B CELLS ARE THE PRINCIPAL CIRCULATING MONONUCLEAR CELLS INFECTED BY DENGUE VIRUS

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Abstract. Although dengue virus infects a variety of cells *in vitro*, little is known about cell types infected *in vivo*. Since blood is a readily accessible tissue, we chose to determine which circulating blood cells are infected by dengue viruses. We collected blood mononuclear cells from acutely ill dengue patients and separated the cells by flow cytometry into subsets for virus isolation. Cells were sorted into groups corresponding to the cluster designations CD3, CD14, CD16 and CD20. Virus was isolated from sorted groups by inoculation into *Toxorhynchites splendens* mosquitos. The majority of the virus was recovered from the CD20 or B cell positive subset. Little virus was isolated from monocytes, NK cells or T cells. Virus was isolated from B cells regardless of the age or sex of the patient, virus serotype isolated, or the patient's history of dengue virus infection. The location of cell associated virus was determined by proteolytic digestion of surface virus. There was an equal distribution of virus between the intracellular compartment and the surface of B cells. The intracellular localization of virus was confirmed by immunocytochemistry. Since this study focused on circulating cells, no inferences were made regarding infection of cells in solid tissues.

INTRODUCTION

Dengue is the most common arthropod-borne virus infection in the world, affecting millions of people annually. Because it is a leading cause of morbidity in some tropical countries, its pathogenesis has been investigated in an attempt to understand events leading to morbidity. The most severe effects of disease including encephalopathy, hemorrhage, hypovolemic shock and liver failure become prominent only after several days of illness when fever and viremia remit. A variety of immune mechanisms have been incriminated as mediating these disease manifestations. On the other hand, the virus-host interactions that trigger these mechanisms are poorly understood. For example, one of the most fundamental aspects of pathogenesis, the range of cells that dengue viruses infect *in vivo*, is poorly characterized.

The only class of cells from which dengue viruses have been repeatedly isolated are peripheral blood mononuclear cells. This was initially accomplished by Halstead and others (Marchette *et al*, 1972) who examined macaque monkeys experimentally infected with dengue virus. The seminal work of Halstead was extended to man by Scott and others who recovered dengue viruses from washed peripheral blood mononuclear cells collected from patients with dengue fever and dengue hemorrhagic fever. Isolations of dengue viruses from peripheral blood mononuclear cells have been reported occasionally by others (Waterman *et al*, 1985). Comparisons of virus recovery from patient's peripheral blood mononuclear cells and serum have suggested that all people with dengue circulate infected leukocytes, if only briefly (Innis, unpublished). Human studies were generally conducted with unfractionated populations of mononuclear leukocytes. In recent years, it has become possible to sort peripheral blood mononuclear cells into more homogenous groups based on their expression of cell surface markers that serve as cluster designations. The objective of the experiments reported herein was to elucidate the peripheral blood mononuclear cell fraction infected with dengue virus during acute disease. Patients with acute dengue infections were systematically selected for study. Their peripheral blood mononuclear cells were washed and fractionated into 4 subsets, each defined by a monoclonal antibody to a cluster designation marker. The subsets and cluster designations were: T lymphocytes (CD3), B lymphocytes (CD20), natural killer cells (CD14) and monocytes (CD16). Comparison of virus content of each cell fraction identified B lymphocytes as the major infected cell of peripheral blood.

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Because these results were unexpected, additional experiments were performed to determine whether B lymphocyte associated virus was intracellular (*ie* the cell was infected) or extracellular alone (not infected).

MATERIALS AND METHODS

Patients

As part of an ongoing program of surveillance, all children identified in the outpatient clinic or admitted to the infectious disease ward of Children's Hospital, Bangkok, Thailand with a suspicion of acute dengue were evaluated for inclusion into this study. Intermittently from April to September 1990, the first patient seen each weekday who had an oral temperature of 38°C or above, a history of fever of 5 days or less, a positive tourniquet test and no localizing signs inconsistent with dengue fever was enrolled in the study. The acute blood specimen was anticoagulated with heparin. A total of 72 patients were entered into the study to characterize infected PBMC subsets. Of those, virus was isolated from serum of 44 patients, virus was isolated from peripheral blood mononuclear cells from 29 patients and enough virus was present in mononuclear cells of 18 patients to include in the study (see criteria below). Control blood specimens were collected from all patients one week after the acute specimen. The convalescent specimen was used for paired serum to provide a diagnosis of primary or secondary dengue.

A second group of patients was enrolled using identical methods in 1992 to furnish specimens for assays of extracellular virus localization. A final group of patients was enrolled using identical methods in 1992-1993 for immunocytochemical staining of peripheral blood mononuclear cells.

Peripheral blood mononuclear cells

Whole, anticoagulated blood was immediately cooled to 4°C. Within two hours of collection, peripheral blood mononuclear cells were separated from 5 ml of peripheral blood by Ficoll-Hypaque (Histopaque 1077, Sigma) density gradient centrifugation. Before the separation, heparinized blood was centrifuged at 400*g* for 10 minutes. The plasma was removed and the cells were resuspended in 20 ml of RPMI 1640 (Gibco) with 5% fetal bovine serum (FBS) and 1 μ/ml Na heparin. The suspension was gently layered over 10 ml of Ficoll-Hypaque and centrifuged at room temperature for 30 minutes. Collected cells were washed thrice by re-suspension of the cells in RPMI 1640 with 5% FBS and centrifugation at 400*g* for 10 minutes.

