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ISOLATION OF GROUP C ARBOVIRUSES IN PANAMA INCLUDING TWO NEW MEMBERS, PATOIS AND ZEGLA*

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During the first five years of an extensive survey for arboviruses in the Amazon region of Brazil, hundreds of virus strains, including nine new types, were isolated from different sources.[1] Five prototypes of these new agents were found to constitute a distinct antigenic group to which the name group C was given.[2] More than 200 strains in this group were obtained from man, mosquitoes and sentinel and wild animals during this study. Three serological complexes within the group, Marituba-Murutucu, Caraparú-Apeu and Oriboca, were also recognized by hemagglutination-inhibition (HI) and neutralization (N) tests.[2] Caraparú was the most frequently encountered group C agent in that study area. A strain closely resembling the prototype Caraparú virus was also isolated repeatedly in Trinidad.[3] Subsequently, another agent, called Itaqui, was isolated in Brazil and was reported as being closely related to Oriboca virus.[4] Results of further studies on the serological relationships among members of this group of arboviruses were reported by Shope and Causey,[5] who also suggested a method of combined complement-fixation (CF) and HI techniques for the rapid identification of group C isolates. A seventh member of group C, Nepuyo virus,[6] was isolated both in Brazil and Trinidad. Two additional distinct types, Madrid and Ossa, were recovered from blood serum of mosquito collectors in Panama.[7]

The present paper reports the isolation of certain group C arboviruses, including two additional new types, from rodents exposed or captured in a tropical rainforest area near Almirante, Panama during 1961 and 1962.

MATERIALS AND METHODS

Detailed descriptions of the study area, collecting stations and field-collecting methods are presented elsewhere.[8] Virus isolation attempts from field specimens were made in white Swiss mice two to four days old using the combined intracerebral (i.c.) and intraperitoneal (i.p.) routes of inoculation. Brain suspensions from sick mice were then serially passed in new litters of suckling mice by the i.c. route of injection.

Virus isolation attempts and preparation of reagents for reference strains were done in two entirely separate sections of the laboratory by different personnel.

Reference virus strains. Dr. Loring Whitman of the Rockefeller Foundation Virus Laboratories in New York kindly furnished us with early passage material for each of the following group C type strains: Oriboca (BeAn 17), Murutucu (BeAn 974), Itaqui (BeAn 12752), Caraparú (BeAn 3994), Apeu (BeAn 848), Marituba (BeAn 15) and Nepuyo (BeAn 10709), all of which were readily established in our laboratory and were used in this study. Another prototype strain, Madrid (BT 4075), was obtained from Dr. Enid de Rodaniche of the University of Panama.

Reagents for type strains other than group C viruses were also prepared for screening identification procedures. Most of these strains were obtained from the Rockefeller Foundation Virus Laboratories in New York, Belem, and the Communicable Disease Center in Atlanta, Georgia. The remaining reference strains utilized in the study were isolated locally.

Antigens. Infective inocula were prepared from infected suckling mouse brain for Oriboca, Murutucu, Marituba, Itaqui and Apeu viruses; from infected suckling mouse serum for Caraparú virus; and from infected suckling mouse liver, after several serial liver passages, for Nepuyo and Madrid viruses. Inocula for infecting mice with suspected group C isolates were prepared from infected suckling mouse brain or liver.

All hemagglutinating (HA) antigens were prepared from infected suckling mouse serum by acetone extraction, following the method described by Clarke and Casals[9] with slight modifications.

Complement-fixing antigens for type strains as

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well as for some of the test strains were made by sucrose-acetone extraction of infected suckling mouse liver or brain.[9] Occasionally, crude liver antigens were used in the case of test strains.

Immune sera. Immune serum for each of the group C type strains was prepared by the i.p. injection of adult mice with two doses of virus prepared from infected suckling mouse liver, administered 10 days apart. Formalin-inactivated virus was used for the first injection, except in the case of Apeu virus in which both inocula contained live virus. Bleeding for immune serum was performed 10 days after the second injection. Group immune ascitic fluid for group C viruses was kindly supplied by Dr. Robert Shope of the Belem Virus Laboratory.

