, 0	0, 0
18	>23, >23	32, >45	>23, >23	>16, >16	>8, 11	>16, >16	0, 8	0, 8

[a] Values for two different roosters. Averages of 2 to 10 tests. 0, <8-fold reduction. HA were from CEC.

E strains, and plasma from one of two Pixuna roosters reacted with strains of subtypes I-B, I-C, I-D, and I-E (Table 4). In reciprocal HI tests with rooster plasmas to subtype I or II strains versus Kubes, Mucambo, and Pixuna HA, Kubes HA reacted with antibodies to strains of subtypes I-A and I-B and to one subtype II strain, and Mucambo and Pixuna HA reacted with antibodies to the subtype I-D and I-E strains (Table 4).

The cross-reactions between subtypes I-A and II strains and of subtypes I-D and I-E strains with subtypes III and IV were unexpected. However, they still existed after virus for inoculation of roosters was purified by centrifugation and passage through sucrose gradients. Rooster plasmas made with purified virus of subtype I-D (strain V209A) cross-reacted with subtypes III and IV strains, and subtype II (strain Fe5-47et) plasmas reacted with strains of subtypes I-A, I-B, I-C, and I-D (Table 5).

HI tests of newly isolated strains of VE virus with rooster antibodies. Epizootic strains that appeared in Middle America during 1969 were readily distinguished from preexisting enzootic strains by HI tests with rooster antibodies. For example, strong cross-reactions occurred between Kubes strain and Guatemalan epizootic strain 69Z1 but not with Guatemalan enzootic strains 68U200, 68U201, or 71U338 (Table 4). Moreover, Mucambo or Pixuna strains cross-reacted with enzootic strains. In other HI tests, antibodies from three roosters inoculated with enzootic strain 68U201 reduced by 32- to 128-fold HA titers of mouse brain antigens to homologous strain 68U201 and three other Middle American enzootic strains (Mexican strains 63U2, 63Z1, and 65U64), but did not significantly reduce HA titers of epizootic strains 69Z1, 69Z1126, and 69T1597. The only cross-reactions observed were with antibodies from two roosters inoculated with epizootic strain 69Z1, which reduced by 32- to 128-fold HA titers of enzootic strains 63U2, 63Z1, and 65U64 as well as the three epizootic strains. Thus, Middle American epizootic strains could be distinguished from enzootic strains by using rooster antibodies to Kubes, Mucambo, Pixuna, or Middle American enzootic strains.

However, newly isolated strains from a single location were not always similar in their cross-reactions. For example, three of four strains of VE virus isolated from mosquitoes or sentinel hamsters at a rain forest near Iquitos, Loreto, Peru, during 1971 (20) displayed cross-reactions with subtypes I-B and III, but a fourth strain, 71D1252, reacted only with Mucambo and Pixuna antibodies and with Mucambo HA (Table 4).

Failure of plaque-reduction N tests with rooster antibodies to distinguish VE virus strains. N antibody titers to homologous and heterologous VE strains were low in day-10 rooster plasmas. A rooster plasma made to strain 69Z1 had N antibody titers of 1:30, 1:90, 1:90, 1:10, and 1:30 versus strains 69Z1, 68U201, Fe5-47et, BeAn8, and BeAr35645, respectively; titers of a plasma to Mucambo strain BeAn8 were 1:10, <1:10, <1:10, <1:10, and 1:10, and of a plasma to Pixuna strain BeAr40403 they were 1:10, 1:10, 1:10, <1:10, and 1:30. Thus N antibody titers in early rooster plasmas were too low, and

Table 4

Results of VE virus-dilution, short incubation HI tests using early rooster antibodies to strains of different Young and Johnson subtypes and to four strains from the Peruvian Amazon of uncertain subtype

