ANTIGENIC VARIANTS OF EASTERN EQUINE ENCEPHALITIS VIRUS

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In preliminary communications from these laboratories (1, 2) it was reported that antigenic differences could be detected among strains of several group A arthropod-borne animal viruses (arboviruses). The results with one of the viruses, eastern equine encephalitis (EEE), as well as the methods used for detecting the variants are described in this paper in some detail.

Materials and Methods

Strains.—Table I lists the 19 strains of EEE virus included in this study, together with pertinent information. In some cases the number of passages in laboratory animals could not be ascertained; however, it was probably high, particularly for the strain designated Prototype. On receipt in these laboratories, the strains were maintained by intracerebral passage of infected brain tissue in newborn mice, usually two to four passages but occasionally only one or two. The stock suspensions of virus were kept lyophilized at −20°C.

Antigens.—Antigens for the hemagglutination-inhibition (HI) test were prepared by the sucrose-acetone method (3). Mice 3 to 4 days old were inoculated intracerebrally with 0.02 ml of dilution 10^{-2} of a stock suspension of virus; 24 to 28 hours later the mice were sacrificed and their brains removed, frozen instantly, and held at −76°C until used. Between 10 and 15 litters of 6 to 8 mice each were sufficient to prepare a batch of antigen of convenient size. One or 2 of the inoculated litters were held for observation, and with all 19 strains these mice were dead within 48 hours. A 20 per cent suspension of infected brain tissue in an 8.5 per cent solution of sucrose was prepared and precipitated with 20 volumes of acetone; the precipitate was washed with acetone, dried under an oil-pump vacuum, and resuspended in a volume of physiological saline equal to 0.4 of that of the 20 per cent suspension of tissue. After centrifugation, the supernatant antigen was dispensed in 0.5 ml amounts into glass ampules, lyophilized, and kept at either −20° or 4°C. In some instances, precipitation with protamine sulfate (3) was carried out to extend the pH range of activity of the antigen toward the acid side.

The antigens agglutinated suspensions of goose erythrocytes within an over-all pH range between 5.9 and 6.4; their titers varied from 1:2000 per 0.2 ml for strain Tr 24443 to 1:40,000 per 0.2 ml for strains Arg M and JW. New Jersey 1945 and T172 antigens were used at pH 6.10, Arg M and Arg B at pH 6.15, Prototype, Panama, Tr 24443, Tr 25714, and Arg LL at pH 6.20, and the rest at pH 6.25.

Immune Sera.—Thirty to 40 mice, 50 to 70 days old, were vaccinated with each strain. Two intraperitoneal inoculations were given. The first consisted of 0.3 ml of a 10 per cent suspension of infected newborn mouse brain tissue in physiological saline, inactivated either with 0.05 per cent beta propiolactone for 1 hour at 37°C or with 0.5 per cent (final concentration) of formalin for 1 week at 4°C; the second, given 18 to 25 days later, consisted of 0.3 ml of a freshly prepared, fully active suspension. The mice were bled 5 or 6 days after the second injection.

In some cases, sera were also prepared in mice given a single intraperitoneal injection of 0.3 ml of a 10^{-2} suspension of active virus. Between 25 and 75 per cent of these animals died within 7 days; the survivors were bled 7 or 8 days after inoculation.

Test.—Comparison of strains was done essentially by means of the HI test as described elsewhere (3), using goose red cell suspensions at optical density 0.750 (approximately equivalent to a concentration of 0.4 per cent of packed cells), lucite plates, and removal of nonspecific inhibitors with acetone. Adsorption of sera with goose erythrocytes was eliminated, since experience soon showed that acetone-treated sera had no agglutinins left for goose erythrocytes at the dilutions used in this study.

During the study, several modifications of the general HI procedure were introduced. The

TABLE 1

Strains of Eastern Equine Encephalitis Virus Studied

Designation	Supplied by	Isolation			No. of passages in laboratory animals	
		Place	Yr.	Host	Mice	Other
Prototype	TenBroeck	New Jersey-Virginia	1933	Horse	200-300?	
New Jersey 1945	Hammon	New Jersey	1945	Pheasant	7–9	1
New Jersey 1959	Shope	New Jersey	1959	Mosquitoes	3–5	
Va-60-1	Byrne	Maryland	1960	Horse	4–6	2
JW	Daniels	Massachusetts	1956	Man	2	4
Florida 08-3-0855	Work	Florida	1960	Red-eyed vireo	5	
Alabama 09-28-0930	Work	Alabama	1960	Carolina wren	2	1
Sanchez	Eklund	Dominican Republic	1949	Donkey	4	3
Jamaica 7761	Belle, Grant	Jamaica, West Indies	1962	Horse	6	3
Panama	Shelokov	Panama	1958	Horse	6–8	
Tr 24443	Downs	Trinidad, West Indies	1959	Mosquitoes	4–6	
Tr 25714	Downs	British Guiana	1959	Horse	4–6	
BeAn 178	Causey	Brazil (Belem)	1955	Monkey, sentinel	4–6	
BeAn 221	Causey	Brazil (Belem)	1955	Monkey, sentinel	4–6	
BeAn 5122	Causey	Brazil (Belem)	1956	Monkey, sentinel	4–6	
Arg M	Rosenbusch	Argentina (Buenos Aires)	1959	Horse	?	
Arg B	Rosenbusch	Argentina (Buenos Aires)	1938	Horse	?	
Arg LL	Rosenbusch	Argentina (Buenos Aires)	1936	Horse	?	
T172	Hammon	Thailand	1958	Mosquitoes	4–6	

