

Dengue Infection at the Children's Hospital of Bangkok

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OBJECTIVE: To provide viral diagnostic and laboratory expertise to the Children's Hospital of Bangkok and to collect specimens for specialized dengue virus isolation and serology.

BACKGROUND: Dengue virus infections continue to be an annoying and potentially critical problem for military forces stationed in many tropical areas. Dengue infections are also a major cause of morbidity and mortality among children in Southeast Asia. As in previous years SEATO Medical Research Laboratory has collaborated with the Children's Hospital of Bangkok in the study of dengue hemorrhagic fever. This has been mutually beneficial allowing for improved patient care through diagnostic and laboratory work provided by SEATO Laboratory and allowing for the collection of specialized samples from dengue patients to allow for investigations of the pathogenesis and clinical expressions of this infection.

DESCRIPTION: Patients with a hospital admission diagnosis compatible with dengue infections (dengue hemorrhagic fever, dengue fever or undifferentiated fever) were selected from the infectious disease wards of the Bangkok Children's Hospital. A standardized chart of pertinent signs, symptoms and laboratory findings was instituted on each patient.

An attempt was made to collect blood on at least the day of diagnosis and on the third, fifth, fifteenth and thirtieth days after hospitalization. Blood was allowed to clot or was collected using heparinized tubes (~20 u heparin/ml blood). Studies were done on either serum or plasma. During the month of July and August plasma was removed from heparinized blood and the cellular components were separated using a dextran sedimentation technique. Peripheral blood leukocytes were used by Dr. Nyven J. Marchette, University of Hawaii, in an investigation of the occurrence and specificity of *in vitro* antibody production, and to study the phenomenon of permissive peripheral blood leukocytes previously identified in monkeys. Platelets and, in a few cases, leukocytes as well as plasma were submitted for virus isolation.

Virus isolation was performed using a direct and delayed plaque technique. Plasma was also inoculated into mosquitoes, both at SEATO Medical Research Laboratory and at the University of Hawaii, to test the mosquito isolation technique of Rosen *et al* (see elsewhere in this report). Sera or plasma were used for serology; hemagglutination inhibition (HI) tests were performed using suckling mouse brain antigens prepared from dengue 1 (Hawaii), dengue 2 (New Guinea C), dengue 3 (H-87), dengue 4 (H-241), Japanese Encephalitis (Nakayama) and Chikungunya (Ross). Sera were extracted with acetone and tested against eight units of antigen. All sera collected from one patient were tested simultaneously.

At the conclusion of hospitalization, a clinical discharge diagnosis was made and the severity of the illness was independently classified by clinicians in charge of the case.

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Grading of severity of dengue hemorrhagic fever used criteria established by one of us.

Grade I: Fever accompanied by non-specific constitutional symptoms. The only hemorrhagic manifestation is a positive tourniquet test.

Grade II: Fever and skin hemorrhage or other bleeding such as epistaxis or gingival hemorrhage.

Grade III: Circulatory failure manifested by weak, rapid pulse with narrowing of pulse pressure (less than 20 mm Hg) or hypotension (systolic pressure 90 mm Hg or less).

Grade IV: Moribund patients with undetectable blood pressure or pulse.

Following grading, isolation and serological data were used to identify those patients infected with dengue and to determine the type of antibody response. Patients were considered to have had a dengue infection if a four-fold rise in HI antibody titer to at least two of the group B antigens was found between the acute and convalescent sera or if convalescent antibody titers to at least two antigens equaled or exceeded 1:640. Criteria for the identification of primary or secondary dengue have been previously reported. Patients with convalescent HI titers of 1:640 or more to at least two dengue antigens were considered to have secondary infections while those with convalescent HI titers of less than 1:640 were considered to have primary infections. Where necessary to clarify the occurrence of a primary or secondary infection, plaque reduction neutralization by selected sera of appropriate Group A and Group B seed viruses were performed.

In a few cases immunoglobulin separations were performed using sucrose gradient ultracentrifugation. The original sera and the fractions obtained from the sucrose gradients were tested for IgM, IgG and B1C/B1A concentrations using radial diffusion plates (Hyland Laboratories). Antibody contained in these fractions was assayed by hemagglutination inhibition with and without treatment with 2 mercaptoethanol (2 ME). The 2 ME treatment was used to reduce IgM antibody activity.

