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DENGUE HEMORRHAGIC FEVER IN CUBA, 1981: RAPID DIAGNOSIS OF THE ETIOLOGIC AGENT[1]

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The 1981 outbreak of dengue-2 in Cuba, which produced cases of hemorrhagic fever, shock syndrome, and death, prompted quick action by Cuban health authorities to diagnose the problem and isolate the responsible disease agent. This article describes that work and its results. It is expected that data relating to the epidemic, derived from this work and other sources, will provide valuable information about dengue etiopathology.

Introduction

Since 1827 many dengue epidemics have been reported in the Caribbean and other parts of the Americas. Initially, of course, diagnosis was based on the disease's clinical characteristics *(1);* but when virologic and serologic methods became available, retrospective studies could be made *(2,3)* to confirm earlier clinical observations. Subsequently, circulation of the four dengue serotypes was demonstrated on a number of Caribbean islands and the American mainland.

Specifically, dengue-3 was found to be circulating in the Caribbean in 1963 *(4,5),* as was dengue-2 in 1968. Dengue-2 predominated in the region in 1969, although dengue-3 was also present *(6).* In 1977 a dengue-1 epidemic occurred in Jamaica; this soon spread to other Caribbean islands and the American mainland *(7).* In 1981 dengue-4 was reported circulating on several islands of the Lesser Antilles—the first time this serotype had been found in the Americas *(8).* As of early 1981, only isolated cases of hemorrhagic fever and dengue shock syndrome had been reported in the Region *(9,10).*

With regard to Cuba, during this century clinically diagnosed dengue cases were reported in 1944 *(11);* but no further cases were notified until 1977, when an outbreak of dengue-1 in the Caribbean produced a large-scale epidemic in Cuba; in all, 477,438 cases of classical dengue were reported, and the presence of the virus on the island *(12)* was confirmed by viral isolation. A serologic survey made in 1975, using dengue-2 antigen, showed 2.6 per cent of the subjects tested to be positive for dengue-2 antibodies. All those yielding positive responses were over 45 years of age *(12),* a result supporting the view that from 1944 to 1977 there was no dengue activity on the island.

In late May of 1981 the health services of Havana Province[8] detected a growing number of patients with symptoms of fever; muscular, postorbital, and abdominal pain; rash; intense headache; and asthenia. In many cases

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these symptoms were accompanied by hemorrhagic manifestations with varying degrees of severity, and in some instances by shock and death.

In view of the urgency of the situation and the need to reach immediate diagnostic conclusions, it was decided to study both single sera from patients experiencing the disease and a group of sera from patients who had had the disease over seven days for the purpose of ascertaining the hemagglutination-inhibition (HI) responses of these sera to dengue, yellow fever, and Chikungunya virus antigens. In addition, serum specimens were immediately collected from patients with acute symptoms for the purpose of virus isolation.

This article reports the results of those initial serologic studies and the circumstances leading to identification of four virus strains isolated within a week of the time the epidemic was recognized. Over the course of a few days, these results made it possible to confirm the presumptive diagnosis initially submitted to the national health authorities. It should also be mentioned that the diagnosis of hemorrhagic dengue cases was carried out according to the criteria established by the Technical Advisory Committee on Dengue Hemorrhagic Fever for the WHO Region of Southeast Asia and the Western Pacific *(13)*.

Materials and Methods

Serology

During the first day the epidemic was reported, serum specimens were obtained from four pediatric patients with manifestations of hemorrhagic dengue; and, on the following day, 16 sera were collected from people in the area where the epidemic began who reported having symptoms compatible with the disease during the preceding 20 days. (Eleven of these 16 subjects manifested hemorrhagic dengue symptoms.) All the sera were treated with acetone; and the hemagglutination-inhibition procedure described by Clark and Casals *(14)*, as adapted to micro-methods, was performed using dengue-1, dengue-2, dengue-3, yellow fever, and Chikungunya virus antigens prepared in mouse brains and extracted by the saccharose-acetone technique.

Virus Isolation

Specimens. The serum specimens used for virus isolation were obtained from patients with clinically diagnosed dengue cases. All were collected within 96 hours of the onset of clinical symptoms. The specimens were transported frozen and were maintained in a frozen state until use.