antigens were rehydrated the day before a test was to be done, diluted to 1:10, and held overnight at 4°C. On the day of the test, the antigens were titrated with extreme care to determine the dilution that would contain 1 unit in 0.2 ml. On this basis a series of six increasing twofold dilutions of the 1:10 stock was prepared, containing from 256 to 8 units of antigen per 0.2 ml; these constituted the master set of antigen dilutions.

A master set of four twofold serum dilutions was also prepared. The highest dilution was that which according to previous experience barely inhibited 8 units of antigen in a standard HI test (overnight incubation at 4°C), and the three lower dilutions were respectively 2, 4, and 8 times the concentration of the highest.

Test tubes, 100 x 13 mm, were placed in racks so that for every serum-antigen combination in the test there were four rows of six tubes each. To the first tube in each row was added 0.8 ml of the highest dilution from the master set of antigens; a similar amount of the second highest dilution was added to the second tube in each row, and so on until antigen had been added to all tubes. Immediately after this was done, the master set of serum dilutions was distributed (0.8 ml per tube), the highest dilution being added to the six tubes in the first

row, the second highest to the six tubes in the second row, and so on. The mixtures were then held at 22°C, the moment at which a set had been completed being designated 0 time. About 25 minutes after 0 time, 0.4 ml of the mixture in each tube was transferred to a corresponding well on a lucite plate, using a short pipette of soft glass attached to a Cornwall syringe with rubber tubing. Since in each row the amount of serum was constant and the amount of antigen increased, a single pipette could be used for an entire row. At exactly 28 minutes after 0 time, 0.4 ml of the red cell suspension was added to the mixtures in the wells, using the same pipetting device. To complete this step with a set of 24 wells usually took 1.5 to 2 minutes. The system was then incubated at 37°C until the reaction was read. This determination was called the 0.5 hour titration.

A second set of 0.4 ml aliquots of the mixtures was treated in the same way at 2 hours after 0 time; this reading was called the 2 hour titration. At the same time, samples of the unmixed antigen dilutions left in the master tubes were retitrated for comparison with the original titration; usually only the dilutions calculated to contain 16 and 64 units were tested.

After the serum-antigen mixtures had been held at 22°C for 18 hours, the procedure was repeated again and a final titration of the antigen was done.

Reading and recording of the results was done in the usual way. In Tables V through VII, + = complete agglutination, ⊕ = partial agglutination, ± = trace agglutination, and 0 = no agglutination. The titer of an antigen was determined by the highest dilution giving either + or ⊕ agglutination, the titer of a serum by the highest dilution giving complete inhibition, or 0 reading. In Tables VIII through XV, results are expressed as the number of units of antigen that had been completely inhibited by the serum; 0 indicates that no inhibition occurred with 8 units of antigen, the lowest amount used.

TABLE II

Definition of Viral Strains as EEE Virus by Hemagglutination Inhibition

Serum	Antigen					
	EEE, 12 strains	WEE	VEE	Chi	May	Sin
Prototype	1280–2560*	80	80	80	20	40
New Jersey 1959	2560–10240	80	80	40	20	40
Tr 25714	5120–20480	80	40	20	0	0
Panama	2560–10240	0	0	0	0	0
Tr 24443	640–2560	40	20	0	0	0
BeAn 5122	1280–5120	80	40	40	20	40
BeAn 178	2560–10240	40	0	0	0	0
Arg B	640–5120	40	20	20	0	20
Arg M	1280–10240	40	40	20	0	0

WEE, Western equine encephalitis; VEE, Venezuelan equine encephalitis; Chi, Chikungunya; May, Mayaro; Sin, Sindbis.

* Reciprocals of titers against 8 units of antigen; 0 indicates no inhibition at dilution 1:20, lowest used.

RESULTS

Definition of Strains as EEE Virus.—All the strains included in the study had been identified as EEE virus by the persons submitting them with the

exception of the Argentinian strains, which were identified in these laboratories (4). In each case the identification had been made in tests other than or including the HI test.

Work in these laboratories (5) had shown that the Prototype EEE strain could be easily separated from prototypes of other group A viruses by HI. For the purposes of this study, however, it was necessary to make sure that all strains were unmistakably recognizable as EEE virus by the HI test. Accordingly, sera obtained during the secondary immune response of mice im-

TABLE III

Definition of Viral Strains as EEE Virus by Hemagglutination Inhibition

Serum		Antigen								
Virus	No. injections	Homologous	EEE strain							
			Prototype	NJ 1945	Panama	Tr 24443	Tr 25714	BeAn 5122	Arg B	Arg LL
Chikungunya	5	5120	40	20	40	0	40	40	20	0
Mayaro	5	2560	80	80	160	80	160	80	80	40
Middelburg	5	2560	20	40	20	0	40	20	20	20
Semliki	4	10240	80	80	160	80	80	80	80	40
Sindbis	4	1280	0	20	20	0	20	40	20	0
VEE	4	5120	20	40	40	20	40	40	20	20
WEE	5	5120	0	0	0	0	0	0	0	0
AMM 2021	5	5120	20	20	20	0	20	20	0	0
AMM 2354	5	5120	20	20	40	20	20	40	20	0
EEE, NJ 1945	3		5120	5120	2560	5120	1280	2560	1280	1280

See Table II for explanations.

munized with a given strain were titrated against 8 units of antigens prepared with each of the presumed EEE strains as well as with other group A agents.

