

Isolation of Viruses from Leukocytes of Dengue Patients

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OBJECTIVE : To determine if dengue virus can be isolated from leukocytes during natural dengue infections and to identify the cells infected.

BACKGROUND : Dengue viruses have classically been isolated from human serum or plasma. Studies on the pathogenesis of dengue virus infections in man and monkeys suggested that these viruses may also be associated with the formed elements of the blood. That peripheral blood leukocytes might be a source of virus has been shown by Marchette *et al.* (1) in dengue infected Rhesus monkeys. In man, fluorescent antibody studies by Boonpucknavig *et al.* have identified leukocyte associated dengue antigens (2). Also, several laboratories have demonstrated the *in vitro* replication of dengue viruses in varying types of human leukocyte cultures (3, 4, 5 and 6). These observations indicated that isolation of viruses from the leukocytes of dengue patients might be rewarding.

METHODS : Clinical histories and blood samples were collected from patients admitted to the Bangkok Children's Hospital. The first day of fever was defined as the first day of clinical illness. Two blood samples were collected on the day of admission and approximately 15 days later.

The severity of illness was graded using the following criteria.

- Grade I : Fever accompanied by non-specific constitutional symptoms; the only hemorrhagic manifestation is a positive tourniquet test.
- Grade II : Fever accompanied by skin hemorrhage or other bleeding such as from the nose or gums.
- Grade III : Circulatory failure manifested by rapid, weak pulse, narrowing of pulse pressure (≤ 20 mm Hg) or hypotension.
- Grade IV : Blood pressure and pulse are undetectable.

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Grades I and II were considered dengue hemorrhagic fever without shock and grades III and IV were dengue shock syndrome. Sera obtained from each individual were tested simultaneously for antibodies by hemagglutination inhibition tests.

Each case was classified as either primary or secondary dengue infection. Patients with convalescent titers less than 1:640 to three or more dengue types were assumed to have primary infections. Those with convalescent titers of 1:640 or greater to two or more dengue antigens were considered to have secondary infections.

A dextran sedimentation method was used to separate the formed components of the blood. Heparinized blood was divided into cell free plasma and a cellular pellet by centrifugation. The pellet was resuspended in Dextran T-250 and the red blood cells were allowed to sediment and were discarded. The supernatant was centrifuged at 150 x g to sediment the leukocytes. Viruses were isolated using a direct and/or delayed plaque technique on LLC-Mk₂ cells depending on the sample. In some cases, aliquots of leukocyte suspensions were transferred to tissue culture flasks. Following incubation, nonadherent cells were removed and adherent cells were vigorously washed. Both types of cells were assayed for virus by a delayed plaque technique. Isolates were confirmed and identified by a plaque reduction neutralization test using monkey antisera prepared against prototype dengue strains.

RESULTS : Isolation from plasma and leukocytes were attempted on 211 patients who were adequately followed and had a clinical picture of dengue infection. Six of these had serological evidence of chikungunya and one patient yielded chikungunya virus from both plasma and leukocytes. Serological evidence of dengue infection was found in 195 of these patients; 47 of them yielded virus. To date, we have identified 32 dengue strains, 14 dengue type 4 and 18 dengue type 2. The 47 viruses were composed of one isolate from plasma alone, 15 isolates from plasma and leukocytes and 31 isolates from leukocytes alone (Table 1). The use of leukocytes allowed over three times the recovery rate compared to that obtained from plasma.

Table 1. Virus Isolations from 195 Dengue Patients
Bangkok 1976 - 1977.

Specimen	Number	Percent of Isolations
Plasma	1	2.1
Leukocytes	31	66.0
Both	15	31.9
Total	47	100.0

The number and percent of isolates by day of clinical illness are shown in Table 2. Samples obtained early in the courses of disease were most likely to yield virus. Plasma served as a source of virus in 8.2% of patients as compared to 23.5% for leukocytes. Plasma yielded virus isolations during the first four days of disease while virus could be recovered from leukocytes through the sixth day of disease. Furthermore in samples collected during the first four days, plasma yielded virus in only 22% in contrast to leukocytes which yielded almost 50%

Table 2. Isolation of Viruses from Human Plasma and Leukocytes during Dengue Infections.

Day of ^a Disease	Patients Studied	Positive Specimens					
		Plasma		Leukocytes		Both	
		No.	%	No.	%	No.	%
2	3	1	33.3	1	33.3	1	33.3
3	21	9	42.9	15	71.4	16	76.2
4	49	6	12.2	18	36.7	18	36.7
5	57	0	0	10	17.5	10	17.5
6	31	0	0	2	6.5	2	6.5
7-10	34	0	0	0	0	0	0
Total	195	16	8.2	46	23.6	47	24.1

^a Day after first day of fever.

