DISCOVERY AND IDENTIFICATION OF GROUP C, NEPUYO ARBOVIRUS IN MEXICO[1]

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The discovery of group C, Nepuyo arbovirus in southeastern Mexico during 1963-66 represents the first group C arbovirus to be isolated from natural sources north of Panama, and should alert Mexican physicians to the possibility of related human disease.

Introduction

To date reported isolations of group C arboviruses from natural sources have been limited to the western hemisphere countries of Brazil, Trinidad and Panama (*1-3*). Since some group C arboviruses are human pathogens (*1*), their geographic distribution is of public health importance. This article therefore describes the discovery and identification of group C, Nepuyo arbovirus strains isolated from southeastern tropical Mexico during 1963-66.

Materials and methods

Study sites were at 7 locations along the Gulf of Mexico coast, 6 in the state of Veracruz and 1 in Tabasco (figure 1). 1) *Santecomapan,* Veracruz, a village of approximately 1,000 people, at sea level 100 miles southeast of Veracruz City, bordered a brackish water lagoon in a narrow strip of coastal lowland between the Gulf of Mexico and the San Andres mountains, a small range of volcanic peaks surrounding Lake Catemaco. Habitats there included tropical wet forest dominated by canopy-forming trees, secondary and recently cut forest, mangrove, small patches of trees near houses, cultivated fields, open pastures and yards (figures 2-4). 2) *Los Laureles,* a small group of thatched houses at the base of the Atlantic slope of the San Andres mountains about 15 miles east of Santecomapan, was at the edge of land being cleared of virgin tropical wet forest (figure 5). 3) The *El Arenal heron colony* was located near a sand and gravel quarry, El Arenal, about 2 miles west of the large oil refining city of Minatitlan (72,000 inhabitants in 1967). This was in a permanently flooded swamp with trees 20-60 feet tall, and a dense understory of emergent aquatic vegetation 1-6 feet tall (figure 6). 4) *El Naranjo* was a cluster of about 15 thatched-roof, mud-wattle houses at the edge of the same lowland area as El Arenal, but about 1 mile farther west from Minatitlan (figures 7-8). 5) Bordered by the coastal dunes, and the Rio Coatzacoalcos, the *Coatzacoalcos Marsh,* over 12 miles in diameter, extended the entire distance between Minatitlan and the port city of Coatzacoalcos (75,000 population in 1960). This fresh water marsh contained predominantly tall emergent aquatic vegetation including cattails, and was bordered by, and contained

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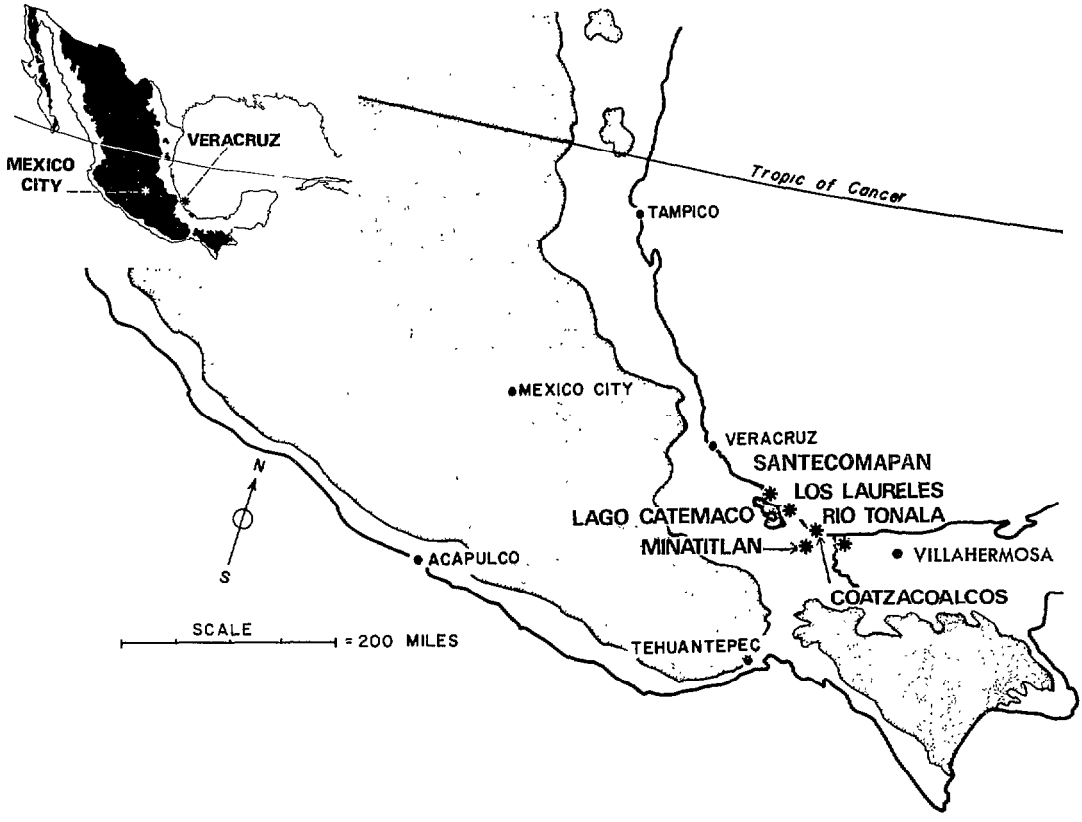
FIGURE 1—Map of study sites in Mexico.