The results obtained, as typified in Table II, indicated that the strains were indeed similar among themselves and easily distinguishable from other group A agents. Table III illustrates the results of additional experiments in which immune sera against group A agents other than EEE strains were prepared in mice inoculated four or five times intraperitoneally, and the sera were tested by HI against 8 units of antigens for several EEE strains.

It can be seen that no antiserum against a strain designated EEE reacted to more than 1:16 of its homologous titer with any of the other prototype group A viruses. In contrast, the titers with the other EEE strains were either the same as that given by the homologous antigen or different from it by at most a factor of 2 or 4; in the last instance, the homologous reaction was not necessarily the highest.

Cross-Reactions among EEE Strains by Titration of Sera with a Constant

Amount of Antigen.—Table IV shows the results of a test in which a number of sera and antigens were tested by the standard HI procedure; *i.e.*, sera in dilutions against a constant amount (8 units) of antigen. These sera and antigens were samples of the specimens used in the rest of the study. The serum-antigen mixtures had been held at 4°C for 18 hours before the red cell suspensions were added.

Although there was some variation in the titers given by a serum against different antigens, this occurred non-systematically and often represented a difference of only one dilution between homologous and heterologous titers.

TABLE IV

Cross-Reactions between EEE Virus Strains by Standard HI Test

Serum	Antigen							
	NJ 1945	NJ 1959	Pan	Tr 24443	BeAn 5122	BeAn 178	Arg M	Arg B
New Jersey 1945	640*	640	320	320	160	320	160	320
New Jersey 1959	10240	10240	5120	5120	5120	5120	10240	10240
Panama	10240	2560	10240	5120	5120	2560	10240	10240
Tr 24443	1280	640	1280	2560	1280	1280	2560	2560
BeAn 5122	5120	2560	5120	2560	5120	2560	5120	5120
BeAn 178	10240	5120	5120	5120	5120	5120	10240	10240
Arg M	5120	2560	10240	5120	5120	5120	10240	10240
Arg B	2560	640	2560	2560	1280	1280	5120	5120

* Titer of serum was 1:640.

These results were considered technically unsatisfactory as a means of discerning possible antigenic differences among the strains, and the newer methods described in this paper were developed.

Influence of pH on the Result of an HI Test.—It is known (3) that the pH of the system, after the suspension of erythrocytes has been added to an antigen, influences the antigen's capacity to agglutinate the cells and thus determines the pH range within which the antigen is usable. Within this range the titer of the antigen can vary, as illustrated in Table V. In general, antigens have been used at an optimal pH selected on the basis of highest titer, together with due provision of enough margin on either side of this pH to protect against minor technical fluctuations.

During the present study it was found that the pH of the system also influences the result of an HI test in terms of the number of units of antigen inhibited.

In a typical experiment, a rabbit-immune serum against the EEE Prototype strain and a normal rabbit serum were diluted to 1:80, and a Prototype-strain antigen was diluted in increasing serial twofold dilutions beginning at 1:25. Two sets of 12 test tubes each were used.

To each tube of the first set was added 2 ml of the EEE serum, and to each tube of the second set the same amount of the normal serum. Next, 2 ml of EEE antigen at the highest dilution (1:51,200) was added to the 12th tube in each set, the same amount of the second highest dilution (1:25,600) to the 11th tubes, and so on. The sets were then held at 4°C for 18 hours, when 0.4 ml was transferred from each tube to the wells of agglutination trays so as to give eight replicas of each series, with immune and with normal serum. Red cell suspension was added to replica No. 1 at pH 6.00, to replica No. 2 at pH 6.05, and so on through increments of 0.05 to pH 6.35.

As shown in Table VI, the titer of the antigen in the presence of normal serum remained nearly constant at pH 6.00–6.30, the value at pH 6.20 being

TABLE V

Influence of pH on the Agglutinating Capacity of EEE Virus Antigens

pH	Strain and reciprocal of dilution																
	Arg LL									New Jersey 1945							
	25	50	100	200	400	800	1600	3200	6400	25	50	100	200	400	800	1600	3200	6400
6.0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	0
6.1	+	+	+	+	+	+	+	⊕	0	+	+	+	+	+	+	+	⊕	0
6.2	+	+	+	+	+	+	+	⊕	0	+	+	+	+	+	+	+	+	0
6.3	+	+	+	+	⊕	⊕	⊕	0	0	+	+	+	+	⊕	⊕	0	0	0
6.4	⊕	⊕	⊕	0	0	0	0	0	0	⊕	⊕	0	0	0	0	0	0	0
6.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Volume of antigen, 0.2 ml.