Labeling of cell subsets

The cells from each patient were divided into four equal groups for sorting. The cells were concentrated by centrifugation at 400*g* and resuspended in 20 μl of phycoerythrin tagged monoclonal antibody. The phycoerythrin labeled monoclonal antibodies Leu-4 (CD3), Leu-M3 (CD14), Leu-11c (CD16), and Leu-12 (CD20) were purchased from Becton Dickinson. Leu-4 recognizes the T cell receptor and is specific for T cells. Leu-M3 recognizes a lipopolysaccharide receptor and is specific for monocytes (and granulocytes in mixed white blood cell suspensions). Leu-11c recognizes the Fc¥ receptor type IIIa and is specific for NK cells in mononuclear cell suspensions and granulocytes and macrophages in other cell populations. Leu-12c recognizes a phosphoprotein on B cells. The cells were incubated for 20 minutes at 4°C then washed twice with RPMI 1640 with 5% FBS (medium) by centrifugation at 400*g*. The cells were resuspended in medium at a concentration of one million cells per ml for sorting.

Sorting of cell subsets

Each of the four groups (starting with an equal number of cells) was sorted into two groups yielding eight groups in total. One group for each sort was positive for the selected subset and the rest of the cells were sorted into a subset negative group. Flow cytometric analysis and sorting were done using a FACSTAR Plus flow cytometer (Becton Dickinson). Data acquisition was done with an HP 9000 series 300 computer in list mode using FACSTAR Plus RESEARCH software. The cells were excited with 488 nm light from a 5 W argon ion laser (Inova 90, Coherent) at a power output of 200 mw. Red fluorescence was observed through a 585 nm longpass filter. The instrument was sterilized prior to sorting with 70% ethanol followed in 15 minutes by sterile medium. Cells were sorted at a sweep trigger rate of 2,000 cells/second using a logarithmic scale red fluorescence gate for the positive selection. The negative selection was all other cells. Cells not clearly identified by the flow cytometer were discarded during the sorting process.

After sorting, the cells were manually counted to verify that the number of cells collected equaled the number counted by the flow cytometer. Cells were transferred to a labeled Eppendorf tube and centrifuged for 5 minutes at maximum speed in an Eppendorf centrifuge. The supernatant was discarded and the cell pellet was frozen and stored at -70°C.

Determination of virus quantity

Due to the labor intensive nature of virus titration, a screening procedure was necessary to reduce analysis of cell samples lacking virus. Initially, the presence of serum viremia was a criteria for analysis of the cells. After the first 5 patients, an additional criteria of cellular viremia in the T cell negative sort (containing B cells, NK cells and monocytes) was added.

Virus quantity was determined by titration in *Toxorhynchites splendens* mosquitos. For cells, the thawed cell pellet was resuspended in 20 µl of medium (RPMI 1640 + 10% FBS) and the suspension was vigorously vortexed to disrupt the cells. Serum was first diluted 1:5 in medium. Serial log10 dilutions of all specimens were made in medium. For the undiluted cell samples, 0.34 µl of the suspension was injected intrathoracically into 8-15 *Toxorhynchites splendens* mosquitos. For the rest of the samples, 1.02 µl of the suspension was injected intrathoracically into 8-15 *Toxorhynchites splendens* mosquitos. The mosquitos were maintained at 30°C in a humid atmosphere for 14 days. Infection of the mosquitos was determined by indirect fluorescent antibody microscopy of a head squash preparation using hyperimmune anti-dengue mouse ascitic fluid followed by fluorescein tagged goat anti-mouse IgG (Sigma) (Rosen and Gubler, 1974). The median mosquito infectious dose (MID_{50}) was calculated by probit analysis using the statistical software SPSS. Cell trophism was determined by comparing the amount of virus isolated from positive cell sorts. Groups were only compared if significant virus was isolated from either the positive or the negative population from every sort from a given PBMC sample. Significant virus was defined as isolation of virus from a sample diluted 1:50.

Location of cell associated virus

It was recognized that quantification of virus from cells measured both intracellular and membrane bound virus. The method used could not distinguish between the two. Since virus can bind to Fc receptor bound immunoglobulin, membrane bound immunoglobulin, and the virus receptor, a method was needed to distinguish externally bound virus from intracellular virus. Although we were interested in determining if the virus was infecting cells, we were equally interested in the potential for cells to carry external virus. We reasoned that dengue virus could be spread throughout lymph nodes by B cells during normal lymphocyte re-circulation. Therefore, an assay was designed to measure a reduction in mononuclear cell virus quantity due to applied protease E treatment of external virus.

Two cell types were used for this assay; patient cells, and control cells. To assess the degree of run to run variation, a uniform control sample was prepared for each patient. An assay was accepted if there was a significant reduction in the control cells. Initially, two sets of control cells were used. One control was human mononuclear cells from a non-infected volunteer. The second control was the mouse hybridoma D141-72-19/6. This hybridoma was chosen because it expressed dengue type 2 anti-envelope protein monoclonal antibody. It was reasoned that dengue 2 virus would bind to membrane bound monoclonal antibody. After it was clear that results were similar for the human and mouse hybridoma cells, the mouse hybridoma cells were used exclusively for the control.

