ISOLATION OF TWO STRAINS OF DENGUE VIRUS TYPE 3 IN SINGAPORE

By Y. C. Chan, PH.D., K. Kanapathipillai and K. S. Chew
(From the Department of Bacteriology, University of Singapore)

Dengue virus was first isolated in 1960 in Singapore when two virus strains (dengue virus types 1 and 2) were recovered from acute phase blood of two patients during an outbreak of haemorrhagic fever (Lim, Rudnick and Chan, 1961). In the following year, three strains of dengue virus type 4 were also isolated from patients' blood (Lim, Chan, Phoon and Hanam, 1964). In 1963, among the eleven strains of dengue virus isolated from acute phase blood of patients, two strains were identified as dengue virus type 3. This is the first report of the isolation of dengue virus type 3 in Singapore. The isolation and identification of the two virus strains and the serological findings on the patients' sera are described in this paper.

VIROLOGICAL METHODS

Infant mice, 1-2 days' old, were used for the isolation of virus. The isolation procedures have been described (Lim, Chan, Phoon and Hanam, 1964).

The virus strains were identified by the complement-fixation (CF), haemagglutination-inhibition (HI), and microprecipitin agar gel diffusion tests. The following prototype virus antigens and hyperimmune mouse sera were used in the identification of the virus strains: dengue type 1 (Hawaiian strain), dengue type 2 (New Guinea "C" strain), dengue type 3 (H-87 strain), dengue type 4 (H-241 strain) and Japanese encephalitis (Nakayama strain). In the CF test, the micro drop method was used, and the results were analysed according to the method of Lim, Chan, Phoon and Hanam (1964) with one modification. Heterologous serum-antigen reactions were compared with the homologous reaction and expressed as the cross-fixation ratios of the homologous reaction. Each pair of virus antigens was then compared by taking the product (not the arithmetic mean) of their cross-fixation ratios (Bradish and Brooksby, 1960). The values of the cross-fixation product indicate the degree of antigenic relationship between virus strains. Identical virus strains have cross-fixation products of 1.0 while antigenically different viruses have products of less than 1.0 depending upon the degree of difference. The HI test of Clarke and Casals (1958) was used. Antigens for the CF and HI tests were prepared by sucrose-acetone extraction, unless otherwise indicated. The microprecipitin agar gel diffusion technique has been described (Chan, 1965).

RESULTS

The S-888/63 virus strain was isolated from acute phase blood of a young adult patient with haemorrhagic fever on November 11. The blood specimen was obtained on the fourth day of illness. The S-1033/63 virus strain was isolated from acute phase blood of a 14-year-old patient with haemorrhagic fever on December 23. His blood was obtained on the second day of illness. Infant mice inoculated with both blood specimens first showed signs of sickness on 8th day after inoculation. The two virus strains were finally adapted to infant mice after ten mouse brain passages, when all the mice became sick regularly on 7th day.

The S-888/63 and S-1033/63 virus strains were compared with the four prototype dengue viruses in a cross CF test. The cross-fixation ratios and products obtained for the two virus strains are shown in Table I. The S-888/63 and S-1033/63 virus antigens have cross-fixation products of 1.26 and 0.49, respectively, with dengue virus type 3 antigen, and these results are the highest obtained between the antigens being compared. The two virus strains are therefore closely related antigenically to dengue virus type 3. The S-1033/63 virus antigen has a cross-fixation product of 0.8 with S-888/63 virus antigen, showing close antigenic relationship of the two virus strains. The identification of S-1033/63 virus strain was confirmed in another CF test (Table II).

TABLE I

The cross-fixation ratios of S-888/63 and S-1033/63 virus antigens and antisera						
Virus antigen	Serum					
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-888/63	S-1033/63
Dengue 1	1.0				10.5	3.0
Dengue 2		1.0			8.5	3.0
Dengue 3			1.0		9.0	2.6
Dengue 4				1.0	14.5	5.2
S-888/63	0.03	0.03	0.14	0	1.0	0.8
S-1033/63	0	0	0.19	0	1.0	1.0
Cross-fixation products showing antigenic relationship						
Virus	Virus					
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-888/63	S-1033/63
S-888/63	0.31	0.25	1.26	0	1.0	
S-1033/63	0	0	0.49	0	0.8	1.0

TABLE II

The cross-fixation ratios of S-1033/63 virus antigen and antiserum					
Virus antigen	Serum				
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-1033/63
Dengue 1	1.0				0.2
Dengue 2		1.0			0.7
Dengue 3			1.0		1.5
Dengue 4				1.0	0.2
S-1033/63	0.23	0.27	0.71	0.22	1.0
Cross-fixation products showing antigenic relationship					
Virus	Virus				
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-1033/63
S-1033/63	0.05	0.19	1.1	0.04	1.0

TABLE III

Identification of S-888/63 and S-1033/63 virus strains by haemagglutination-inhibition test

Virus (8 units antigen)	Serum **					
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-888/63	S-1033/63
Dengue 1	160	40	80	40	40	160
Dengue 2	20	160	80	40	80	160
Dengue 3	80	160	320	160	160	320
Dengue 4	40	80	160	320	40	80
S-888/63*	40	80	320	80	160	320
S-1033/63*	20	40	160	40	80	160

* Antigens were 20% crude infant mouse brain suspensions.

