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Serological Comparisons Among Hart Park Virus and Strains of Flanders Virus

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The antigenic relationships among Hart Park (HP) virus strain AR 70, Flanders virus strain 61-7484, and other related strains were investigated. Strains AR 70 and 61-7484 were shown to be antigenically related but different from each other in neutralization, complement fixation, and double-diffusion tests. Two strains isolated in Texas and one strain each, isolated in Illinois and Florida, appeared to be closely related if not identical to Flanders virus (61-7484) and antigenically related but different from HP virus AR 70. Ten additional strains isolated in 10 different states were compared to the two prototype strains. Seven of the 10 could not be distinguished from Flanders virus, and all 10 strains were different from HP virus. The results indicate that a virus-type consisting of viruses antigenically related if not identical to Flanders virus is widely distributed within the United States, whereas HP virus is antigenically related but different from this type.

Hart Park (HP) virus strain AR 70 and Flanders virus strain 61-7484 are two antigenically related but different viruses that have been isolated from mosquitoes. HP virus AR 70 was isolated from a pool of 56 adult female *Culex tarsalis* mosquitoes collected 5 August, 1955, at Hart Park, Calif. (6). The prototype of Flanders virus, strain 61-7484, was isolated from a pool of 25 unengorged adult female *Culiseta melanura* mosquitoes collected 8 August 1961, in Flanders, Long Island, N.Y. (14). Similar agents have been isolated from mosquitoes collected in the southeast United States (9), Kentucky (8), Tennessee (13), Texas (12), Illinois (7), Missouri (7), Saskatchewan (5), and Utah (4). Flanders virus has also been isolated from diluted blood obtained from house sparrows and red-winged blackbirds collected in Illinois (7) and the spleen of an oven bird collected in New York (14).

Whitney (14) suggested that the strains isolated in New York and those isolated in Texas in 1961 may be members of a new, widely distributed arbovirus group. This investigation was undertaken to study the antigenic relationships among viruses isolated in different areas of the United States and thought to be related to HP virus or Flanders virus, or both, and to determine which of three standard serological tests might be most satisfactory in studying these strains.

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MATERIALS AND METHODS

Mice. The CFW strain of albino Swiss mice obtained from Carworth Farms, Inc., New City, N.Y. was used. Adult females were bred, and the newborn mice were used for propagation of virus, neutralization tests, and as the source of viral-infected brain material for stock viruses and sucrose-acetone antigens. Adult mice were used in the preparation of immune reagents.

Virus. Virus strains used in these experiments are listed in Table 1. Virus stocks were prepared as 5% suckling mouse brain suspensions (w/v) in 100% normal rabbit serum.

Immune reagents. Immune reagents were 7-day sera obtained from mice that had received one intracerebral (ic) inoculation and were bled after 7 days, 14-day sera obtained from mice that received two ic inoculations 7 days apart and were bled 14 days after the first inoculation, and 28-day sera obtained from mice that had received 4 intraperitoneal (ip) inoculations at 7-day intervals and were bled 28 days after the first inoculation. All sera were obtained by cardiac puncture. Immune ascitic fluids were prepared in mice that received four ip inoculations 7 days apart and one ip injection of 0.2 ml of fresh, whole ascitic fluid containing sarcoma 180 TG cells the same day the third inoculation was administered. Once acute ascites had occurred, the fluid was collected by abdominal paracentesis.

Serological tests. The complement fixation (CF) test as described by Casals et al. (2) was used. Sucrose-acetone-extracted antigens were prepared by the method of Clarke and Casals (3). The neutralization (NT) test described by Smithburn et al. (11) was performed in 1- to 2-day-old mice. Neutralization indices