One hundred and thirty four patients with admission diagnoses compatible with dengue infection were seen on the ward. One hundred and twenty seven of these were adequately followed and 114 (90%) were diagnosed as dengue infection by viral isolation, by serological criteria or both. Sixteen strains of dengue virus were isolated by direct or delayed plaque technique from either the plasma or the cellular components from the blood of the 114 patients with evidence of dengue infection (Table 1). In some cases isolations were made from the cellular components only. This represents an isolation rate of 14%. Five strains were dengue 1, five were dengue 2, three were dengue 3 and three are as yet unclassified. No dengue 4 was identified in Bangkok in 1974. Further details of isolation by the direct and delayed plaque technique and the mosquito isolation procedure will be found elsewhere in this report.

Of the 114 patients diagnosed as dengue, 13 (11.4%) patients had low level antibody responses detected by HI and were considered primary dengue infections, 94 (84%) had high HI titers and were considered secondary infections. Of the latter, 10 patients exhibited high fixed titers and 84 showed rising titers. Seven (6%) patients died, usually before the fifth day of disease. As no convalescent sera could be collected in these cases, the patients could not be classified on serological grounds as having primary or secondary infections. Laboratory findings on the 107 patients on whom classification could be completed may be found in Table 2. Dengue hemorrhagic fever occurred in patients with either primary or secondary infections and the distribution of clinical grades was essentially similar to that found in previous studies of hospitalized patients with one major exception.

This year, close clinical observation allowed for the detection of shock in three patients (D74-77, 91 and 103) exhibiting an antibody pattern characteristic of primary dengue infection (Table 3).

These three patients were investigated further. Figure 1 is a flow diagram of the clinical course of one of them (D74-77). In this patient shock occurred in the morning of the sixth day at a time when the fever was subsiding. The rising hematocrit and the falling platelet counts occurred over a short period of time just prior to the onset of shock. HI antibody titers at this time were between <1:20 and 1:80

against dengue 1 virus and were lower against other dengue types. Complement factor three, as estimated from the B1C/B1A concentrations, was 74 mg% on day 5; it fell to 54 mg% on day 7 and rose slightly to 64 mg% by day 9. It was measured again on days 19 and 34 when levels had returned to 136 mg%; a value within the normal range of 143 ± 22 mg% reported by Hyland Laboratories. The clinical course of D74-77 was essentially similar to those of the other two cases investigated.

From one of these patients (D74-103) a dengue 3 virus was isolated. No virus was identified in the other two (D74-77 and 91). Plaque reduction neutralization tests of all four dengue types and Chikungunya were performed on acute and convalescent sera from each patient. The results are currently available from two of them (D74-77, 91) (Table 3). There was no appreciable antibody to any of the viruses in either of the acute sera obtained on the fifth day of disease. In the convalescent sera from both cases high titered antibody to dengue 1 was found. In one (D74-77), low level antibody was also found to dengue type 3 and to Chikungunya. This patient received several units of plasma which may have contained antibody to group A and B arboviruses and might have caused the serological findings.

In order to determine whether dengue specific IgM was produced in these patients as would be expected in primary infection, sucrose gradient ultracentrifugation was performed on convalescent sera (Table 4). IgM antibody was found in the second through the fourth sucrose fraction (35-31% sucrose) of each serum studied. Specific IgM (reduced by 2 ME) was found against all four dengue types when tested by HI. In D74-77 and 91 the highest titers were found against dengue 1. In a convalescent serum from a case of secondary dengue (identified by HI antibody titers $\geq 1:20480$), which was centrifuged simultaneously, IgM antibody was again found in the second, third and fourth fraction. A low level of antibody activity was also found in these fractions against all four dengue types but it could not be reduced by 2 ME indicating that the antibody activity was possibly due to IgG contamination. In none of the sera tested in this manner was any 2 ME reducible antibody to Chikungunya detected in the IgM portion of the gradient. In the convalescent serum from D74-77, low level antibody to Chikungunya was found in the IgG portion of the gradient; this activity was not reduced by 2 ME.

Because of the association of shock in dengue with low complement levels, B1C/B1A concentrations were determined on the acute and convalescent sera of all three patients. In all cases the B1C/B1A concentrations were reduced to less than 50% of normal in the acute sera and in sera taken at the time of shock. The concentration increased to the normal range during convalescence. B1C/B1A levels in five patients with primary disease without shock were either in the normal range or only slightly depressed throughout the course of the disease.