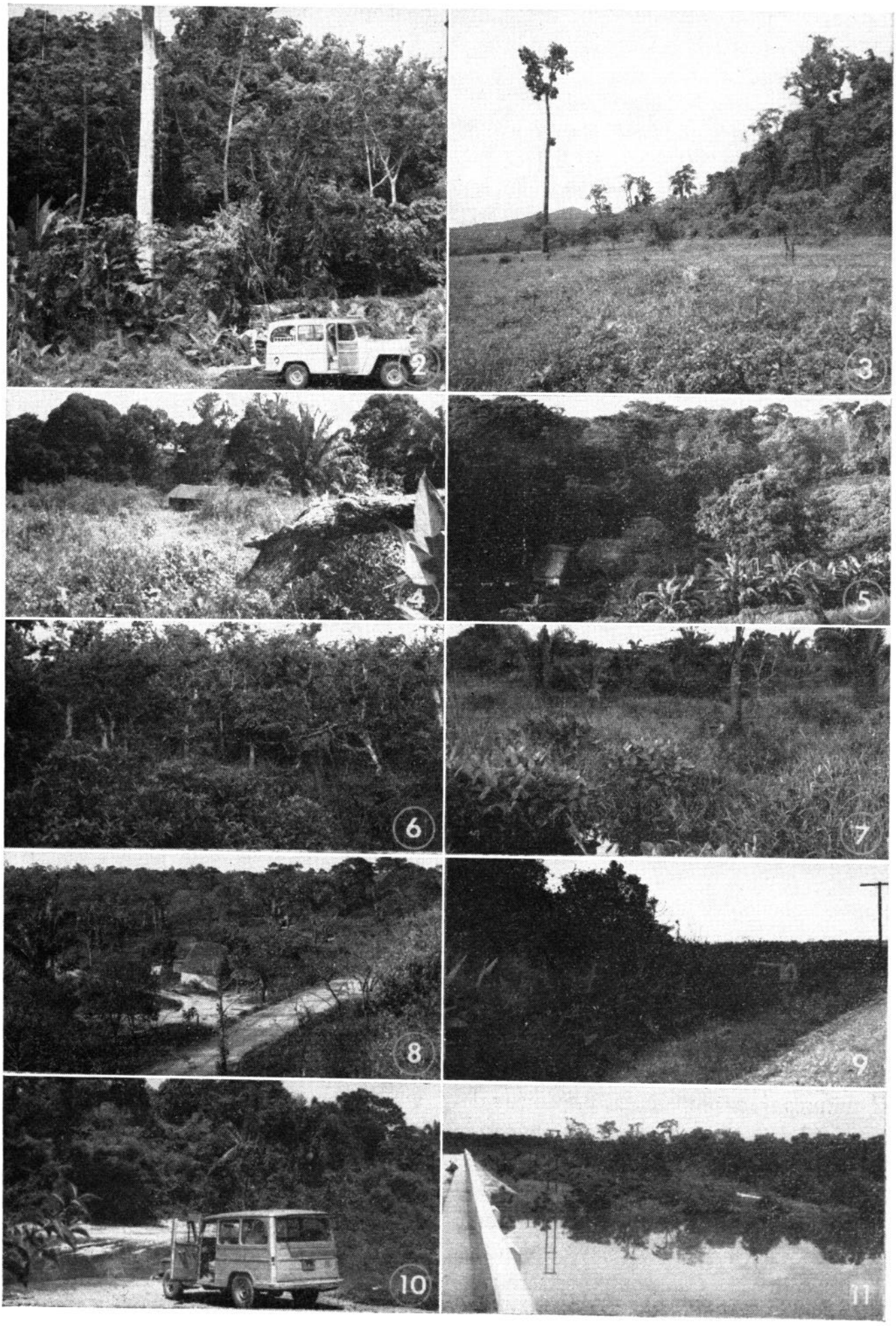


islands of permanently flooded swamp forest, such as the area utilized in these studies (figure 9). This patch of swamp forest was 0.1-0.2 square miles in area and was located about 3 miles from Minatitlan on the west side of the road to Coatzacoalcos, and approximately 0.5 miles from another heron colony and rookery located within a mile of the Minatitlan airport. 6) Two miles east of the *Rio Coatzacoalcos bridge,* about 3 miles from Coatzacoalcos city, there was an intermittently swampy, second-growth forest, with abundant moisture-adapted palms and permanently saturated soils (figure 10). 7) On the east side of the *Rio Tonala,* just into the state of Tabasco and about 2 miles inland from the Gulf of Mexico, sentinel animals were placed in a tall mangrove forest with a thick undergrowth of shrubs and 6-8 foot ferns (figure 11). This forest was abolished by cutting between summer 1965 and spring 1966. During 1963-66, locality 1) was the principal study area, localities 3)-5) constituted part of a comparative investigation of arboviruses in areas with and without breeding herons, and the other localities were studied only sporadically.

Mosquitoes were collected and identified as described elsewhere (*4*) except that modified Chamberlain light traps and various styles of traps baited with adult hamsters were also used. Isolations of arboviruses from mosquitoes were made using suckling mice as described elsewhere (*4*).

Sentinel animals. Suckling Swiss albino *mice,* 1-4 days old, used as virus sentinels (SSM) were exposed overnight without

FIGURES 2-11—See "Materials and methods" text for identification and descriptions of study habitats.



mothers; after return to the mother, they were observed for illness and/or death for 10-14 days. A 10% suspension of brains from ill or dead sentinel mice was made in 1% bovine albumin in Hanks' solution with penicillin, 100 units/ml and streptomycin, 100 micrograms/ml (BA); 0.01 ml of the centrifuged supernatant (10^3g, 10 minutes) was inoculated intracranially (ic), and 0.01 ml subcutaneously (sc), into 8 suckling mice which were observed for 2 weeks.

Adult golden *hamsters* were used as sentinels (SH) as previously described (*5*). Dead hamsters were stored on dry ice and tested in New York for virus; some hamsters were autopsied at the field laboratory in Mexico and only heart, lung, kidney or brain frozen in sealed glass ampules on dry ice. A 10% suspension of heart, pooled heart and kidney, pooled heart, lung and kidney, or brain was prepared in BA, centrifuged at 10^4g for 1 hour at 5°C, and the supernatant fluid passed through a 450 millimicron Millipore filter to eliminate bacteria before inoculation of primary chicken embryonic cell cultures, 0.1 ml per tube culture, to detect VE virus. When no cytopathic effects (CPE) occurred, aliquots of hamster organ suspensions stored in sealed glass ampules at −60°C, were inoculated into suckling mice like the sentinel mouse brain suspensions described above. A few hamster organ suspensions were inoculated in 0.1 ml volume into HeLa cell cultures and fluid harvested when CPE occurred after 3-7 days at 37°C.

