Further studies on the serological relationships of group C arthropod-borne viruses and the application of these relationships to rapid identification of types*

Shope, R. E. Causey, O. R.

Previous studies have established the serological relationships of six types in group C of the arthropod-borne viruses. Using hemagglutination-inhibition (HI) and neutralization (NT) testing, Casals and Whitman[1] distinguished five prototype viruses: Oriboca, Murutucu, Marituba, Apeu and Caraparu. They showed that Murutucu and Marituba viruses, on the one hand, and Apeu and Caraparu viruses, on the other, were closely allied antigenically, and that these two subgroups were easily separable from each other and from Oriboca virus. Shope et all.[2] distinguished a 6th group C virus, Itaqui, which by HI testing was closely allied to Oriboca.

Using the complement-fixation (CF) test, Casals and Whitman found that four of the prototypes – Oriboca, Murutucu, Marituba and Apeu – showed broad cross-reactions, a fact which led them to state that "the CF test added a great deal of evidence to bring out the group relationships, but proved unsatisfactory as a means of specific identification"[1]. Only Caraparu (An 3994) failed to show this CF cross-reaction, although another Caraparu strain, H 5546, cross-reacted strongly with the other prototypes by CF and did not react with

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An 3994. In their investigations, Shope et all.[2] found that Itaqui shared a common CF antigen with Caraparu An 3994 and likewise failed to cross-react significantly with the other prototypes.

These studies, based on experience with the prototypes and few other strains, provoked several questions:

a) is the antigenic composition of group C virus uniform, or are new natural variants continually appearing?

b) is the CF antigen a group antigen shared by most of the agents or a type-related antigen with some degree of specificity?

c) what is the serological relationship of H 5546 to the other group C types?

In an effort to answer these questions, HI and CF studies were undertaken at the Belém Virus Laboratory on 205 group C strains that had been isolated between 1954 and mid 1959. The present paper reports the results obtained and describes a method derived from them for the rapid identification of new strains in this group.

MATERIALS AND METHODS

Viruses

The six prototype group C strains used were Oriboca (An 17), Murutucu (An 974), Marituba (An 15), Apeu (An 848), Caraparu (An 3994) and Itaqui (An 12797)

Of the 205 test strains, 13 had been isolated from workers at a rubber plantation near Belém during the acute phase of a febrile illness; the remainder were obtained from sentinel and wild animals and mosquitoes[3]. The group classification of all 205 was based on the results of CF tests using mouse brain and liver antigens in reaction with hyperimmune serum made against several types in group C[3].

Sera

Immune sera against the six prototype strains were produced in adult mice, which were given one injection subcutaneously of a 10% suspension of infected suckling mouse brain and were bled one to three weeks later. In general, sera satisfactory for HI and CF identification could be obtained one week after vaccination, although higher titers were found in later bleedings. Antisera for the test strains were produced in adult mice with use of a single subcutaneous injection of lyophilized virus reconstituted at 1:80.

In addition, paired acute-phase and convalescent sera were available from ten of the 13 rubber workers from whom virus had been isolated. These ten patients were considered to have suffered a primary infection with group C virus since in each instance the acute-phase serum was free of antibody.

HI Tests

HI testing was done in plastic plates according to methods described by Clarke and Casals[4]. Hemagglutinins were made by acetone extraction of suckling mouse sera. Tests were performed with a constant amount of antigen, either four or eight units, against serial two fold dilutions of serum starting at 1:10. To facilitate comparison of results, those from tests done with four units have been corrected to eight units on the assumption that a reciprocal relationship exists between antigen dilution and serum dilution; thus, a serum inhibiting four units of antigen at the 1:320 dilution is recorded as 1:160.

CF Tests

CF testing was done by a microtechnique modified from Fulton and Dumbell[5], using two units of complement and primary incubation overnight at 4°C. Prototype antigens were prepared by sucrose-acetone extraction of suckling mouse liver[4] that had previously been homogenized in a blender and centrifuged at 8,000 rpm for one hour at 4°C. All other antigens were 10% suspensions of liver or brain

in veronal buffered saline, centrifuged at 2,500rpm for 15 minutes. The dilutions of antigen selected for testing contained approximately four units of antigen; when less than four units were present, results were inconsistent and the test was repeated. The initial serum dilution was 1:4.

