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ISOLATION OF TWO ANTIGENICALLY DISTINCT ARTHROPOD-BORNE VIRUSES OF GROUP C IN PANAMA*

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In the present report the isolation of two arthropod-borne viruses both belonging to group C is described. The two viruses are antigenically different one from the other but are more closely related to Caraparu than to any other member of group C so far studied. These agents are of interest because they are the first isolates of group C viruses from Central America and because both were recovered from human blood. The infections from which these agents were obtained show a number of similarities. Both were contracted in the early part of the year 1961 in a tropical rain forest area of the Province of Bocas del Toro, near the town of Almirante. Both persons affected were men employed to capture blood-sucking insects during the course of investigation into the ecology of arboviruses in the area previously mentioned. Both suffered moderately severe self-limited febrile illnesses which required hospitalization, and virus was recovered from each by the injection of suckling mice with blood drawn during the early acute febrile phase.

The first isolation was made from Ossa (BT 1820), a 56-year-old man, who began work collecting hematophagous insects in the upper canopy of the study area on January 5, 1961. His illness began on January 18, fixing the period of incubation at no more than 13 days. Onset was sudden with fever, chills, malaise and severe headache. He was hospitalized on January 19. Physical findings were essentially negative. The white blood cell count was 8,000 with a differential count of 62% neutrophils, 37% lymphocytes and 1% eosinophils. No malaria parasites were observed. The fever was diphasic, a temperature of 101.8°F being registered on admission, which soon dropped to normal levels and then rose again on January 21 reaching a maximum of 102.5°F in the second hump of the "saddleback." Recovery was uneventful and he was dismissed from the hospital on January 25.

The second isolation was made from Madrid (BT 4075), a 36-year-old man, who became acutely ill on March 2, 1961, approximately two months after beginning work as a mosquito collector. The principal symptoms were chills, fever, severe headache, prostration and pain in the right upper quadrant. He was hospitalized on March 3, when a temperature of 101°F was recorded. Smears for malaria were negative. Except for a slight pharyngitis there were no significant physical findings. The fever declined rapidly and he was dismissed from the hospital on March 5, three days after onset.

The acute-phase bloods were forwarded on ice from Almirante to Panama by airplane. Serum of each was injected into two litters of suckling mice, one litter by the combined intracerebral and subcutaneous route and the other by the combined intracerebral and intraperitoneal route. Both viruses were reisolated in suckling mice by the intracerebral injection of serum stored in sealed glass ampules in a dry-ice chest. Ossa virus was also reisolated by the intracerebral injection of two hamsters. Ossa convalescent serum showed a neutralization index of more than 3.5 logs as against 0 for acute-phase serum and Madrid convalescent serum of more than 4.6 as against 0 for acute-phase serum.

ANIMAL PATHOGENICITY

Ossa BT1820. In suckling mice 2 to 3 days old, injected intracerebrally with 10% mouse brain or liver suspensions, the incubation period is from 24 to 36 hours. Death follows soon after symptoms appear, the average survival time (AST) being about 2 days. Titer in infant mouse brain has varied from $10^{-3.2}$ to $10^{-5.3}$. In infant mouse serum higher titers, $10^{-7.0}$ and $10^{-7.4}$, are obtained. The incubation period and AST are essentially the same after intraperitoneal or

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subcutaneous injection as after intracerebral injection. Results in weanling mice have been very variable. After intracerebral injection the incubation period has averaged 4 days (shortest 2 days and longest 5) and the AST has been 5.1 days, 16% to 83% of the animals in the different groups being noticeably affected. Hamsters are very susceptible. Two were sacrificed when prostrate 2 days after intracerebral injection with 0.03 ml of the original acute-phase serum and two, subinoculated with a 10% emulsion of the brains of these, died in 3 days. Two guinea pigs inoculated intracerebrally with acute-phase serum remained asymptomatic but produced antibodies.

Madrid (BT4075). The incubation period in 2- to 3-day-old mice injected intracerebrally with 10% mouse brain suspensions is 2 to 4 days, usually 2. The AST is 2.9 days. After intraperitoneal or subcutaneous inoculation the average incubation period is 3.4 and 3.7 days, respectively. Titer in infant mouse brain has been from $10^{-4.3}$ to $10^{-5.6}$ and in infant mouse serum, $10^{-6.0}$ to $10^{-6.2}$. In weanling mice, as with Ossa virus, results are variable. The incubation period shows an average of 3.6 days and the AST is 4.2 days (limits 2 and 7 days). About 80% of the weanlings inoculated intracerebrally have shown symptoms. Symptoms are variable but paralyses are not manifest with this or Ossa virus. There seems little doubt that these viruses could be adapted successfully to adult mice. Of two hamsters injected intracerebrally with 0.03 ml of a 10% mouse brain suspension, one died at the end of a 2-day period and the other in 3 days. Two guinea pigs inoculated intracerebrally with 0.1 ml of a 10% suspension of infected hamster brain remained well.