Cell cultures. Mouse L, human HeLa, primary chicken embryo and hamster kidney cell cultures were prepared as previously described (*7-10*). L cells were maintained in 10% newborn, precolostrum, calf serum in maintenance solution (MS) (*7*), HeLa cells in 10% human serum in MS, chicken embryo cells in MS and hamster kidney cells in 2% calf serum in MS. Calf and horse sera were from animals residing in Colorado or the middle eastern coastal states of the USA respectively, and human sera were from residents of Minnesota or New York.

Virus suspensions and infectivity assays. For further studies of a virus, 10% suspensions of brain (and sometimes also hind limb muscle) from sick or dead suckling mice were made as described above for brains from sentinel mice and were stored in sealed glass ampules on dry ice or in screw capped vials in an electric −60°C box. Infectious virus assays were done by ic inoculation of suckling mice with decimal dilutions of virus suspension, and 50% lethal doses ($SMicLD_{50}$) were calculated (*6*) in terms of mouse tissue after observation of mice for 2 weeks.

Preparation of antisera. 63U11 antibody in mouse ascitic fluids was prepared by two ip inoculations of adult albino mice with virus as 10% mouse brain suspension (suckling mouse passage 3) 36 days apart; sarcoma 180/TG cells were also given ip on day 36, and ascitic fluid was obtained on days 45-52 for fluid A (5 mice) and days 41-43 for fluid B (2 mice). 63U11 antiserum was also prepared in guinea pigs given 5 intraperitoneal (ip) inoculations at 0, 7, 14, 25 and 35 days and bled 8 days after the last inoculation. For 63R79 virus, mouse ascitic fluids were used from days 60 and 110 following 3 ip virus inoculations on days 0, 9 and 44 (the latter with Freund's adjuvant). Immune mouse ascitic fluids for other virus strains were prepared by single ip injections of virus as 10% suckling mouse brain suspensions representing SM-pl-3 except for 64U68, 64U76, 64U81, and 64U89 which were first or second passage HeLa cell culture harvests. 65U11 virus was used as 10% sentinel hamster brain suspension or suckling mouse brain suspensions from SM-pl, and both single-injection and 5-injection, mouse ascitic fluids were employed. Ascitic fluids were produced by sarcoma 180/TG cells and were taken 9-45 days (average 27 days) after single virus inocu-

lation or 6-20 days after the fifth injection of 65U11 virus (given on days 0, 8, 18, 29 and 44).

Identification of group C arboviruses was done by complement-fixation (CF) test (*11*) and for two strains, by neutralization (N) of viral CPE in HeLa cell cultures using 63U11 guinea pig antiserum. CF antigens were prepared as supernatant fluids from 20% suckling mouse liver in normal saline, refrigerated at 5°C for 18 hours and centrifuged at 10^4g for 60 minutes.

Other procedures employed to characterize strain 63U11 virus are described elsewhere as follows: sensitivity of viral infectivity to sodium deoxycholate (*12*), N tests in suckling mice (*4*), preparation of hemagglutinating (HA) antigens and performance of hemagglutination-inhibition (HI) tests (*13*). Some CF and all HI tests were done in microtiter plastic wells with reduced volumes of 0.025 ml antigen, 0.025 ml serum, 0.05 ml complement (2 units) and 0.05 ml sensitized sheep erythrocyte suspension for CF tests, or 0.05 ml of goose erythrocyte suspension for HI tests. Before filtration of infected mouse brain suspension of strain 63U11 virus, the suspension was centrifuged at 10^4g for 30 minutes at 5°C.

Results

Discovery of group C, Nepuyo arbovirus in Mexico. A virus designated 63U11 was recovered from a sentinel hamster which was exposed on 16 August 1963 in a wire cage about 5 feet above ground in a tropical wet forest at Santecomapan, Veracruz. The hamster was dead on 19 August, and following inoculation of a suspension of pooled heart, lung, and kidney tissues into suckling mice on 16 December 1963, mice died within 3 days.

Initial identification of 63U11 virus as a group C arbovirus was made by CF test using polyvalent arbovirus group A, group B, Group C, Venezuelan encephalitis and Tlacotalpan virus antisera. The test was positive only with 3 different group C antisera. Next, 63U11 CF antigen was tested against antibodies to Bunyamwera and California group viruses, to a Mexican strain of Patois virus (63A49), and to group C viruses; positive reactions were again obtained only with polyvalent group C antiserum.[5] Subsequently 63U11 CF antigen was found to react with individual group C, Caraparu, Marituba and Murutucu antisera, but not with Oriboca, Itaqui or Apeu antibodies. Polyvalent group C, Caraparu, Marituba and Murutucu antisera also inhibited 63U11 HA whereas Oriboca, Itaqui, Apeu and polyvalent California group antisera did not. Polyvalent group C serum also neutralized 63U11 virus in HeLa cell cultures. In contrast Marituba, Murutucu, and Caraparu antisera failed to neutralize 63U11 virus in HeLa cell cultures. Thus it seemed that 63U11 virus was a group C arbovirus antigenically distinct from Oriboca, Itaqui, and Apeu and probably different from Caraparu, Marituba, and Murutucu since although it reacted by CF and HA tests with antisera to the latter 3 viruses, it was not neutralized by these antisera.