** HI antibody titre expressed as highest dilution of serum showing complete inhibition by antigen.

TABLE IV

The cross-inhibition ratios of S-888/63 and S-1033/63 virus antigens and antisera

Virus antigen	Serum					
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-888/63	S-1033/63
Dengue 1	1.0	0.25	0.5	0.25	0.25	1.0
Dengue 2	0.12	1.0	0.5	0.25	0.5	1.0
Dengue 3	0.25	0.5	1.0	0.5	0.5	1.0
Dengue 4	0.12	0.25	0.5	1.0	0.12	0.25
S-888/63	0.25	0.5	2.0	0.5	1.0	2.0
S-1033/63	0.12	0.25	1.0	0.25	0.5	1.0

Cross-inhibition products showing antigenic relationship

Virus	Virus					
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-888/63	S-1033/63
Dengue 1	1.0					
Dengue 2	0·03	1.0				
Dengue 3	0.12	0.25	1.0			
Dengue 4	0.03	0.06	0.25	1.0		
S-888/63	0.06	0.25	1.0	0.06	1.0	
S-1033/63	0.12	0.25	1.0	0.06	1.0	1.0

TABLE V

Antibody responses of patients by complement-fixation test

Patient	Blood taken on day	Antigen*					
		Dengue 1	Dengue 2	Dengue 3	Dengue 4	JE	Chik
S-888/63	4	ND	ND	ND	ND	ND	ND
	45	128	<8	8	256	64	<8
	50	64	<8	16	256	128	<8
S-1033/63	2	<4	<4	<4	<4	16	ND
	42	32	<4	4	4	128	ND

* In the test with S-888/63, the following Singapore virus strains were used: Dengue 1(S-601/60), Dengue 2 (S-521/62), Dengue 4 (S-554/61) and JE (Muar). JE = Japanese encephalitis. Chik.= chikungunya. ND = not done.

TABLE VI

Antibody responses of patients by haemagglutination-inhibition test

Patient	Blood taken on day	Antigen*					
		Dengue 1	Dengue 2	Dengue 3	Dengue 4	JE	Chik
S-888/63	4	ND	ND	ND	ND	ND	ND
	45	⩾2560	⩾2560	⩾2560	⩾2560	⩾2560	<20
	50	⩾2560	⩾2560	⩾2560	⩾2560	⩾2560	<20
S-1033/63	2	<20	<20	<20	<20	160	ND
	42	2560	2560	2560	2560	1280	ND

* In the test with S-888/63, the following Singapore virus strains were used: Dengue 1; (S-601/60), Dengue 2 (S-521/62). Dengue 4 (S-554/61), JE (Muar).

The two virus strains and the four prototype dengue viruses were also compared in a HI test (Table III). Taking a four-fold difference in antibody titre as a criterion, S-888/63 and S-1033/63 virus strains are shown to be dengue virus type 3. When the HI results are expressed in the form of cross-inhibition ratios and products, the antigenic relationship of the viruses becomes more apparent (Table IV).

The identification of S-888/63 and S-1033/63 virus strains as dengue virus type 3 was finally confirmed by the microprecipitin agar gel diffusion test. In this test, homotypic precipitation was obtained between the two virus strains and dengue virus type 3 antiserum, but not with antisera to dengue virus types 1, 2 and 4.

The antibody responses of the two patients are shown in Tables V and VI. Although the acute phase blood of patient S-888/63 was not available for testing, his convalescent phase serum taken 45 and 50 days after onset of disease showed high CF and HI antibody titres to both dengue and Japanese encephalitis virus antigens. A CF antibody titre of 1:256 and a HI antibody titre of equal or greater than 1:2560 in a single blood specimen, in our experience, is suggestive of a recent group B arbovirus infection. No antibody to chikungunya virus antigen was detected in the patient's sera.

In patient S-1033/63, a significant rise in CF and HI antibody titres to dengue and Japanese encephalitis virus antigens was demonstrated between the acute and convalescent phase sera. This patient probably had a group B arbovirus infection previous to the present infection, as shown by the presence of CF and HI antibodies to Japanese encephalitis virus antigens in the acute phase serum.

DISCUSSION

With the isolation of dengue virus type 3 strains in 1963, all the four types of dengue virus have been shown to occur in Singapore.

The use of the cross-fixation or cross-inhibition products in demonstrating antigenic relationship between viruses in the complement-fixation and haemagglutination-inhibition tests has been shown to be of value in the identification of dengue viruses. In the present investigation, the use of the mean antigenic relationship (MAR) of Lim, Chan, Phoon and Hanam (1964) in analysing the complement-fixation results did not demonstrate clearly the antigenic relationship of the two virus strains to the prototype dengue viruses. The MAR values and the cross-fixation products obtained in Table I are tabulated in Table VII for comparison. The high MAR values obtained in this instance appears to be due to the low potency of S-888/63 and S-1033/63 virus antigens and the high antibody titres of their antisera to the prototype dengue virus antigens. This is seen in the high cross-fixation ratios in Table I. By using the

TABLE VII

The mean antigenic relationship and the cross-fixation products of S-888/63 and S-1033/63 virus antigens and antisera

Virus	Virus					
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	S-888/63	S-1033/63
S-888/63						
MAR	5·26	4.26	4.57	7.25	1.0	
CFP	0.31	0.25	1.26	0	1.0	
S-1033/63						
MAR	1.5	1.5	1.39	2.6	0.9	1.0
CFP	0	0	0.49	0	0.8	1.0

MAR = mean antigenic relationship
CFP = cross-fixation product.

cross-fixation products, it has been possible to identify the two virus strains as dengue virus type 3. The use of the cross-fixation products in the complement-fixation test to measure the minor antigenic differences between strains of foot-and-mouth disease virus of the same immunological type has recently been reported (Davie, 1964).

The haemagglutination-inhibition test in the identification of dengue viruses has been known to be of little value, owing to extensive cross-reactions occurring among these viruses. When the haemagglutination-inhibition antibody titres are expressed as the cross-inhibition products, it is possible to demonstrate antigenic differences between dengue viruses.

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