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Serologic Distinctness of Eastern, Western, and Venezuelan Equine Encephalomyelitis Viruses.*

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Infections produced in animals by the viruses of eastern, western, and Venezuelan equine encephalomyelitis are so similar in their symptoms and course that they constitute, from a practical standpoint, a single clinical entity.

Nevertheless, cross-neutralization and cross-protection tests have, on the whole, been rather consistent in showing that these viruses are immunologically distinct. A possible relationship, however, was suggested by Gallo and Vogelsang,[1] who found that eastern and western equine encephalomyelitis immune sera exerted a slight neutralizing action against the Venezuelan virus, and by Soriano Lleras and Figueroa[2] who, using a Colombian virus presumably of the Venezeulan type,[3] reported similar results.

On the other hand, the results of cross-immunity experiments have been at variance with interpretations derived from neutralization tests. Records and Vawter[4] reported that 6 of 8 horses immune to western equine encephalomyelitis virus resisted cerebral or nasal inoculation with the eastern virus. Meyer, Wood, Haring, and Howitt[5] reported that 12 horses hyperimmunized to western equine encephalomyelitis virus strains resisted intracutaneous inoculation of the eastern virus, and 1 of 3 other animals survived cerebral inoculation of eastern virus; 2 horses hyperimmunized to the eastern virus resisted cerebral injection of western virus. Howitt[6] found that 40% of 115 guinea pigs immune to the western virus were resistant to inoculation with the eastern virus. Beck and Wyckoff[7] observed that while guinea pigs immune to the western virus were fully susceptible to inoculation with the Venezuelan virus, animals immune to either the eastern or Venezuelan viruses showed some resistance to inoculation with the heterologous virus, outliving the controls by a day or more; the results were considered as suggestive of some relationship between the eastern and Venezuelan viruses. Carneiro and Cunha[8] reported that guinea pigs solidly immune to the Venezuelan virus were afforded a small amount of protection to inoculation with a Brazilian (eastern type) virus, and guinea pigs immune to the eastern virus regularly resisted inoculation with the Brazilian virus; in the reverse direction, animals immune to the Brazilian virus were fully susceptible to the Venezuelan virus and only partially resistant to the eastern virus.

In view of the relationships suggested by cross-immunity tests, it was considered not impossible that the failure of neutralization tests to substantiate the results of cross-immunity experiments may have been due to the severity and lack of sensitivity of the usual intracerebral neutralization technics. Since the extraneural neutralization test con-

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1 Gallo, P., and Vogelsang, E. G., *Rev. Med. Vet. y Parasitol., Caracas,* 1940, **2**, 143.

2 Soriano Lleras, A., and Figueroa, L., *Bol. Inst. Nac. Hig. Samper Martinez,* 1942, No. 8, 3.

3 Kubes, V., *Rev. Med. Vet. (Bogota),* 1943, **12**, 108.

4 Records, E., and Vawter, L. R., *J. Am. Vet. Med. Assn.,* 1934, n. s. **38**, 89.

5 Meyer, K. F., Wood, F., Haring, C. M., and Howitt, B. F., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 89.

6 Howitt, B. F., *J. Immunol.,* 1935, **29**, 319.

7 Beck, C. E., and Wyckoff, R. W. G., *Science,* 1938, **88**, 530.

8 Carneiro, V., and Cunha, R., *Arq. Inst. Biologico, São Paulo,* 1943, **14**, 157.

stitutes a much more highly sensitive method for the detection of antibodies to these viruses than does the intracerebral test,[9,10] this technic was used to restudy the possibility that serologic relationships exist between these viruses. Because of the impression gained in previous work[10] that the amount of virus neutralized by an immune serum tended to be somewhat greater in 3-day-old mice than in older animals, serum-virus mixtures were tested by the subcutaneous route in animals of this age.