+, complete agglutination; ⊕, partial agglutination; 0, no agglutination.

just slightly higher. In contrast, the inhibitory action of the serum increased with the pH so that the units of antigen inhibited rose from 4 at pH 6.00 and 6.05, to 8 at pH 6.10 and 6.15, to 32 at pH 6.20, 6.25, and 6.30. At the highest pH, 6.35, the antigen titer began to drop off because this value was at the edge of the active range. (Similar observations were made with other group A viruses and also with viruses of groups B and C, although in the latter two groups the effect was not studied sufficiently to determine its magnitude or generality.)

The pH of the system after addition of the red cell suspension was therefore established for each strain. First, the pH range of the antigen was determined by titrating it at 0.05 levels from pH 6.00–6.40. Within this range, the pH of optimum sensitivity for inhibition was selected, as illustrated in Table VI. However, since the antigens differed somewhat in their pH range, the pH of maximum sensitivity for inhibition selected for an antigen was to some extent dependent on that at which the other antigens could be used. Accordingly, a number of exploratory tests were made in which red cells were added to re-

peated sets of sera and antigens for two or three strains at different pH. The pH thus selected was the highest at which the homologous titer of the serum was equal to or greater than the heterologous titers. This meant that some antigens had to be used at a pH lower than their optimum for inhibition because at that level a heterologous serum had a greater titer against them than against its own antigen.

Effect of the Use of High Dilutions of Serum on Discrimination of Strains.— The possibility that titration of antigens in the presence of constant amounts of serum might reveal sharper differences between strains than those observed in

TABLE VI

Influence of pH on Inhibition of Agglutination by EEE Prototype Strain

pH	Serum and reciprocal of dilution of antigen																Units inhibited
	EEE							Normal									
	25	50	100	200	400	800	1600	25	50	100	200	400	800	1600	3200	6400	12800	
6.00	+	+	+	+	⊕	0	0	+	+	+	+	+	+	+	⊕	0	0	4
6.05	+	+	+	+	⊕	0	0	+	+	+	+	+	+	+	+	0	0	4
6.10	+	+	+	⊕	0	0	0	+	+	+	+	+	+	+	+	±	0	8
6.15	+	+	+	⊕	0	0	0	+	+	+	+	+	+	+	+	±	0	8
6.20	+	+	+	0	0	0	0	+	+	+	+	+	+	+	+	⊕	0	32
6.25	+	+	0	0	0	0	0	+	+	+	+	+	+	+	⊕	0	0	32
6.30	+	⊕	0	0	0	0	0	+	+	+	+	+	⊕	⊕	⊕	0	0	32
6.35	⊕	0	0	0	0	0	0	+	+	+	+	+	±	±	0	0	0	—

±, trace agglutination. For explanation of remaining symbols see Table V.

the standard HI test was investigated. So as to cover a sufficiently wide range of dilutions, both sera and antigens were used simultaneously in serial dilutions.

In a representative experiment, antigens for New Jersey 1959 and BeAn 5122 strains were titrated at pH 6.25, both titers being 1:10,240 per 0.2 ml. Master dilutions of the antigens were prepared in twofold steps from 1:40 to 1:1280, and on the basis of the preliminary titration were calculated to contain from 256 to 8 units, in descending order. Immune sera were similarly diluted, beginning at dilution 1:320 for New Jersey 1959 and at 1:160 for BeAn 5122. Sera and antigens were mixed as described under Materials and Methods and, after incubation at 22°C for 2 hours, the red cell suspension was added. Unmixed samples of the two antigens in dilutions 1:80, 1:160, 1:320, and 1:640 were titrated at this time and found to contain the calculated numbers of units.

The results of the HI test (Table VII) indicate a marked difference between the strains. They also show that at dilution 1:640 New Jersey 1959 serum inhibited 128 units of its own antigen and 64 units of BeAn 5122 antigen, a difference of 64 units, while at dilution 1:2560 the same serum inhibited 32 units of its own antigen and less than 8 units of the other, a difference at most of 32

units. The difference at dilution 1:640, although greater, is based on a single sequential twofold dilution; that at dilution 1:2560, however, involves at least three twofold dilutions and hence is technically more satisfactory because less susceptible to error and more reproducible. These and similar results were the grounds for use of the high serum dilutions in preference to the lower ones.

This test illustrated another important point, one that can best be observed with simultaneous use of twofold dilutions of antigen and serum near their end-points, namely, that in this range of dilutions there is not always an indirect twofold ratio between serum titer and amount of antigen used.

TABLE VII

Influence of Variation in Serum and Antigen Dilutions on Inhibition of Agglutination by EEE Virus Strains (Reaction Time, 2 Hours)

Serum		Antigen												
		New Jersey 1959							BeAn 5122					
		Dilution and units							Dilution and units					
Strain	Dilution, 1:	1:40	80	160	320	640	1280	Units inhibited	1:40	80	160	320	640	1280	Units inhibited
		256	128	64	32	16	8		256	128	64	32	16	8	
NJ 1959	320	0	0	0	0	0	0	256+	⊕	0	0	0	0	0	128
	640	±	0	0	0	0	0	128	+	+	0	0	0	0	64
	1280	+	⊕	0	0	0	0	64	+	+	+	⊕	0	0	16
	2560	+	+	+	0	0	0	32	+	+	+	+	+	±	<8
BeAn 5122	160	0	0	0	0	0	0	256+	0	0	0	0	0	0	256+
	320	+	0	0	0	0	0	128	0	0	0	0	0	0	256+
	640	+	+	⊕	0	0	0	32	+	0	0	0	0	0	128
	1280	+	+	+	+	⊕	0	8	+	+	0	0	0	0	64

For explanation of symbols see Tables V and VI.