Further HI tests of 63U11 virus against 10 currently reported group C arboviruses showed it to be Nepuyo virus (*2, 3*); minor cross reactions occurred with Marituba and Ossa viruses (a known relationship for Nepuyo virus), but no reaction was found with 6 other group C viruses or with Patois

[5] Arbovirus antisera were kindly supplied by Dr. J. Gibbs (group C polyvalent, monkey and guinea pig, Apeu, mouse, and Itaqui, rabbit), Dr. T. Work (group B polyvalent, monkey), Dr. J. Casals (group A polyvalent, mouse ascitic fluid, Caraparu, Marituba and Murutucu, mouse), and Dr. Robert Shope (polyvalent group C, Bunyamwera and California mouse). The polyvalent group C sera had I.NI ranging from 3.1 to >4.5 versus 5 group C viruses, Apeu was 3.5 and Itaqui was 3.2; the group B serum had CF and HI antibody titers 1:64-1024 and 1:640-5120 respectively versus 11 group B viruses, the group A serum had CF titers of 1:8-128 versus 14 group A viruses, and the California serum had HI titers 1:160-320 versus 4 California group viruses. Oriboca guinea pig antiserum, made at the University of Minnesota, had a homologous CF titer of 1:64.

and Zegla viruses (table 1).[6] The HI relationship between 63U11 virus and Nepuyo virus was such that both antisera strongly inhibited both hemagglutinins. CF tests did not clearly distinguish 63U11 virus from Nepuyo, Marituba and Oriboca, but N tests established its essential identity with Nepuyo virus (table 2).

Filtration, host range, sensitivity to sodium deoxycholate and antigenic properties of the 63U11 strain of Nepuyo virus. At SM-p7 infectious virus passed 450, 100 and 50, but not 10 millimicron Millipore cellulose filters; $SMicLD_{50}$ titers per 0.01 ml of brain for each filtrate were: $450m\mu$-$10^{-6.7}$, $100m\mu$-$10^{-6.6}$, $50m\mu$-$10^{-5.6}$ and $10m\mu$-$<10^{-2.6}$.

63U11 virus was lethal for *suckling mice* within 3-4 days of ic inoculation; titers of virus were $10^{-5.3}$ $SMicLD_{50}$/0.01 ml of pooled mouse brain and muscle tissues at SM-p2 (average of 3 titrations), and $10^{-6.4}$ and $10^{-6.2}$ $SMicLD_{50}$/0.01 ml of liver at SM-p6 and 11 respectively. *Weanling mice* died within 6-10 days after ic inoculation of $>10^{3.2}$ $SMicLD_{50}$ of virus at SM-p1. *Adult mice* did not become ill after 2 monthly ip inoculations of 10% infected suckling mouse brain suspension at SM-p3, but made CF and HI antibodies. Three of 3 *hamsters* over

[6] Through the kindness of Drs. Robert Shope, W. Downs and others at the Yale Arbovirus Unit and WHO World Arbovirus Reference Center, the studies of 63U11 virus involving infectious antigens of other than the Mexican group C viruses were performed by Dr. Zarate in the Yale laboratory of Dr. Shope who generously supplied the known group C virus antigens and antisera. This was done to avoid working with known group C viruses in the Cornell laboratory where there had been none except Oriboca virus prior to recovery of 63U11 virus, and Oriboca virus was used only as a CF antigen (made in 1961 at the University of Minnesota) in the initial tests described in the text to identify 63U11 virus as a group C virus.

TABLE 1—Immunologic relationships between 63U11 virus and group C arboviruses by hemagglutination-inhibition test.

Antibodies vs. virus (strain)	No. virus injections per mouse	Reciprocal of HI antibody titer versus each virus antigen (No. of HA units in test)								
		63U11 (4)	Nepuyo (2)	Marituba (4)	Oriboca (4)	Murutucu (4)	Apeu (4)	Caraparu (2)	Itaqui (4)	(4)
63U11	1[a]	*160*	320	40	0[b]	0	0	0	0	0
Nepuyo (An 10709)	5	>320	*>320*		40		80	320	40	
Marituba (An 15)	4	20	160	*640*	0		40	160	20	
Ossa (BT 1820)	?[a]	40	160		40		320	>320	20	
Madrid (BT 4075)	?[a]	0	20		0		20	80	0	
Oriboca (An 17)	2[a]	0	0		*80*		0	0	0	
Murutucu (An 974)	?	0	0		0	*320*	0	0	0	
Apeu (An 848)	5	0	0		0		*>320*	320	0	
Caraparu (An 3994)	2	0	40		0		320	*>320*	20	
Itaqui (12752)	?									*80*
Patois (BT4971)	2[a] ?	0,0 0								
Zegla (BT 5012)	2[a] ?	0,0 0								

[a] Indicate antibodies as mouse ascitic fluids induced by sarcoma 180/TG cells; no footnote denotes mouse serum. 63U11 antibody was from Cornell, the second Patois and Zegla antisera were from Dr. Sunthorn Srihongse at the Gorgas Memorial Laboratory, Panama, and the rest were from Dr. R. Shope at Yale.
[b] $0 = <20$.

TABLE 2—Immunologic relationships between 63U11 virus and group C arboviruses by complement-fixation and neutralization tests.

Antibodies vs. virus (strain)	No. virus injections per mouse	Antibody titers versus each virus antigen Complement-fixation test [a]					Neutralization test [b]	
		63U11	Nepuyo	Marituba	Oriboca	Caraparu	63U11	Nepuyo
63U11	1 [c]	*16*	<8 [d]	8	<8	<8	*>5.0*	4.2
Nepuyo (An 10709)	5	128	*512*				>5.0	*>4.9*
Marituba (An 15)	4 ?	16		*128*			negative	
Oriboca (An 17)	2	8			*32*			
Caraparu (An 3994)	2 ?	<8				*32*	negative	

[a] Reciprocal of serum dilution reacting with 4 CF antigen units except for Nepuyo antigen which was 1 unit.
[b] Log_{10} neutralization index in suckling mice inoculated ic or negative neutralization in HeLa cell cultures as described in text.
[c] Mouse ascitic fluid induced by sarcoma 180/TG cells or mouse serum. 63U11 antibody was from Cornell, Marituba and Caraparu with ? injections were from Dr. J. Casals and the others were from Dr. R. Shope at Yale.
[d] Titer 16 vs 16 units of Nepuyo antigen.