For the control cells, five ml of hybridoma cell culture suspension at log phase containing from 2 to 6 million cells was divided into two groups. Each group was centrifuged at 400*g* for 10 minutes. Each group's cells were resuspended in 100 µl of dengue type 2 virus strain D80-100 at a concentration of 1.1 X 10^8 plaque forming units per ml yielding a pfu to cell ratio of from 2 to 10 pfu/cell. Each group was incubated for 30 minutes at 4°C. The cells were washed twice before protease digestion.

For the patient's sample, peripheral blood mononuclear cells were separated from 5 ml of whole blood as described above. Washed cells from the patient's sample were divided into two groups. One group of patient's sample and one group of control cells was incubated for one hour at 4°C in 500 µl of a solution of protease E (Sigma) at a concentration of 2 mg/ml. The remaining groups were incubated in PBS alone for the same time and temperature. The cells were washed thrice in RPMI 1640 + 10% FBS by centrifugation at 400*g* for 10 minutes. On the last resuspension, the cells were transferred to an Eppendorf tube. The cells were centrifuged for 5 minutes at maximum RPM in an Eppendorf centrifuge. The supernatant was decanted and the cell pellet was stored at -70°C. Virus quantity was determined by titration in mosquitos as described. Virus titration in mosquitos was blinded. Loss of virus from the cell surface was calculated by subtracting the log of the amount of virus in the protease digested cells from the amount of virus in the non-digested group. For all patient groups from which virus was isolated in at least the second log dilution, the log drop of virus in the patient sample was compared by paired Student's *t*-test with the log drop of virus in the hybridoma cells.

Immunocytochemistry

Test slides containing peripheral blood mononuclear cells and control slides containing dengue virus type 2 infected and uninfected Vero cells were fixed for 10 minutes in -20°C acetone. After drying for 5 minutes, slides were stained at 37°C in a humidified chamber with approximately 50 µl of each reagent as follows. Slides were incubated for 20 minutes with napthol AS-B1 phosphate and diazotized 4′-amino-2′, 5′-diethoxybenzanilide (Histomark Blue, Kirkegard Perry Laboratories, Gaithersburg, MD, USA) to stain endogenous alkaline phosphatase blue. This was followed by a 15 minute incubation with blocking buffer of 2% (w/v) bovine serum albumin and 2% (v/v) horse serum (Gibco-BRL, Grand Island, NY, USA) and a 30 minute incubation with hyper-immune mouse ascitic fluid (HMAF) to dengue type 2, diluted 1:1,000 in phosphate buffered saline, pH 7.4 (PBS) containing 40% (v/v) human plasma lacking hemagglutination-inhibiting antibodies to dengue types 1-4 (normal human plasma (NHP) obtained from out-dated frozen plasma, Walter Reed Army Medical Center Blood Bank). Slides were washed in PBS containing 0.1% (v/v) Tween-20 and then incubated for 30 minutes with biotinylated horse anti-mouse IgG (Vector Laboratories, Inc Burlinggame, CA, USA) diluted 1:200 in PBS. After washing in PBS-Tween-20, slides were incubated for 30 minutes in streptavidin-alkaline phosphatase (Gibco-BRL, Grand Island, NY, USA) diluted 1:50 in PBS. Unbound enzyme conjugate was removed with 3 washes of 0.1 M Tris, pH 7.4; bound enzyme was stained red with AS-B1 phosphate, hexazotized new fuchsin and levamisol (Histomark Red, Kirkegard Perry Laboratories, Gaithersburg, MD, USA). Finally, the slides were washed in tap water, counter-stained with Mayer's hematoxylin, and dipped in Scott's tap water (0.027 M magnesium sulfate. 0.016 M sodium bicarbonate) to enhance nuclear detail. Slides were again rinsed in tap water then mounted with aqueous media (Immuno-mount, Shandon, Inc Pittsburgh, PA, USA).

Serology

Levels of serum IgG and IgM to dengue were determined by ELISA as previously published (Innis *et al*, 1989). Results were used to confirm or establish a diagnosis of dengue infection and to characterize the infection as primary or secondary (*ie* with an anamnestic response). These assays are semi-quantitative and the kinetic responses are reproducible from patient to patient. Kinetics for primary infection differs from that of secondary infections. We categorized patients as early infection if the acute serum level of IgM and IgG anti-dengue antibody was < 40 EIA units. Infections were characterized as primary or secondary according to established criteria.

Data analysis and criteria for inclusion

The quantity of virus among mononuclear cell subsets was compared if virus was isolated by mosquito inoculation in the second dilution (1:50) of mononuclear cell lysate from either the positive or negative sort of every subset of any given collection. Differences among groups were determined by analysis of variance and differences between groups were determined by Student's *t*-test.

RESULTS

B cells are the principal circulating mononuclear cells infected by dengue virus

The largest quantity of virus was isolated from B cells (Table 1). This was true for all 18 of the patients included in this study. An average of 95% of the virus was isolated from B cells and less than 3% was isolated from any other single type of mononuclear cell. Little virus was isolated from monocytes, NK cells and T cells.

The quantity of virus was highest in B cells whether the infection was primary or secondary dengue. The majority of patients had an anamnestic serological response (Table 2) during the current infection. However three of the patients had serological evidence of a primary infection. In all three primary dengue patients, the greatest quantity of virus recovered was from B cells, as was true for patients with secondary dengue.