Effect of Reaction Time on the Inhibitory Activity of Immune Sera.—Investigation of the effect of duration of contact between antigen and serum was carried out as described under Materials and Methods. This procedure also made it possible to observe the influence of serum dilution on the degree of specificity of the reaction.

Table VIII shows the results of an experiment with antigens and antisera for three strains, Prototype, Tr 24443, and BeAn 5122. At 0.5 hours, Prototype serum reacted at dilution 1:1280 with its own antigen only, and BeAn 5122 serum reacted similarly at dilution 1:640. At 2 hours, the homologous reactions had increased at these dilutions or had appeared at new ones. Thus, Prototype serum at dilution 1:1280 inhibited 64 units of homologous antigen but showed

lesser titers with the other two antigens; the Tr 24443 system gave a homologous reaction only; and BeAn 5122 serum, while easily separable from the Prototype, reacted with Tr 24443 antigen as well as it did with its own. At 18 hours, Prototype serum still reacted best with its own antigen, but the reaction with the other two was now represented by a difference of only one dilution in the series, technically a result of little value but significant as continuing the trend established by the results at 0.5 and 2 hours. The difference between the Tr

TABLE VIII

Comparison of EEE Virus Strains by HI Test Using Simultaneous Dilutions of Serum and Antigen and Rate of Reaction

Serum		Antigen, reaction time in hrs. and units inhibited								
		0.5			2			18		
Strain	Dilution, 1:	Prototype	Tr 24443	BeAn 5122	Prototype	Tr 24443	BeAn 5122	Prototype	Tr 24443	BeAn 5122
Prototype	1280	32	0	0	64	32	16	128	64	64
	2560	0	0	0	16	0	0	64	32	16
	5120	0	0	0	0	0	0	16	8	8
	10240	0	0	0	0	0	0	0	0	0
Tr 24443	640	0	0	0	0	32	0	0	64	32
	1280	0	0	0	0	0	0	0	32	8
	2560	0	0	0	0	0	0	0	8	0
	5120	0	0	0	0	0	0	0	0	0
BeAn 5122	640	0	0	32	0	128	128	32	128	128
	1280	0	0	0	0	16	32	8	64	32
	2560	0	0	0	0	0	0	0	16	16
	5120	0	0	0	0	0	0	0	0	8

0 indicates that the minimum number of units present in the test, 8, was not inhibited.

24443 and Prototype strains shown by Tr 24443 serum was well maintained; BeAn 5122 serum likewise gave a consistently better (fourfold) reaction with its homologous antigen than with the Prototype. It is questionable whether at this point the Tr 24443 and BeAn 5122 strains could be distinguished.

In this and other tests, the homologous reaction appeared first, at either 0.5 or 2 hours, and was then of such specificity that the strains could be easily separated. With time, 2 or 18 hours, cross-reactions developed which in the more closely related systems rendered slight differences almost impossible to evaluate and often were not diagnostic of strain difference. Easily separable strains, Prototype and either Tr 24443 or BeAn 5122 in Table VIII, showed a consistent cross-reacting pattern that facilitated their separation. With more

closely related systems, separation was possible, if at all, only after the shortest interval of incubation.

In addition to illustrating the progress with time of the HI reaction, both homologous and heterologous, Table VIII shows that the homologous rate of reaction was the faster, even though at 18 hours both reactions had nearly the same or equal titers.

Types of Cross-Reactions among EEE Strains.—The preceding sections have shown that distinction of strains was facilitated by employing high pH of re-

TABLE IX

Reactions of Identity among Three EEE Strains from the United States

Serum		Antigen, and time in hrs.								
Strain	Dilution, 1:	0.5			2			18		
		Prototype	NJ 1945	NJ 1959	Prototype	NJ 1945	NJ 1959	Prototype	NJ 1945	NJ 1959
Prototype	640	64	32	32	64	64	64	128	64	128
	1280	16	8	16	32	16	32	64	32	64
	2560	0	0	0	8	8	8	16	16	16
	5120	0	0	0	0	0	0	0	0	0
NJ 1945	2560	16	16	16	32	32	32	64	64	64
	5120	0	0	0	16	16	16	16	16	32
	10240	0	0	0	0	0	0	8	8	16
	20480	0	0	0	0	0	0	0	0	0
NJ 1959	1280	32	32	32	64	64	64	64	64	128
	2560	8	16	16	16	16	32	32	32	32
	5120	0	0	0	0	0	8	16	16	16
	10240	0	0	0	0	0	0	0	0	0

action, high dilutions of serum, and several time intervals of reaction, particular attention being paid to the shorter intervals. In previous work (5) it had been found that the specificity of immune sera in the HI test was increased when only one or two vaccinating injections were given and the animals were bled early in the course of an immune response. When EEE strains were compared by methods that included all these conditions, it soon became apparent that reproducible differences existed among some of the strains. As these differences varied quantitatively, models or types of reactions were established according to which strains were considered identical, easily separable because of marked differences, or separable, if at all, with difficulty because the differences were slight.