6 months of age, died between 48-66 hours after sc inoculation of $10^{5.2}$ $SMicLD_{50}$ of virus at SM-p11. *Guinea pigs* given 0.1 ml of 10^{4}g supernatant fluid from a 20% suspension of infected suckling mouse brain at SM-p3 or 4, sc on days 0, 7, 14, 25 and 35 or ip on days 0, 7, 14, showed no signs of illness and developed CF antibody titers in serum to 1:64 on day 43 or 1:128 on day 22 respectively. The virus grew and produced CPE in mouse *L cell cultures* at 37°C. CPE did not appear until day 6 and continued to progress as late as days 13-16. The titer of virus in second passage mouse brain suspension based on CPE ($10^{-4.5}$/0.1 ml) equalled its infectivity endpoint for L cell cultures (determined by ic inoculation of cultural fluids into suckling mice). Cultured virus representing a cumulative dilution of 10^{-9} of the original suckling mouse brain suspension, was neutralized in L cell cultures by 63U11 guinea pig antiserum. 63U11 virus produced CPE in human *HeLa cell cultures* at 37°C within 2-5 days of inoculation, and CPE did not progress beyond day 5. The CPE titer of virus as brain suspension from SM-p2 was $10^{-4.2}$/0.1 ml of brain; fluid harvested from cultures inoculated with $10^{-3.7}$ dilution titrated $10^{-3.5}$/0.1 ml in other HeLa cell cultures, and this virus was neutralized by 63U11 guinea pig antiserum. 63U11 virus as 10% mouse brain-muscle suspension from SM-p1 or SM-p2, in dosages of $10^{0.3-4.3}$ $SMicLD_{50}$ per culture, failed to produce transmissible CPE in primary *chicken embryonic* (CEC) or *hamster kidney* cell cultures maintained in MS or 2% calf serum in MS respectively for up to 8 days at 37°C. Some CPE was seen with $10^{4.3}$ $SMicLD_{50}$ in CEC on days 1-5 after inoculation, but fluid harvested on day 5 failed to produce CPE in other CEC within 5 days at 37°C. Repeat inoculation was done by Dr. F. Austin of $10^{5.2}$, $10^{3.2}$ and $10^{1.2}$ $SMicLD_{50}$ into primary hamster kidney cell cultures maintained in either 2.5% calf serum, 0.5% lactalbumin hydrolysate in Hanks' solution with 0.1% sodium bicarbonate or this medium plus 0.1% final concentration of yeast extract and 420 mg% additional glucose. CPE involving 60-90% of cells was seen with the largest virus dose by the sixth day of incubation at 37°C in the latter medium, but less than 30% of cells were affected in the former medium. With the middle virus dose, CPE was seen only with the latter me-

dium, and no CPE occurred with the smallest virus dose.

63U11 virus was not dangerous to laboratory personnel, provided aseptic technic was employed, nor did human infections occur during field work. No inapparent infections were detected by CF test of sera from a) 4 persons who worked with the virus in the laboratory for ½-2 years, b) 3 persons exposed in endemic Mexican habitats during 2-3 month periods during 1963-66, or c) 10 persons exposed in endemic areas during one of the summers of 1963-66.

63U11 virus from SM-p2 was inactivated by sodium deoxycholate in BA, but not by SDC in human serum (1:1000 final concentration of SDC, 37°C, 1 hour). $SMicLD_{50}$/0.01 ml of brain representing SM-p2 were: human serum control $10^{-4.6}$; SDC 1:1000 in BA, $<10^{-3.0}$; and SDC 1:1000 in human serum, $10^{-3.8}$.

CF antigens of 63U11 virus could be made by sucrose-acetone extraction of suckling mouse brain, serum or liver. Antigen titers at SM-p3 were two- to four-fold higher in liver than in brain, and were equal to four-fold higher in brain than in serum. Liver extracted in saline overnight as a 20% suspension and then centrifuged at 10^4g for 60 minutes at 5°C also provided high titer (1:128-512) CF antigens at SM-p3 and at several succeeding mouse passage levels.

Goose erythrocyte agglutinins (HA) could not be made by sucrose-acetone extraction of infected brain, liver or serum at SM-p3. At SM-p5, serum yielded HA of low (1:8) titer. Additional, rapid serial passages in suckling mice of virus as infected liver suspensions eventually, by passages 10 or more, produced high enough titers of virus in serum and liver to produce usable HA by the sucrose-acetone extraction method. HA with titers usually less than 1:128 were obtained from liver or serum 24-32 hours after inoculation of mice when tested at the optimal pH of 6.0 and at room temperature. Both liver and serum HA were inhibited by 63U11 antibody, though antibody titers were slightly higher with 4 units of serum-HA than with 4 units of liver-HA. Hemagglutinins have also been prepared similarly from serum of adult hamsters.

Isolation and antigenic analysis of 22 additional group C arboviruses from Mexico. Forty-six additional viruses were identified as group C arboviruses by positive reactions in CF tests of 1:4-32 dilutions of liver antigens versus 4-8 units of 63U11 virus guinea pig antibody or in neutralization tests in mice or HeLa cell cultures using 63U11 guinea pig antiserum (table 3). Thirteen of these viruses came from sentinel adult hamsters which were exposed in nature for 2-21 days (mean 7.8 days), and survived a total, including exposure, of 3-21 days (mean 8.4 days) before becoming ill or dying of virus infections (table 4). Eight strains were from sentinel suckling mice which were exposed overnight without mothers and survived 2-9 days (mean 3.9 days) (table 4). Two virus strains were recovered from *Culex* mosquitoes collected in August 1963 and 1964 (table 4); a third strain (table 3) came from 13 female *Culex opisthopus* collected in August 1964 from a hamster-baited trap where virus feedback was considered to be unlikely.

Twenty-two of the 46 strains were selected for detailed antigenic analysis, study of host range in weanling mice and chicken embryonic cell cultures, and comparison with the 63U11 strain of Nepuyo virus; these strains were chosen to represent significant proportions of viruses from different locations, hosts, months and years (tables 3 and 4). When 63U11 antisera were tested against HA of 16 of these viruses, HI antibody titers were essentially the same as with homologous 63U11 HA (table 5, part A). When 63U11 HA was tested versus antisera to the 22 viruses, inhibition of 63U11 HA occurred with all but 65U11 virus antiserum. In general, heterologous 63U11 HI antibody titers were similar to homologous titers; for con-

TABLE 3—Group C arboviruses from various habitats in southeastern Mexico, 1963-1966.

