DENGUE VIRUSES AND MONONUCLEAR PHAGOCYTES
II. Identity of Blood and Tissue Leukocytes Supporting In Vitro Infection*

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In the previous paper we demonstrated for peripheral blood leukocytes (PBL)[1] from nonimmune primate donors the dependence of dengue 2 virus (D2V) permissiveness upon the presence of non-neutralizing anti-dengue IgG in culture medium (1). Infection occurs in the absence of complement and is dependent upon a protease-resistant leukocyte Fc receptor. When the infectious dose is at or above a multiplicity of infection (MOI) of 0.001 and mononuclear PBL separated from anti-coagulated blood are standardized to 1×10^6/ml, antibody-dependent D2V infection is highly reproducible. The significance of antibody-enhanced infection as a possible mechanism in the pathogenesis of severe dengue infections in infants born in dengue endemic areas was discussed. In this paper we report studies on the identification and the enumeration of human and simian blood and simian tissue leukocytes supporting antibody-enhanced dengue infection, in vitro.

Materials and Methods

Preparation of virus, antibody, separation of leukocytes, and methods of leukocyte propagation are as previously described (1). Unless otherwise specified, PBL were infected with D2V at a MOI between 0.1 and 0.01 in a final concentration of 1:200 of H-187 anti-dengue 4 (aD4) and were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine, and antibiotics (complete RPMI).

Leukocyte Counts and Identification. Leukocyte suspensions diluted in trypan blue and in leukocyte diluent were counted in a hemacytometer. Viable cells were those which excluded 0.2% trypan blue after 5 min incubation at 37°C. Methods for counting monocytes and sheep erythrocyte (SRBC)-rosetted (T) and membrane immunoglobulin-bearing (B) lymphocytes are described separately.[2] Briefly, 1×10^5 PBL in 0.1 ml medium were incubated with an equal volume of 0.5% neuraminidase-treated SRBC at 37°C for 5 min, then centrifuged at 100 *g* for 5 min, and incubated

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[1] *Abbreviations used in this paper*: aD4, anti-dengue 4; B lymphocyte, membrane Ig-bearing lymphocyte; D2V, dengue 2 virus; FA, fluorescent antibody; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; K cell, cell-mediating antibody-dependent cytotoxicity; MOI, multiplicity of infection; PBL, peripheral blood leukocytes; PBS, phosphate-buffered saline; PWM, pokeweed mitogen; T lymphocyte, SRBC-rosetted lymphocyte.

[2] O'Rourke, E. J., S. B. Halstead, A. C. Allison, and T. A. E. Platts-Mills. 1977. Specific lethality of silica for human mononuclear phagocytes, *in vitro*. *J. Immunol. Methods*. In press.

further 18 h at 4°C. Cells were resuspended by gentle rocking and counted after adding a final concentration of 0.0002% acridine orange (Euchrysine 3R; G. T. Gurr, London, England) to facilitate identification of nonrosetted lymphocytes when viewed simultaneously under transmitted white and incident blue light. Only cells with three or more rosetted SRBC were counted. For B-cell counts, 10 μl of undiluted fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin (Nordic Diagnostics, Sera Service Limited, Maidenhead, Berkshire, England) was added to 0.1 ml L-15 medium containing 2×10^5 washed PBL. After 60 min incubation at 4°C, cells were washed three times in cold L-15 medium and counted under phase and UV microscopy. Cells having monocyte morphology (see below) with surface immunoglobulin (Ig) were not counted as B lymphocytes. Counts were expressed as percent of total mononuclear cells. For monocyte counts, PBL were incubated with polystyrene beads (Bacto-Latex, 0.81; Difco Laboratories, Detroit, Mich.) at a bead to cell ratio of 500:1 for 90 min at 37°C. The cells were then treated with 0.0002% acridine orange for 5–10 min. Under blue light illumination this stained DNA a brilliant green and lysosomal enzymes yellow to orange (2). Monocytes were visualized under both phase microscopy and UV illumination. Monocytes are defined as cells with a ruffled cell membrane containing ingested polystyrene particles. In acridine orange-treated preparations, a bright orange lysosomal fluorescence seen in over 95% of cultured monocytes facilitated recognition. Monocytes were considered viable if they exhibited nuclear acridine orange fluorescence. Differential counts were done on at least 500 cells per slide. Cells exhibiting antibody-dependent cell-mediated cytotoxicity (K cells) were assayed by a microtechnique based on the method of MacLennon et al. (3), using human PBL as effectors and ^{51}Cr-labeled Chang cells incubated with or without rat anti-Chang serum as targets.