Inoculation in mice. The sera were inoculated into newborn mice by the intracerebral and subcutaneous routes. Pure sera and sera diluted 1:10 and 1:50 with Medium 199 containing 10 per cent calf serum were used for these inoculations. One litter of mice was used for each inoculation at each dilution, an uninoculated mouse in each litter being left as a control. The inoculated and control mice were then observed for 21 days.

Inoculation in tissue culture. Clone 9 of the LLC-MK_2 cell line was obtained from Cuba's National Institute of Hygiene, Epidemiology, and Microbiology in Havana. This cell line was maintained by serial passages in Medium 199 with 20 per cent calf serum, penicillin (100 I.U. per ml), and streptomycin (100 micrograms per ml). The cells were sown in plastic flasks, and 0.5 ml of each serum specimen was inoculated onto a confluent cell layer and allowed to remain in contact for 15 minutes at room temperature. Subsequently, the same medium with 1 per cent fetal calf serum was used for maintenance. The incubation temperature was 36°C, and the flasks were examined daily for cytopathic effects.

In cases where cytopathic effects were observed, the cells were frozen, thawed, and centrifuged. The resulting supernatant was then used for virus identification.

Virus Identification

Plaque reduction. Virus strains isolated in both mice and tissue cultures were identified by means of the plaque-reduction neutralization test according to the method of Russell and McCown *(15)*. For this purpose we used 1:10, 1:20, 1:40, and 1:80 dilutions of ascitic fluids hyperimmune to dengue virus types 1, 2, 3, and 4 received from the United States Centers for Disease Control (CDC) in Atlanta. These various ascitic fluid dilutions were mixed with equal volumes of each virus suspension (to yield final concentrations of 30-100 plaque formation units (PFU) per 0.1 ml) and were placed in a water-bath for 45 minutes at 37°C. Some of the mixture (0.3 ml) was then inoculated onto LLC-MK_2 cell cultures and was permitted to remain in contact with the cells for 15 minutes. The cells were then overlaid with agar and incubated for five days, after which they were again overlaid—this time with agar containing a 1:7,500 dilution of neutral red stain. Each culture was then read 24 hours after staining.

Immunofluorescence. This method was used for rapid identification of the virus strains isolated by inoculation into mice. Brain tissue specimens from the inoculated mice were mounted on slides, and the indirect immunofluorescence test was performed using 1:20, 1:40, 1:80, and 1:160 dilutions of ascitic fluids hyperimmune to dengue virus types 1, 2, 3, and 4, and to yellow fever virus (received from the Military Institute of Epidemiology and Microbiology of Prague) in combination with mouse antiserum conjugated with fluorescein isothiocyanate.

Results

Table 1 shows the HI test results obtained with the single serum specimens from four children manifesting symptoms of hemorrhagic dengue. As may be seen, the titers were extraordinarily high, with titers to dengue types 1 and 3 and to yellow fever being the highest. No antibodies to Chikungunya virus were detected.

Table 2 shows HI test results obtained with 16 single sera collected approximately 48 hours after the epidemic became known. Most of these single sera, obtained between nine and 21 days after onset of the disease, were found to yield very high titers against the three dengue serotypes tested and against yellow fever virus. They also tended to show a certain predominance of high antibody titers against the dengue-2 serotype. Subject 14, a child two years of age, showed an antigenic response of the primary type (the last previous dengue epidemic in Cuba occurred in 1977). Subject 16, a child five years of age who died with symptoms of shock and hemorrhage, presented a secondary type of antibody response.

Four strains of dengue-2 virus were isolated, the first two in newborn mice inoculated

Table 1. HI test results obtained with single sera from four children with manifestations of hemorrhagic dengue. The specimens tested were all collected within a week of onset of disease symptoms.

No.	Sex	Age (in years)	Days after onset	Observed HI titers with: Dengue-1	Dengue-2	Dengue-3	Yellow fever	Chikungunya
1	F	14	7	10,240	640	20,480	20,480	– *
2	F	10	4	2,560	160	10,240	5,120	–
3	M	10	2	80	20	160	80	–
4	F	14	5	1,280	20	10,240	5,120	–

* – = No reaction.

Table 2. HI test results obtained with single sera from convalescents who were examined within approximately 48 hours of the time the epidemic became known. These sera were procured during the second and third weeks of illness.