Material and Methods. Viruses. The strains used were those employed in previous work.[10,11]

Fresh mouse brain virus was used for the neutralization tests. Mice were inoculated intracerebrally with 0.03 ml of source virus diluted to the desired concentration with nutrient broth containing 10% of normal sheep serum. When signs of infection appeared, the animals were killed with chloroform. The brains were removed, cultured, and stored overnight in the freezing compartment of a refrigerator. The next day 5 bacteriologically sterile brains were weighed, triturated without abrasive, and made into a 20% suspension, by weight, in 10% serum-broth. The suspension was centrifuged for 15 minutes at 1500 r.p.m. in an International centrifuge equipped with an angle head. The supernatant fluid was drawn off and used to prepare a series of tenfold dilutions in serum-broth; dilutions were always made by serial transfer of 0.5 ml amounts into 4.5 ml amounts of diluent.

Sera. Immune sera were prepared in rabbits. The animals were bled at weekly intervals until a large amount of normal serum had been accumulated from each; the serum specimens were then pooled according to animals, filtered through a Seitz EK pad, and stored at 4° without preservative.

Pairs of rabbits were immunized against each virus. For the eastern, western, and French neurotropic yellow fever viruses the following method was used. Two freshly removed infectious mouse brains were triturated in a mortar with gradual addition of 10 ml of 0.85% salt solution, and the whole of the resulting suspension (uncentrifuged) was injected intraperitoneally into a rabbit. The animals were bled out 10 days later, and the serum was filtered and stored.

Immunization with the Venezuelan virus necessitated the use of inactive material because of the high virulence of this virus for rabbits.[12] The animals were given a total of 4 intraperitoneal injections consisting of 5 ml of formalized mouse brain vaccine[10] weekly; after a rest period of 2 weeks, 5 ml of a 10^{-4} suspension in saline of active mouse brain virus was given subcutaneously. The animals were bled out 10 days later, and the serum was filtered and stored in the same manner as the specimens mentioned above.

Neutralization Tests. In order more accurately to evaluate the results obtained with an immune serum should cross-neutralization occur, normal serum taken from the animal furnishing the immune serum was always included in the same test.

Undiluted sera were distributed into 75 x 12 mm tubes, and an equal volume of virus dilution was added. Virus was added to the normal serum first and then to the immune serum, beginning with the lowest virus dilution and ending with the highest. Homologous serum-virus mixtures were prepared last and inoculated first. The tubes were shaken to mix the contents, which were inoculated without incubation into 3-day-old mice in 0.03 ml amounts; the inoculum was administered subcutaneously between the shoulders, the needle being inserted parallel to the vertebral column and withdrawn slowly during the injection. Six mice were used for each serum-virus mixture.

The serum-virus mixtures were kept on the table in a container of ice and water during the inoculations.

In each experiment immune sera to each of the 3 equine encephalomyelitis viruses were tested against one of the viruses, and serum from a rabbit immunized against the

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[10] Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 375.

[11] Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 175.

[12] Lennette, E. H., and Koprowski, H., *J. Am. Med. Assn.*, 1943, **123**, 1088.

TABLE I.

Serologic Distinctness of Eastern, Western, and Venezuelan Equine Encephalomyelitis Viruses as Shown by Subcutaneous Cross Neutralization Tests in 3-Day-Old Mice.