Preparation of Leukocyte Suspensions from Lymphoid Organs. Lymphatic organs were obtained from adult rhesus monkeys by surgery or immediately after pentothal euthanasia. Bone marrow was removed from the femur and suspended in phosphate-buffered saline (PBS). Lymph node, spleen, and thymus were minced with scissors and expressed through a stainless steel wire mesh (gauge 200) using a disposable 10-ml plastic syringe plunger. Cell suspensions were washed three times in PBS, counted, and suspended at 1×10^6 mononuclear cells/ml in RPMI-1640 with 10% FBS. Daily counts of viable mononuclear cells and phagocytic cells were done.

Infectious Center Assay. D2V was incubated with PBL in an appropriate dilution of test antibody or control for 90 min at 37°C. Cells were then washed in 50 volumes of PBS and resuspended to 1 ml in 1:10 monkey anti-dengue 2 for 30 min at 37°C. Anti-dengue 2 was removed by washing three times in PBS. Viable cells were counted and the concentration adjusted to 1×10^6 ml. Between 5×10^5 and 1×10^3 PBL were applied to LLC-MK2 monolayers or added to 0.4 ml PS cell suspensions (1). With the addition of 10^6 or more PBL, toxic changes in kidney cells were observed. Immediately after inoculating PBL on LLC-MK2 monolayers an agar overlay was added; with PS cells, L-15-carboxymethyl cellulose was added after 4 h incubation.

Fluorescence Microscopy. Dengue antigen(s) in infected PBL was localized by fluorescence microscopy using the direct staining technique. Convalescent human serum from a secondary dengue infection was supplied by Dr. Duane Gubler, Department of Tropical Medicine and Medical Microbiology, University of Hawaii, Honolulu. This serum had a hemagglutination-inhibition titer vs. dengue 1–4 antigens of 1:20,480 and a dengue 2 serum had a 50% plaque reduction neutralization titer of 1:10,000. Globulins from this serum were precipitated with saturated ammonium sulfate, dialyzed, and adjusted to 25 mg/ml. To 1.8 ml of Ig solution, 0.2 ml of a carbonate-bicarbonate buffer, 0.5 M, pH 9.0, was added. The pH was corrected to 9.0 and FITC (Nordic Diagnostics, Sera Service Limited), 20 μg/ml protein, was added. The preparation was incubated and stirred for 18 h at 4°C and then exhaustively dialyzed with PBS, pH 7.2, to remove unconjugated FITC. Conjugated Ig was deaggregated by ultracentrifugation at 100,000 g for 60 min. PBL suspensions were counted and 0.2 ml containing 20,000 cells were sedimented (Cytospin; Shandon Southern Instruments, Camberly, Surrey, England) on alcohol-cleaned or gelatin-coated glass slides, air-dried, fixed in acetone at −20°C for 10 min, and then stored at −70°C until stained. Approximately 0.05 ml of a 1:10 dilution of FITC-conjugated anti-dengue was incubated on PBL for 30 min at 37°C. Cells were then washed three times in PBS, pH 7.4, rinsed with distilled water, and counter-stained with 0.005% Evan's Blue for 10 min at room temperature. Slides were again rinsed with distilled water, air dried, and a cover slip mounted in 50% glycerol, pH 9.0. To visualize FITC, a Zeiss microscope, model WL equipped for photomicrography (Carl Zeiss, Inc., New York), with transmitted light phase-contrast microscopy and incident blue light from a UV

light source passing through KP 490, KP 500, Rfl 510, and LF 528 filters was used. Noninfected PBL were stained with anti-dengue FITC, infected PBL were stained with FITC-conjugated antibodies to nondengue antigens, and blocking experiments with human and monkey anti-dengue 2 were performed to test the specificity of FITC localization to dengue 2 antigen.

Silica Treatment of Leukocytes. Silica particles, <5 μm in diameter (Quartz DQ No. 12, Dörentrop, from Dr. A. C. Allison) were suspended at 1 mg/ml in RPMI-1640 with 10% fetal bovine serum. The suspension was sonicated for 2 min, then kept at 4°C for 18 h, and resonicated just before adding to leukocyte suspensions. Silica suspensions were always freshly prepared as their effectiveness in killing macrophages in vitro declined rapidly on storage either in liquid or frozen state.