Immune serum for each of the suspected group C isolates, unless otherwise indicated was obtained from adult mice one to two weeks after a single i.p. inoculation of live virus suspension prepared from infected suckling mouse liver.

Serological tests. Hemagglutination (HA) and HI tests were performed according to the techniques described by Clarke and Casals,[9] the tests being held at room temperature (22° to 24°C) after the addition of goose erythrocytes. All immune sera were treated with kaolin for removal of non-specific inhibitors. HI antibody titers were corrected, when necessary, to those expected with four units of antigen.

CF tests were performed in plastic plates using a modification of the Fulton and Dumbell microtechnique.[10]

Neutralization testing was done in infant mice by the i.c. route of injection, employing a constant-serum, varying-virus dilution technique, and stock virus suspensions prepared from infected suckling mouse brain. Tests were incubated at 37°C for one hour prior to injection in mice. Titration end-points were calculated by the method of Reed and Muench.[11]

RESULTS

Virus isolations. Fifteen virus strains, distributed in five antigenically distinct types of group C, including two hitherto undescribed members of the group, were obtained from rodents. Five of these agents were recovered from the cotton rat (*Sigmodon hispidus*), one from the spiny rat (*Proechimys semispinosus*), and nine from sentinel mice. Multiple isolations of the same virus from a litter of sentinel mice are considered as a single isolation in this report. These isolates were obtained from a total of 273 serum samples of mammals other than man and 436 litters of sentinel mice tested for the presence of arboviruses.

Of the six rodent sera which yielded group C viruses, only one (BT 5012) was available for reisolation attempts, which proved to be successful.

Virus identifications. Brain and liver antigens of new isolates were first tested by CF technique with mouse hyperimmune sera of the following antigenic types of arboviruses: Venezuelan equine encephalitis (VEE), Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), Pixuna, Mayaro, Aura, Una, Guamá, Guaroa, Wyeomyia, Kairi, Cache Valley and California viruses. No fixation of complement was observed in these screening tests. However, positive results were obtained with some of the seven group C typing antisera also used in these preliminary tests.

Attempts were made to prepare HA antigens from infected suckling mouse sera for all of the suspected group C isolates under study. Although difficulties were frequently encountered, many workable HA antigens were obtained, thus making possible the performance of HI tests toward preliminary characterization of these isolates. Inhibition of hemagglutinins of some new isolates was demonstrated in the presence of group C immune ascitic fluid. In cross HI tests employing specific immune sera, test strains were compared with eight group C type strains with the following results:

Caraparú. Four isolates from sentinel mice (BTSM 122B, 147B, 411A and 435A) were found to be closely related to Caraparú virus (Table 1).

TABLE 1

Results of hemagglutination-inhibition tests with Caraparú virus and four isolates from sentinel mice

Antiserum	Antigen				
	Caraparú	BTSM 122B	BTSM 147B	BTSM 411A	BTSM 435A
Caraparú	*640**	160	320	320	320
BTSM 122B	160	*160*			
BTSM 147B	160		*>160*		
BTSM 411A	320			*320*	
BTSM 435A	320				*640*

* Reciprocal of highest serum dilution inhibiting four units of antigen.

TABLE 2

Results of hemagglutination-inhibition tests with Madrid virus and two isolates from sentinel mice

Antiserum	Antigen		
	Madrid	BSTM 116B	BTSM 127A
Madrid	*1280**	1280	
BTSM 116B	1280	*1280*	>320
BTSM 127A	1280	1280	*>320*

* Reciprocal of highest serum dilution inhibiting four units of antigen.

Madrid. Two strains from sentinel mice (BTSM 116B and 127A) showed close antigenic relationships with Madrid virus (Table 2).

Nepuyo. One agent obtained from the spiny rat (BT 4968) proved to be closely related to Nepuyo virus (Table 3).

Undescribed viruses. The remaining eight strains were found to differ from the prototype group C viruses utilized in this study and to belong to two distinct antigenic types. The first type, to be designated Patois virus, includes two isolates from the cotton rat (BT 4971 and 4972) and one isolate from sentinel mice (BTSM 117A). The second type, which will be referred to as Zegla virus, is represented by three isolates from the cotton rat (BT 4975, 5012 and 5015) and two isolates from sentinel mice (BTSM 109A and 389A).