Table 1 – Hemagglutination-inhibition tests of group C prototype and test strains against prototype antigens

(continua)

Serum	Antigen					
	Oriboca	Itaqui	Murutucu	Marituba	Caraparu	Apeu
Oriboca						
Prototype (An 17)	320*	10	0	0	0	0
An 913	320+	40	0	0	0	10
An 9216	320	20	0	0	0	0
H 9240	320+	40	0	0	0	0
An 10991	160	40	0	0	0	20
An 13867	40	0	0	0	0	0
Itaqui						
Prototype (An 12797)	0	320+	0	0	0	0
An 4538	20	160	0	10	0	0
An 8629	0	80+	0	0	0	0
An 8920	80	320+	0	0	0	0
An 10870	80	320+	0	0	0	0
An 11127	80	320+	0	0	0	20
Murutucu						
Prototype (An 974)	0	10	160	40	20	20
An 191	0	0	80	10	0	10
An 4970	0	10	320+	80	0	10
An 6064	0	0	160	80	20	20
An 7023	0	0	80	40	20	20
An 10702	0	0	160	80	20	20
Marituba						
Prototype (An 15)	0	0	40	640+	20	0
An 5591	0	0	80	320+	20	0
An 6010	0	0	40	160	40	20
An 7243	0	0	10	80	0	0

* Reciprocal of serum dilution giving complete inhibition of agglutination of eight units of antigen.

+ = endpoint not reached.

0 = negative at 1:10 dilution.

Table 1 – Hemagglutination-inhibition tests of group C prototype and test strains against prototype antigens

(conclusão)

Serum	Antigen					
	Oriboca	Itaqui	Murutucu	Marituba	Caraparu	Apeu
An 7639	0	0	20	320	10	10
An 12293	0	0	20	320+	40	40
Caraparu						
Prototype (An 3994)	0	10	10	40	1280	320
An 931	0	0	10	20	1280	320
An 4901	0	0	0	0	320+	160
An 7880	0	0	0	20	320+	160
An 9133	0	0	0	20	640	160
An 10816	0	0	0	0	320+	160
Apeu						
Prototype (An 848)	0	0	0	0	320	2560
An 4604	0	0	0	0	40	320+
An 4746	0	0	0	0	20	320+
An 5856	0	0	20	0	80	320+
An 8966	0	0	0	0	20	160
An 9126	0	0	0	0	20	320+

+ = endpoint not reached.
0 = negative at 1:10 dilution.

HI and CF Tests

The HI relationships of the test strains were first explored, antiserum for each strain being tested against each of the six prototype HA antigens. Five strains, isolated from a single mouse group, failed to conform to any of the prototype patterns and were set aside for future study as a possible new type. Antisera for the remaining 200, however, inhibited hemagglutination of prototype antigens according to one of the six prototype patterns previously established in HI and NT tests[1,2]. It was thus verified, on a larger scale, that Oriboca shares cross-reactivity with Itaqui, Murutucu with Marituba and Apeu with Caraparu. The data in Table 1 indicate the similarity of HI reaction patterns among strains of each type.

Table 2 – Hemagglutination-inhibition tests of human acute and convalescent sera against group C prototype antigens

Serum		Antigen						Virus isolated
Nº	Days after reported onset	Oriboca	Itaqui	Murutucu	Marituba	Caraparu	Apeu	
UR 145	1	0	0	0	0	0	0	Marituba
	121	0	0	0	10	0	0	
UR 385	1	0	0	0	0	0	0	Apeu
	66	0	0	10	10	80	160	
UR 388	4	0	0	0	0	0	0	Apeu
	67	80	20	20	40	320+	320+	
UR 394	2	0	0	0	0	0	0	Oriboca
	42	80	0	0	0	0	0	
UR 406	2	0	0	0	0	0	0	Apeu
	134	160	40	40	40	320+	320+	
UR 411	2	0	0	0	0	0	0	Caraparu
	26	0	0	0	0	160	20	
UR 413	2	0	0	0	0	0	0	Oriboca
	56	80	0	0	0	0	0	
UR 425	2	0	0	0	0	0	0	Caraparu
	55	0	0	0	0	20	10	
UR 465	2	0	0	0	0	0	0	Caraparu
	23	0	0	0	0	10	0	
UR 506	5	0	0	0	0	0	0	Caraparu
	40	0	0	0	0	20	0	