Radiation. Separated and washed human PBL were cultured in RPMI-1640 with 10% FBS for 24 h then exposed for varying times to a cobalt-60 source emitting 1.65 rads/s. PBL were either infected immediately after radiation or 24 h later.

Separation of Leukocytes on Nylon Wool Columns or Glass or Plastic Surfaces. Loose, sterile nylon wool was prepared and packed in 10-ml plastic disposable syringes as described by Dickler (4). Up to 5×10^7 cells were suspended in 5 ml of 10% FBS in RPMI-1640 or in 5 ml of 100% autologous serum and loaded onto nylon columns. After incubation at 37°C for 60 and 30 min, respectively, cells were eluted from columns with 20 cm^3 of cold PBS. Cells were counted and identified as described above. Monolayers of leukocytes adherent to 50-mm plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) were obtained with cells suspended in 10% FBS in RPMI-1640 according to the method described by Koller et al. (5).

Results

Peripheral Blood. Identification of leukocytes supporting antibody-enhanced D2V replication was approached by attempting to isolate, remove, or destroy specific leukocyte types.

Adherence. A large number of studies was done to characterize the glass- or plastic-adherence properties of dengue-permissive cells. A typical experimental result is diagrammed in Fig. 1. After 1 h incubation of 2×10^6 mononuclear leukocytes in 2 ml complete RPMI, nonadherent cells were removed by vigorous pipetting of plastic surface with large volumes of PBS. Cells adherent to each 50-mm Falcon plastic Petri dish were counted using an inverted microscope. In companion Petri dishes, adherent cell populations were differentially counted after incubation with latex beads using phase microscopy and observing acridine orange stain under fluorescence microscopy. Infected cells as measured by the infectious center method were most numerous in the strongly adherent population, but were also found among cells washed from the plastic surface. Monocytes were found in both populations.

Further attempts were made to remove adherent cells on nylon wool columns treated with 10% fetal bovine serum or 100% autologous serum. In the former method, both monocytes and B lymphocytes were depleted (Fig. 2). In the latter, monocytes were selectively depleted while B lymphocytes and K cells passed through the column (Fig. 3). D2V-permissive cells were always absent when 99% monocytes were removed from suspension. No plaques were seen in three replicate wells inoculated with 5×10^5 cells. The number of infected cells measured by the infectious center method in unfractionated PBL was always very small. Results shown are representative of three experiments with each type of column.

Radiation. Portion of PBL suspensions from two nonimmune human donors were exposed to 300, 600 and 1,200 rads. These cells and nonirradiated controls were incubated immediately and 24 h after radiation with D2V plus aD4 incu-

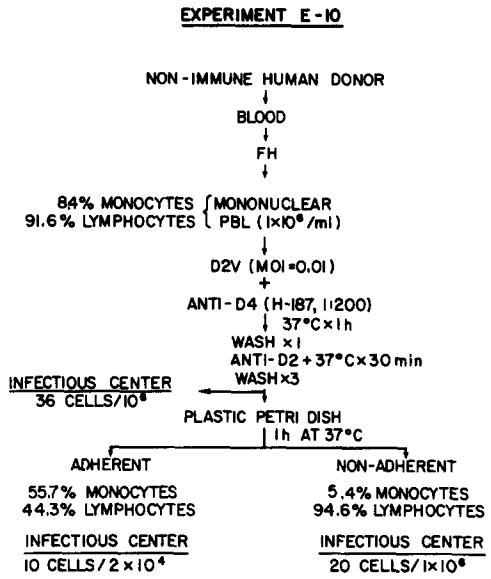

FIG. 1. Infectious center assay of D2V-infected human PBL among plastic-adherent and nonadherent cells. See text for leukocyte identification techniques and experimental methods. Mononuclear leukocytes separated on Ficoll-Hypaque (FH) and assayed on PS cells. Infectious centers (cells) expressed as a fraction of total assayed.