Tables IX and X illustrate reactions of "identity" among three strains iso-

lated in the northeastern United States and among three strains isolated in Argentina. The greatest difference noted between any two strains was represented by only a single serial dilution, and even this was not necessarily in the direction of the homologous system. In the interpretation of these results, an occasional difference of inhibition amounting to only one dilution was considered to be within the technical limitations of the method; however, when such differences were observed consistently between two strains and always in the direction of the homologous system, they were considered significant.

TABLE X

Reactions of Identity among Three EEE Strains from Argentina

Serum		Antigen, and time in hrs.								
Strain	Dilution, 1:	0.5			2			18		
		M	LL	B	M	LL	B	M	LL	B
Arg M	640	16	32	32	128	128	64	128	128	128
	1280	0	0	0	32	32	32	64	64	32
	2560	0	0	0	0	0	0	32	16	16
	5120	0	0	0	0	0	0	8	8	8
Arg LL	640	0	0	0	32	64	32	64	64	64
	1280	0	0	0	0	0	8	32	32	16
	2560	0	0	0	0	0	0	16	8	8
	5120	0	0	0	0	0	0	0	0	0
Arg B	640	8	8	16	64	64	64	128	64	64
	1280	0	0	0	8	8	16	32	32	32
	2560	0	0	0	0	0	0	16	8	16
	5120	0	0	0	0	0	0	0	0	0

Tables XI and XII illustrate reactions of "distinctiveness" among strains that were therefore considered easily separable.

An example of a reaction of "ambiguity" is given in Table XIII. In this test, the Panama strain serum reacted equally with its own antigen and that for BeAn 5122 at all three readings; in the two instances where the titer of the homologous system exceeded that of the cross-reaction, the difference amounted to only one dilution and was not considered significant. In the reciprocal test, BeAn 5122 serum reacted better with its own antigen than with the heterologous one at 0.5 and 2 hours, but this difference was largely obliterated after 18 hours' incubation. On the basis of these results, the two strains were considered slightly dissimilar.

Separation of EEE Strains into Two Main Divisions.—Thirteen strains of

EEE virus were subjected to complete cross-testing, including determination of rate of reaction at three time intervals and use of four twofold serial dilutions of serum. Since the number of serum-antigen combinations obviously precluded complete simultaneous testing, the antigens and their corresponding antisera were tested simultaneously in groups of three or four. Enough repeat determinations were done to insure that the results of different tests could be brought together in a tabular summary, Table XIV.

To simplify the presentation in Table XIV, each serum is represented by only one dilution, which is the same against all the antigens; different sera, however,

TABLE XI

Reactions of Distinctiveness between EEE Strains from the United States and Argentina

Serum		Antigen, and time in hrs.					
Strain	Dilution, 1:	0.5		2		18	
		Fla	LL	Fla	LL	Fla	LL
Florida	1280	0	0	16	0	32	0
	2560	0	0	8	0	16	0
	5120	0	0	0	0	0	0
	10240	0	0	0	0	0	0
Arg LL	640	0	32	16	64	32	128
	1280	0	8	0	16	8	32
	2560	0	0	0	8	0	16
	5120	0	0	0	0	0	8

may have different dilutions. The number of units inhibited is that for the 2 hour titration test except in the case of BeAn 221 serum, for which the 18 hour titration is given.

As the table shows, these 13 strains divide themselves into two main groups. The five strains in the first group were isolated in the northeastern United States, from Massachusetts to Maryland. By the present method they are completely identical, but are easily separable from the eight strains in the second group.

This second group is less homogeneous, as indicated by the amounts of antigen inhibited by their respective sera. For example, it was repeatedly noted that while Panama serum reacted with all other antigens to a titer similar to that with its homologous antigen, sera for the seven other strains reacted at a lower level with Panama antigen. Tr 24443 was also slightly distinctive in that under adequate conditions its serum reacted at a better titer with its own antigen than with the heterologous ones, while the other sera reacted nearly to

titer with Tr 24443 antigen. Such differences as occurred among the remaining strains were of the order described above as inconsequential.

These two groups have been arbitrarily designated North American and

TABLE XII

Reactions of Distinctiveness between EEE Strains from the United States and British Guiana

Serum		Antigen, and time in hrs.					
Strain	Dilution, 1:	0.5		2		18	
		JW	Tr	JW	Tr	JW	Tr
JW	1280	64	0	64	8	64	16
	2560	8	0	32	0	32	8
	5120	0	0	16	0	16	0
	10240	0	0	0	0	8	0
Tr 25714	640	32	128	128	128	128	128
	1280	0	16	32	64	32	64
	2560	0	0	8	16	16	32
	5120	0	0	0	0	8	16

TABLE XIII

Reactions of Ambiguity between EEE Strains from Panama and Brazil

Serum		Antigen, and time in hrs.					
Strain	Dilution, 1:	0.5		2		18	
		Pan	BeAn	Pan	BeAn	Pan	BeAn
Panama	640	32	16	64	64	128	128
	1280	0	0	16	8	32	32
	2560	0	0	0	0	16	16
	5120	0	0	0	0	0	0
BeAn 5122	640	0	32	16	128	64	128
	1280	0	0	0	16	16	32
	2560	0	0	0	0	0	16
	5120	0	0	0	0	0	0

South American types. With the less homogeneous South American type, there may be a subgroup represented by the strains from Argentina, Brazil, and British Guiana, which, although not reacting among themselves as uniformly as the North American strains, are nevertheless considered identical. It is also possible that the Panama and Tr 24443 strains each represent another subgroup.