As shown in Table 2, qualitatively similar results were obtained in HI tests with the ten pairs of human sera. The very broad cross-reaction shown by two convalescent sera (UR 388 and UR 406) was perhaps caused by a double infection. The characteristic HI reaction pattern of each prototype against sera of a given species may be extremely useful in making a serological diagnosis of an illness or in survey studies to determine which viruses are active in an area.

The relative uniformity of HI reactions among the 200 test strain antisera having been established, reciprocal cross CF testing was

undertaken in which the homologous test antigen and serum were reacted against each prototype antigen and serum. With one exception, H 5546 (to be discussed later), the test strains again reacted in the manner of their prototypes. Examples of CF reaction patterns for each type are given in Table 3. In tests with human sera, three pairs gave no CF response, but with the other seven pairs the reactions were qualitatively comparable to those of the mouse sera (Table 4)

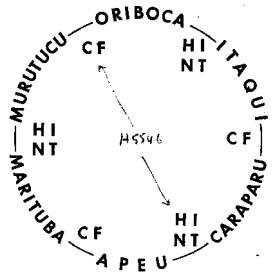

Fig. 1 – The serological relationships of group C virus types.

These CF studies bring out a phenomenon not previously emphasized, i.e., that within the limits of the CF technique used Apeu strains are indistinguishable from those of Marituba, Murutucu strains from those of Oriboca, and Itaqui strains from those of Caraparu. Casals and Whitman used the terms “group” and “common” antigen in describing the CF cross-reactions, although noting that Oriboca and Murutucu, on the one hand, and Marituba and Apeu, on the other, were closer to one another than to the other members of the group. In view of the consistency of the reactions among 199 test strains, it would seem more precise now to consider that Apeu and Marituba share a common CF antigen, as do Oriboca and Murutucu, and that the two antigens are different. (It is possible that soluble CF antigens are involved and that by use of another technique a type specific antigen may be found)

Table 3 – Reciprocal cross complement-fixation tests with group C prototype and test strains

(continua)

Strains identified by HI as Oriboca

Antigen	Serum										
	Oriboca	Murutucu	Marituba	Apeu	Caraparu	Itaqui	An 913	An 9216	H 9240	An 10991	An 13867
Oriboca	16*	64	0	4	0	4	16	32	64	64+	32
Murutucu	16						8	16	16	32	16
Marituba	4						4	8	16	16	8
Apeu	0						4	8	8	16	8
Caraparu	0						0	0	0	0	0
Itaqui	4						0	0	0	0	0
An 913	16	64	4	4	0	0	16				
An 9216	32	32	4	0	0	0		32			
H 9240	32	64+	4	4	0	4			64+		
An 10991	32	64+	8	0	4	4				64+	
An 13867	32	64+	4	0	0	0					64+

Strains identified by HI as Murutucu

Antigen	Serum										
	Oriboca	Murutucu	Marituba	Apeu	Caraparu	Itaqui	An 191	An 4970	An 6064	An 7023	An 10702
Oriboca		64					8	32	64	64+	64+
Murutucu	16	32	0	0	0	0	8	16	32	32	32
Marituba		16					0	8	8	16	32
Apeu		16					4	16	16	16	16
Caraparu		0					0	0	0	0	0
Itaqui		0					0	0	0	0	4
An 191	16	32	0	0	4	0	16				
An 4970	16	32	0	0	4	0		64			
An 6064	16	32	0	0	4	0			64		
An 7023	16	32	4	0	4	0				32	
An 10702	16	64+	4	4	0	0					64+

* Reciprocal of the serum dilution giving 3-4+ fixation of complement in the presence of four units of antigen. 0 = less than four.