Dengue virus serotypes 1 and 2 were isolated during this study. The quantity of virus was highest in B cells irrespective of the serotype of virus isolated, indicating a similar B cell trophism for both virus serotypes.

Cell sorting enriched for mononuclear cell subsets

The proportion of total mononuclear cells represented by each cell type was recorded during sorting. The proportion was highly variable, particularly for the NK cells and monocytes (data not shown). There were too few observations to determine the significance of exceptionally high NK or monocyte populations in some dengue patients. Further FACS analyses in dengue patients are warranted. The fractional

Table 1
Amount of dengue virus isolated from mononuclear cell subsets.

Patient No.	T Cells	NK Cells	Monocytes	B Cells
510-90	1[a,b]	1	0	139
	(0-27)[c]	(0-69)	(0-27)	(77-237)
454-90	2	10	8	154
	(0-140)	(0-1938)	(0-788)	(81-260)
459-90	1	1	2	431
	(0-78)	(0-180)	(0-368)	(239-1,627)
607-90	15	12	10	462
	(5-36)	(4-34)	(2-46)	(249-875)
339-90	1	1	1	466
	(0-416)	(0-597)	(0-380)	(202-992)
394-90	4	8	4	510
	(0-66)	(0-210)	(0-216)	(278-958)
555-90	3	13	29	704
	(1-12)	(4-36)	(13-60)	(348-1,528)
453-90	48	84	44	708
	(6-241)	35-191)	(8-167)	(287-1,569)
802-90	6	9	5	913
	(0-2,146)	(2-30)	(0-334)	(535-1,618)
520-90	6	59	23	1,147
	(2-18)	(29-119)	(9-49)	(540-2,507)
533-90	27	102	29	2,719
	(10-65)	(54-181)	(14-55)	(1,541-4,877)
521-90	22	49	29	3,286
	(10-41)	(17-122)	(9-76)	(1,464-7,387
624-90	41	178	137	4,023
	(21-74)	(96-309)	(65-267)	(2,302-7,065)
700-90	63	202	43	6,930
	(33-115)	(117-346)	(21-81)	(3,463-13,947)
543-90	52	14	24	7,201
	(25-103)	(6-32)	(10-52)	(3,456-14,141)
901-90	1	44	54	8,629
	(0-5)	(19-90)	(22-120)	(4,897-15,593)
535-90	62	46	57	14,136
	(28-137)	(19-108)	(23-138)	(5,120-39,234)
480-90	46	89	81	16,153
	(21-94)	(45-164)	(34-178)	(8,031-32,722)

[a]Each row represents an individual patient
[b]Virus quantity as 50% mosquito infectious dose per 100,000 cells.
[c]95% confidence interval for virus quantity (probit analysis).

sum of cells sorted was approximately 100% for each group. This gives some assurance that the monoclonal antibodies used did not over identify or under identify any mononuclear cell type. The mean total was slightly less than 100%, indicating some contamination of granulocytes or other non-mononuclear cell after the Ficoll-Hypaque separation.

Cell counts (total cells recovered) for each subset were determined during sorting. Cells were manually counted after sorting to determine if the number of cells collected was the same as the number identified during sorting. This was done to detect either a loss of cells during sorting or a gain of contaminating cells. Comparison of manual counts and flow cytometric cell counts using Student's *t*-test revealed no statistical significance within any

Table 2
Patient description and clinical data.

Patient No.	Age	Sex	Day of fever[a]	Fever[b]	Grade[c]	Serotype[d]	Serology[e]
339-90	15	F	4	38.7	DF	2	S
394-90	12	M	2	38.8	DF	2	S
453-90	11	M	3	39.6	3	2	S
454-90	7	F	3	38.5	2	2	S
459-90	6	M	3	39	DF	2	S
480-90	12	M	3	38.2	3	1	S
510-90	11	M	4	40	2	2	S
520-90	4	M	4	38.6	DF	1	P
533-90	8	F	3	38.8	3	2	S
543-90	13	M	4	38.6	1	2	S
535-90	5	M	4	39	1	2	S
555-90	7	F	4	39	DF	1	P
521-90	10	M	3	38.3	DF	2	S
607-90	3	F	4	38	3	2	S
624-90	7	M	4	38	2	1	S
700-90	12	F	5	39	1	1	P
802-90	9	M	4	38.1	1	2	S
901-90	13	M	2	38.5	DF	2	S

[a]Number of days of fever when acute blood samples for virus quantitation was collected.
[b]Temperature in degrees celcius (when acute).
[c]Grade of disease according to WHO classification.
[d]Serotype of virus isolated from patient.
[e]Primary or secondary dengue.

Table 3
The quantity of virus in B cells is independent of the quantity of virus in serum. (Spearmans rho correlation coefficient = -0.16, p = 0.51).

B Cells (MID_{50}/100,000 cells)	Viremia (MID_{50}/µl)
9	790,679
34	177,828
55	295,121
139	38
139	1,072
154	0
431	15,849
435	1,148
466	4,140
703	380,189
1,147	24
1,597	794
2,719	18,197
3,286	2,754
4,023	2,570
7,201	16,982
14,136	282
16,153	34,674

mononuclear cell type. (p=0.255 for CD3, 0.723 for CD14, 0.794 for CD16 and 0.439 for CD20).