Studies with Additional EEE Strains.—Six strains listed in Table I but not included in Table XIV were subsequently studied. After a basic pattern had been established, as described in the preceding section, these isolates were characterized by comparison with representatives from the North American and South American types. The results of these tests have been combined in Table XV on the same basis as was used in Table XIV.

It can be seen that the isolates from Florida, Alabama, Jamaica, the Dominican Republic (Sanchez), and Thailand (T172)[1] were indistinguishable from the

TABLE XIV

Separation of EEE Virus Strains into Two Main Groups

Serum	Antigen												
	Proto.	NJ 1945	NJ 1959	JW	Va-60-1	Pan	Tr 24443	Tr 25714	BeAn 178	BeAn 221	BeAn 5122	Arg M	Arg B
Prototype	32	32	32	32	16								
NJ 1945	32	32	32	32	64								
NJ 1959	32	32	32	32	64				8				
JW	32	32	64	64	128								
Va-60-1	16	32	16	16	16								
Panama			8			32	64	64	32	—	32	32	32
Tr 24443				—			32	8	8	8	8	8	8
Tr 25714			8			16	32	32	16	8	16	64	32
BeAn 178									16	16	16	16	16
BeAn 221						16	32	16	64	32	32	32	32
BeAn 5122			8				16	16	16	32	32	16	16
Arg M			—				32	32	32	32	16	32	32
Arg B						8	32	32	8	8	16	32	32

Units of antigen inhibited by a constant dilution of serum. Blank spaces indicate no inhibition of 8 units, lowest amount used; — indicates not tested.

North American strains used, and that Arg LL was identical with either Arg M or B in the South American group and had a relationship with Tr 24443 not unlike that shown in Table XIV for the latter and the Argentinian strains.

HI Tests with Human Sera and Antigens Derived from Different EEE Strains.—An attempt was made to determine whether the antigenic distinctiveness between the North and South American types would be observable in antibodies present in human sera.

[1] The isolation of a strain of eastern equine encephalitis virus in Thailand by Dr. William McD. Hammon and his associates will be described elsewhere (Hammon, personal communication). Dr. Hammon kindly supplied the author with this strain and allowed him to mention it here before a description had appeared in the literature.

TABLE XV

Identification of Additional EEE Virus Strains as North American or South American Variants

Serum	Antigen								
	Proto., JW	Arg M, B	Tr 24443	T172	Fla	Ala	Jam	San-chez	Arg LL
Prototype or JW	32			32	16	32	32	32	
Arg M or B		32	—						32
Tr 24443	—	—	16						
T172	32			32	—	—	—	—	—
Florida 08-3-0855	32		—	—	32	—	—	—	
Alabama 09-28-0930	16			—	—	32	—	32	
Jamaica 7761	64	—	—	—	32	—	32	—	—
Sanchez	16			—	—	32	—	32	
Arg LL		16	16	—					16

See Table XIV for explanations.

TABLE XVI

HI Titers of Human Sera with Different EEE Virus Strains

Serum origin and No. tested	Antigen, 8 units	No. of sera giving indicated titer								Geometric mean titer antigen	
		0	20	40	80	160	320	640	1280	Individual	Combined
New Jersey 2 sera	Prototype							1	1	900	1080
	NJ 1945								2	1280	
	BeAn 221						2			320	
	BeAn 5122					2				160	250
	Arg M						2			320	
	Panama						2			320	
	Tr 24443						2			320	
Brazil 20 sera	Prototype	4	5	5	5	1				20	25
	NJ 1945	3	3	7	4	2	1			30	
	BeAn 221		4	4	6	2	3		1	80	
	BeAn 5122	1	1	5	4	5	4			79	78
	Arg M		4	4	5	4	3			75	
	Panama	1	3	7	3	4	2			54	
	Tr 24443		4	4	4	4	4			80	

Reciprocal of titers given.

Twenty-two sera were available: two collected in 1959 from persons convalescing from infection with EEE virus in New Jersey, and 20 from residents of the Amazon valley in Brazil who had been found to have antibodies capable of neutralizing EEE virus (6). These sera were tested in a standard HI test against 8 units of various antigens representing the North American and South American types of EEE virus.

From the results given in Table XVI, it is evident that in the case of the Brazilian sera the number positive and also the mean serum titers were higher with the South American strains. Similarly, the two sera from New Jersey showed a higher reactivity with the North American strains.