The grouping of these isolates by HI technique is indicated in Table 4. As may be noted, antigens prepared for Patois virus strains reacted at very low titers or not at all with Zegla antisera. Low HI titers were also detected when Zegla antigens were reacted with Patois antisera, showing that Patois and Zegla are distinct antigenic entities. However, the results of CF titrations shown in Table 5 demonstrate a close relationship between these two viral types. Neutralization testing with Patois and Zegla prototype strains confirmed the results of HI tests, demonstrating conclusively that Patois and Zegla viruses are related but distinct (Table 6).

In HI screening tests, Patois and Zegla hemagglutinins were not inhibited by hyperimmune sera or ascitic fluid of the following arbovirus

TABLE 3

Comparison of five antigenically distinct rodent isolates with eight group C type strains by hemagglutination-inhibition test

Antiserum	Antigen											
	Oriboca	Marituba	Murutucu	Itaqui	Apeu	Caraparú	Nepuyo	Madrid	BTSM 116B	BTSM 147B	BT 4971	BT 5012
Oriboca	*320**	0†	0	20	0	0	0	0	0	0	0	0
Marituba	0	*160*	80	0	20	0	40	20	20	0	0	0
Murutucu	0	80	*640*	0	40	40	40	40	40		20	0
Itaqui	0	0	0	*80*	0	0	0	0	0	0	0	0
Apeu	0	0	0	0	*640*	80	0	40	40	20	0	0
Caraparú	0	80	40	0	320	*640*	40	160	80	320	0	0
Nepuyo	0	40	40	0	40	40	*640*	20	20	0	0	0
Madrid	0	20	20	0	0	40	40	*1280*	1280	20	0	0
BTSM 116B	0	40	20	0	80	80	20	1280	*1280*		0	0
BTSM 147B	0	20	0	0	40	160	0	40		*>160*	0	0
BT 4971	40	80	0	20	40	20	0	40		20	*2560*	80
BT 5012	0	0	0	0	0	0	0	0		0	20	*640*
BT 4968‡	0	20	0	0	0	40	640	0	0		0	0
Polyvalent group C immune ascitic fluid	40	320	160	320	640	640	160	20			40	20

* Reciprocal of highest serum dilution inhibiting four units of antigen.

† HI titer less than 1:20.

‡ Sufficient quantities of BT 4968 antigen were not available for inclusion in this series of tests.

TABLE 4

Grouping of Patois and Zegla virus isolates by hemagglutination-inhibition test

Antiserum	Antigen							
	Patois			Zegla				
	BT 4971	BT 4972	BTSM 117A	BT 5012	BT 5015	BT 4975	BTSM 109A	BTSM 389A
Patois								
BT 4971	*2560**	2560	2560	80				80
BT 4972		*1280*	2560		40		40	40
BTSM 117A	320	320	*640*	20	20	40	20	20
Zegla								
BT 5012	20		40	*640*	640		640	320
BT 5015	0†		0	320	*640*	$>$160	320	320
BT 4975	20	0	20	640	640	$>$*160*	1280	320
BTSM 109A	20	0	20	160	320	320	*320*	
BTSM 389A	0	0	0	320	160	320	$>$80	*160*
Polyvalent group C immune ascitic fluid‡	40		40	20	20	20	20	

* Reciprocal of highest serum dilution inhibiting four units of antigen.

† HI titer less than 1:20.

‡ Patois and Zegla viruses were not included in the preparation of polyvalent group C immune ascitic fluid.

TABLE 5

Results of complement-fixation tests with several Patois and Zegla virus isolates

Antiserum	Antigen				
	Patois		Zegla		
	BT 4971	BTSM 117A	BT 5012	BTSM 109A	BT 4975
Patois					
BT 4971	*64**	64	128	128	128
BTSM 117A	64	*128*	128	128	128
Zegla					
BT 5012	32	64	*128*	128	64
BTSM 109A	128	256	256	*256*	256
BT 4975	128	128	256	128	*128*

* Reciprocal of highest serum dilution showing 3 or 4 + fixation of complement in the presence of two units of antigen.

