

## Isolation of Dengue Viruses from Plasma and Cellular Components of Blood

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**OBJECTIVE :** To determine the relative frequency of isolation of dengue viruses from plasma and cellular components of the blood of dengue patients.

**BACKGROUND :** Studies of the pathogenesises of dengue hemorrhagic fever (DHF) have implicated human peripheral blood leukocytes (PBL), particularly the phagocytic monocyte, as the primary site of dengue virus replication. Evidence that supports this proposition includes the association of dengue viruses and/or dengue virus antigens with PBL of the blood of dengue patients and of experimentally infected monkeys (1, 2, 3, 4, 5). In vitro studies have shown that dengue viruses replicate in both monkey and human PBL cultures (6, 7). In addition cultured human B type lymphoblastoid cell lines and macrophages have been shown to support replication of dengue-2 virus (7, 8, 9).

However, attempts to infect polymorphonuclear (PMN) cells, T type human lymphoblastoid cell lines, and T lymphocytes, have not been successful (6, 7) Despite these findings, direct association of dengue viruses with PBL during human dengue virus infection remains to be proven.

**METHODS :** Heparinized blood was obtained from dengue patients as described previously (10). Blood specimens were centrifuged at 2000 rpm for 15 minutes and plasma was stored at  $-20^{\circ}\text{C}$  and at  $-70^{\circ}\text{C}$  for serological and virological studies.

The dextran sedimentation technique employed in a preliminary comparative study with the Isopaque Ficoll (IP-Ficoll) flotation technique for isolating leukocytes was performed as described previously (5).

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The IP-Ficoll flotation technique (IP-Ficoll) for separating cellular fractions of the blood of dengue patients was employed as shown in Figure 1. Each cellular fraction was mixed with 4.0 ml of RPMI 1640 medium and carefully layered over IP-Ficoll medium. After centrifugation at 1800 rpm for 30 minutes at 4°C, the top layer of RPMI 1640 medium was carefully removed with a pasteur pipette. The mononuclear cell and platelet fraction of the interface region was transferred to a 15 ml conical centrifuge tube. Cells were then suspended in 5.0 ml of RPMI 1640 medium and centrifuged at 500 rpm for 15 minutes.

The supernatant was transferred to a 15 ml conical centrifuge tube and the pelleted mononuclear cells were resuspended and centrifuged again as above. Supernatants were pooled and centrifuged at 2000 rpm for 10 minutes at 4°C and the pelleted platelets were resuspended in 2.0 ml of RPMI 1640 and a 1.0 ml and a 0.2 ml aliquot was stored at -70°C. Sedimented mononuclear cells were suspended in 2.5 to 3.0 ml of RPMI 1640 medium, with 10% heat inactivated fetal calf serum (FCS). Cell concentration was adjusted to approximately  $1 \times 10^6$  cells per ml and a 1.0 ml and a 0.2 ml aliquot was stored at -70°C.

Adherent and nonadherent cells were obtained from the remaining portion of mononuclear cell fractions on the basis of glass adherence properties of monocytes. Each mononuclear cell-RPMI 1640 medium fraction (0.5 ml) was transferred to 1 oz glass bottles and placed at 37°C for 3 hours to allow monocytes to adhere to glass. The non-adherent cell fraction that remained suspended in the RPMI 1640 medium was transferred to 1 oz bottles. Adherent cells were rinsed 2 to 3 times with RPMI 1640 medium. Four ml of a LLC-Mk2 cell suspension,  $2 \times 10^6$  cells/ml was then added to each bottle that contained adherent and/or non-adherent cells. After 24 hours incubation at 37°C, 2.5 ml of a standard agar overlay was added to each bottle of cells. A similar volume of a second agar overlay that contained a 1:10,000 dilution of neutral red was added on day 6 post inoculation. Plaque forming units (PFU) were counted and recorded on the following day.