Ninety-five percent of infectious dengue virus was recovered from mononuclear cell sorts that included B cells. Therefore, the following four sorted samples yielded dengue virus in all patients: B cell positive, T cell negative (B cells, NK cells, monocytes), NK negative (B cells, T cells, monocytes), and monocyte negative (B cells, T cells, NK cells). The quantity of virus recovered in negative cell sorts containing B cells should have equaled the amount recovered in B cell positive sorts. We wanted to know whether characteristics of non-B mononuclear cells in the negative sorts, such as lysosome content and number or intracellular cytokines could affect virus quantitation. Monocytes, for instance, have a greater quantity and activity of lysosomal contents than do lymphocytes. To evaluate this potential effect, the quantity of virus in the negative and positive sorts for each mononuclear cell type was summed. Any differences due to a toxicity of a particular cell subset would manifest as a loss of virus in the negative populations containing that subset. For example the T cell negative sort tests the toxicity of macrophages and NK cells on the virus derived from the

B cells contained in that negative sort. There was a statistically significant difference among groups. The largest negative influence was by the presence of T cells. The reason or significance for the reduced virus isolation in T cell containing groups could not be determined. However, in spite of the minor, statistically significant, reduction seen in some groups, the virus recovered from B cell containing groups was overwhelmingly larger than that recovered from groups without B cells.

Serum viremia was measured, as well as cell-associated viremia. Table 3 shows a comparison of virus quantity isolated from both serum and cells. There was no correlation between the two types of viremia (Spearman's rho correlation coefficient = -0.16, p = 0.51). Even so, in most cases, serum viremia was much greater than cell associated viremia. The greater quantity of virus in serum is evident because of differences in the amount of material assayed. Serum viremia was measured as mosquito MID_{50}/µl while cell associated viremia was measured as mosquito MID_{50}/100,000 B cells. There are at least 100-fold fewer B cells per µl of blood than 100,000.

Virus associated with B cells is both intracellular and extracellular. B cells have membrane bound antibody and Fc receptor bound antibody on their surface. B cells also may have specific viral receptors other than antibody. Virus bound to B cells by any of these means may be on the surface of the B cell and could account for the bulk of the virus isolated. Virus location, therefore, needed to be determined.

The method used to determine virus location was virus quantitation after proteolytic digestion of virus from the surface of B cells. Proteolytic digestion was done at 4°C to inhibit endocytosis of virus. The concentration of proteolytic enzyme used in the assay was determined by titrating the amount of enzyme needed to digest virus from control cells coated with extracellular virus. Digestion of virus from non-infected control cells was done concurrently with patient samples.

The loss of virus from proteolytic digestion of the cell surface was greater in the control non-infected cells than in patients cells. Table 4 shows that the ratio of virus retained after proteolytic digestion is higher in patient's cells than that retained in hybridoma controls. To analyze the difference, the log of the after digestion MID_{50} was subtracted from the log of the before digestion MID_{50}. The resulting value was compared to values obtained from hybridoma controls. The average decrease (±SD) in log titer of virus digested from the control cells was 1.47+ 0.21. The average drop in log titer of the seven patients cells was 0.25 +0.09. The statistical significance using a paired Student's *t*-test was >0.001.

There was an average of 51.5% reduction of virus in the PBMCs from patient's samples indicating that roughly half of the virus was on the cell surface. Thus, virus was equally distributed between the surface of patient's cells and within cells.

To further verify that a portion of virus was intracellular, PBMC obtained from dengue patients were collected for immunocytochemical localization of virus. The individuals were part of a separate study and not the individuals whose cells were originally collected for virus isolation. Fig 1 shows a

Table 4
Location of virus in patient cells. Dengue virus was evenly distributed between the intracellular and extracellular compartments in patient's mononuclear cells. Numbers shown are 50% mosquito infectious doses.

Patient No.	Patient PBMC			Hybridoma control		
	Before[a]	After[b]	Ratio[c]	Before[a]	After[b]	Ratio[c]
96-92	24	14	0.58	37	0	0.00
112-92	23	6	0.26	323	90	0.28
134-92	91	67	0.74	13	0	0.00
130-92	368	160	0.43	415	15	0.04
127-92	1,099	658	0.60	45	3	0.07
128-92	21	10	0.48	45	3	0.07

[a]Virus isolated from cells before cell surface proteolytic digestion.
[b]Virus isolated from cells after cell surface proteolytic digestion.
[c]Percent of virus resisting proteolytic digestion.

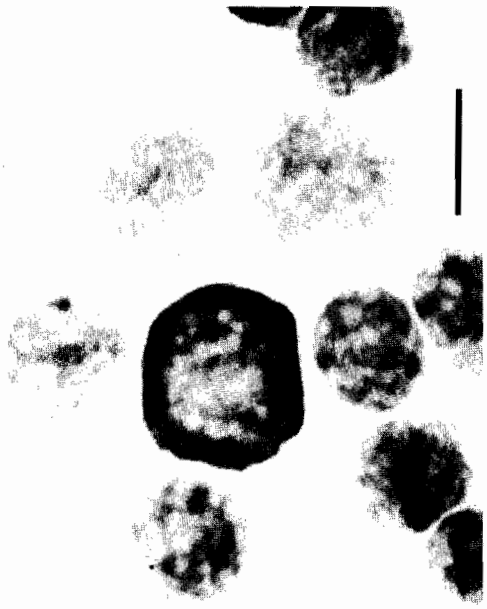

Fig 1–Intracellular dengue antigen. Peripheral blood mononuclear cells were stained using a dengue specific immunoalkaline phosphatase procedure. The center cell shows dengue antigen in the cytoplasm indicating an infection of that cell. Surrounding cells are negative. The bar indicates a length of approximately 10 μ.