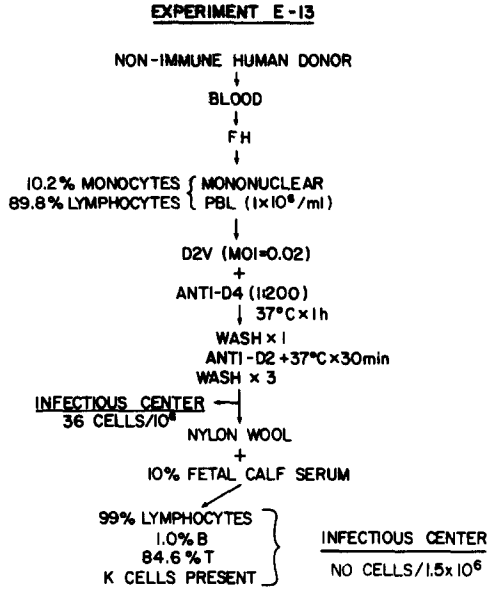

FIG. 2. Infectious center assay of D2V-infected human PBL after application to a 10% fetal bovine serum nylon wool column.

bated at 37°C and assayed for virus content. No difference was observed in virus replicated in irradiated cells or controls.

Selective Killing of Mononuclear Phagocytes. We have shown that silica destroys human mononuclear phagocytes but has no effect on B- or T-lympho-

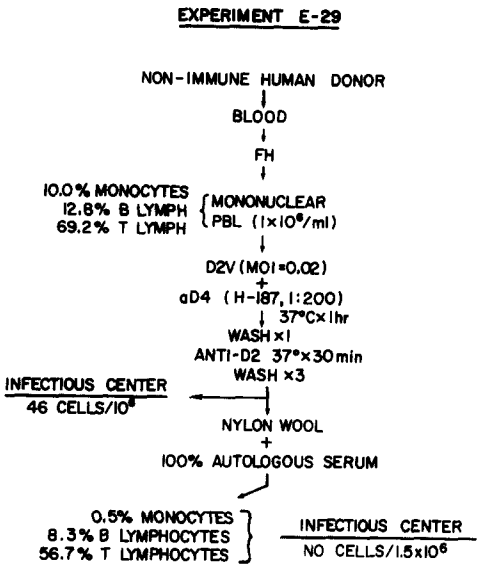

FIG. 3. Infectious center assay of D2V-infected human PBL after application to a 100% autologous serum nylon wool column.

cyte numbers or on B- or T-lymphocyte or K-cell function.[2] Many of these published observations accompanied studies reported here. Freshly prepared silica suspensions were added to PBL before or after inoculation of D2V plus aD4 at concentrations ranging between 0.1 and 100 μg/ml. Monocyte counts and D2V titers in silica-treated human PBL and controls are given for a range of silica concentrations in Table I. The lethal effects of silica on human monocytes and reduction in D2V replication compared with controls were approximately parallel. A summary of representative experiments using rhesus PBL is given in Table II.

Fluorescent Antibody (FA) Identification of Dengue Antigen in PBL. Human PBL infected with D2V were removed at 12-h intervals during 96 h of culture. Cells were counted then incubated with latex beads for 90 min at 37°C. Air-dried PBL were stained with human anti-dengue FITC. Noninfected PBL were stained in similar fashion. In addition, infected PBL were stained with a FITC-conjugated antiserum to an irrelevant antigen. Distinct focal perinuclear fluorescence seen only in D2V-infected cells stained with FITC anti-dengue was first observed at 24 h. Focal areas of fluorescence gradually enlarged to maximum size at 60 h. Only rarely was the entire cytoplasm stained with FITC. From 60 h on, degenerative changes and degenerated cells were noted and the number of infected cells decreased.

Correlations between fluorescence-positive cells, infectious center assay, and virus titers are shown in Table III. Infectious center enumeration performed after 90 min incubation of virus antibody with PBL showed poor correlation with virus content of PBL suspension 24–96 h later. However, the correlation between the concentration of FA-positive cells and viral titer was good. In these experiments, fluorescent-positive cells comprised less than 1% of blood mononuclear phagocytes. In observations not shown, FA-positive cells achieved but did

TABLE I

Effect of Particulate Silica on Human Mononuclear Phagocytes and D2V Replication in PBL Cultures