TABLE 1. *Hart Park-Flanders and related virus strains studied*

Strain designation	Source	Collection date	Location	Suckling mouse passage level of stock viruses
AR 70[a]	*Culex tarsalis*	Aug. 1955	Hart Park, Calif.	35
61-7484[b]	*Culiseta melanura*	Aug. 1961	Flanders, N. Y.	7, 8
TH4-47f[a]	*Culex quinquefasciatus*	Aug. 1964	Houston, Texas	2
65V-78[c]	*C. tarsalis*	June 1965	Hale County, Texas	2
AR 714[d]	*Culex pipiens complex*	June 1965	McLeansboro, Ill.	4
F1-210A[a]	*Culex nigripalpus*	July 1961	Southern Florida	3
A9-155L[a]	*C. melanura*	Aug. 1960	Southern Alabama	3
NJO-60C[a]	*C. melanura*	July 1960	New Jersey	4
WX3-1AQ[a]	*C. melanura*	Aug. 1963	Southern Georgia	3
A-264[a]	*C. pipiens*	Sept. 1964	Massachusetts	3
NCJ5-6AQ[a]	*C. melanura*	Aug. 1965	North Carolina	3
V5-13A[a]	*C. melanura*	Sept. 1965	Virginia	3
AP6-11X[a]	*C. quinquefasciatus*	Sept. 1966	Arkansas	3
TnM6-27C[a]	*Culex restuans*	Sept. 1966	Tennessee	4
TCC7-24A[a]	*C. quinquefasciatus*	June 1967	Texas	3
W-18905[a]	*C. pipiens*	July 1965	Ohio	4

[a] Received from the Center for Disease Control, Atlanta, Ga.
[b] Received from E. Whitney, New York State Department of Health, Albany, N.Y.
[c] Received from the NCDC, Fort Collins, Colo.
[d] Received from the Illinois Center for Zoonoses Research, University of Illinois, Urbana, Ill.

were calculated by the method of Reed and Muench (10). Agar for Ouchterlony plates was prepared as a 0.8% solution of Noble agar (Difco Laboratories, Detroit, Mich.) in a tris(hydroxymethyl)amino-methane-barbital-sodium-barbital buffer, *p*H 8.8, obtained from Gelman Instrument Company, Ann Arbor, Mich. The plates were prepared by pouring 6 ml of agar onto precleaned glass slides (2 by 3 inches; ca. 5.1 by 7.6 cm). Wells 6 mm in diameter were cut in the agar, the antigens and immune reagents were added to the wells, and the slides were incubated at 29 C.

RESULTS

Initial tests were conducted to determine an immunization procedure that would demonstrate differences between the two prototype strains, AR 70 and 61-7484, in NT and CF tests. Results of testing 7-day sera, 14-day sera, and 28-day sera are shown in Table 2.

Strains AR 70 and 61-7484, as well as four other virus strains, were selected for comparison in reciprocal-cross CF tests (Table 3) and in reciprocal-cross NT tests (Table 4) so that, with the two prototype strains, the six represented the northeastern, southeastern, middlewestern, southwestern, and far western areas of the United States. The F1-210A virus was not included in the NT tests because the virus stocks prepared with this strain failed to develop an adequate titer.

These six strains were compared with the Ouchterlony-double-diffusion technique using immune ascitic fluids. Each immune ascitic fluid was tested against each of the six antigens and sucrose-acetone-extracted normal mouse brain (Fig. 1). No reactions were observed between any of the immune ascitic fluids and the normal antigen. AR 70 immune ascitic fluid reacted only with its homologous antigen. The reaction was represented by one line approximately midway between the reactant wells. AR 714, F1-210A, and 61-7484 immune ascitic fluids each formed a uniform and consistent line of identity with all the antigens except AR 70. The reaction with AR 70 antigen was

TABLE 2. *Complement fixation test (CF) and neutralization test (NT) results of comparisons between Hart Park (AR 70) and Flanders (61-7484) viruses*

Antigen	Sera	CF		NT	
		AR 70	61-7484	AR 70	61-7484
AR 70	7-day	0[a]	0	0[b]	>0.4
61-7484		0	4/≥32[c]	≤1.0	2.0
AR 70	14-day	16/≥64	8/32	≥3.4	2.4
61-7484		0	16/≥64	≤1.0	3.4
AR 70	28-day	32/≥256	8/128	≥3.1	2.8
61-7484		8/64	32/≥256	2.6	≥3.6

[a] No positive reaction observed at lowest serum dilution of 1:4.
[b] Neutralization index $\log_{10}$.
[c] Reciprocal of highest serum dilution with 3+ or 4+ complement fixation/reciprocal of highest antigen dilution with 3+ or 4+ complement fixation.

represented by a faint line, situated close to the immune ascitic fluid well, that did not meet the line of identity formed by the other antigens present. Each immune ascitic fluid for strains TH4-47f and 65V-78 formed a line of identity with all the antigens present except AR 70 antigen. No reactions were visible with AR 70 antigen.