slide of immunocytochemically stained cells. Intracellular virus is represented by diffuse dark intracytoplasmic staining clearly seen in one of the cells in a field of otherwise negative cells. Of the 48 individuals whose cells were examined by this method, 39 had cells with this pattern of staining. These results confirm that most patients with dengue circulate mononuclear cells that contain intracellular virus.

DISCUSSION

In this study, we achieved our experimental objective to elucidate the peripheral blood mononuclear cell fraction infected with dengue virus. In every patient studied, the highest quantity of dengue virus was isolated from B cells. This was true regardless of the serotype isolated or of the patientís history of exposure to other dengue virus serotypes. This was the first definitive demonstration of an *in vivo* trophism of dengue virus for cells although *in vivo* tissue trophisms have been described.

Blood is a complex tissue containing mononuclear cells that recirculate and other mononuclear cells on a one way path to tissues. One must consider the findings in this study in light of cell circulation patterns. Each mononuclear cell subset has its own circulation pattern to and from the vascular compartment and each has its own transit time through the vascular compartment. Monocytes, for instance, are produced in bone marrow and enter the circulation from the bone marrow. After spending some time in the vascular compartment, monocytes exit the vessels to reside in tissues as mature macrophages and do not routinely return to the blood.

In contrast, lymphocytes (T cells and B cells) have a more complex circulatory pattern. Immature lymphocytes are produced in the bone marrow. After bone marrow production, T cells and B cells have separate maturation patterns. Following maturation, each has the ability to divide in lymphoid tissue in response to stimulation and to circulate between lymphoid tissue and blood (Sprent, 1973; Gowans, 1959). Thus, in blood, lymphocytes represent a sample of cells recirculating from solid tissue while monocytes represent a sample of cells in transit from bone marrow to peripheral tissue. As a result, more lymphocytes than monocytes are seen in peripheral blood having a solid tissue origin other than bone marrow.

Because of the recirculating pattern of B cells, infected B cells may spread the virus from lymph node to lymph node. This role may be especially important before serum viremia when dengue virus could not otherwise disseminate to lymph tissue other than the regional lymph node draining the site of initial infection. The efficiency of virus spread by B cells would depend upon the location of virus associated with B cells, intracellular or extracellular. As lymphocytes exit the vascular compartment through the high endothelial venule, there is some scrubbing of the cell membrane (Anderson and Ward, 1988). Scrubbing could limit extracellular virus transport while not affecting intracellular virus transport to lymph nodes. The actual role of B cells in the spread of dengue virus is unknown.

Extracellular virus can attach to B cell surfaces in any of four ways, via membrane bound immunoglobulin, via Fc receptor bound immuno-

globulin, via virus specific receptors or nonspecifically due to charge or other molecular interactions. Since monocytes and NK cells possess Fc receptors some of these binding mechanisms also may apply to them. Binding through specific interactions (immunoglobulin or Fc receptor) should lead to endocytosis of virus, reduction of cell surface virus and possibly infection of the cell. Serum viremia would counteract this effect by providing more virus for binding to the cell surface.

Three observations support the intracellular location of dengue virus in B cells: failure of surface protease to remove a significant quantity of virus from infected cells; immunocytochemical identification of dengue virus in PBMC and inability to find virus associated with other Fc receptor bearing cells (circulating monocytes and NK cells). The most convincing of the three observations was that a protease digestion sufficient to digest virus from the surface of control cells failed to remove more than half of dengue virus from the surface of patient's cells. Protease treatment almost completely eliminated virus from the surface of control hybridoma cells (see results) and control human B cells (data not shown) where it remained external because of the 4°C incubation. Yet the same protease treatment failed to eliminate up to half of the virus from patient cells. This result demonstrated that virus was both intracellular and extracellular. Intracellular virus could have been either in the cytoplasm or in phagocytic vacuoles of infected cells. The latter is unlikely to account for a significant quantity of virus because virus viability would be reduced in phagocytic compartments over time.

In addition to quantitative evidence for intracellular virus, virus was observed in the cytoplasm of infected cells stained using dengue antigen specific immunocytochemical techniques. This observation supplements an earlier observation by Boonpucknavig *et al* (1976a) showing dengue virus only on the surface of B cells. The difference could be explained by differences in technique. The earlier study used immunofluorescence and the present study used immuno-alkaline phosphatase. The enzymatic assay is more sensitive and has less background than the immunofluorescence technique. More importantly, for the current study, cells were fixed and permeabilized prior to staining, allowing access to the cytoplasm of the infected cells. In the earlier study, cells were not fixed until after staining, thus access to the cytoplasm was not achieved during staining.

Additional evidence for an intracellular location of virus was that dengue virus was not found in circulating monocytes or NK cells. If virus was adherent to Fc receptor bound surface immunoglobulin, the bound dengue virus should have been recovered from monocytes and NK cells as well as B cells. Both cell types have membrane immunoglobulin Fc receptors. Virus was not recovered from either group of cells.