Silica	No. exps.	Mean ± SD, mononuclear phagocytes × 10^5				Mean PFU × 1,000/ml	
		Day of culture				Day of culture	
		0	1	2	3	2	3
μg/ml							
None	14	8.9 ± 0.8	9.3 ± 1.1	10.8 ± 0.9	10.6 ± 0.8	3.1 ± 0.8	1.7 ± 0.4
0.1	4		8.1 ± 1.7	12.1 ± 2.2	14.6 ± 3.2	3.7 ± 0.4	2.8 ± 0.4
1.0	4		8.0 ± 1.4	10.8 ± 2.3	10.2 ± 2.1	3.5 ± 0.5	2.0 ± 0.8
10.0	4		3.7 ± 1.3	4.1 ± 1.2	3.3 ± 1.5	1.7 ± 0.2	0.5 ± 0.1
25.0	1		7.4	5.2	5.3	0.03 ± 0.0	0.2 ± 0.1
50.0	1		0.7	2.2	3.8	0.04 ± 0.0	0.04 ± 0.0
100.0	10		2.9 ± 0.8	1.3 ± 0.4	1.0 ± 0.5	0.07 ± 0.0	0.04 ± 0.0

TABLE II

Effect of 100 μg/ml Particulate Silica on Rhesus Monkey Mononuclear Phagocytes and D2V Replication in PBL Cultures

Monkey	Silica	Mononuclear phagocytes × 10^5					PFU × 1,000/ml			
		Day of culture					Day of culture			
		0	1	2	3	6	2	3	4	5
Rh 113	None	3.2	4.8	6.2	10.6	20.8	2.2	3.5	3.5	3.5
	100 μg/ml		1.0	0.4	0.2	0.8	0.15	0.03	0.05	0.05
Rh 114	None	6.8	8.0	10.8	9.2	20.4	3.8	3.5	5.0	5.0
	100 μg/ml		3.4	1.2	1.2	0.2	0.03	0.03	0.1	0.1

not exceed 1% of the monocyte population. In seven human leukocyte preparations examined, 73% of fluorescence-positive cells contained ingested latex. All dengue fluorescence-positive cells were large, had ample cytoplasm, and had ruffled cytoplasmic margins.

Infectious Center Assay. Numerous attempts were made to count dengue-infected cells by infectious center techniques. The method most commonly used involved infecting PBL with D2V, washing to remove unabsorbed virus, incubating with anti-dengue 2 to neutralize virus not internalized, again washing, counting, and inoculating onto LLC-MK2 monolayers or in PS cell suspensions. In 25 attempts, the largest number of D2V-infected cells enumerated 90 min after incubation with immune complex was 100 per 10^6 mononuclear PBL and the mean was $31/10^6$. A larger number of infected PBL was measured when cells were allowed to adhere to a plastic surface before washing and neutralizing the virus. The number of infected PBL enumerated after 1 or more days incubation was irregularly higher than the number measured shortly after initial infection. PBL incubated for 48 h or longer are productively infected and release dengue virus into culture medium. It is theoretically impossible to quantitate infected cells by the infectious center assay at this time.

Tissue Leukocytes. Leukocyte suspensions were prepared from lymphatic tissues freshly obtained from five nonimmune rhesus monkeys. After standardizing suspensions to 1×10^6 mononuclear leukocytes/ml, D2V plus aD4 or D2V

TABLE III

Enumeration of Infected Human PBL by Fluorescent Antibody and Infectious Center Techniques Compared with Plaque Assays of Sampled Cultures

Exp.	Hour of study	% Monocytes	Mean D2V-positive cells/10^6 PBL		PFU × 1,000/ 10^6 PBL
			Infectious center*	FA	
1	0	8.1	26	ND‡	ND
1	24	8.4	ND	24	0.4
1	48	9.6	ND	454	2.9
1	72	11.8	ND	563	3.3
2	0		0	ND	
2	48	6.2	ND	50	0.6
2	72	6.8	ND	67	0.7
3	0		38	ND	
3	60	17.0	ND	1,327	15.6
4	60	13.0	ND	849	7.4

* Infected cells/10^6 PBL, assayed in PS cells (1). Infectious center measured after 90 min incubation of D2V and aD4 with PBL (time = 0 hours).

‡ ND, not done.