Table 3 – Reciprocal cross complement-fixation tests with group C prototype and test strains

(continua)

Strains identified by HI as Marituba											
Antigen	Serum										
	Oriboca	Murutucu	Marituba	Apeu	Caraparu	Itaqui	An 5591	An 6010	An 7243	An 7639	An 12293
Oriboca			0				8	16	8	16	16
Murutucu			0				4	4	4	8	4
Marituba	4	16	8	8	0	0	32	16	32	32	32
Apeu			8				32	16	32	32	32
Caraparu			0				0	0	0	0	0
Itaqui			0				0	0	0	0	0
An 5591	8	4	32	32	0	0	64				
An 6010	8	32	16	16	8	0		32			
An 7243	8	16	16	8	4	0			64+		
An 7639	8	16	8	8	4	0				64	
An 12293	8	16	8	16	4	0					32

Strains identified by HI as Apeu											
Antigen	Serum										
	Oriboca	Murutucu	Marituba	Apeu	Caraparu	Itaqui	An 4604	An 4746	An 5856	An 8966	An 9126
Oriboca				4			4	0	4	8	16
Murutucu				0			0	0	0	4	4
Marituba				8			8	8	16	16	32
Apeu	0	16	8	16	0	0	16	16	32	32	64+
Caraparu				0			0	0	0	0	0
Itaqui				0			0	0	0	0	0
An 4604	8	16	8	8	0	0	16				
An 4746	8	16	8	8	4	0		16			
An 5856	4	16	16	16	4	0			32		
An 8966	8	16	8	8	4	0				32	
An 9126	8	16	16	16	8	0					64+

* Reciprocal of the serum dilution giving 3-4+ fixation of complement in the presence of four units of antigen. 0 = less than four.

Table 3 – Reciprocal cross complement-fixation tests with group C prototype and test strains

(conclusão)

Strains identified by HI as Caraparu											
Antigen	Serum										
	Oriboca	Murutucu	Marituba	Apeu	Caraparu	Itaqui	An 931	An 4901	An 7880	An 9133	An 10816
Oriboca					0		0	0	0	0	8
Murutucu					0		0	0	0	0	0
Marituba					0		0	0	0	0	8
Apeu					0		4	4	0	4	0
Caraparu	0	0	0	0	64	64+	16	16	8	64	64+
Itaqui					64+		32	32	32	64	64+
An 931	0	0	0	0	64+	32	64				
An 4901	0	0	0	0	32	64+		32			
An 7880	0	0	0	0	32	64+			64		
An 9133	0	0	0	0	32	64+				64+	
An 10816	0	4	0	0	64+	64+					64+

Strains identified by HI as Itaqui											
Antigen	Serum										
	Oriboca	Murutucu	Marituba	Apeu	Caraparu	Itaqui	An 8629	An 8920	An 11127	An 14214	
Oriboca						4	0	0	0	0	
Murutucu						4	0	0	0	0	
Marituba						0	0	0	0	0	
Apeu						0	4	4	0	0	
Caraparu						64+	16	16	8	32	
Itaqui	4	0	0	0	64+	64+	32	32	32		
An 8629	0	0	0	0	64+	64	64				
An 8920	0	0	0	0	64+	64		32			
An 11127	0	4	0	0	64+	64+			64		
An 14214	0	0	0	0	64+	64+				64+	

* Reciprocal of the serum dilution giving 3-4+ fixation of complement in the presence of four units of antigen. 0 = less than four.