The finding that B cells are infected by dengue virus appears to contradict previous conclusions that macrophages are the predominant cell type infected by this virus. Monocytes and macrophages are the same cell with differences in maturation, activation and location within the body. Infection of one (macrophages) does not imply that the other (monocytes) can become infected. Several observations with human tissue support the possibility that dengue virus infects tissue resident macrophages. The evidence, summarized by Halstead, is supportive but not definitive (Halstead, 1989).

In vitro, macrophages support the growth of dengue virus. With mouse monocytes, it is clear that activation increases the number of cells infected and the amount of virus produced by those cells (Hotta and Homma, 1994; Hotta and Hotta, 1982). With human monocytes, activation using interferon gamma enhances Fc receptor mediated infection (Kontny *et al*, 1988). The role of activation in human monocyte/macrophage infection in the absence of enhancing antibody is not clear. A receptor for dengue virus adherence to cells in the absence of antibody has recently been determined (Chen *et al*, 1997). Studies of receptor density in response to activation may clarify this issue.

In vitro, dengue virus can infect a wide variety of cells. Mammalian cell lines that have been infected *in vitro* include: monocytes (Hotta *et al*, 1984; Brandt *et al*, 1982), myelomonocytic cells (Kurane *et al*, 1990), B cells (Theofilopoulos *et al*, 1976), T cells, kidney cells (Koff *et al*, 1981; Eckels *et al*, 1976; Matsumura *et al*, 1971) and mastocytoma cells (Legrand *et al*, 1986). Primary cell lines infected *in vitro* include macrophages (Halstead and O'Rourke, 1977), peripheral blood mononuclear cells (PBMC), kidney cells (Hotta *et al*, 1966; Halstead *et al*, 1984; Westaway, 1966), fibroblasts (Kurane *et al*, 1992), lung cells (Eckels *et al*, 1980) and bone marrow myeloid and erythroid precursors (Nakao *et al*, 1989). It is unclear whether the *in vivo* cell trophism is as broad. The results of this study argue against a broad *in vivo* trophism of circulating cells because T cells were not infected but this study does not address infection of non-circulating cells.

In natural infections, dengue virus has been isolated from human tissues (Dasaneyavaja *et al*, 1961; Nisalak *et al*, 1970). At autopsy, virus was isolated from liver, heart, lymph node, lung, and bone marrow. Other autopsy or biopsy specimens of human tissue, examined by fluorescent antibody, showed virus in "reticulum cells" of the liver (Bhamarapravati *et al*, 1967), Kupffer cells, mononuclear phagocytes of the dermal papillae (Boonpucknavig *et al*, 1979) and splenic, thymic and pulmonary macrophages (Yoksan and Bhamarapravati, 1984). Infected renal macrophages were identified by electron microscopy (Boonpucknavig *et al*, 1976b). Most of these tissues have few, if any, B cells and macrophages are common in most of the tissues mentioned.

In summary, a B cell trophism was demonstrated for dengue virus. The cell associated virus was both intracellular and on the cell surface. This finding did not contradict previous findings of dengue virus trophism for other cells, especially macrophages. The role of dengue infected B cells in the dissemination and pathogenesis of virus is unknown but there is a potential for early virus dissemination prior to serum viremia and persistence of cell-associated viremia for hours to days after cell-free virus is cleared or complexed with immunoglobulin.

ACKNOWLEDGEMENTS

The authors thank Somkiat Changnak for his assistance in processing blood cells and preparing them for flow cytometry analysis, Nonglak Ongsakorn and Sanguan Boonak for performing the mosquito inoculations and fluorescent antibody analyses for virus isolations, and Panor Srisongkram for performing ELISA analyses. The authors also wish to thank the nurses that assisted with this study: Nathada Plavooth, Sumetha Hengprasert, Sumolvadee Saravasee, Pranom Vangnai and Somnuk Lumjiak.

REFFERENCES

Anderson AO, Ward JM. Endocytic stripping of ligands from migrant lymphocytes in high endothelial venules (HEV): implications for immunomodulation vs viral pathogenesis. *Adv Exp Med Biol* 1988;237;525-31.

Bhamarapravati N, Tuchinda P, Boonyapaknavik V. Pathology of Thailand haemorrhagic fever: a study of 100 autopsy cases. *Ann Trop Med Parasitol* 1967; 61;500-10.

Boonpucknavig S, Bhamarapravati N, Nimmannitya S, Phalavadhtana A, Siripont J. Immunofluorescent staining of the surfaces of lymphocytes in suspension from patients with dengue hemorrhagic fever. *Am J Pathol* 1976a; 85: 37-48.

Boonpucknavig V, Bhamarapravati N, Boonpucknavig S, Futrakul P, Tanpaichitr P. Glomerular changes in dengue hemorrhagic fever. *Arch Pathol Lab Med* 1976b; 100: 206-12.

Boonpucknavig S, Boonpucknavig V, Bhamarapravati N, Nimmannitya S. Immunofluorescence study of skin rash in patients with dengue hemorrhagic fever. *Arch Pathol Lab Med* 1979; 103: 463-6.

Brandt WE, McCown JM, Gentry MK, Russell PK. Infection enhancement of dengue type 2 virus in the U-937 human monocyte cell line by antibodies to flavivirus cross-reactive determinants. *Infect Immun* 1982; 36: 1036-41.