TABLE IV

Replication of D2V in Nonimmune Rhesus Tissue Leukocyte Cultures in the Presence (aD4) and Absence (FCS) of aD4

Monkey	Mean PFU, days 2-5, × 1,000/10^6 mononuclear leukocytes							
	Source of leukocytes							
	Bone marrow		Spleen		Thymus		Lymph node	
	aD4	FCS	aD4	FCS	aD4	FCS	aD4	FCS
Rh 113	2.1 ± 0.5*	0.04	0.8	0.1	ND	ND	0.2	0.1
Rh A	<0.02	<0.02	<0.02	<0.02	0.3	0.04	0.4	0.1
Rh B	0.1	0.1	0.1	0.03	0.2	0.04	0.1	0.1
Rh C	71.5 ± 6.0‡	0.2	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Rh D	3.3 ± 0.5	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02

Virus antibody added to freshly prepared leukocyte cultures.

* SE of mean shown for values >1.0.

‡ Day 5 culture contained 189,000 PFU/ml.

only was added to cells. After incubation at 37°C, virus content was assayed on days 1–6. Table IV shows mean plaque counts from days 2 to 5 of cultures. When D2V-aD4 was added on day 0, virus replicated in three of five bone marrow cultures. In two animals (Rh 113, Rh D) virus titers approximated those of peripheral blood. The third culture (Rh C), produced the highest in vitro D2V titer of the experimental series. Low level replication was observed in some spleen, thymus, and lymph node suspensions. When D2V plus aD4 was added to tissue leukocytes after 48–96 h in vitro incubation, virus consistently replicated in bone marrow cells but not in leukocytes from other lymphatic tissues (Table V).

TABLE V
Replication of D2V in Nonimmune Rhesus Tissue Leukocyte Cultures in the Presence and Absence of aD4

Monkey	Day infected	Mean PFU days 3-5 × 1,000/10^6 mononuclear leukocytes							
		Source of leukocytes							
		Bone marrow		Spleen		Thymus		Lymph node	
		aD4	FCS	aD4	FCS	aD4	FCS	aD4	FCS
Rh 113	2	1.3 ± 0.3*	0.3	0.2	0.09	ND	ND	0.17	0.1
Rh A	2	0.9 ± 0.5	0.1	0.04	0.04	0.05	0.03	0.07	0.04
Rh B	2	1.3 ± 0.6	0.2	0.06	0.06	0.2	0.05	0.6	<0.02
Rh C	4	67.1 ± 6.0	1.3 ± 0.7	0.15	0.01	0.1	0.07	0.06	<0.01
Rh D	4	3.7 ± 0.8	0.5	0.1	0.08	ND	ND	<0.01	<0.01

Virus antibody added after leukocytes held for 48 or 96 h at 37°C.
* SE of mean shown for values >1.0.

TABLE VI
Mononuclear Phagocytes in Rhesus Tissue Leukocyte Cultures

Monkey	Mononuclear phagocytes × 10^5							
	Bone marrow		Spleen		Thymus		Lymph node	
Day	0	3	0	3	0	3	0	3
Rh 113	1.3	1.8	0.5	0.7	ND	ND	0.05	<0.01
Rh A	ND	1.0	ND	0.8	ND	0.01	ND	0.02
Rh B	ND	2.4	ND	0.4	ND	0.02	ND	0.02
Rh C	1.3	1.7	0.3	0.4	0.08	0.08	0.07	0.06
Rh D	1.0	1.4	0.3	0.4	ND	ND	<0.01	<0.01

On day 0 all cultures contained 1 × 10^6 mononuclear leukocytes.

Virus replication occurred in leukocyte populations with the highest proportion of mononuclear phagocytes (Table VI). Silica treatment reduced D2V replication in tissue leukocytes, although not as completely as in peripheral blood cultures (Table VII). This may have been related to the use in these experiments of stored silica suspensions. When bone marrow, spleen, and thymus leukocyte suspensions incubated with latex were stained with anti-dengue FITC, fluorescence-positive cells were observed to have phagocytosed latex and to have mononuclear phagocyte morphology.