TABLE 6

Comparison of Patois and Zegla prototype strains by neutralization test

Serum	Virus			
	Patois (BT 4971)		Zegla (BT 5012)	
	Titer*	NI†	Titer	NI
Normal rabbit serum	4.6		4.5	
BT 4971 (4i)‡	$<$1.0	$>$*3.6*	2.2	2.3
BT 5012 (4i)	2.4	2.2	$<$1.0	$>$*3.5*

* $\log_{10}$ LD_{50}/0.02 ml of virus-serum mixture after incubation at 37°C for one hour.

† $\log_{10}$ neutralization index of undiluted serum.

‡ Antiserum prepared in mice given four i.p. inoculations of 10% infected liver suspension.

types: VEE, EEE, Pixuna, Mayaro, Aura, Ilheus, St. Louis encephalitis, Yellow fever, Bussuquara, Dengue II, Cache Valley, Guaroa, Wyeomyia, Maguari, Kairi, California, Capim, Guajara, Oropouche, Turlock, Bwamba, Anopheles A, Anopheles B, Tacaribe, Changuinola, Cocal, Vesicular stomatitis (Indiana type), Sicilian Sandfly fever, Icoaraci and Chagres viruses. Similarly, Patois and Zegla hyperimmune sera failed to inhibit hemagglutinins of Maguari, Turlock and Icoaraci viruses as well as several members of arbovirus groups A and B. In the same tests a

polyvalent group C immune ascitic fluid as well as certain hyperimmune Guamá group sera reacted at low titers with Patois and Zegla HA antigens. However, the latter sera failed to react with Patois and Zegla antigens in CF tests, whereas members of the Guamá group characteristically show a high degree of cross-reactivity in this type of test.

The antigenic relationships of Patois and Zegla viruses, represented by prototype strains BT 4971 and BT 5012, respectively, with certain group C arboviruses are shown in Table 3. As may be noted, Patois and Zegla viruses differed significantly in HI tests from all known members of group C, with the exception of Ossa virus which was not available for testing. However, Rodaniche *et al.* have shown that Ossa is closely related by HI technique to Madrid and Caraparú viruses,[7] while Patois and Zegla viruses show very slight or no antigenic relationship in this type of test with the latter agents. As shown in Table 3, Patois antigen and antiserum reacted at low titers with antisera and antigens, respectively, of a number of other group C agents, while Zegla antigen and antiserum (one injection) were completely non-reactive with the other known members of group C utilized in this study.

From the evidence presented above it is concluded that Patois and Zegla viruses represent two closely related hitherto undescribed members of group C. Some properties of Patois and Zegla prototype strains are described below:

Patois virus. Prototype strain BT 4971 of Patois virus which is named after Patoistown, a sector of Almirante, Bocas del Toro Province, Republic of Panama, was isolated from the blood serum of an adult male cotton rat, *Sigmodon hispidus*, trapped two miles north of Almirante on June 16, 1961. Of six suckling mice inoculated with a 1:2 dilution of this serum, two were found sick and four dead on the second post-inoculation day. Brain material from the sick mice was inoculated intracerebrally in a litter of suckling and weanling (21-day-old) mice. All of the infant mice were ill or dead two days after injection, whereas five of six weanling mice inoculated survived for at least 15 days. Stock virus for this isolate was prepared from the brains of infected suckling mice, at the third or fourth passage level, sacrificed about 48 hours after inoculation. Titers of $10^{6.0}$ and $10^{4.6}$ 50% lethal doses (LD_{50}) per 0.02 ml of stock virus suspension were observed in three-day-old mice inoculated i.c and i.p., respectively. No significant loss in infectivity was observed after filtration through a Seitz EK pad. The average survival time (AST) of infant mice inoculated i.c. or i.p. with 0.02 ml of a 1% suspension of stock virus was 2.5 days. Weanling and adult mice similarly inoculated with a stock virus suspension survived a two week period of observation without development of overt illness. This prototype strain was almost completely inactivated after 30 minutes of incubation at 56°C (loss of approximately five log LD_{50}), whereas exposure of the virus suspension to diethyl ether[12] resulted in a drop in titer exceeding three log LD_{50} for infant mice injected intracerebrally. The titer of an HA antigen prepared by acetone extraction of infected suckling mouse serum was 1:2560 when tested within the optimal pH range of activity (5.75 to 6.0) and incubated at 22° to 24°C. Patois virus can be propagated with cytopathogenic effects in primary hamster kidney tissue cultures. No original blood serum was available for virus re-isolation attempts.

