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CROSS-NEUTRALIZATION STUDIES WITH GROUP C ARBOVIRUSES*

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Summary. — Eleven group C arboviruses as well as high passage tissue culture preparations of most of the Serogroup C viruses were studied by cross-neutralization testing conducted under specified standardized conditions. None of the viruses showed apparent antigenic changes as a result of high passage in tissue culture. The serological relationships determined here on the basis of two-way neutralization crosses were in general accordance with relationships previously established by hemagglutination inhibition (HI) testing. In our hands, Gumbo Limbo did not exhibit any neutralization relationship with any of the other known group C viruses.

Introduction

Initial studies concerned with serological relationship among group C arboviruses were based primarily on results obtained with the HI test (Casals, 1957; Casals and Whitman, 1961; Shope and Causey, 1962). Neutralization testing has been conducted on a limited basis as an adjunct to the HI testing. There is need for a comprehensive study of the neutralization relationships of group C arboviruses. Such a study will provide serological information which can be used to additionally characterize already established relationships and perhaps clarify certain other relationships.

This report will present results of cross-neutralization testing with 11 group C arboviruses. The neutralization testing was standardized so that all immune ascitic fluids were heat inactivated prior to use, and they were reacted with the test viruses in the absence of fresh normal serum.

Materials and Methods

Virus stocks. The group C viruses used were Apeu (Be An 848), Caraparu (Be An 3994), Gumbo Limbo (FE 371H), Itaqui (Be An 12797), Madrid (BT 4075), Marituba (Be An 15), Mututucu (Be An 974), Nepuyo (Be An 10709), Oriboca (Be An 17), Ossa (BT 1820), Restan (Tr 51144) and Tr 34503-1. All virus stocks were prepared as 10% suspensions of infective liver or brain in 0.75% bovine albumin dissolved in phosphate buffered saline, pH 7.3. The 10% suspensions were clarified by centrifugation at 10,000 rev/min for 30 minutes and were stored wet frozen in sealed ampoules at approximately —65° C.

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Tissue culture. Details concerning the handling of the baby hamster kidney (BHK-21) cell cultures as well as the growth and maintenance of African green monkey kidney (Vero) cell cultures and their use under agar overlay have been reported previously (Karabatsos and Buckley, 1967; Karabatsos, 1969).

Virus passage in tissue culture. The viruses under study were serially passaged every 48 hours in stationary BHK-21 tube cultures. The multiplicity of infection during serial passage was approximately 1.

Immune ascitic fluids. Mouse immune ascitic fluids were obtained from the collection of the WHO International Reference Centre in the Yale Arbovirus Research Unit. Specific immune ascitic fluids were prepared in mice by the following immunization schedules: 4 injections of live virus (Gumbo Limbo); 3 injections of live virus (Oriboca, Apeu); 2 injections of live virus (Marituba, Itaqui, Murutucu, Ossa, Madrid, and Nepuyo); 2 injections of inactivated virus followed by an injection of live virus (Caraparu, Restan); and finally 1 injection of inactivated virus followed by 2 injections of live virus (Tr 34053-1). All immunizing antigens were inoculated intraperitoneally and/or subcutaneously. Sarcoma 180/TG (Sartorelli *et al.*, 1966) was used in all the immunization regimens.

Neutralization testing. Inhibition of plaque formation was taken as an index of neutralization. Immune or normal ascitic fluid was mixed with an equal volume of varying log dilutions of virus, and the ascitic fluid-virus mixtures were incubated at 37° C for 1 hour. Tissue culture bottles were then inoculated, the inoculum adsorbed for 1 hour, and all bottles overlaid. A $\log_{10}$ neutralization index (LNI) of 1.0 or greater, and differences between indices of 1.0 or greater, were considered significant.

In those instances where normal serum was employed in an attempt to enhance neutralization (Morgan, 1945; Whitman, 1947), the control and immune ascitic fluids were heat inactivated at 56° C for 30 minutes and then diluted in the normal serum. This was then mixed in equal volumes with the virus dilutions. Normal serum was freshly obtained and stored at −65° C in aliquots sufficient for one day's testing.