Ten additional strains were compared to strains AR 70 and 61-7484 with 14-day sera. Eight were studied in CF tests (Table 5) and nine in NT tests (Table 6). The homologous neutralization index for sera from six of the nine strains (A9-155L, WX3-1AQ, A-264, AP6-11X, TnM6-27C, and W-18905) was equivalent to that obtained when each serum was tested against 61-7484 virus. None of these sera neutralized AR 70 virus. However, three of the nine strains (NJO-60C, V5-13A, and NCJ5-6AQ) seemed to vary from the other six strains in their reactions with strain 61-7484, but did not vary from the other six strains in their reactions with strain AR 70. Consequently, reciprocal-cross NT tests were performed among these three strains. The homologous neutralizing antibody titer of NCJ5-6AQ was too low to permit evaluation of its reactivity with AR 70, 61-7484, and the other two viruses. However, the 61-7484 serum did not react as much with NCJ5-6AQ virus as it did with the other viruses. V5-13A serum neutralized >2.9 log of V5-13A virus and >2.7 log of NJO-60C virus, but failed to significantly neutralize NCJ5-6AQ, 61-7484, or AR 70 viruses. NJO-60C serum neutralized 2.9 log of NJO-60C virus but failed to significantly neutralize AR 70, 61-7484, NCJ5-6AQ, or V5-13A viruses.

TABLE 3. *Complement fixation test results of comparisons among six strains with 14-day sera*

Antigen	Sera					
	AR 70	61-7484	TH4-47f	65V-78	AR 714	F1-210A
AR 70	*32*[a]	16	8	8	16	8
61-7484	8	*64*	32	32	32	32
TH4-47f	8	32	*32*	32	32	32
65V-78	8	64	32	*32*	64	32
AR 714	8	64	32	32	*64*	32
F1-210A	8	64	32	32	64	*32*

[a] Reciprocal of highest serum dilution with 3+ or 4+ complement fixation. Italicized numbers = homologous reactions.

DISCUSSION

CF and NT tests are known to vary in their sensitivity in demonstrating antigenic differences among closely related viruses. The method of preparation of immune reagents used in these tests also influences the degree of cross-reactivity observed between related viruses. In this study, 14-day sera were found to be specific towards the immunizing strain and possess an adequate titer to permit use in CF and NT tests.

Results from CF, NT and double-diffusion tests all indicate that strain AR 70 of HP virus and strain 61-7484 of Flanders virus, although antigenically related, are not identical to each other. This relationship is the same as that described by Whitney and Roz (15) and Kokernot et al. (7). The degree of cross-reactivity between the two strains varied within CF and NT tests, depending on the method used to prepare the immune reagents. Antigenic differences between the two strains were demonstrated in NT tests with 14-day sera and in CF tests with either the 14-day sera or the 28-day sera. Differentiation between the two strains was not possible in NT tests with 28-day sera, demonstrating the close antigenic relationship of these two strains. The two strains were also clearly differentiated from each other in Ouchterlony-double-diffusion tests.

Five of the six strains compared in reciprocal-cross comparisons could not be distinguished from each other by any of the three techniques employed. These five strains were 61-7484, TH4-47f, 65V-78, AR 714, and F1-210A. In addition, all but strain F1-210A were readily distinguished

TABLE 4. *Neutralization test results of comparisons among six strains with 14-day sera*

Virus	Sera					
	AR 70	61-7484	TH4-47f	65V-78	AR 714	F1-210A
AR 70	*2.6*[a]	1.8	0.8	1.2	2.1	1.5
61-7484	<0.2	*>3.7*	>3.3	>3.7	>3.7	2.7
TH4-47f	<0.2	>3.3	*>3.3*	>3.3	>3.3	2.9
65V-78	0.7	>3.2	3.0	*>3.2*	>3.7	2.6
AR 714	<0.6	>3.6	2.9	>3.6	*>3.6*	2.0

[a] Neutralization index $\log_{10}$. Italicized numbers = homologous reactions.