Rooster plasma antibodies to VE strains		Average fold reduction of HA titers of VE strains by subtype																			
HI subtype	Strain	I-A		I-B		I-C	I-D	I-E				II		III	IV	?I-D			?V		
		Kubes	TC83	69Z1	52/73	CBS1-9	V209A	63A216	63U2	68U201	71U338	Fe3-7c	Fe5-47et	BeAn8	BeAr35645	70U1129	71D1249	71D1316	71D1252		
I-A	Kubes	28,20**	32,23	90,110	110,90	250,78	28,32	0,0	0,0	0,8	0,0	55,39	0,0	0,0	0,0						
	TC83	11,0	64,32											0,0	0,0						
I-B	69Z1	0,23		23,78										0,0	0,0	0,11	0,0	10,8	0,0		
	52/73	13,0			45,23									0,0	0,0	28,45	23,45	28,32	0,0		
I-C	CBS1-9	0,0				32,64								0,0	0,0						
I-D	V209A	0,0					23,23							23,45	0,28						
I-E	63A216	0,0						32,11	45,8	45,11	45,16			32,0	32,8						
	68U200	0,0						16,16	8,8	16,11	16,0			8,0	0,0						
	68U201	0,0						32,32	45,16	28,32	45,45			155,78[x]	90,10[x]						
	71U338	0,0						8,0	0,0	0,0	23,16			0,0	0,0						
II	Fe3-7c	0,0										28,23		0,0	0,0						
	Fe5-47et	23,16											32,39	0,0	0,0						
III (Mucambo)	BeAn8	0,0	0,0	0,0	0,0	0,0	0,28	11,16	11,8	0,8	11,11	0,0	0,0	32,32	11,8	8	16	16	23		
	52049													64	23						
IV (Pixuna)	BeAr35645	0,0	0,0	0,10	0,10	0,11	0,16	0,45	0,45	0,32	0,45	0,0	0,0	0,0	23,29	0	0	0	8		
	BeAr40403													11,0	32,45						
Uncertain subtypes from Peru																					
?I-D	70U1129			0,0	0,11									32,0	8,0	45,16					
	71D1249			0,16	8,11									0,64	0,0		23,45				
	71D1316			0	0									11	0			23			
?V	71D1252			0,0	0,0									16,16	0,0				16,16		

[a] Values for two different roosters. Averages of two to four tests. 0, less than 8-fold reduction.

[b] Averages of four tests. Averages of two additional tests for BeAn8 were 23, 32 and for BeAr35645 16, 11.

TABLE 5. *Results of VE virus-dilution, short-incubation HI tests using early rooster antibodies obtained after inoculation of unpurified or purified virus*

Rooster plasma antibodies to VE strain	Avg-fold reduction of HA titers of VE strains by HI subtype:										
	I-A		I-B		I-C	I-D	I-E	II		III	IV
	Kubes	TC83	69Z1	52/73	CBSI-9	V209A	68U201	Fe3-7c	Fe5-47et	BeAn8	BeAr35645
V209 A (subtype I-D)											
Unpurified virus	0, 0	0, 0	0, 8	11, 11	8, 0	16, 23	0, 0	0, 0	0, 0	16, 23	0, 0
Purified virus	0, 0	0, 0	8, 0	11, 16	0, 0	23, 32	0, 0	0, 0	0, 0	16, 16	8, 11
Fe5-47et (subtype II)											
Unpurified virus	16, 11	0, 0	64, 64	45, 45	65, 45	0, 0	0, 0	45, 45	23, 16	0, 0	0, 0
Purified virus	11, 11	8, 8	64, 64	45, 45	64, 64	8, 8	0, 0	32, 45	32, 32	0, 0	0, 0
Control plasma to homologous virus	32	45	32	11	23		23	32		32	11

there were insufficient differences between homologous and heterologous titers to distinguish strains of VE virus.

DISCUSSION

By using rooster plasmas and short-incubation, virus-dilution HI tests, strains of VE virus could be distinguished but not clearly divided into distinct subtypes. Rather, there appeared to be a spectrum of complex antigenic interrelationships. At one extreme of the spectrum were five strains of VE virus classified by Young and Johnson as subtypes I-ABC and at the other end were those of subtypes I-E (four strains), III (Mucambo) (two strains), and IV (Pixuna) (two strains). Two subtype II strains were related to subtype I-ABC strains. A single strain of subtype I-D was in the middle of the spectrum because it cross-reacted with viruses at both ends. Three strains of VE virus from the Amazon region of Peru also cross-reacted with viruses at both ends of the spectrum. These three Peruvian strains have been tentatively categorized as I-D (20). Antibodies made to purified viruses cross-reacted similarly to those made with unpurified viruses.

Although characterization of a newly isolated VE strain could begin with the easier procedure of testing its HA versus a bank of rooster antibodies to known virus strains, some cross-reactions did not appear, and thus a strain could not be fully characterized, until rooster antibodies were made to it and tested against HA of standard strains. Usually plasmas from two roosters reacted similarly, but plasmas from two roosters inoculated with the BeAr35645 strain of subtype IV (Pixuna) virus were significantly different in cross-reactivities.