Granulocytes and erythrocytes that comprised the bottom layer of the IP-Ficoll medium were suspended in 2 volumes of 2.4% dextran (T-250), Hank's balanced salt solution (HBSS) and 0.15% EDTA. The mixture was left at 4°C for 45 minutes to one hour, and the supernatant that contained the granulocytes was transferred to a 15 ml conical centrifuge tube. Supernatant was centrifuged for 10 minutes at 1000 rpm and the pelleted granulocytes were resuspended in HBSS plus 0.02% EDTA. This step was repeated and granulocytes were resuspended in 3.0 ml of RPMI 1640 medium, with 10% heat inactivated FCS, and a 1.0 ml and a 0.02 ml aliquots were stored at -70°C.

Plasma, platelets, whole leukocyte and whole mononuclear cell, and polymorphonuclear cell fractions were assayed for virus at 32°C by the mosquito inoculation technique employing *Toxorhynchites splendens* (11). The direct fluorescent antibody technique was used to test mosquito heads for virus (11). Corresponding thorax-abdomen portions of mosquitoes, and aliquots of the above plasma, platelets, etc. fractions were tested for virus at 35°C in LLC-Mk2 cells by direct and delayed plaque assay (12). The volume of inoculum per cell culture was 0.3 ml and 0.85 ul per mosquito. Viruses were identified by plaque reduction neutralizing tests employing monospecific dengue virus types 1, 2, 3

and 4 antisera prepared in rhesus monkeys. A differential cell count was performed on smears of whole leukocytes, mononuclear, nonadherent, polymorphonuclear and platelets fractions using Wright's stain.

Dengue virus types 1, 2, 3, and 4 were used in plaque reduction neutralization tests (PRNT) to determine antibody titers for dengue patients. Plasma were heat treated at 56°C for 30 minutes. Mixtures of equal volumes of four fold dilutions of plasma and approximately 30 to 60 PFUs of each virus were incubated at 37°C for 90 minutes. Medium free monolayers of LLC-Mk2 cells propagated in 24 well plastic plates were inoculated with virus plasma mixtures, 0.1 ml per each of 2 cultures. After incubation at 37°C for 90 minutes, cell monolayers were rinsed with HBSS and overlaid with 0.5 ml of a standard agar overlay. The remaining procedures for plaque assay were similar to those described for adherent and nonadherent cells. Cell cultures were incubated at 35°C during the assay period. Fifty percent plaque reduction neutralization titers were estimated by probit analysis.

Severity of illness of dengue patients was graded according to previously established criteria (13).

The results of a comparative study to evaluate the dextran sedimentation and the IP-Ficoll flotation technique for fractionating cellular components of the blood of dengue patient for virus isolation studies are presented in Table 1. Dengue viruses were isolated from 9 of 27 leukocyte fractions obtained from blood of 27 patients by both leukocyte fractionating techniques. Although the number of virus isolations from leukocytes fractions obtained by the 2 techniques was comparable, viruses were not always isolated from the same leukocyte fractions. Possibly, this was associated with the difference in the relative proportions of the types of cells in fractions obtained by the 2 techniques. As shown in Table 2, the predominate cell type in the fractions obtained by the IP-Ficoll technique was mononuclear cells (93%) whereas fractions associated with the dextran sedimentation technique were comprised of mononuclear (59%) and polymorphonuclear.

The relative proportion of 37 dengue virus isolations associated with one or more of the different types of fractions of the blood of dengue patients is presented in Table 3. Dengue viruses were associated with all types of blood fractions but most frequently with mononuclear cell fractions. Although the data was based on an unequal number of fractions assayed per blood specimen, similar results were obtained by analysing the data for 26 patients for whom all types of fractions were assayed for virus (Table 4).

As shown in Table 5 the adherent cell portion of the mononuclear fraction was responsible for the majority of the additional dengue virus isolations. Of the blood fractions other than leukocytes, dengue viruses were associated more frequently with plasma than with platelets fractions (Table 6).