We broke down the 195 patients with serological evidence of dengue infection by age, sex, primary or secondary infection and severity of disease (Table 3, 4). There was no apparent relationship between dengue isolation and sex or age group with the exception that viruses were more frequently recovered in older children.

There were 14 patients with primary dengue infection. The isolations from these primary patients by severity of illness are illustrated in Table 5. Three of these primary patients were older children of grade III severity. Due to later hospital admission, virus was recovered from the leukocytes of only 35% of the patients. Because of the small number of isolates, patterns were difficult to discern.

Table 6 shows the virus isolation rates and the severity of disease in 181 patients with secondary dengue infection. Here there appeared to be no relationship between the virus isolation rates and the severity of disease.

Table 3. Dengue Patients by Grade of Severity and Sequence of Infection.

Grade of Severity	Sequence of Dengue Infections		
	Primary	Secondary	Total Infections
	7 (50.0) ^b	7 (3.9)	14 (7.2)
I	0 (0)	6 (3.3)	6 (3.1)
II	4 (28.5)	69 (38.1)	73 (37.4)
	3 (21.4)	83 (45.9)	86 (44.1)
IV	0 (0)	16 (8.8)	16 (8.2)
Total	14	181	195

^a Undifferentiated fever

^b Percent of total in primary or secondary group

Table 4. Virus Isolations from Leukocytes by Age and Sex, Bangkok Children's Hospital, 1976 - 1977.

Age	Male			Female			Total		
	No. Tested	Isolations		No. Tested	Isolations		No. Tested	Isolations	
		No.	%		No.	%		No.	%
2	4	0	0	9	2	22.2	13	2	33.3
3	9	2	22.2	8	2	40.0	17	4	23.5
4	9	0	0	9	2	22.2	18	2	71.1
5	9	0	0	8	0	0	17	0	0
6	8	1	12.5	15	4	26.6	23	5	21.7
7	9	1	11.1	9	1	11.1	18	2	11.1
8	4	0	0	10	1	10.0	14	1	7.1
9	6	1	16.7	12	5	41.7	18	6	33.3
10	7	2	28.5	10	5	50.0	17	7	41.2
11	5	3	60.0	7	1	14.3	12	4	33.3
12	8	5	62.5	9	3	33.3	17	8	47.0
13	5	3	60.0	4	2	50.0	9	5	55.5
14	1	0	0	0	0	0	1	0	0
15	1	0	0	0	0	0	1	0	0
Total	85	18	21.2	110	28	25.5	195	46	23.6

Table 5. The Relationship of Severity of Disease to the Virus Isolation Rate in Primary Dengue Cases, Bangkok 1976-1977.

Grade of Severity	Number Studied	Positive Specimens					
		Plasma		Leukocytes		Both	
		No.	(%) ^b	No.	(%)	No.	(%)
UF ^a	7	1	(14.3)	2	(28.6)	2	(28.6)
I	0						
II	4	1	(25.0)	2	(50.0)	2	(50.0)
III	3	1	(33.3)	1	(33.3)	1	(33.3)
IV	0						
Total	14	3	(21.4)	5	(35.7)	5	(35.7)

^a UF = Undifferentiated fever

^b Percent of number studied

Table 6. The Relationship of Severity of Disease to the Virus Isolation Rate in Secondary Dengue Cases, Bangkok 1976-1977.

Grade of Severity	Number Studied	Positive Specimens					
		Plasma		Leukocytes		Both	
		No.	(%) ^b	No.	(%)	No.	(%)
UF ^a	7	0		1	(14.3)	1	(14.3)
I	6	2	(33.3)	1	(16.7)	2	(33.3)
II	69	6	(8.7)	15	(21.7)	15	(21.7)
III	83	4	(4.8)	21	(25.3)	21	(25.3)
IV	16	1	(6.3)	3	(18.8)	3	(18.8)
Total	181	13	(7.3)	41	(22.9)	42	(23.5)

^a UF = Undifferentiated fever

^b Percent of number studied

We have begun to identify the cells which are infected with viruses. Isolations from adherent and non-adherent cells of 18 patients are presented in Table 7. Virus was obtained from adherent cells in ten patients, from adherent and non-adherent cells in two patients. This suggests that the phagocytic monocyte might be the site of virus infection, however, recovery of virus from non-adherent cells indicates that virus might be associated with other white blood cells as well. Identification of the leukocytes infected with dengue virus in vitro is continuing.

Table 7. Recovery of Virus from Adherent and Non-Adherent Leukocytes.

Leukocytes	Isolations	
	No.	(%)
Adherent	10	(55.5)
Adherent & Non-Adherent	6	(33.3)
Non-Adherent	2	(11.1)
Total	18	(100.0)

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