The scientific value of an antigen-antibody test, in this case HI, to distinguish strains of VE virus was considered carefully by Young and Johnson (14, 28). They emphasized a correlation with geographic distribution, but they also pointed out a defect in the hypothesis of foci of divergent evolution of VE virus, namely, the occurrence of antigenically distinguishable viruses in very nearly the same localities at the same time (i.e., Mucambo and Pixuna viruses near Belem, Brazil). Moreover, since their publication in 1969, VE virus of the South American subtype I-ABC appeared in Middle America (11, 14), and we found in these studies distinguishable strains from one small rain forest in the Amazon region of Peru. In fact, one of these Peruvian strains, 71D1252, has additional antigenic and virulence differences from coexisting strains (20). Thus, variations among VE virus strains do not necessarily correlate with geographic location.

In view of a changing situation with VE virus due to events in nature and developments in laboratory methods and information, it is hazardous to attempt to relate geographic distribution to our distinctions of VE strains by HI tests with rooster antibodies. It can only be noted that the strains most closely related to the prototypic Kubes strains have existed in the northern South American countries of Venezuela, Trinidad, and Peru, the southern tip of Florida, and presumably Colombia and Ecuador. Strains at the Mucambo-Pixuna end of the spectrum exist in Brazil and Middle America, and those between the ends of the spectrum have been found in Colombia and Peru and presumably eastern Panama.

Another useful correlation has been made between antigenic subtypes and equine virulence of VE strains (14, 28). To date, only subtype I-ABC strains, except the attenuated TC83 vaccine, have regularly produced encephalitis and death in equines (14). A similar correlation can be made between equine virulence and the clusters of VE strains distinguishable by HI tests with rooster antibodies since all equine-virulent strains are at the end of the spectrum with the prototypic, equine-virulent Kubes strain. How-

ever, it must be noted that strains from Florida are related to Kubes and other similar strains by HI tests with rooster antibodies, yet they are benign for equines (12).

A major value of an HI test that distinguishes VE strains is in tracing progress of an outbreak and possibly relating epizootic strains to strains from another region. This was beautifully exemplified by Franck and Johnson during the outbreak that appeared suddenly and unexpectedly in Middle America during 1969 (11). HI tests clearly showed this strain to be different from existing, recognized Middle American strains and similar to strains concurrently active in Ecuador. HI tests as described herein with rooster antibodies should function similarly since they also distinguished the Middle American pre-1969 enzootic strains from the 1969–1971 epizootic strains.