Place [a]	Source [b]	No. of group C arboviruses isolated by year and *no. compared in these studies*								Total isolated and *studied* per location	
		1963		1964		1965		1966			
Santecomapan	SH	2	*2*	7	*3*	1	*0*	6	*0*	16	*5*
	SSM	2	*1*	2	*2*					4	*3*
	M	1	*1*	2	*1*					3	*2*
Los Laureles	SH			1	*1*					1	*1*
El Arenal heronry	SH			1	*0*	1	*0*	5	*0*	7	*0*
(Minatitlan)	SSM			5	*5*					5	*5*
El Naranjo	SH							2	*1*	2	*1*
Coatzacoalcos marsh	SH					2	*1*	3	*2*	5	*3*
Coatzacoalcos bridge	SH			1	*1*					1	*1*
Rio Tonala	SH					2	*2*			2	*2*
Total isolated and *studied* per year		5	*4*	19	*13*	6	*3*	16	*3*	46	*23*

[a] Rio Tonala is in Tabasco State; other locations are in Veracruz State.
[b] SH = sentinel adult hamster, SSM = sentinel suckling mice, M = adult female mosquitoes.

trol purposes, 23 antisera were tested with Venezuelan encephalitis virus HA and found not to react (table 5, part B). Further HI tests revealed that the results with 65U11 virus were peculiar to one immune mouse ascitic fluid and that other fluids from the same mouse as well as 2 additional hyperimmune fluids reacted with 63U11 HA (table 6). Moreover 65U11 virus was indistinguishable from prototype Nepuyo virus (Belem, Brazil strain An10709) by HI test (table 6), and all 3 viruses were indistinguishable by N tests although, CF test again did not clearly differentiate the Mexican Nepuyo virus strains from Marituba and Oriboca (table 7). Thus it seemed that the 22 selected group C arboviruses were sufficiently similar antigenically to the Mexican 63U11 strain of Nepuyo virus to consider them Nepuyo virus strains.

Pathogenicities of 22 Mexican group C, Nepuyo arboviruses for weanling mice and chicken embryonic cells in culture (CEC). Sixteen strains of virus were inoculated ic and ip into weanling mice and 12 into CEC with fluid medium; all killed mice by both routes within 2-11 days of inoculation, and none produced cytopathic effects in CEC (table 8).

Discussion

Group C arboviruses are known to cause sporadic, systemic disease in man, but to date their geographic distribution as evidenced by virus isolations has been limited to equatorial regions of the western hemisphere. The discovery of Nepuyo virus in Mexico initially in 1963 represents the first evidence of group C arboviruses north of Panama, and extends the geographic distribution of Nepuyo virus from its previous known locations in Trinidad, Brazil and Panama (*2, 3, 14*). Its continued presence in Veracruz during 1963-66 indicates that it is endemic there.

Although group C arbovirus disease is not yet considered a major public health problem in any country, this may reflect lack of suspicion, diagnosis and recognition of these diseases. Therefore it is important that physicians suspect them as etiologic agents of systemic febrile disease in man, and that field and laboratory investigations designed

TABLE 4—Background information for the 23 group C arboviruses compared in these studies.

Location	Year	Source of virus	Dates of exposure or collection	Days sentinels exposed/survived		Initial group C identification		Virus strain
				Hamsters	Mice	CF[a]	Neut.[b]	
Santecomapan	1963	Sentinel hamster	16-19 Aug.	3/3		Nepuyo		63U11
	"	"	7- 9 Aug.	2/4		+	> 2.8	63U5
	"	Sentinel suckling mouse	12-13 Aug.		1/9	+		63R79
	"	*Culex spp* [c]	8- 9 Aug.			+		63A108
	1964	Sentinel hamster	15 Jul.-5 Aug.	21/21		+		64U81
	"	"	20 Jul.-8 Aug.	19/19			> 2.6	64U89
	"	"	18-29 Aug.	11/11		+		64U106
	"	Sentinel suckling	17-18 Sept.		1/4	+		64R25
	"	mice	17-18 Sept.		1/4	+		64R26
	"	*C. iolambdis* [d]	4-10 Aug.			+		64A180
Los Laureles	1964	Sentinel hamster	24 Jul.-2 Aug.	9/11		+		64U76
El Arenal heronry (Minatitlan)	1964	Sentinel suckling	23-24 July		1/2	+		64R27
	"	mice	"		1/3	+		64R28
	"	"	"		1/3	+		64R29
	"	"	"		1/3	+		64R30
	"	"	"		1/3	+		64R31
El Naranjo	1966	Sentinel hamster	12-17 Feb.	5/5		+		66U33
Coatzacoalcos marsh	1965	"	6-14 Sept.	8/8		+		65U244
	1966	"	25 Apr.-2 May	7/7		+		66U117
	"	"	25-28 Apr.	3/3		+		66U121
Coatzacoalcos bridge	1964	"	23-28 July	5/7			> 2.6	64U68
Rio Tonala	1965	"	18-22 Feb.	4/6		+		65U8
	"	"	1- 5 May	4/4		+		65U11

[a] + = titer of suckling mouse liver antigen ≧ 32 vs 2–4 units of 63U11 guinea pig antibody and < 4 vs 2–8 units of 63A49 (a Mexican strain of Patois virus) guinea pig antibody.
[b] $\log_{10}$ neutralization index in suckling mice inoculated ic (63U5) or HeLa cell cultures.
[c] 110 females and males from a light trap.
[d] 33 females from a hamster-baited trap where virus feedback was unlikely.

to recover virus and detect serum antibodies be carried out in humans and domestic and wild animals to establish diagnoses, understand the ecology and epidemiology of these viruses and evaluate their health hazard in Mexico and tropical American countries. Moreover since multiple types of group C arboviruses coexist in Trinidad, Brazil and Panama, it is also important to continue studies in Mexico to learn if group C arboviruses other than Nepuyo virus exist there.