SEROLOGICAL RELATIONSHIPS

A neutralization test was run using Madrid human convalescent serum, Ossa guinea pig hyperimmune serum and Madrid and Ossa viruses in suckling mouse brain suspension with the following results:

Antiserum	Virus (logs neutralized)	
	Madrid	Ossa
Madrid human convalescent.....	4.6	2.0
Ossa hyperimmune guinea pig....	0	3.2

We do not know whether the neutralization of Ossa virus by Madrid convalescent serum is the result of the antigenic relationship between the two viruses or the result of a previous infection with this or a related virus. As Madrid had been engaged in collecting hematophagous insects in the study area for two months prior to the onset of the illness, it is quite possible that he may have suffered a previous subclinical infection with Ossa virus. Sufficient convalescent Ossa serum was not available for a similar cross-neutralization test.

Neither virus was found to correspond by neutralization or complement-fixation testing to yellow fever, St. Louis, Ilhéus, Una, EEE, Vesicular stomatitis, Indiana type, Wyeomyia, Guama, Guaroa, Melao or Changuinola Valley virus.

Madrid and Ossa hyperimmune mouse sera were sent to the Belém Virus Laboratory in Brazil where complement-fixation tests were negative for eastern equine encephalitis, Mucambo, Mayaro, Una, and Aura viruses in group A; yellow fever, Ilhéus, St. Louis, and Bussuquara in group B; Cache Valley, Kairi, and Wyeomyia in the Bunyamwera group; Melao in the California complex; Guama, Catu, and BE AN20525 in the Guama group, Oropouche, BE AN8582, BE AN24232, BE AN27639, BE AN27326, BE H22511, and BE AR32149. A relationship was established however with viruses of group C by both complement-fixation and hemagglutination-inhibition studies.

Definitive serological identification studies were undertaken by hemagglutination-inhibition testing, complement-fixation, and neutralization testing. Hemagglutination-inhibition was carried out by the techniques of Clarke and Casals,[5] using antigens made from acetone-extracted baby mouse sera. Nonspecific inhibitors of immune serum were removed by Kaolin. Goose cells were added at a final pH of 6.0 at room temperature. Complement-fixation was done in plastic trays using a microtechnique, with two units of complement and overnight primary incubation at 4°C. Grid titrations were carried out. Neutralization testing was done in baby mice by the intracerebral route. Infected baby mouse serum was used as virus source. The serum-virus mixtures were incubated for one hour prior to inoculation. A constant serum, varying virus dilution technique was employed.

The most extensive studies were done by

TABLE 1

Hemagglutination-inhibition testing of Madrid and Ossa with group C viruses

Sera*	Antigens								
	Madrid	Ossa	Caraparu	Apeu	Marituba	Murutucu	Itaqui	Oriboca	Nepuyo
Madrid BT4075	*640*†	80	160	80	10	20	0	0	20
Ossa BT1820	80	*1280*	160	640	40	80	0	0	20
Caraparu BE AN3994	40	160	*320*	160					
Apeu BE AN848	40	80	160	*640*					
Marituba BE AN15	160	—			*>320*				
Murutucu BE AN974	0	—				*160*			
Itaqui BE AN12797	0	—					*80*		
Oriboca BE AN17	0	—						*320*	
Nepuyo BE AN10709	0	—							*>320*

* Sera from mice receiving one injection of live virus, or in some cases, two injections of formol-inactivated virus followed by live virus. Ossa serum is hyperimmune.

† Reciprocal of serum dilution completely inhibiting 8 units of antigen.

TABLE 2

Comparison by hemagglutination-inhibition testing of five Caraparu strains with Madrid and Ossa

Sera*	Antigens						
	Madrid	Ossa	Caraparu				
			1	2	3	4	5
Madrid	*80*†	0	0	0	0	0	0
Ossa	0	*40*	0	0	0	0	10
Caraparu 1	0	0	*80*	20	20	20	40
2	0	0	0	*10*	10	10	20
3	0	10	40	40	*40*	40	80
4	10	40	80	80	80	*160*	320
5	0	40	40	80	80	80	*160*

* Mice received two formalin-inactivated inoculations followed by live virus intraperitoneally and were bled 12 days after the live inoculation.