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LITERATURE CITED

1. **Berge, T. O., I. S. Banks, and W. D. Tigertt.** 1961. Attenuation of Venezuelan equine encephalomyelitis virus by *in vitro* cultivation in guinea pig heart cells. Am. J. Trop. Med. Hyg. **73**:209–218.
2. **Calisher, C. H., K. S. C. Maness, R. D. Lord, and P. H. Coleman.** 1971. Identification of two South American strains of eastern equine encephalomyelitis virus from migrant birds captured on the Mississippi delta. Am. J. Epidemiol. **94**:172–178.
3. **Casals, J.** 1964. Antigenic variants of eastern equine encephalitis virus. J. Exp. Med. **119**:547–564.
4. **Chamberlain, R. W., R. E. Kissling, D. D. Stamm, D. B. Nelson, and R. K. Sikes.** 1956. Venezuelan equine encephalomyelitis in wild birds. Am. J. Hyg. **63**:261–273.
5. **Chamberlain, R. W., W. D. Sudia, P. H. Coleman, and T. H. Work.** 1964. Venezuelan equine encephalitis virus from South Florida. Science **145**:272–274.
6. **Chamberlain, R. W., W. D. Sudia, T. H. Work, P. H. Coleman, V. H. Newhouse, and J. G. Johnston.** 1969. Arbovirus studies in south Florida, with emphasis on Venezuelan equine encephalomyelitis virus. Am. J. Epidemiol. **89**:197–210.
7. **Clarke, D. H., and J. Casals.** 1958. Techniques of hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. **7**:561–573.
8. **Dickerman, R. W.** 1972. Venezuelan encephalitis, p. 133–134. Pan American Health Organization Scientific Publication no. 243. Washington, D.C.
9. **Dickerman, R. W., and C. M. Bonacorsa.** 1975. Venezuelan equine encephalomyelitis viral infection of newly hatched chickens and embryonated eggs. Am. J. Vet. Res. **36**:1231–1234.
10. **Dickerman, R. W., C. M. Bonacorsa, and W. F. Scherer.** 1976. Viremia in young herons and ibis infected with Venezuelan encephalitis virus. Am. J. Epidemiol. **104**:678–683.
11. **Franck, P. T., and K. M. Johnson.** 1971. An outbreak of Venezuelan equine encephalomyelitis in Central America. Am. J. Epidemiol. **94**:487–495.
12. **Henderson, B. E., W. A. Chappell, J. G. Johnston, Jr., and W. D. Sudia.** 1971. Experimental infection of horses with three strains of Venezuelan equine encephalomyelitis virus. I. Clinical and virological studies. Am. J. Epidemiol. **93**:194–205.
13. **Hess, A. D., and P. Holden.** 1958. The natural history of the arthropod-borne encephalitides in the United States. Ann. N.Y. Acad. Sci. **70**:294–311.
14. **Johnson, K. M., and D. H. Martin.** 1974. Venezuelan equine encephalitis. Adv. Vet. Sci. Comp. Med. **18**:79–116.
15. **Jonkers, A. H., L. Spence, W. G. Downs, T. H. G. Aitken, and C. B. Worth.** 1968. Arbovirus studies in Bush Bush Forest, Trinidad, W. I. VI. Rodent-associated viruses (VEE and agents of group C and Guama): isolations and further studies. Am. J. Trop. Med. Hyg. **17**:285–298.
16. **Kubes, V., and F. A. Rios.** 1939. The causative agent to infectious equine encephalomyelitis in Venezuela. Science **90**:20–21.
17. **Monath, T. P., C. H. Calisher, M. Davis, G. S. Bowen, and J. White.** 1974. Experimental studies of rhesus monkeys infected with epizootic and enzootic subtypes of Venezuelan equine encephalitis virus. J. Infect. Dis. **129**:194–200.
18. **Ordonez, J. V., W. F. Scherer, and R. W. Dickerman.** 1971. Isolation of eastern encephalitis virus in Guatemala from sentinel hamsters exposed during 1968. Bol. Of. Sanit. Panam. **70**:371–375.
19. **Scherer, W. F.** 1964. Inapparent viral infection of cells in vitro. I. Conversion of inapparent to apparent infection by environmental alteration of chicken embryonic cells in cultures inoculated with Japanese encephalitis virus. Am. J. Pathol. **45**:393–411.
20. **Scherer, W. F., and K. Anderson.** 1975. Antigenic and biologic characteristics of Venezuelan encephalitis virus strains including a possible new subtype, isolated from the Amazon region of Peru in 1971. Am. J. Epidemiol. **101**:356–361.
21. **Scherer, W. F., K. Anderson, B. A. Pancake, R. W. Dickerman, and J. V. Ordonez.** 1976. Search for epizootic-like Venezuelan encephalitis virus at enzootic habitats in Guatemala during 1969–1971. Am. J. Epidemiol. **103**:576–588.
22. **Scherer, W. F., R. W. Dickerman, A. Diaz-Najera, B. A. Ward, M. H. Miller, and P. A. Schaffer.** 1971. Ecologic studies of Venezuelan encephalitis virus in southeastern Mexico. III. Infection of mosquitoes. Am. J. Trop. Med. Hyg. **20**:969–979.
23. **Scherer, W. F., R. W. Dickerman, and J. V. Ordonez.** 1970. Discovery and geographic distribution of Venezuelan encephalitis virus in Guatemala, Honduras, and British Honduras during 1965–1968, and its possible movement to Central America and Mexico. Am. J. Trop. Med. Hyg. **19**:703–711.
24. **Scherer, W. F., C. A. Ellsworth, and A. K. Ventura.** 1971. Studies of viral virulence. II. Growth and adsorption curves of virulent and attenuated strains of Venezuelan encephalitis virus in cultured cells. Am. J. Pathol. **62**:211–219.
25. **Scherer, W. F., J. V. Ordonez, P. B. Jahrling, B. A. Pancake, and R. W. Dickerman.** 1972. Observations of equines, humans and domestic and wild vertebrates during the 1969 equine epizootic and epidemic of Venezuelan encephalitis in Guatemala. Am. J. Epidemiol. **95**:255–266.
26. **Scherer, W. F., W. C. Reeves, J. L. Hardy, and T. Miura.** 1972. Inhibitors of western and Venezuelan encephalitis viruses in cattle sera from Hawaii. Am. J. Trop. Med. Hyg. **21**:189–193.
27. **Shope, R. E., O. R. Causey, A. H. Paes de Andrade,**

and M. Theiler. 1964. The Venezuelan equine encephalomyelitis complex of group A arthropod-borne viruses, including Mucambo and Pixuna from the Amazon region of Brazil. Am. J. Trop. Med. Hyg. **13**:723–727.

28. **Young, N. A., and K. M. Johnson.** 1969. Antigenic variants of Venezuelan equine encephalitis virus: their geographic distribution and epidemiologic significance. Am. J. Epidemiol. **89**:286–307.
29. **Zarate, M. L., and W. F. Scherer.** 1969. A comparative study of virulences, plaque morphologies and antigenic characteristics of Venezuelan encephalitis virus strains. Am. J. Epidemiol. **89**:489–502.