According to results of infectious center assay of adherent and nonadherent cell fractions, both monocytes and lymphocytes appeared to support dengue virus replication (Table 7). Of the culture for which infectious centers could be enumerated, the average yield of virus for the 2 types of cell fractions was

comparable; however, more infectious centers than could be counted was associated more frequently with the adherent cell fractions. No apparent relation was noted between the number of infectious centers and the grade of disease or the day of disease of the patients.

The distribution of dengue virus isolations among the different blood fractions of dengue patients by day of disease is presented in Table 8. Overall, the pattern of the rate of virus isolation for the blood fractions was similar as indicated by a rapid decrease in rates subsequent to day 3 of disease. Although the rates for different fractions did not appear to differ significantly, the pattern of decrease for plasma and platelets fractions was more similar and faster than that of the leukocyte, especially the adherent cell fractions.

The relation of antibody titer to the isolation of dengue viruses from plasma and leukocyte fractions of the blood of dengue patients is presented in Table 9. Dengue viruses were isolated from each type of fraction of patients with HI and plasma neutralizing antibody titers that ranged from <10 to >10,240 and from undetectable to >640, respectively. Significantly more virus isolation, however, were obtained from leukocytes than from plasma fractions of patients with high neutralizing antibody titers ( $X^2 = 5.619$ ,  $p = .018$ ). A similar relationship was observed between HI antibody titers and the pattern of isolation of dengue viruses from the 2 types of blood fractions.

The distribution of dengue virus isolations among the different blood fractions in relation to the immune response of dengue patients is presented in Table 10. No striking pattern was noted in the distribution of virus isolations among the different fractions of blood of patients who serconverted. In contrast, a marked reduction occurred in the rate of isolation of dengue viruses from platelet fractions of the patients with high fixed antibody titers.

The pattern of virus isolation did not appear to be related to the severity of disease, but the decrease in the rate of isolation of dengue viruses from platelets fraction tended to be associated with the late phase of illness of the patients.

This is a final report

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Table 1. Dengue Virus Isolations from Leukocyte Fractions Prepared by the Dextran Sedimentation and the Isopaque Ficoll Flotation Techniques.

No. of Patients studied <sup>1</sup>	Virus Isolations					Total
	IP-Ficoll	DS	Both <sup>2</sup>	IP-Ficoll Only	DS Only	
27	06	07	04	02	03	03

<sup>1</sup> All 27 patients studied by both techniques.

<sup>2</sup> Virus isolated from the same leukocyte suspension by both techniques.

Table 2. Percentage of Leukocytes and Number of Platelets in Cell Fractions Assayed for Dengue Viruses

Cell Fractions	No. of Fractions	Differential cell count				Platelets
		Polymorpho-nuclear	Lymphocytes	Monocytes	Atypical Lymphocytes	
Mononuclear	28	1.0%	79.0%	12.0%	8.0%	1.6 <sup>1</sup>
Nonadherent cells	18	3.0	56.0	1.0	40.0	0.5
Polymorphonuclear	25	84.0	15.0	1.0	0.4	1.7
Leukocytes <sup>2</sup>	17	41.0	41.0	4.0	14.0	0.3

<sup>1</sup> Average count per high powered field

<sup>2</sup> Obtained by dextran sedimentation, others obtained by combination of IP-Ficoll, dextran sedimentation and differential centrifugation.

Table 3. Dengue Virus Isolations from Plasma and Cellular Fractions Based on an Unequal Number of Fractions Assayed Per Blood Specimens

Blood Fractions	No. of Fractions	Total virus Isolation	Virus isolation Alone	% of Total
Plasma	37	25	00	00.0
Mononuclear cells	36	29	05	71.4
Polymorphonuclear cells	27	14	01	14.3
Platelets	27	12	00	00.0
Adherent cells	28	22	01	14.3
Nonadherent cells	28	17	00	00.0
Total		30	07	100.0

Table 4. Dengue Virus Isolations from Blood Fractions of Each Patients Ranked in Decending Order According to the Number of Patients with all Fractions Positive to the Number of Patients with only One Virus Positive Fraction