Virus	Rabbit sera		Mortality ratio* of mice inoculated with serum plus virus dilution										Effective virus titer	LD_{50} doses of virus neutralized by serum
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
Eastern Equine Encephalomyelitis	Eastern E. E.	268	6/6	0/6	0/5	0/6	0/6						$10^{-1.5}$	50,000,000
	Normal	268					6/6	6/6	6/6	6/6	4/6	0/5	$10^{-9.2}$	
	Eastern E. E.	301	5/6	2/6	0/6	0/6	0/6						$10^{-1.6}$	50,000,000
	Normal	301						6/6	6/6	6/6	4/6	1/6	$10^{-9.3}$	
	Western E.E.	295					6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or >	
	Normal	295						6/6	6/6	6/6	4/6	1/6	$10^{-9.3}$	0
	Western E. E.	298				6/6	6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or >	
	Normal	298					6/6	6/6	6/6	6/6	4/6	0/6	$10^{-9.2}$	0
	Ven. E.E.	337				6/6	6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or >	
	Normal	337					6/6	6/6	6/6	6/6	6/6	1/6	$10^{-9.6}$	2.5 or <
	Ven. E.E.	375					6/6	6/6	6/6	6/6	3/6		$10^{-9.0}$ or >	
	Normal	375						6/6	6/6	6/6	5/6	1/6	$10^{-9.5}$	3 or <
	French Neuro.†	306					6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or >	
	Normal	306						6/6	6/6	6/6	4/6	0/6	$10^{-9.2}$	0
	French Neuro.	308					6/6	6/6	6/6	6/6	4/6	1/6	$10^{-9.3}$	
	Normal	308					6/6	6/6	6/6	6/6	6/6	0/6	$10^{-9.5}$	1.5 or <
Western Equine Encephalomyelitis	Western E. E.	295	2/6	0/6	0/6	0/6	0/6	0/5					$<10^{-1.0}$	$>100,000,000$
	Normal	295						6/6	6/6	6/6	3/6	0/6	$10^{-9.0}$	
	Western E. E.	298	1/6	0/6	0/6	0/6	0/6						$<10^{-1.0}$	> 31,600,000
	Normal	298					6/6	6/6	6/6	6/6	0/6	0/6	$10^{-8.5}$	
	Ven. E. E.	337				6/6	6/6	6/6	6/6	4/6	2/6		$10^{-8.5}$	
	Normal	337					6/6	6/6	6/6	6/6	0/6	0/6	$10^{-8.5}$	0
	Ven. E. E.	375					6/6	6/6	6/6	5/6	1/6		$10^{-8.5}$	
	Normal	375						6/6	6/6	5/6	1/6	0/6	$10^{-8.5}$	0
	Eastern E. E.	268					6/6	6/6	6/6	6/6	0/5		$10^{-8.5}$	
	Normal	268					6/6	6/6	6/6	6/6	1/6	0/6	$10^{-8.6}$	0
	Eastern E. E.	301					6/6	6/6	6/6	2/6	3/6		$10^{-8.2}$	
	Normal	301						6/6	6/6	5/6	2/6	1/6	$10^{-8.7}$	3
	French Neuro.	306					6/6	6/6	6/6	6/6	3/6		$10^{-9.0}$ or >	
	Normal	306						6/6	6/6	6/6	3/6	0/6	$10^{-9.0}$	0
	French Neuro.	308					6/6	6/6	6/6	4/6	1/6	0/6	$10^{-8.3}$	
	Normal	308					6/6	6/6	6/6	2/6	2/6	0/6	$10^{-8.0}$	0

Venezuelan	Ven. E. E.	337	6/6	4/6	1/6	0/6	0/6	0/6					10-2.3	12,600,000
Equine	Normal	337					6/6	6/6	6/6	6/6	5/6	0/6	10-9.4	
Encephalomyelitis	Ven. E. E.	375	0/6	0/6	0/6	0/6	0/6	0/6					<10-1.0	> 31,600,000
	Normal	375						6/6	6/6	6/6	0/6	0/6	10-8.5	
	Eastern E. E.	268				6/6	6/6	6/6	6/6	6/6	1/6		10-8.6	
	Normal	268					6/6	6/6	6/6	6/6	0/6	0/6	10-8.5	0
	Eastern E. E.	301					6/6	6/6	6/6	6/6	4/6		10-9.2	
	Normal	301						6/6	6/6	6/6	2/6	1/6	10-8.8	0
	Western E. E.	295					6/6	6/6	6/6	6/6	6/6		10-9.5 or >	
	Normal	295						6/6	6/6	6/6	3/6	2/6	10-9.3	0
	Western E. E.	298				6/6	6/6	6/6	6/6	6/6	4/6		10-9.2	
	Normal	298					6/6	6/6	6/6	6/6	0/6	0/6	10-8.5	0
	French Neuro.	306					6/6	6/6	6/6	5/6	6/6		10-9.4 or >	
	Normal	306						6/6	6/6	6/6	6/6	0/6	10-9.5	0
	French Neuro.	308					6/6	6/6	6/6	6/6	2/6	0/6	10-8.7	
	Normal	308					6/6	6/6	6/6	5/6	1/6	0/6	10-8.5	0
Yellow Fever	French Neuro.	306	0/6	0/6	0/6	0/6	0/5						<10-1.0	>200,000,000
(French	Normal	306						6/6	6/6	4/6	1/6	0/6	10-9.3	
Neurotropic	French Neuro.	308	0/6	0/6	0/6	0/6							<10-1.0	> 10,000,000
Strain)	Normal	308					6/6	6/6	6/6	3/6	0/6	0/6	10-8.0	
	Ven. E. E.	337				6/6	6/6	6/6	6/6	6/6	1/6	0/6	10-8.6	
	Normal	337				6/6	6/6	6/6	6/6	5/6	2/6	0/6	10-8.6	0
	Ven. E. E.	375						6/6	6/6	5/6	0/5		10-8.4	
	Normal	375						6/6	6/6	3/6	0/6	0/5	10-8.0	0
	Eastern E. E.	268				6/6	6/6	6/6	6/6	5/6	1/6	0/6	10-8.5	
	Normal	268				6/6	6/6	6/6	6/6	6/6	1/6	0/6	10-8.6	0
	Eastern E. E.	301						6/6	5/6	3/6	1/6		10-8.0	
	Normal	301						6/6	6/6	4/6	0/6	0/6	10-8.2	1.5
	Western E. E.	295						6/6	6/6	4/6	0/6		10-8.2	
	Normal	295						6/6	6/6	4/6	3/6	0/6	10-8.7	3
	Western E. E.	298				6/6	6/6	6/6	6/6	5/6	6/6	0/6	10-9.4	
	Normal	298				6/6	6/6	6/6	6/6	4/6	0/6	0/5	10-8.3	0