No. of subject	Sex	Age	Days since onset of illness	Observed HI titers with:			
				Dengue-1	Dengue-2	Dengue-3	Yellow fever
1	M	32	15	1,280	2,560	1,280	2,560
2	F	45	17	640	1,280	640	640
3	F	34	15	2,560	2,560	1,280	1,280
4	F	25	21	640	2,560	640	320
5	F	37	16	2,560	2,560	10,240	2,560
6	M	54	9	640	2,560	1,280	640
7	F	23	12	2,560	2,560	5,120	640
8	F	22	9	640	2,560	320	1,280
9	M	17	17	640	320	1,280	320
10	M	4	9	5,120	2,560	5,120	2,560
11	M	35	10	320	2,560	1,280	1,280
12	F	17	10	5,120	2,560	5,120	2,560
13	M	5	9	2,560	2,560	1,280	320
14	M	2	9	20	40	20	20
15	F	31	9	10,240	2,560	20,280	1,280
16	F	5	8	640	640	2,560	640

with serum taken from two patients on the third day of their illness. Strain one was isolated from a mouse on the third day following its inoculation, after the mouse showed typical disease symptoms. This mouse had been inoculated with a 1:50 dilution of the serum. (Subsequently, sickness was observed in other mice that had received this serum pure, diluted 1:10, and diluted 1:50.) Dengue was therefore diagnosed on the third day after mouse inoculation, and the serotype involved was determined on the fourth day (see Table 4) by means of the indirect immunofluorescence method. The second dengue-2 strain was isolated 16 days after mouse inoculation from a mouse receiving a 1:50 dilution of serum.

The third and fourth dengue-2 strains were isolated directly in LLC-MK_2 cell cultures inoculated with the sera of patients who had been sick for four and two days, respectively. The cytopathic effect, evidenced by a rounding off and detachment of cells, began to appear on the fifth day after inoculation.

The patients yielding dengue-2 strains one and two were women 31 and 25 years of age, respectively, and those yielding strains three and four were boys 14 and 15 years of age, respectively. HI tests with sera from the first two patients failed to detect dengue antibodies; sera from the last two patients were not subjected to HI testing.

Tables 3 and 4 show data obtained with plaque reduction testing of these four isolates and indirect immunofluorescence testing of the first two. Plaque reduction testing of all four strains yielded very similar results. That is, 50 per cent plaque reduction was produced in each case by a 1:80 dilution of the dengue-2 hyperimmune mouse ascitic fluid, while the yellow fever and other dengue ascitic fluids yielded results that were essentially negative.

In a similar fashion, isolates one and two yielded positive immunofluorescence test responses with dengue-2 hyperimmune ascitic fluid dilutions of 1:160 and 1:80. However, much lower dilutions (no greater than 1:20) were required to produce detectable immunofluorescence with ascitic fluid sensitized to yellow fever and the other dengue serotypes.

Table. 3 Identification of the four isolated dengue strains by the plaque reduction method.

Isolate strain number	Highest dilutions of hyperimmune ascitic fluid for the indicated virus producing a plaque reduction of at least 50 per cent				
	Dengue-1	Dengue-2	Dengue-3	Dengue-4	Yellow fever
1	1:10	1:80	1:10	1:10	1:10
2	1:10	1:80	1:10	1:10	Not done
3	1:10	1:80	1:10	1:10	Not done
4	1:10	1:80	1:10	1:10	Not done

Table 4. Immunofluorescence test results obtained with the first two dengue-2 strains isolated.

Dengue strain tested	Highest dilutions of ascitic fluid for the indicated virus at which immunofluorescence was apparent				
	Dengue-1	Dengue-2	Dengue-3	Dengue-4	Yellow fever
Isolate 1	1:20	1:160	1:20	–	Not done
Isolate 2	–	1:80	1:20	–	1:20
Dengue-2 (from New Guinea)	1:20	1:640	1:20	–	Not done

– = Negative result.

Discussion and Conclusions

Overall, these data clearly suggest that the virus causing the epidemic of dengue hemorrhagic fever which occurred on Cuba in mid-1981 was dengue-2. As in the 1977 epidemic *(12),* it was not possible to determine the circulating dengue serotype by means of HI testing, a difficulty that was confirmed as the epidemic progressed. However, correlation of the clinical, epidemiologic, and serologic findings made it possible to determine within hours that we were facing an outbreak of disease cases caused by a new dengue serotype, together with an epidemic of hemorrhagic fever. On the basis of these findings, a campaign to control this epidemic and *Aedes aegypti,* the responsible mosquito vector, was launched immediately.