Number of Patients (N = 26)	Number of Fractions Positive/No. of Fractions	Blood Fractions					
		Plasma	Mono-nuclear	Polymorpho-nuclear	Platelets	Adherent	Non-adherent
5	6/6	5	5	5	5	5	5
5	5/6	5	5	4	5	4	2
4	4/6	4	4	1	0	4	3
5	3/6	2	2	2	1	4	4
3	2/6	1	2	0	0	2	1
4	1/6	0	2	1	0	1	0
Total		17	20	13	11	20	15

Table 5. Distribution of Dengue Virus Isolations Among the Adherent and Nonadherent Leukocytes Fractions of Dengue Patients

Cell Fractions	No. Virus Isolations	Percent of Total Isolates
Adherent cells alone	07	29
Nonadherent cells alone	02	08
Both fractions	15	63
	24	100

Table 6. Isolations of Dengue Viruses from Blood Fractions Other than Leukocyte Fractions

Blood Fractions <sup>1</sup>	No. Virus Isolations
Plasma alone	06
Platelets alone	00
Both fractions	12

<sup>1</sup> Twenty-seven of each fraction assayed for virus.

Table 7. Range and Average Number of Dengue Virus Infectious Center for Adherent and Non-adherent Cells

Mononuclear Cell Fractions	Cellular Fractions <sup>1</sup>	Plaque forming units <sup>2</sup>		
		Range	Average	TNTC <sup>2</sup>
Adherent Cells	22	1-64(14) <sup>3</sup>	15.7	8
Non-adherent Cells	17	5-52(14)	17.9	3

<sup>1</sup> Includes fractions that yielded one or more PFU

<sup>2</sup> Too numerous to count

<sup>3</sup> ( ) Number of fractions from which 1 to 64 plaque forming units were obtained.

Table 8. Dengue Virus Isolations from Plasma and Cellular Components of the Blood of Dengue Patients by Day of Disease

Day of Disease	Blood Fractions					
	Plasma(%)	Mono-nuclear Cells(%)	Polymorpho-nuclear Cells(%)	Platelets(%)	Adherent Cells(%)	Non-adherent Cells(%)
2	4/05(80) <sup>1</sup>	5/05(100)	3/05(60)	4/05(80)	3/05(60)	4/05(80)
3	2/02(100)	2/02(100)	2/02(100)	2/02(100)	2/02(100)	2/02(00)
4	8/21(38)	9/21(43)	3/12(25)	3/11(27)	4/10(40)	3/10(30)
5	9/38(24)	8/36(22)	3/25(12)	2/19(11)	9/25(36)	7/25(28)
6	2/29(07)	5/24(21)	3/13(23)	1/12(08)	3/13(23)	3/13(23)
7	0/18(00)	0/16(00)	0/11(00)	0/10(00)	1/07(14)	0/07(00)
8	0/06(00)	0/06(00)	0/02(00)	0/02(00)	0/03(00)	0/03(00)
9	0/04(00)	0/03(00)	0/02(00)	0/03(00)	0/03(00)	0/03(00)
10						
11	0/01(00)	NT <sup>2</sup>	NT	NT	NT	NT
Total	25/124(20)	29/113(26)	14/72(19)	12/64(19)	22/68(32)	19/68(28)

<sup>1</sup> Number virus isolations/total number of blood fractions assayed

<sup>2</sup> Not tested.