Results and Discussion

Prior to the actual cross-neutralization testing, studies were undertaken to determine whether fresh normal serum was required in the neutralization test. For this purpose, an Oriboca goat antiserum known to be enhanced in its neutralizing activity by the presence of normal serum was chosen as one of the reactants in a model test system. Oriboca virus constituted the other reactant. Freshly obtained normal cat, guinea pig, Rhesus monkey, sheep and human sera were tested for (1) enhancement of neutralization, and (2) for lack of a "direct inactivating" effect on the test virus. Homologous neutralization tests were then performed with each group C virus, using both the liver or brain source and the high passage tissue culture material. The immune ascitic fluid was heat inactivated and tested for its homologous neutralization activity in the presence and absence of normal guinea pig serum. These results indicated that normal guinea pig serum did not significantly enhance neutralization by any of the various group C immune ascitic fluids. Thus, all subsequent neutralization testing was performed with heat inactivated immune ascitic fluids which were not supplemented with any fresh normal serum.

The cross-neutralization results are presented in Table 1. It is important to note that, without exception, both the liver and brain source virus and high passage tissue culture virus were equally neutralized in homologous or heterologous tests. These findings indicate that the tissue culture preparations of the group C viruses did not manifest any apparent antigenic

Table 1. Cross-neutralization of high passage tissue culture and liver or mouse brain source of group C viruses in Vero cell cultures

Viruses		Mouse immune ascitive fluid											
		Oriboca	Marituba	Restan	Murutucu	Caraparu	Itaqui	Ossa	Madrid	Apeu	Tr 34053-1	Nepuyo	Gumbo Limbo
Oriboca	L	4.3	0.2	0.2	0.0	0.5	1.4	0.3	0.1	0.1	0.1	0.1	0.0
	TC	≧3.7	0.1	0.3	0.1	0.5	1.4	0.2	0.0	0.0	0.3	0.0	0.0
Marituba	L	0.1	4.2	0.1	0.7	0.7	0.2	1.2	0.5	0.0	0.5	0.3	0.0
	TC	0.3	*4.0*	0.3	0.8	0.3	0.0	0.5	0.4	0.0	0.5	0.3	0.0
Restan	L	0.0	≧1.6	3.0	≧1.6	0.3	0.0	0.8	0.3	0.2	0.1	0.1	N. T.
	TC	0.2	3.2	*3.1*	3.2	0.8	0.6	1.1	0.5	0.8	0.1	0.0	0.2
Murutucu	L	0.2	2.2	1.5	3.0	0.7	0.1	1.6	0.9	0.3	0.2	0.0	0.4
	TC	0.2	2.7	1.2	*3.2*	0.7	0.4	1.9	1.4	0.8	0.3	0.0	0.5
Caraparu	L	0.1	0.9	0.0	0.4	4.3	0.0	3.5	0.0	2.4	1.5	0.0	0.3
	TC	0.1	1.2	0.2	0.1	*4.6*	0.0	3.3	0.2	3.1	2.0	0.0	0.0
Itaqui	L	1.7	0.0	0.0	0.0	1.3	2.7	0.1	0.0	0.1	0.0	0.0	0.0
	TC	1.9	0.1	0.0	0.1	0.7	*2.8*	0.4	0.5	0.3	0.2	0.1	0.1
Ossa	L	0.1	1.8	0.1	0.6	2.8	0.1	≧3.6	1.1	2.1	3.3	0.3	0.1
	TC	0.2	1.9	0.2	0.6	3.1	0.2	*3.5*	1.0	2.2	3.3	0.3	0.2
Madrid	L	0.4	0.3	0.2	0.5	0.9	0.0	1.7	4.5	0.4	0.2	0.0	0.0
	TC	0.4	0.8	0.3	0.3	0.9	0.2	1.6	*4.5*	0.2	0.3	0.1	0.0
Apeu	L	0.1	0.2	0.1	0.5	1.8	0.0	2.1	1.2	*3.0*	1.8	0.2	0.1
Tr 34053-1	L	0.1	1.7	0.1	0.6	3.0	0.2	2.7	0.8	1.5	4.0	0.1	0.2
	TC	0.0	1.5	0.2	0.2	≧3.5	0.0	≧3.5	1.1	2.1	*4.0*	0.1	0.0
Nepuyo	L	0.3	1.7	0.5	0.9	1.0	0.0	0.4	0.1	0.3	1.5	*3.3*	0.5
Gumbo Limbo	MB	0.3	0.0	0.0	0.1	0.3	0.0	0.3	0.0	0.0	0.0	0.2	*3.5*