Discussion

In preliminary studies using leukocytes from dengue-immune monkey donors, it had been noted that virus replication was associated with weakly rather than strongly glass-adherent PBL (6). In addition, virus replicated in mitogen-stimulated non-immune PBL (7). From these data we surmised that lymphocytes, possibly B cells, might be the site of dengue virus synthesis. This hypothesis was reinforced when we and others observed D2V replication in human B lymphoblastoid cells (8, 9). The present results do not support this hypothesis. When D2V and appropriate concentrations of dengue antibodies are added to human and simian PBL within 24 h of their removal from the host,

TABLE VII
Effect of 100 μg/ml Particulate Silica on the Replication of D2V in Cultured Tissue Leukocytes

Monkey	Day infected	Mean PFU days 2-5 × 1,000/10⁶ mononuclear leukocytes							
		Bone Marrow		Spleen		Thymus		Lymph node	
		Control	Silica	Control	Silica	Control	Silica	Control	Silica
Rh 113	0	2.1 ± 0.5*	0.4	0.8	0.06	ND	ND	0.2	<0.02
Rh C	0	71.5 ± 6.0	3.9 ± 0.9	<0.02	ND	<0.02	ND	<0.02	ND
Rh D	0	4.1 ± 0.7	1.0 ± 0.3	<0.02	ND	<0.02	ND	<0.02	ND
Rh 113	2	1.3 ± 0.3	0.2	ND	ND	ND	ND	ND	ND
Rh A	2	0.9 ± 0.5	0.08	ND	ND	ND	ND	ND	ND
Rh B	2	1.3 ± 0.6	0.2	ND	ND	ND	ND	ND	ND
Rh C	4	67.1 ± 6.0	2.7 ± 0.7	0.15	0.2	0.1	0.02	0.06	<0.02
Rh D	4	3.7 ± 0.8	0.7	0.08	<0.02	ND	ND	<0.01	ND

* SE of mean for values >1.0.

virus enters and replicates exclusively in mononuclear phagocytes. Evidence supporting this conclusion is that D2V replication is associated with a surface-adherent, radiation-resistant cell and that D2V replication does not occur when PBL cultures were selectively depleted of mononuclear phagocytes by silica treatment or by application to a 100% autologous serum nylon wool column. When FITC-stained dengue-infected leukocytes were visualized under UV light all fluorescence-positive cells had abundant cytoplasm, resembled mononuclear phagocytes in size and cell membrane character, and a large majority had been actively phagocytic.

Theofilopoulos and co-workers have recently presented suggestive evidence of low level dengue infection in unfractionated human PBL and in monolayers of plastic-adherent human leukocytes, all cultured for 3 days before addition of D2V (9). These workers also described D2V replication in unfractionated PBL stimulated for 3 days by phytohemagglutinin or pokeweed mitogen (PWM) then infected and in PWM-stimulated PBL which had been depleted of T lymphocytes and of colloidal iron-adsorbant cells. Many of these data are difficult to interpret. The number of replicate experiments, the number and dengue-immune status of PBL donors, and the confidence limits of measurements of virus concentrations are not given. In only one experiment does peak virus production exceed the 24-h postinoculation titer by more than fourfold, a change in virus concentration which is less than the experimental error of the dengue plaque assay. Although the authors argue that dengue virus replication was demonstrated in mitogen-treated B lymphocytes, no evidence was provided to show how or whether lymphocyte-like monocyte precursors were excluded by the separation techniques employed. Monocytes demonstrate EAC rosetting and can possess membrane immunoglobulin, criteria which were used to demonstrate the purity of putative B lymphocytes.

We have performed a small number of experiments in which PBL were held for 3 days in culture before adding D2V. As reported (1), PBL from nonimmune human donors did not become spontaneously permissive to D2V through 96 h. It was noted, however, that rhesus monkey PBL to which D2V had been added

within a few hours of collection supported virus replication beginning on day 4 or 5 of incubation (1). Except for the difference in time of addition of virus, this delayed growth of dengue virus resembles viral growth described by Theofilopoulos et al. (9). The late replication of D2V in nonimmune monkey PBL was observed sporadically. Attempts to study the phenomenon were impeded by our inability to attain reproducibility. We have not determined which cell type supports delayed dengue virus replication in monkey PBL. This phenomenon is seemingly at variance with observations in human PBL which showed a decreasing permissiveness of mononuclear phagocytes to infection by immune complexes with time in culture.

D2V replicates in PBL which demonstrate weak surface adherence. As shown in Fig. 1, when preinfected PBL were allowed to attach to a plastic surface, virus-containing cells were found among both adherent and nonadherent populations. This same experiment, with minor modifications, has been repeated numerous times using monkey and human cells with similar results. This suggests the existence of a dengue-permissive monocyte subpopulation. This hypothesis is supported by additional observations: (*a*) When held for 2 or more days in culture before adding immune complexes, human and monkey PBL become relatively insusceptible to antibody-mediated dengue infection. During the same period the absolute number of mature monocytes increased (Table I). (*b*) By contrast, even after 4 days in culture, simian bone marrow leukocytes supported antibody-mediated dengue infection at undiminished levels compared with day 0. (*c*) As evidenced by the number of fluorescence-positive cells, a very small proportion of mononuclear phagocytes are infected by D2V, usually less than 1%.