Summary

Twenty-three virus strains isolated during 1963-66 from sentinel adult hamsters, sentinel suckling mice and *Culex* mosquitoes collected in the States of Veracruz and Tabasco, Mexico, were identified as group C, Nepuyo arbovirus. More strains were isolated from sentinel hamsters than from mosquitoes or sentinel mice. One strain (63U11) studied in detail, passed 50 millimicron but not 10 millimicron filters, killed suckling and weanling mice after intracranial inoculation, and hamsters but not guinea pigs, after subcutaneous inoculation. It produced cytopathic effects in mouse L, human HeLa and primary hamster kidney cell cultures, but not in primary chicken embryonic cell cultures. The virus was inactivated by

TABLE 5—Antigenic relationships by HI test between Nepuyo virus (Mexican strain 63U11) and 22 additional group C viruses isolated in southeastern Mexico.

Hemagglutinins (part A) or antisera (part B) of 22 virus strains	A Reciprocals of HI antibody titers of 63U11 antisera vs. indicated HA for tests 1–5 [a]					B Reciprocals of HI antibody titers of indicated virus antisera vs.		
	1	2	3	4	5	Homologous HA	63U11 HA	VE HA [c]
63U11	40	80	40	160	20			< 10
63U5	40		40		40	40	40	< 10
63R79	40					160	80	< 10
64U81		20			10	40, 40	40, 20	< 10
64R25		20	20			40, 40	40, 40	< 10
64R26		80	80		40	160, 80	160, 40	< 10
64U76		80			20	160, 80	160, 40	< 10
64R29		40			20	80, 80	80, 40	< 10
64R30		40			20	20, 20	20, 20	< 10
64R31		40			< 10	80, 80	40, 40	< 10
65U8		40				40, 40	20, 40	< 10
65U11		20	40		< 10	80, 40	< 10, < 10	< 10
64R28			40			80	80	< 10
64R27				80		nt [b]	nt	< 10
64U89					20	20	20	< 10
64U106					20	20, 20	20, 20	< 10
64U68					20	40, 40	20, 40	< 10
63A108						nt	80	
64A180						nt	80	
65U244						nt	80	
66U33						nt	80	
66U117						nt	40	
66U121						nt	40	

[a] 63U11 antisera were mouse ascitic fluid A for tests 1–3, B for test 4 and guinea pig serum for test 5.
[b] nt = not tested.
[c] Venezuelan encephalitis virus, strain 63U2.

TABLE 6—Immunologic relationship by HI test between 65U11 virus and Nepuyo viruses, strains An10709 and 63U11.

Antibodies (source and no. of virus injections)	HI antibody titers versus each virus HA [a]									
	Test a			Test b			Test c		Test d	
	65U11	63U11	Nepuyo	65U11	63U11	Nepuyo	65U11	63U11	65U11	63U11
65U11 (1 mouse, 1 inj) [b]	*>320*	<10	160	*80*	<40	320	*80, 80*	40, 20	*40, 80*	40, 20
(3 mice, 1 inj)							*10*	<10	*20*	10
							20	<10	*20*	10
							10	<10	*20*	10
(2 mice, 5 inj)							*20*	80	*10*	40
							20	80	*20*	40
63U11 (several mice pooled, 1 inj)	>320	*160*	>320	80	*80*	640				
(6 guinea pigs pooled, 5 inj)	>320	*160*	>320	80	*80*	640				
Nepuyo (mouse, 5 inj)	2560	640	*>5120*	640	640	*>10240*				

[a] Reciprocal of serum dilution inhibiting 2-4 HA units for 65U11 and 63U11 and 1-2 units for Nepuyo in test a, and 16 units for 65U11, 2-4 for Nepuyo and 4-8 for 63U11 in test b.
[b] Ascitic fluids from this mouse were taken on day 39 for tests a and b and on days 25 and 30 for the 2 results presented for each test c and d.

TABLE 7—Immunologic relationship by CF and N tests between 65U11 virus and Nepuyo viruses, strains An10709 and 63U11.

Antibodies vs. virus (strain)	No. virus injections per mouse	Antibody titers versus each virus antigen: Complement-fixation test [a] 65U11	Nepuyo (An10709)	Nepuyo (63U11)	Marituba	Oriboca	Caraparu	Neutralization test [b] 65U11	Nepuyo (An10709)	Nepuyo (63U11)
65U11	1 [c]	*16*	8	16	8	<8	<8	*>4.7*	>4.9	4.3
Nepuyo (An10709)	5	128	*512*	128				>5.7	*>4.9*	>5.0
(63U11)	1	16	<8 [d]	*16*	8	<8	<8	>4.0	4.2	*>5.0*
Marituba (An15)	4	16		16	*128*					
Oriboca (An17)	2	8		8		*32*				
Caraparu (An3994)	2	<8		<8			*32*			

[a] Reciprocal of serum dilution reacting with 4 CF antigen units except for Nepuyo, which was 1 unit.
[b] Log_{10} neutralization index in suckling mice inoculated ic.
[c] Mouse ascitic fluid induced by sarcoma 180/TG cells or mouse serum. 65U11 and 63U11 antibodies were from Cornell, and the others from Dr. R. Shope at Yale.
[d] Titer 16 vs 16 units of Nepuyo antigen.

sodium deoxycholate. Sixteen other strains also killed weanling mice after intracutaneous or intraperitoneal inoculation, and in this respect, Mexican Nepuyo virus strains were unlike the original strains from Trinidad and Brazil.

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TABLE 8—Pathogenicities of Mexican group C arbovirus strains for weanling mice inoculated ic and ip and for primary chicken embryonic cells in culture.