† Reciprocal of serum dilution inhibiting equivalent of 8 antigen units.

hemagglutination-inhibition as it was this technique that Casals and Whitman[3] originally used as a basis for classification in group C. Both Madrid and Ossa viruses cross-reacted with Caraparu and Apeu viruses, which had been described by Casals and Whitman[3] as forming a complex within group C. Table 1 shows results chosen from several hemagglutination-inhibition tests to illustrate the closeness of the relationship of both Madrid and Ossa to Caraparu virus using mouse immune sera. It is evident that the Madrid and Ossa strains are not the same and differ from each other to about the extent that they differ from Caraparu.

To establish the amount of variation among strains to be expected in the hemagglutination-inhibition test, five Caraparu strains, isolated from humans, arthropods, and sentinel animals near Belém, Brazil, were compared with each other and with Madrid and Ossa. The tester did not know the identity of the strains until after the preparation of immune sera and testing were completed. The results of hemagglutination-inhibition testing (Table 2) established a degree

of confidence that the observed differences between the Panamanian and Brazilian viruses represent real differences and not technical variations. The Caraparu strains isolated in Brazil were relatively uniform in reactivity and all were distinguished from Ossa and from Madrid.

Neutralization testing (Table 3) confirmed the close relationship of Madrid and Ossa to Caraparu, and indicated that the neutralization technique may separate the viruses also.

Complement-fixation testing (Table 4) confirmed the close relationship of Madrid and Ossa to Caraparu virus.

DISCUSSION

Causey *et al.*[1] in 1961 described the first isolations of group C viruses. More than 200 strains obtained from man, sentinel and wild animals and mosquitoes were classified in 5 entities by hemagglutination-inhibition and neutralization tests: Oriboca, Marituba, Murutucu, Caraparu, and Apeu. Later two additional members of the group were defined: Itaqui,[2] and Nepuyo. Casals and Whitman[3] in 1961 and Shope and Causey[4] in 1962 established the antigenic interrelationships between members of the group and the serological criteria for their identification. To our knowledge group C viruses have been recovered only in Brazil and Trinidad prior to our isolations in Panama.

These viruses belong to the Caraparu-Apeu complex of group C, and are most closely related to Caraparu. The differences among Ossa, Madrid, and prototype Caraparu are sufficient by hemagglutination-inhibition testing that for purposes of serological survey, diagnostic, and epidemiologic studies, they should be considered distinct.

TABLE 3

Neutralization testing of Madrid, Ossa, and Caraparu viruses

Guinea pig sera diluted 1:16	Virus (log neutralization index)		
	Caraparu	Madrid	Ossa
Caraparu, 1 injection	*4.6*	<0.6	1.0
Madrid, 2 injections	>5.4	*>4.6*	>3.4
Ossa, 2 injections	2.6	<0.5	*>3.6*

SUMMARY

Isolation of two new group C viruses, for which the names Madrid and Ossa are suggested, is reported. They are immunologically distinct from each other, but both are more closely related to Caraparu than to any other known group C virus. Each was obtained from the blood of a male entomological worker in a tropical rain forest in the Province of Bocas del Toro, R.P., suffering from a self-limited acute febrile illness. These are the first reported isolations of group C viruses from Central America.

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TABLE 4

Complement-fixation testing of Madrid and Ossa with group C viruses

Hyperimmune mouse sera	Antigens (liver, sucrose-acetone extracted)								
	Madrid	Ossa	Caraparu	Itaqui	Marituba	Apeu	Oriboca	Murutucu	Nepuyo
Madrid	*>32**	>32	32	32	0	4	0	0	0
Ossa	>32	*>32*	>32	>32	0	0	0	0	0
Caraparu	8	8	*16*						
Itaqui	32	8		*32*					
Marituba	0	0			*>32*				
Apeu	0	0				*8*			
Oriboca	0	0					*16*		
Murutucu	0	0						*16*	
Nepuyo	0	0							*16*

* Reciprocal of serum dilution giving 3 or 4 plus fixation at optimal antigen dilution.

comparison and characterization of these viruses made possible their identification.

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