DISCUSSION: As in previous years SEATO Medical Research Laboratory has provided laboratory and diagnostic support to the Children's Hospital of Bangkok in an attempt to delineate dengue infection in Bangkok. This year, through close clinical observation, shock was detected in three patients whose HI antibody titers indicated a primary dengue infection. Efforts to firmly establish the nature of these infections are presently underway.

Table 1. Dengue Isolation by Direct and Delayed Plaque Technique — 1974

Patient	Source ^a	Plaque Technique ^b			Identification
		Direct	Delayed	Secondary Delayed	
D74-16	Plasma	11 ^c	—	—	D2
D74-33	Plasma	28	—	—	D3
	Platelets	106	—	—	
D74-38	Plasma	16	—	—	D2
	Platelets	15	—	—	
D74-44	Plasma	7	—	—	D1
D74-61	Platelets	0	6	—	D1
D74-63	Plasma	0	22	—	D1
	Platelets	2	—	—	
D74-66	Platelets	0	TNTC ^d	—	D2
D74-74	Leukocytes	2	—	—	D2
D74-90	Plasma	0	72	—	?
D74-95	Plasma	63	—	—	D1
D74-103	Plasma	121	—	—	D3
D74-104	Plasma	0	0	66	?
D74-112	Plasma	118	—	—	D1
D74-137	Plasma	0	17	—	?
D74-150	Plasma	TNTC	—	—	D2
D74-151	Plasma	TNTC	—	—	D3

^a — Isolation from plasma was attempted in every case.

^b — Direct, Delayed and Second delayed plaque techniques were used; using 0.3 ml of plasma

^c — Number of plaques counted.

^d — TNTC = too numerous to count.

Table 2. Hemagglutination Inhibition Antibody Levels in Convalescent Sera from 107 Patients with Dengue Infection

Grade of Disease	Primary Infection Titer <1:640	Secondary Infection Titer ≥1:640
UC ^a	0	1 (0.9%)
UF ^b	5 (4.7%)	8 (7.4%)
I & II	5 (4.7%)	50 (46.7%)
III	3 (2.8%)	29 (27.1%)
IV	0	6 (5.6%)
TOTAL	13 (12.1%)	94 (87.8%)

a. UC indicates that the patient was unclassified.

b. UF indicates undifferentiated fever.

Table 3. Hemagglutination Inhibition and Plaque Reduction Neutralization Tests of Selected Dengue Hemorrhagic Fever Patients Seen in 1974

Study Number	Day of Disease	Reciprocal Hemagglutination Inhibition Titer				Reciprocal Plaque Reduction Neutralization Titer				
		D1	D2	D3	D4	D1	D2	D3	D4	Chitk
D74-77	5	<20	<20	<20	<20	<20	<20	<20	~40	<10
	17	80	40	160	160	>1280	~40	~120	>40	~160
	36	40	20	40	40	>1280	~40	~230	≥40	~120
D74-91	5	40	<20	20	<20	<20	<20	<20	<20	ND
	11	160	40	80	160	<20	<20	<20	<20	ND
	67	160	80	80	160	500	<40	<40	<40	<10
D74-103	3	<20	<20	<20	<20	NC	NC	NC	NC	NC
	7	<20	20	40	<20	NC	NC	NC	NC	NC
	58	20	20	160	80	NC	NC	NC	NC	NC

ND = Not done

NC = Not complete

Table 4. IgM Antibody Titrations of Sera Taken from Selected Dengue Hemorrhagic Fever Patients Seen in 1974

Patient No.	Day of Disease	Sucrose Fraction	IgM* mg %	IgG* mg %	Reciprocal HI Antibody Titer							
					D 1*** B** 2ME		D 2 B 2ME		D 3 B 2ME		D 4 B 2ME	
D74-77	9 (1725)	2	15	0	16	0	0	0	4	0	8	0
		3	24	0	32	0	4	0	16	0	8	0
		4	> 50	0	64	0	32	0	8	0	64	0
		5	20	0	16	0	8	0	8	0	16	0
D74-91	6 (1993)	2	20	0	32	0	0	0	8	0	4	0
		3	44	0	64	0	4	0	8	0	8	0
		4	7	0	32	0	0	0	4	0	8	0
		5	0	0	32	0	0	0	4	0	8	0
D74-103	7 (2112)	2	11	0	0	0	0	0	0	0	0	0
		3	> 50	0	0	0	0	0	32	4	0	0
		4	14	0	0	0	0	0	8	0	0	0
		5	0	4.5	0	0	0	0	0	0	0	0
D74-6	11 (1087)	2	18	0	8	8	8	4	16	16	8	4
		3	35	0	16	16	16	8	32	32	16	16
		4	12.5	0	64	32	16	16	64	64	32	32
		5	0	8.5	256	512	128	256	256	512	256	256