Location	Virus strain	Suckling mouse passage from isolation	No. mice dying (and survival range in days) after inoculation of 5 weanlings [a]: ic	ip	No. CEC with CPE of 3 inoculated [a]
Santecomapan	63U11	5	4 (6-7)	3 (3-6)	
	63U5	3	5 (3-10)	5 (3-5)	
	64U81	2 [b]	5 (2-5)	5 (3-6)	0
	64U89	1 [b]	4 (3-9)	5 (8-11)	0
	64U106	3	5 (3-5)	5 (3-10)	0
	64R25	3	5 (3-6)	5 (4-8)	0 [c]
	64R26	3	5 (3-6)	5 (3-9)	[c]
Los Laureles	64U76	2 [b]	5 (4-7)	5 (3-4)	0
El Arenal heronry (Minatitlan)	64R27	3	5 (3-6)	5 (3-8)	0
	64R28	3	5 (4-7)	5 (3-7)	0
	64R29	3	5 (5-6)	5 (3-7)	0
	64R30	3	5 (3-10)	4 (4-10)	0
	64R31	3	5 (5-6)	5 (3-6)	0
Coatzacoalcos bridge	64U68	2 [b]	5 (3-7)	5 (3-7)	0
Rio Tonala	65U8	2	5 (3-8)	5 (4-9)	
	65U11	1	5 (3-7)	5 (3-8)	0 [c]

[a] Inocula for mice were 10% suspensions of infected suckling mouse brain (except for 63U11 which was liver), 0.02 ml ic and 0.02 ml ip, and mice were observed for 11 days. Inocula for CEC were 1:2 or 1:10 dilutions of the same suspensions, 0.1 ml per tube culture containing 0.9 ml of MS; cultures were were kept at 36°C for 6 days.
[b] Actually, SM-pl, HeLa-p2, SM-pl for 64U81 and 64U76; HeLa-pl, SM-pl for 64U89; and CEC-pl, SM-pl, HeLa-pl, SM-pl for 64U68.
[c] Also no plaques in 5 or 6 days under agar overlay.

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Descubrimiento e identificación de *Nepuyo Arbovirus*, Grupo C, en México (*Resumen*)

Veintitrés cepas de virus, aisladas durante el período 1963-1966, de cricetos centinelas adultos, ratones lactantes centinelas y mosquitos *Culex* capturados en los estados mexicanos de Veracruz y Tabasco, fueron identificadas como de *Nepuyo arbovirus*, Grupo C. Se aislaron más cepas de cricetos centinelas que de mosquitos o ratones centinelas. Una de las cepas (63U11), estudiada detenidamente, pasó por filtros de 50 milimicrones, pero no de 10 milimicrones; mató ratones lactantes y destetados, después de inoculación intracraneana, y cricetos, pero no cobayos, después de inoculación subcutánea. Produjo efectos citopáticos

en cultivos de células L de ratón y células HeLa humanas y en cultivos primarios de células de riñón de cricetо, pero no en cultivos celulares primarios de embrión de pollo. El virus fue inactivado con deoxicolato sódico. Otras 16 cepas también mataron ratones destetados después de inoculación intracraneana o intraperitoneal y, a este respecto, las cepas mexicanas de virus Nepuyo se diferenciaron de las cepas originales de Trinidad y del Brasil.

Descobrimento e identificação do Grupo C, *Nepuyo Arbovirus*, no México (*Resumo*)

Vinte e três famílias de vírus, isolados no período de 1963-66, de criceto adulto sentinela, de camundongo sentinela em período de amamentação e de mosquito *Culex* captados no Estados de Veracruz e Tabasco, no México, foram identificadas como Grupo C, *Nepuyo Arbovirus*. Foram isoladas mais famílias de criceto sentinela do que de mosquito ou camundongo sentinela. Uma família (63U11) que foi estudada minuciosamente atravessou filtros de 50 milimicros mas não de 10 milimicros, matou camundongos em período de amamentação e recém-desmamamdos após inoculação intracraniana, e cricetos, mas não cobaia, após inoculação subcutânea. Produziu efeitos citopáticos em culturas de células L de camundongo e de células humanas HeLa e em culturas primárias de células de rim de criceto, mas não em culturas primárias de células de embraião de galinha. O vírus foi inativado por deoxicolato de sódio. Dezesseis outras famílias mataram também camundongos recém-desmamados após inoculação intracraniana ou intraperitoneal e, nesse particular, as famílias do vírus mexicano Nepuyo eram diferentes das famílias originais de Trinidad e do Brasil.

Dépistage et identification de *Nepuyo arbovirus*, groupe C, au Mexique (*Résumé*)

Vingt-trois souches virales, isolées pendant la période 1963-1966 à partir de hamsters sentinelles adultes, de souriceaux sentinelles à la mamelle et de moustiques *Culex* recueillis dans les Etats de Veracruz et de Tabasco (Mexique) ont été identifiées comme appartenant à *Nepuyo arbovirus*, groupe C. On a isolé un plus grand nombre de souches à partir de hamsters sentinelles que de moustiques ou de souris sentinelles. Une des souches (63U11), étudiée en détail, a traversé des filtres de 50 millimicrons mais non des filtres de 10 millimicrons; elle a tué les souriceaux à la mamelle et les souriceaux sevrés, après inoculation intracranienne, ainsi que les hamsters, mais non les cobayes, après inoculation sous-cutanés. Elle a produit des effets cytopathiques dans les cultures cellulaires L de la souris, les cellules HeLa humaines et les cultures cellulaires primaires de rein de hamster, mais non dans es cultures cellulaires primaires d'embryon de poulet.

Le virus a été rendu inactif avec du désoxycholate de sodium. Seize autres souches ont également tué des souriceaux sevrés, après inoculation intracranienne ou intrapéritonéale, et, à cet égard, les souches virales mexicaines Nepuyo étaient dissemblables des souches originales de la Trinité et du Brésil.

Anticuerpos sintéticos

"Grande es, sin duda, el porvenir de la inmunología. Siendo así que nos es perfectamente conocida la estructura molecular de los anticuerpos naturales, con sus cuatro cadenas, la hilación en serie de sus aminoácidos, etc., pronto podremos estar en condiciones de fabricar anticuerpos artificiales. Y estos anticuerpos, obra de la bioquímica al servicio del hombre, no irán acompañados de las reacciones y alergias que siguen siendo características de los sueros actuales."

Prof. Bernard Halpern, Instituto de Inmunobiología, París, (en una estrevista de radio de la OMS).