Zegla virus. Prototype strain BT 5012 of this virus which is named after Zegla, a sector of Almirante, was recovered from the blood serum of an adult male *Sigmodon hispidus* trapped two miles north of Almirante on June 23, 1961. Two days after injection with a 1:2 dilution of this serum, two sick mice were sacrificed for passage in suckling mice. The remaining five mice in the litter were found dead on the third post-inoculation day. During the second passage, brain material from two mice sacrificed on the day after inoculation was passed to a fresh litter of suckling mice, all of which subsequently became sick or died on the second post-inoculation day. Stock virus was prepared during the third or fourth passage from infected suckling mouse brain harvested about 40 hours after inoculation. Titers of 5.7 and 5.3 log LD_{50} per 0.02 ml of stock virus suspension were obtained in suckling mice inoculated by the i.c. and i.p. routes, respectively. At the fourth mouse passage level, the agent was shown to be filterable through a Seitz EK pad. The AST of three-day-old mice inoculated i.c. or i.p. with 0.02 ml of a 1% suspension of stock virus was two days. Weanling and adult mice similarly inoculated with fourth passage stock virus survived for at least two weeks and developed homologous HI antibody. A 10% brain suspension from suckling mice infected with this agent was totally inactivated after 30 minutes of incubation at 56°C. This strain was also shown to

be sensitive to diethyl ether, the titer of the virus before and after exposure being $10^{5.7}$ and $<10^{2.0}$ LD_{50}, respectively, per 0.02 ml of stock virus suspension administered intracerebrally to suckling mice. A hemagglutinin for Zegla virus was prepared from infected baby mouse serum twice extracted with acetone. Goose red blood cells were used to demonstrate hemagglutination over a pH range of 5.75 to 6.0, the maximum HA titer being 1:640 after incubation at room temperature. Zegla virus can be propagated with concomitant cytopathogenic effects in primary hamster kidney tissue cultures. The virus was reisolated from the original serum specimen after nearly three years of storage at −65°C.

DISCUSSION

Although group C arboviruses have been but recently discovered, numerous infections have been encountered in humans, mosquitoes and wild animals inhabiting areas of tropical America where extensive surveys have been conducted.[1, 3, 4, 6, 7] Little is known regarding the ecology of this group of viruses which have not been found as yet in other continents. Members of this group were first recovered from Central America by Rodaniche *et al.*,[7] who isolated Madrid and Ossa viruses from humans engaged in the collection of mosquitoes in Almirante, Panama. In our study, additional isolations of Madrid virus were made from sentinel mice exposed to the bites of hematophagous insects in the same area. The recovery of Caraparú and Nepuyo viruses from rodents in Panama represents the first isolations of these group C agents in Central America. The isolation of two new members of group C, Patois and Zegla viruses, brings the total number of described group C types to 11, six of which are known to occur in Panama.

Experience in Panama, where many strains of group C viruses have been isolated from man, wild rodents and sentinel mice, but not from large numbers of mosquitoes tested,[8] points to the need for further investigations of group C virus infections in arthropods. Detailed studies on insect-rodent arbovirus cycles in Almirante, Panama are currently in progress.

SUMMARY

Fifteen strains of group C arboviruses belonging to five distinct antigenic types were isolated from rodents in Almirante, Panama during 1961 and 1962. Caraparú and Madrid viruses were recovered from sentinel mice. One isolation of Nepuyo virus was made from a spiny rat. Two new members of the group, Patois and Zegla, were obtained from sentinel mice and cotton rats. Descriptions of these new types are included.

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