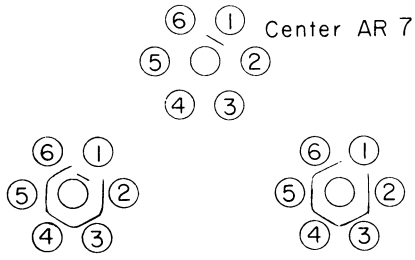

FIG. 1. *Ouchterlony double-diffusion showing the reaction between three immune ascitic fluids and the following antigens: AR 70 (well 1), 61-7484 (well 2), TH4-47f (well 3), 65V-78 (well 4), AR 714 (well 5), and F1-210A (well 6).*

from strain AR 70 in each of the three techniques. Strain F1-210A was distinct from strain AR 70 in CF and double-diffusion tests. All five strains demonstrated cross-reactivity with strain AR 70 in CF tests, yet in all cases a fourfold difference existed between the homologous reactions and the reactions with AR 70 antigen or serum. In the NT tests, 61-7484 and AR 714 sera demonstrated neutralizing activity against AR 70 virus (Table 4). In each case, however, the homologous titer for these two strains was significantly greater than that obtained with AR 70 virus. Such cross-reactivity might be expected among such closely related strains.

Seven of the 10 additional strains studied were found to be closely related if not identical to

TABLE 5. *Complement fixation test results of comparisons between eight virus strains and strains AR 70 and 61-7484 with 14-day sera*

Virus	Sera									
	AR 70	61-7484	V5-13A	A9-155L	WX3-1AQ	A-264	AP6-11X	TnM6-27C	W-18905	TCC7-24A
AR 70	*16*[a]	0	0	0	0	8	0	8	0	0
61-7484	0[b]	*32*	16	32	16	32	32	32	16	16
V5-13A	0	16	*16*							
A9-155L	0	64		*32*						
WX3-1AQ	0	32			*16*					
A-264	0	16				*32*				
AP6-11X	0	32					*32*			
TnM6-27C	0	16						*32*		
W-18905	0	32							*16*	
TCC7-24A	0	32								*16*

[a] Reciprocal of highest serum dilution with 3+ or 4+ complement fixation. Italicized numbers = homologous reactions.

[b] No positive reaction observed at lowest serum dilution of 1:8.

TABLE 6. *Neutralization test results comparing strains AR 70 and 61-7484 with nine other strains using 14-day sera*

Virus	Sera										
	AR 70	61-7484	NJO-60C	V5-13A	NCJ5-6AQ	A9-155L	WX3-1AQ	A-264	AP6-11X	TnM6-27C	W-18905
AR 70	>*3.7*[a]	1.2	<0.2	<0.2	<0.7	0.5	<0.2	1.0	1.1	0.7	1.2
61-7484	<0.2	>*4.4*	1.0 (0.8)[b]	1.5 (1.2)	1.1 (1.1)	2.5	2.3	>3.7	3.7	3.7	>3.7
NJO-60C	1.4	>3.9	*2.9* (2.5)	>2.7	1.7						
V5-13A	1.2	>2.7	1.5	>*2.7* (2.9)	>2.9						
NCJ5-6AQ	1.1	1.9	1.1	1.2	*1.7* (1.1)						
A9-155L	0.2	>3.6				*2.3*					
WX3-1AQ	0.5	>3.3					*2.5*				
A-264	<0.7	3.3						>*3.7*			
AP6-11X	1.6	3.8							*4.5*		
TnM6-27C	1.0	>3.5								>*4.0*	
W-18905	1.0	2.6									*2.4*

[a] Neutralization index $\log_{10}$. Italicized numbers = homologous reactions.

[b] Numbers in parentheses, Results of additional reciprocal-cross tests.

strain 61-7484. These were TCC7-24A, A9-155L, WX3-1AQ, A-264, AP6-11X, TnM6-27C, and W-18905. The other three strains (NJO-60C, V5-13A, and NCJ5-6AQ) isolated in New Jersey, Virginia, and North Carolina, respectively, appeared to vary from strain 61-7484 in NT tests but did not show any increased activity with strain AR 70. Of these three strains, only V5-13A was tested in CF tests, and it could not be distinguished from strain 61-7484. Strains NJO-60C and NCJ5-6AQ may be antigenic variants of Flanders virus. This must be confirmed by additional testing.

Casals (1) described a virus-type as a cluster of different individualities grouped around and resembling a prototype or model. This study suggests that there is a wide distribution within the United States of virus strains that are closely related if not identical to Flanders virus. All 14 strains that were compared to the two prototype strains appear to be members of a virus-type represented by the prototype strain of Flanders virus. Although certain variations may exist within this type, Flanders virus appears to represent an antigenically homogeneous virus-type with little if any variation associated with geographical distribution. HP virus, although related to this type, was found to differ antigenically from all other strains studied.

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