Subsequently, the responsible dengue-2 virus was quickly isolated and identified. As already noted, this was done four days after mouse inoculation by means of the indirect immunofluorescence test, the results of which were confirmed by plaque reduction.

The sudden emergence of this epidemic in Cuba, without dengue-2 activity having been reported in the Americas or in the countries with which Cuba maintains close relations, shrouds the origin of this serious outbreak in mystery. However, as a result of these developments, the health authorities of the Caribbean area and of potentially affected mainland portions of the hemisphere should be alerted to the presence of dengue hemorrhagic fever and shock syndrome, which have manifested themselves in an epidemic fashion for the first time in this part of the world. In addition, study of the data obtained in this case, where the affected country has experienced two epidemics caused by different dengue serotypes (1 and 2) within a three-year period, should prove of great interest and should provide important information that will help with interpretation of the etiopathology of this disease.

SUMMARY

In late May of 1981 health services in Cuba's Havana Province recorded increasing numbers of disease cases with dengue-like symptoms, together with cases of apparently dengue-related hemorrhage, shock, and death.

Responding to this threat, health authorities obtained sera from patients currently manifesting dengue-like symptoms as well as from others who had had the disease for over seven days. These were subjected to hemagglutination-inhibition (HI) tests for the purpose of disease diagnosis, and to various other procedures designed to isolate and identify the responsible virus.

Sera from four children with hemorrhagic symptoms yielded high antibody titers against yellow fever, dengue-1, and dengue-3 viruses and relatively low antibody titers against dengue-2. However, 15 of 16 convalescent sera showed strongly positive antibody responses to all four viruses.

One of various mice inoculated with patients' sera in an attempt to isolate the virus became sick three days after inoculation. The following day, immunofluorescence testing of the mouse's brain tissue, using ascitic fluids hyperimmune to dengue virus types 1, 2, 3, and 4 and to yellow fever virus, indicated that dengue-2 was responsible for the epidemic. Subsequent testing of this and other virus isolates provided confirmation.

Data gathered during the course of this epidemic, where cases of hemorrhagic fever and shock syndrome occurred during the second of two successive epidemics caused by different dengue serotypes, are expected to provide valuable information about dengue etiopathology.

Because no other dengue-2 activity had previously been reported in the Americas or in countries with which Cuba maintains close relations, the origins of the epidemic are unclear. However, the outbreak has served to alert health authorities in the Caribbean and other vulnerable portions of the Americas that dengue hemorrhagic fever and shock syndrome have been occurring in an epidemic fashion for the first time in this part of the world.

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SURVEY OF ACUTE RESPIRATORY INFECTIONS IN CHILE

Chile's Institute of Public Health in Santiago has been actively involved in surveillance of acute respiratory infections, including influenza, for several years. Data for 1981 indicated that acute respiratory infections in children were responsible for about 50 per cent of all consultations, the prevailing pathogens detected being respiratory syncytial virus and parainfluenza virus type 3.

A study on viral respiratory infections among children under two years of age was carried out in the period May-December 1982; 199 disease cases were investigated. The highest number of cases was found during the winter months (June-August) when an outbreak of respiratory syncytial virus occurred; this outbreak reached a peak during the last week of July.

Positive laboratory results were obtained in 88 of the 199 investigated cases, over half of which occurred in infants under six months old. Respiratory syncytial virus was the most commonly diagnosed agent. It alone was detected in 56 cases, and in seven more cases it was found in conjunction with other viruses. Parainfluenza virus types 1, 2, or 3 were detected in 10 cases, adenoviruses in nine cases, and herpes simplex and *Mycoplasma pneumoniae* in one case each. In 11 cases more than one agent was detected; these multiple infections usually involved respiratory syncytial virus in combination with adenovirus, parainfluenza virus, or enterovirus, or else infection with two or three types of parainfluenza virus.

Source: Virology Section, Institute of Public Health of Chile, as reported in the World Health Organization *Weekly Epidemiological Record* 58(22):171, 1983.