* The mortality ratio is expressed by a fraction in which the numerator indicates the number of mice which died and the denominator the number of mice which were inoculated (less those destroyed by the mother of the litter).

† French Neuro. = French Neurotropic strain of yellow fever virus.

French neurotropic strain of yellow fever virus was included as a specificity control. Each experiment was subsequently repeated, using a different set of immune sera, to check the results of the first test.

Presentation of Results. The 50% mortality end point titer[13] of the virus in the presence of immune and of normal sera was calculated and is shown in the table under the heading "Effective virus titer"; the difference in the logarithms of the titer of the virus in the presence of an immune serum and of the corresponding normal serum is expressed arithmetically in the last column as the number of LD_{50} neutralized.

Results. The results of the cross-neutralization tests are presented in Table I. It is apparent that while the immune sera neutralized millions of LD_{50} of their homologous virus, their neutralizing effect on the heterologous viruses was nil—neutralization either was totally undemonstrable, or ranged from 1.5 to 3 LD_{50}, an amount of no significance since it falls within the range of experimental error of the method.

Sera which have a questionable, or definite but weak, neutralizing capacity by the intracerebral test usually, if not always, show a much greater neutralizing power by the subcutaneous or intraperitoneal test,[9,10] and if the 3 equine encephalomyelitis viruses possess any antigenic components in common, it would be expected that the extraneural test would reveal similarities in their antigenic constitution. The absence of cross-neutralization despite the high sensitivity of the test indicates, therefore, that the eastern, western, and Venezuelan equine encephalomyelitis viruses are serologically unrelated.

Why the results of cross-immunity tests should be at variance with those of cross-neutralization tests is not evident. The situation with the equine encephalomyelitis viruses is analogous to that which obtains with the West Nile-St. Louis-Japanese group, except that the reverse holds true—the viruses appear to be related by cross-neutralization tests,[14,15] but not by cross-immunity tests.[14,15] While it is possible that the existence of common antigens may be more readily demonstrable for one group of viruses by one method and for another group by a different method, the antithetic results of the immune reactions within a group stresses the need for further investigation of these reactions. With regard to the equine encephalomyelitis group, the consistent failure to demonstrate by neutralization tests any unequivocal relationship between these viruses points to the desirability of restudying the problem from the standpoint of the specificity of the cross-immunity tests, *i.e.*, has the cross-resistance demonstrated been due to an interference effect,[16] to the existence of paraimmunity,[17] or to an antibody response broadened by repeated antigenic stimuli, or is it due to an acquired specific cellular immunity whose breadth is not reflected by humoral antibodies.

Summary. The relationships suggested by cross-immunity tests to exist between the eastern, western, and Venezuelan equine encephalomyelitis viruses were investigated by means of cross-neutralization tests done in 3-day-old mice by the subcutaneous route. Despite the high sensitivity of the test, there was no evidence that these viruses possess any antigenic components in common.

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