Table 9. Dengue Virus Isolations from Plasma and Leukocyte Fractions of Dengue Patients in Relation to the Day of Disease and Homologous Hemagglutination Inhibition and Plaque Reduction Neutralization Antibody Titers

Patient Number	Fractions		Antibody titer	
	Plasma	Leukocytes	HAI	PRNT
D78-009	0 <sup>1</sup>	+ <sup>2</sup>	320	640
D78-014	0	+	2560	640
D78-055	0	+	2560	640
D78-097	0	+	10240	640
D78-130	0	+	5120	640
D78-091 <sup>3</sup>	0	+	10	25
D78-052	+	+	1280	096
D78-054	+	+	80	160
D78-059	+	+	2560	640
D78-044	+	+	20	32
D78-050	+	+	10	00
D78-051	+	+	40	40
D78-078	+	+	160	190
D78-080	+	+	2560	640
D78-099	+	+	10	35
D78-108	+	+	80	180
D78-112	+	+	10	00
D78-117	+	+	10	130
D78-133	+	+	10	350
D78-145	+	+	160	640
D78-159	+	+	640	640

<sup>1</sup> Virus not isolated

<sup>2</sup> Virus Isolated

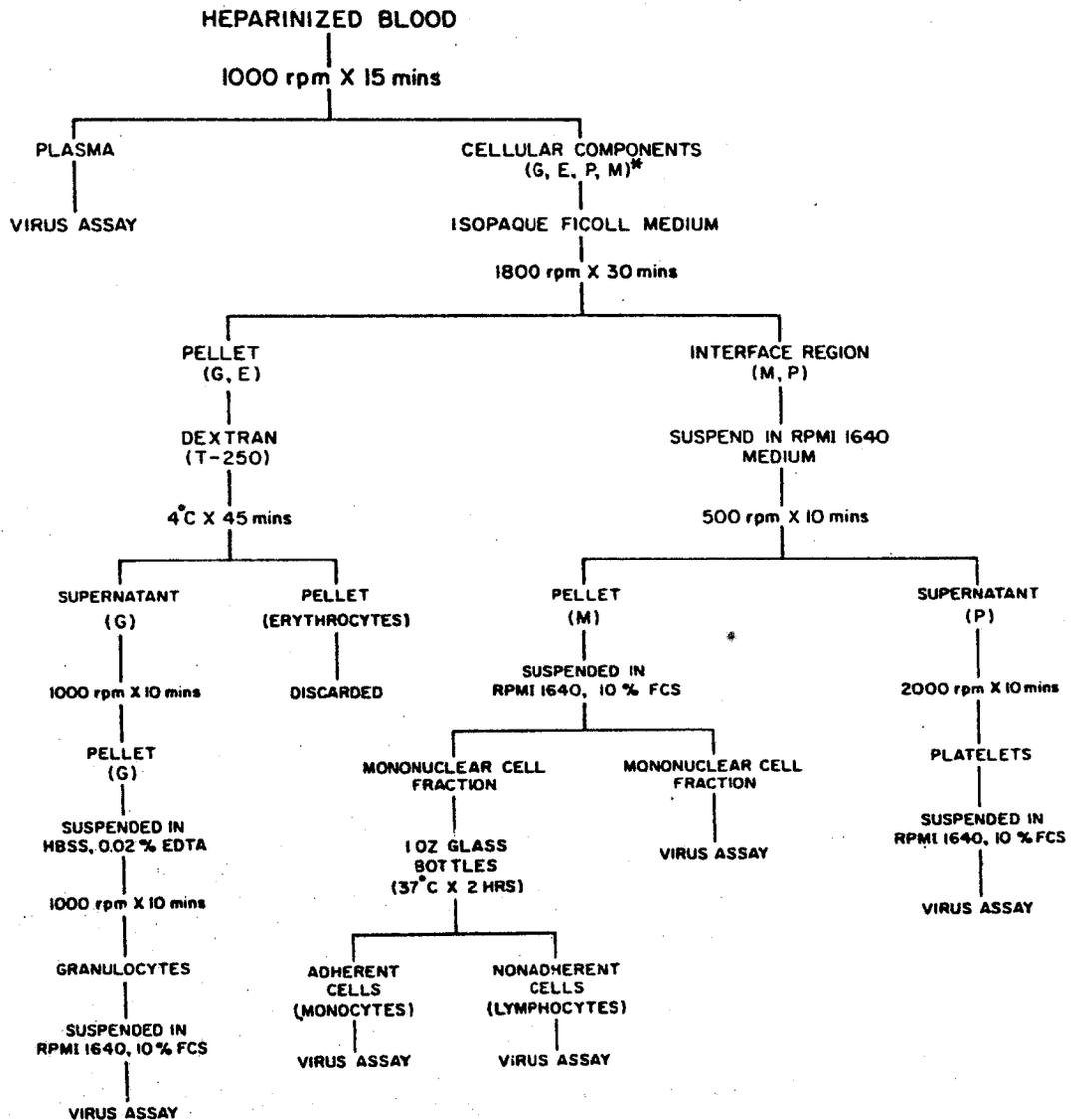
<sup>3</sup> Primary infection

TABLE 10 Dengue virus isolations from plasma and cellular fractions of the blood of dengue patients according to the immunological response

Patient Number	Day of Disease	Grade of Illness	Blood Fractions						HA.I. Antibody Titers <sup>1</sup>
			Plasma	Mono-Nuclear	Polymorpho-Nuclear	Platelets	Adherent	Non-Adherent	
D78-091	04	PUO	0	+	0	0	0	0	<10 <sup>2</sup>
D78-108	04	PUO	+	+	0	0	+	+	80
D78-114	02	PUO	+	+	+	+	+	+	<10 <sup>3</sup>
D78-117	03	PUO	+	+	+	+	+	0	10
D78-118	02	PUO	0	+	0	0	0	0	<10 <sup>3</sup>
D78-132	04	PUO	+	+	0	0	+	+	160 <sup>3</sup>