L, MB: liver or mouse brain source of virus. TC: virus from 55th passage in BHK-21 cell cultures. N. T. = Not tested.

changes as a result of high passage. It may be considered that these viruses are apparently antigenically stable under the conditions utilized in this study. This is in contrast to findings with certain group A arboviruses which showed antigenic variation during host passage (Henderson *et al.*, 1967*a*, *b*; Henderson, 1968). It is possible that, although BHK-21 is relatively homogeneous with respect to cell type, it is inefficient in or incapable of selecting antigen subpopulations (Henderson *et al.*, 1967*b*) from within a given group C

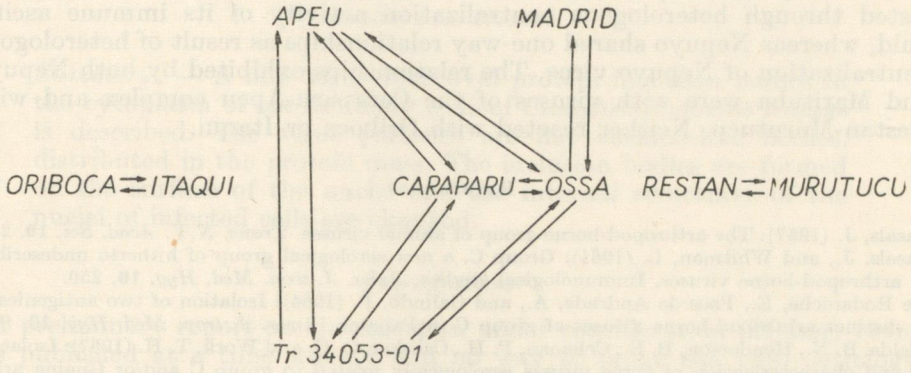

Fig. 1.
Neutralization relationships of group C viruses as determined by significant two-way crossings

virus strain. Thus, it would appear that antigenic variation of arboviruses as a result of host selection processes is probably not common to all arbovirus serogroups. The operation of such a mechanism must be verified by direct experimentation on a serogroup to serogroup basis.

The two-way neutralization relationships determined in our study also indicate a "clustering" of group C viruses. The close relationship both by HI and neutralization testing of Itaqui to Oriboca and Caraparu to Apeu has been noted previously (Casals and Whitman, 1961; Shope *et al.*, 1961; Shope and Causey, 1962). Fig. 1 depicts a diagramatic representation of significant two-way relationships among group C viruses as determined by our neutralization testing. Itaqui and Oriboca are readily distinguished from each other in both directions. Our findings show that Caraparu and Tr 34053-1 are probably not identical and both Tr 34053-1 and Ossa are closely related to Caraparu as well as to one another. Madrid is readily distinguishable from Ossa in both directions while Restan was found to be very closely related to Murutucu. Previous studies have noted that Ossa and Madrid belong in the Caraparu-Apeu complex and that Restan was very close to both Murutucu and Marituba (de Rodaniche *et al.*, 1964; Jonkers *et al.*, 1967). Additional refined antigenic studies may be necessary to resolve the relationships between Caraparu, Tr-34053-1 and Ossa viruses. It is conceivable that the latter two might ultimately be considered as antigenic variants.

Considerable specificity was evidenced under the standardized conditions in which the cross-neutralization testing was conducted. Marituba, Nepuyo and Gumbo Limbo did not exhibit two-way relationships among themselves nor with any of the other group C viruses. Neither Gumbo Limbo virus nor its immune ascitic fluid significantly reacted with any of the other group C reagents. Data obtained by neutralization, complement-fixation and HI testing have previously placed Gumbo Limbo in group C (Fields *et al.*, 1967). One-way neutralization relationships of Marituba were manifested through heterologous neutralization activity of its immune ascitic fluid, whereas Nepuyo shared one-way relationships as result of heterologous neutralization of Nepuyo virus. The relationships exhibited by both Nepuyo and Marituba were with viruses of the Caraparu-Apeu complex and with Restan-Murutucu. Neither reacted with Oriboca or Itaqui.

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