Horwitz and Steagall have described as a macrophage precursor a large lymphocyte found in the blood of patients with infections (10). We have noted consistently in our experiments an absolute increase in cultured human mononuclear phagocytes over a 48–96 h period which is accompanied by an absolute decrease in lymphocytes. Monocytes originate in the bone marrow from precursors (11). Monocytes and small numbers of promonocytes are released into the blood (12). It is likely that any increase in the number of blood monocytes with time in culture must result from a maturational change since little or no division of these cells has been observed (12). By contrast, cultured bone marrow colonies continuously produce monocytes and monocyte precursors (13, 14). Thus, the gradual loss of dengue-permissive cells in cultured PBL, the steady state of permissiveness in bone marrow leukocyte cultures over the same time period, and the very small number of dengue-infected cells are consistent with the hypothesis that dengue virus replicates in an immature monocyte. Similarly, the survival of D2V after phagocytosis suggests as a possible explanation the infection of a cell possessing the property of immune phagocytosis but lacking an efficient intracellular-killing mechanism.

In the preceding paper we suggested as a test of the enhancement hypothesis, the measurement of the size of the dengue-permissive leukocyte population in groups of human beings who respond to dengue infection with mild or severe disease (1). Data presented here suggest that the most accurate method for enumerating dengue-infected leukocytes is the FA technique. The standard infectious center assay applied to virus-infected PBL, in our hands, consistently

underestimated the number of infected cells. We have no insight into this phenomenon, but it may be speculated that the methods used to separate PBL may render them temporarily insusceptible to dengue infection. The repeated centrifugations required for the infectious center assay for example might deplete permissive cells or in some way inhibit virus replication. Such an hypothesis would be consistent with the observation that virus replicates to a higher titer if virus and antibody are added to PBL after 24 h of cultivation rather than immediately after the separation procedure (1). Alternatively, anti-D2 may inactivate leukocytes with exteriorized dengue antigen. Finally, in the environment of the assay system infected PBL may not survive. We have shown that D2V-aD4-infected mononuclear leukocytes incubated in L-15 medium will not sustain viral replication (1). Further studies are required to clarify problems in the assay of isolated dengue-infected leukocytes.

Employing the methodology described, antibody-mediated dengue infection of normal human and simian mononuclear phagocytes is highly reproducible. The method allows use of relatively abundant dengue nonimmune persons as PBL donors and allows antibody, virus, and cell experimental controls. Further studies on this phenomenon may contribute toward an understanding of pathogenetic mechanisms in dengue.

Summary

Studies were made on the identity of human and monkey mononuclear leukocytes permissive to antibody-enhanced dengue 2 virus (D2V) infection. In cultures of peripheral blood leukocytes (PBL) inoculated immediately after separation, it was concluded that only mononuclear phagocytes support dengue infection. This is based upon observations that D2V-permissive cells were resistant to 1,200 rads, were both plastic adherent and nonadherent, were removed when passed through nylon wool columns in 10% fetal bovine serum or 100% autologous serum, and were destroyed by incubation with 100 μg/ml particulate silica. On direct immunofluorescence staining, perinuclear dengue antigen was visualized at 24 h, becoming maximal at 60 h. Antigen-containing cells had ample cytoplasm, ruffled cytoplasmic membrane, and 73% were actively phagocytic. As further evidence of the infection of mononuclear phagocytes, antibody-enhanced D2V replication was observed in bone marrow cultures from five of five rhesus monkeys, but not in cell cultures of spleen, thymus, or lymph nodes prepared from the same animals. It is hypothesized that dengue virus complexed with non-neutralizing antibody is internalized by immune phagocytosis in a mononuclear phagocyte with a defective virus-destroying mechanism. Dengue permissiveness may depend upon cellular immaturity since bone marrow leukocytes could be infected even when held for 4 days before infection while PBL held for this time decreased in permissiveness. In vitro antibody-dependent infection of mononuclear phagocytes should prove useful as a model for study of immunopathologic mechanisms in human dengue.

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