Table 4 – Complement-fixation test with paired human sera and group C prototype strains

Serum		Antigen						Virus isolated
Nº	Days after reported onset	Oriboca	Itaqui	Murutucu	Marituba	Caraparu	Apeu	
UR 385	1	0	4	4	4	4	4	Apeu
	66	0	4	4	8	4	8	
UR 388	4	0	0	0	0	0	0	Apeu
	67	32+	0	32+	32+	0	32+	
UR 394	2	0	4	4	4	4	4	Oriboca*
	42	8	16	8	16	16	16	
UR 406	2	0	0	0	0	0	0	Apeu
	134	16	0	16	32+	0	32+	
UR 411	2	0	0	0	0	0	0	Caraparu
	26	0	4	0	4	8	0	
UR 413	2	0	4	0	4	4	0	Oriboca
	56	32+	4	32+	16	4	16	
UR 465	2	0	0	0	0	0	0	Caraparu
	23	0	4	0	0	4	0	

*Anticomplementary 1:4.

The HI and CF relationships thus established among the six prototypes and 199 test strains link group C viruses in a closed circle in alternate pairs, as illustrated in Figure 1. The one exception, as mentioned above, is H 5546, which must be considered an aberrant strain since although it is closely related to Caraparu An 3994 in HI studies, it fails to conform to the prototype CF pattern. Table 5 shows the results of reciprocal cross CF testing in block titration and cross HI testing. It may be noted here that since the original isolation of H 5546 from man, two further strains have been isolated from sentinel mouse groups in the Belém area.

Table 5 – Serological relationships of H 5546 strain of Caraparu

Hemagglutination-inhibition testing							
Serum	Antigen						
	H 5546	Caraparu	Apeu	Oriboca	Itaqui	Murutucu	Marituba
H 5546	640	160	20	0	0	0	0
CR 37*	160	80	10				
Caraparu	2560	5120	640	0	10	10	40
Apeu	320	640	2560	0	0	0	0

Complement-fixation testing							
Serum	Antigen						
	H 5546	Oriboca	Murutucu	Caraparu	Itaqui	Marituba	Apeu
H 5546	16/64**	16/128	16/32	0/0	0/0	4/256	4/256
Oriboca	32/128	32/64					
Murutucu	32/128		32/32				
Caraparu	0/0			16/64			
Itaqui	0/0				32/128		
Marituba	0/0					8/256	
Apeu	0/0						16/128

* Convalescent serum of patient from whom H 5546 virus was isolated.
** Serum titer/antigen titer.

IDENTIFICATION PROCEDURE

On the basis of the serological relationships described above, the following method has been devised for rapid identification of new isolates belonging to group C.

Three immune sera, consisting either of an Oriboca-Murutucu pool, a Marituba-Apeu pool and a Caraparu-Itaqui pool, or of one serum from each pair, are standardized according to their CF reaction with the prototype antigens. Antigen from the new isolate is then reacted with the three sera or serum pools to determine in which of the CF subgroups it belongs. Since low passage mouse liver or brain consistently yields potent CF antigen in group C, preliminary identification can thus be made within 48 hours of the time a sentinel mouse acquires infection from forest vectors. When CF antigen is harvested, adult mice are inoculated and bled ten days later for immune sera to be used in HI testing against the two antigens of the CF-positive

pair to obtain a type diagnosis. For example, if the antigen from the new isolate reacts by CF with the Caraparu-Itaqui serum pool, the antiserum for the isolate is tested by HI with Caraparu and Itaqui antigens. Since there is no common HI antigen between Caraparu and Itaqui, the type identification of the isolate is clear-cut.

This identification procedure has been successfully applied in the Belém Laboratory to 170 group C strains isolated in the latter half of 1959 and to all of 406 isolates obtained in 1960. That it is also useful in the determination of exceptions or new varieties has been demonstrated in the case of the two new strains of H 5546 mentioned above. It must be remembered, however, that diagnoses obtained by this method are presumptive and that definitive identification requires the more complex procedure of reciprocal cross HI testing with antigen and serum of the new strain and each of the prototypes.

SUMMARY

In studies by HI and CF tests of the serological reactions of 200 strains of group C virus, 199 strains reacted similarly to one of the six prototype viruses; examples are given of HI and CF reaction patterns for each type. The one exception, strain H 5546, reacted by HI like Caraparu and by CF like Oriboca and Murutucu.

These serological relationships have suggested a method of combined CF and HI techniques for the rapid identification of new group C isolates. Since 1959, 576 isolates have been identified to type by this method.

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