D78-133	02	PUO	+	+	+	+	0	+	<10
D78-157	05	PUO	+	+	NT <sup>4</sup>	NT	NT	NT	<10 <sup>3</sup>
D78-009	04	III	NT	+	NT	NT	NT	NT	320
D78-025	04	III	+	+	NT	NT	NT	NT	20
D78-044	04	II	+	+	+	+	+	+	20
D78-050	03	III	+	+	+	+	+	0	<10
D78-051	05	III	+	+	0	0	+	+	40
D78-054	05	II-III	+	0	0	0	+	0	80
D78-069	02	II	+	+	0	+	+	+	10 <sup>3</sup>
D78-078	05	III	+	+	0	+	0	0	160
D78-099	06	III	+	+	+	+	+	+	<10
D78-112	05	II-III	+	+	+	+	+	+	<10
D78-135	02	II	+	+	+	+	+	+	10
D78-145	05	III-IV	+	+	NT	NT	+	+	160
D78-168	05	II	+	+	NT	NT	NT	NT	80
D78-014	04	III	NT	+	NT	NT	NT	NT	2560
D78-017	04	II	+	+	NT	NT	NT	NT	1280
D78-026	06	II	0	+	NT	NT	NT	NT	5120
D78-042	04	III	+	+	+	+	+	+	5120
D78-048	05	III	0	0	+	0	+	+	640
D78-052	05	II-III	0	NT	NT	NT	+	+	1280
D78-055	05	III	0	0	+	0	+	+	2560
D78-059	05	III	+	0	0	0	+	+	2560
D78-074	06	III	0	+	0	0	+	+	5120
D78-077	05	III	0	+	0	0	+	0	2560
D78-080	06	III	+	+	+	0	+	0	2560
D78-084	07	I	0	0	0	0	+	0	2560
D78-097	06	II	0	0	+	0	0	0	>10240
D78-130	06	II	0	+	0	0	0	+	5120
D78-136	04	III	+	+	+	+	+	+	1280
D78-159	04	III	+	+	NT	NT	NT	NT	640

1. Reciprocal homologous HA.I. antibody titers
2. Primary immune response, all others secondary immune response except for unpaired plasma specimens
3. Unpaired plasma specimens
4. Not tested
5. High fixed antibody titer

Figure 1. Separation and isolation of plasma and cellular components of the blood of dengue patients.



\* G = GRANULOCYTES, E = ERYTHROCYTES, P = PLATELETS, M = MONONUCLEAR.