Chen Y, Maguire T, Hileman RE, *et al.* Dengue virus infectivity depends on envelope protein binding to target cell heparin sulfate. *Nature Med* 1997; 3: 866-71.

Dasaneyavaja A, Charansri U. First known isolation of a dengue virus from other human source than blood SEATO medical research monograph no 2. Symposium on haemorrhagic fever, Bangkok. 1961; 2.

Eckels KH, Brandt WE, Harrison VR, McCown JM, Russell PK. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. *Infect Immunol* 1976; 14: 1221-7.

Eckels KH, Harrison VR, Summers PL, Russell PK. Dengue-2 vaccine: preparation from a small-plaque virus clone. *Infect Immunol* 1980; 27: 175-80.

Gowans JL. The recirculation of lymphocytes from blood to lymph in the rat. *J Physiol* 1959; 146: 54.

Halstead SB, O'Rourke EJ. Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* 1977; 265: 739-41.

Halstead SB, Marchette NJ, Diwan AR, Palumbo NE, Putvatana R. Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells II Attributes of virus cloned at different dog kidney passage levels. *Am J Trop Med Hyg* 1984; 33: 666-71.

Halstead SB. Antibody macrophages dengue virus infection shock and hemorrhage: a pathogenetic cascade. *Rev Infect Dis* 1989;11 (suppl 4): S830-9.

Hotta H, Hotta S. Dengue virus multiplication in cultures of mouse peritoneal macrophages: effects of macrophage activators. *Microbiol Immunol* 1982; 26: 665-76.

Hotta H, Wiharta AS, Hotta S, Homma M. Dengue type 2 virus infection in human peripheral blood monocyte cultures. *Microbiol Immunol* 1984; 28: 1099-109.

Hotta H, Homma M. Lectin-mediated enhancement of dengue virus infection in a mouse macrophage cell line Mk1. *Arch Virol* 1994; 134: 51-9.

Hotta S, Fujita N, Maruyama T. Research on dengue in tissue culture: I Plaque formation in an established monkey kidney cell line culture. *Kobe J Med Sci* 1966; 12: 179-87.

Innis BL, Nisalak A, Nimmannitya S. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg* 1989; 40: 418-27.

Koff W, Elm JLJr, Halstead SB. Propagation of dengue viruses in murine cell cultures. *Am J Trop Med Hyg* 1981; 30: 461-4.

Kontny U, Kurane I, Ennis FA. Gamma interferon augments Fc gamma receptor-mediated dengue virus infection of human monocytic cells. *J Virol* 1988; 62: 3928-33.

Kurane I, Kontny U, Janus J, Ennis FA. Dengue-2 virus infection of human mononuclear cell lines and establishment of persistent infections. *Arch Virol* 1990; 110: 91-101.

Kurane I, Janus J, Ennis FA. Dengue virus infection of human skin fibroblasts *in vitro* production of IFN-beta IL-6 and GM-CSF. *Arch Virol* 1992; 124: 21-30.

Legrand LF, Hotta H, Hotta S, Homma M. Antibody-mediated enhancement of infection by dengue virus of the P815 murine mastocytoma cell line. *Biken J* 1986; 29: 51-5.

Marchette NJ, Halstead SB, Nash DR, Stenhouse AC. Recovery of dengue viruses from tissues of experimentally infected rhesus monkeys. *Appl Microbiol* 1972; 24: 328-33.

Matsumura T, Stollar V, Schlesinger RW. Studies on the nature of dengue viruses. V Structure and development of dengue virus in Vero cells. *Virology* 1971; 46: 344-55.

Nakao S, Lai CJ, Young NS. Dengue virus a flavivirus propagates in human bone marrow progenitors and hematopoietic cell lines. *Blood* 1989; 74: 1235-40.

Nisalak A, Halstead SB, Singharaj P, Udomsakdi S, Nye SW, Vinijchaikul K. Observations related to pathogenesis of dengue hemorrhagic fever 3. Virologic studies of fatal disease. *Yale J Biol Med* 1970; 42: 293-10.

Rosen L, Gubler D. The use of mosquitos to detect and propagate dengue viruses. *Am J Trop Med Hyg* 1974; 23: 1153-60.

Sprent J. Migration of T and B lymphocytes in the mouse. I Migratory properties. *Cell Immunol* 1973;7:10.

Theofilopoulos AN, Brandt WE, Russell PK, Dixon FT. Replication of dengue-2 virus in cultured human lymphoblastoid cells and subpopulations of human peripheral leukocytes. *J Immunol* 1976; 117: 953-61.

Waterman SH, Kuno G, Gubler DJ, Sather GE. Low rates of antitgen detection and virus isolation from the peripheral blood leukocytes of dengue fever patients. *J Trop Med Hyg* 1985; 34: 380-4.

Westaway EG. Assessment and application of a cell line from pig kidney for plaque assay and neutralization tests with twelve group B arboviruses. *Am J Epidemiol* 1966; 84: 439-56.

Yoksan S, Bhamarapravati N. Localization of dengue antigens in tissue specimens from fatal cases of dengue hemorrhagic fever. In: Procedings of International Conference on Dengue Hemorrhagic Fever. Kuala Lumpur, Malaysia 1984.