* Concentrations of IgM and IgG as detected by radialimmunodiffusion

** Sucrose gradient fractions pretreated with buffer

*** Sucrose gradient fractions pretreated with 2 mercaptoethanol

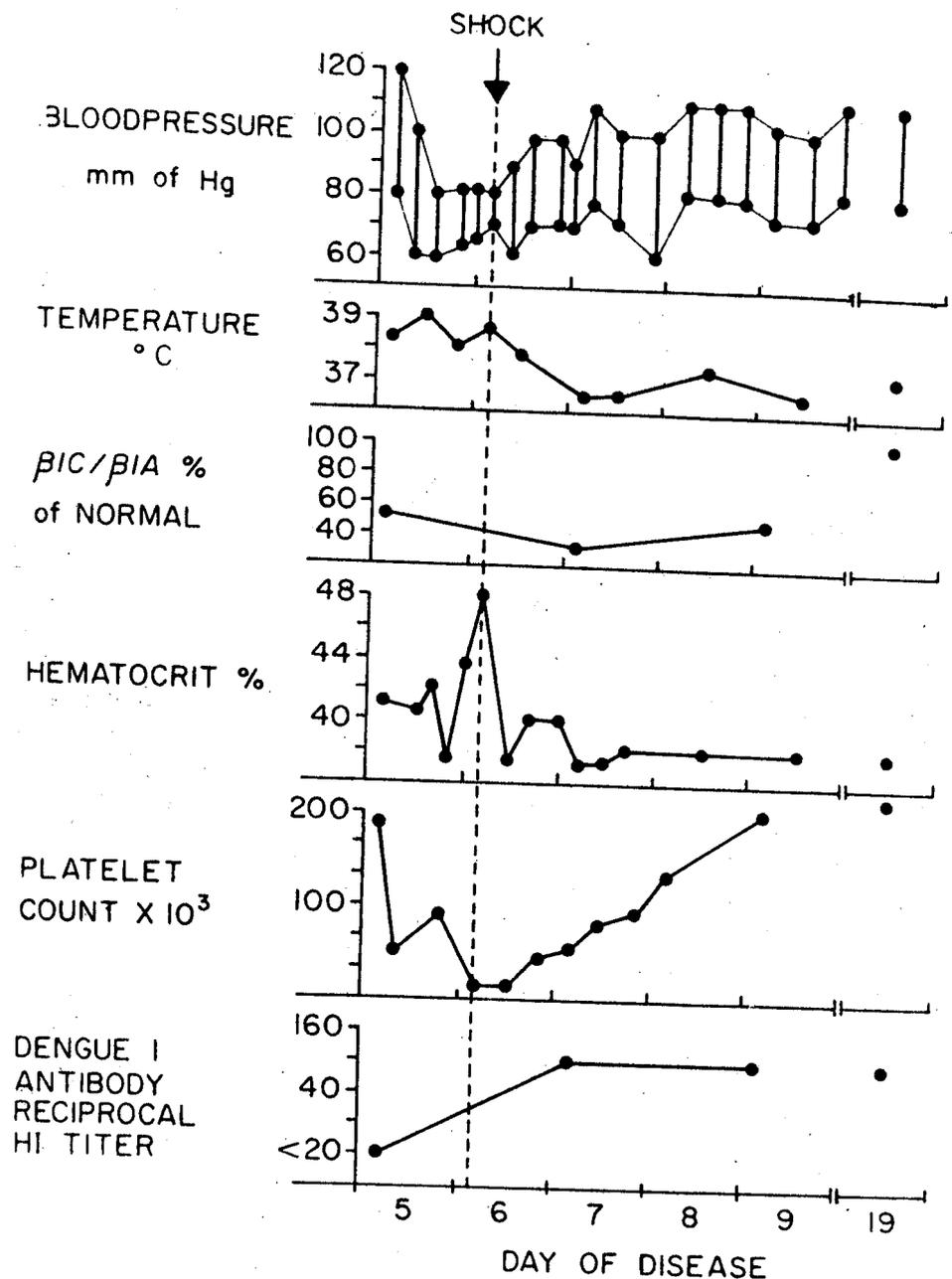


Figure 1. Diagram of the clinical course of patient D74-77 showing the relationship of several clinical and laboratory variables to the onset of shock.