DISCUSSION

Although recognized for some time among other groups of animal viruses, antigenic variation in the arboviruses has been investigated only recently and with relatively few agents. Using the HI test with adsorbed sera, Clarke (7) studied several group B arboviruses and showed that strains of yellow fever differed according to whether they originated in Africa or America. With the methods described in this paper, we have found (1, 2) antigenic variants with Chikungunya and Sindbis, as well as EEE, in group A. Similar findings with western equine encephalitis have recently been reported by Karabatsos *et al.* (8) using several serological tests.

The present study showed that 19 EEE strains fell into two distinct groups, a North American and a South American type. The North American type is represented by ten strains, seven isolated in the eastern half of the United States and one each in the Dominican Republic, Jamaica, and Thailand. The South American type consists of nine strains all isolated in more southerly areas of America,—Panama, British Guiana, Trinidad, Brazil, and Argentina.

The inclusion of strain T172 from Thailand in the North American group was somewhat unexpected, in view of the great distance involved and also the sharp distinctiveness of the two American types. The complete identity of this strain with those from the northeastern United States (in particular, it was repeatedly compared with New Jersey 1945) is susceptible of several interpretations: (*a*) strains from Thailand and the United States are, in fact, identical; (*b*) differences do exist but the method used was not sensitive enough to detect them; (*c*) T172 is an artifact, the result of a laboratory contamination. No choice can be made among these alternatives until additional strains from outside the American continent and adjacent islands become available for study. In this connection, it is of interest that isolation of one strain of EEE virus has been reported in the Philippines (9) and, more recently, of several strains in Kazakhstan, USSR (10). The relationship of these strains to the two types described is not yet known.

With the single exception noted, the determining factor in the distinction

between EEE strains was geographical distribution rather than animal host from which the strain was isolated or year of isolation. The North American type strains were isolated over a span of 30 years from man, horses, birds, and mosquitoes, yet they form a remarkably homogeneous group. The South American type strains, isolated over nearly 25 years from a variety of species, are less uniform as a group but nevertheless are much more closely related to one another than to North American isolates. As noted earlier, it is possible that subdivisions may be established in this group.

It has been observed by Clarke (7) with yellow fever virus and by ourselves with Chikungunya (1) that repeated passage of a virus in an experimental host can alter to some extent the antigenic make-up of the virus. Some of the EEE strains studied had undergone numerous passages in mice, others very few, and some had been passaged in experimental hosts other than mice. Their passage history did not stamp the strains with any distinctive antigenic property recognizable by the present methods, nor was it sufficient to obliterate the difference between the two main groups.

A question that arises during studies of antigenic variation in the arboviruses is what criteria are to be followed in deciding whether a given strain is a variant of a virus or a new virus. The problem has occurred repeatedly in the case of Russian spring-summer encephalitis and Central European tick-borne viruses and more recently with strains of western equine encephalitis virus (8), to mention only two instances. In the standard HI tests, EEE strains are uniform. By other tests, such as neutralization test, Carneiro and Cunha (11) found slight differences between strains of presumed EEE virus, and in limited studies with the present strains, similar slight differences were noted in one instance. Thus, a New Jersey 1959 antiserum had a homologous neutralization index of 4.8 and an index of 4.2 against the BeAn 5122 strain, while a BeAn 5122 antiserum had a homologous index of 5.0 and an index of 3.4 against the New Jersey 1959 strain. These differences, however,—and only the second was at all significant,—occurred between the two extremes of the EEE strains studied and were hardly sufficient, without repeated confirmation, for separation of strains, let alone of viruses. It appears, therefore, that EEE virus is easily separable from all other group A viruses, and that antigenic differences detectable among isolates of EEE are, at most, of the order expected among variants rather than between viruses.

With the arboviruses, at least, the decision as to whether or not two strains differ enough antigenically to be considered distinct viruses is still somewhat subjective and pragmatic. If numerous and repeated observations using different serological tests and known experimental sera show that two strains are very closely related; if tests with sera collected for serological surveys indicate that little would be lost by substituting one for the other; if other strains are isolated that fall, antigenically, between the two, then one would be inclined to

consider the strains as variants of one virus rather than as two different viruses.

A conclusion to be drawn from the present study is that, even though differences between strains are small, some advantage may be gained from using local strains for serological surveys by HI test.

One of the reasons for initiating the present study, and other similar ones, was to determine whether the antigenic evidence would strengthen or weaken the concept of rapid spread of a virus over a wide geographic area, for example, through the agency of bird migration. While the antigenic similarity of the strains from Argentina, Brazil, and British Guiana is not against a rapid spread's having occurred among these countries, the antigenic difference between the North and South American types does not favor such a spread between these two land masses, at least for the time period covered by the isolations. If a rapid spread is postulated, a valid explanation will have to be found for the observed antigenic difference.

SUMMARY

A study by hemagglutination-inhibition test showed that 19 strains of eastern equine encephalitis virus grouped themselves in two main types, which have been designated North American and South American. The former consists of ten strains from the eastern half of the United States, from Massachusetts to Florida; Jamaica, the Dominican Republic, and, subject to confirmation, Thailand. The South American type comprises nine strains from Panama, Trinidad, British Guiana, Brazil, and Argentina. The strains were isolated from different natural hosts over a period of 30 years.

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