

DENGUE VIRUSES AND MONONUCLEAR PHAGOCYTES

I. Infection Enhancement by Non-Neutralizing Antibody*

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During the past two decades, dengue viruses have been a major cause of severe illness and death among children in tropical Asia (1). Dengue viruses are mosquito-borne flaviviruses which occur as four antigenically distinct types. Homotypic immunity is thought to be life long, but after a brief period of cross-protection, heterotypic infections occur and are accompanied by secondary-type antibody responses (2, 3). Severe dengue, the shock syndrome (DSS),¹ is characterized by abnormalities of hemostasis, and hypovolemic shock (4-7). Studies in Thailand have documented a significant correlation between disease severity and the immune status of the host before infection. The risk of shock is greater during secondary compared with primary dengue infections (6, 8, 9). In a large experience, more than 95% of shock cases over the age of 1-yr had secondary-type dengue antibody responses (4, 6). DSS and the milder, but pathogenetically similar dengue hemorrhagic fever without shock also occur frequently in infants in dengue endemic areas (8). Usually these infections are of the primary type (6). In a few instances the mothers of such infants have been studied and all have had dengue antibodies presumably from infections which antedated pregnancy (7, 10). From these and similar observations it was hypothesized that severe dengue illness is the result of a hypersensitivity phenomenon, pre-illness antibody "sensitizing" individuals to subsequent dengue infection (11).

Clinical and experimental studies on humans and monkeys have provided data from which were developed new and provocative concepts of possible immunopathologic mechanisms resulting in vascular permeability and hemorrhage in dengue infection. Several groups of workers have shown that during the shock stage of dengue, complement (C) is activated, in some instances by both the alternate and classical pathways (7, 10). In the heterologously immune host, virus replication occurs in the presence of cross-reacting antibody. Under these conditions large amounts of C-fixing virus-anti-dengue IgG complexes should be produced. In this model, it has been predicted that vascular permeability is mediated by histamine released by immune complex-activated peptides,

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¹ Abbreviations used in this paper: aD4, anti-dengue 4 serum; BME, basal medium Eagle's; CPD, citrate-phosphate-dextrose; D, dengue virus; DSS, dengue shock syndrome; D2V, dengue type 2; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HI, hemagglutination inhibition; PBL, peripheral blood leukocytes; PBSA, phosphate-buffered saline without Ca⁺⁺ or Mg⁺⁺; PFU, plaque-forming units; PRNT, plaque reduction neutralization test.

C3a and C5a (12). This hypothesis does not satisfactorily explain how C depletion and shock can occur in 8- or 9-mo-old infants who presumably have extremely small amounts of residual maternal anti-dengue IgG.

We believe that antibody contributes to the severity of dengue infection by an entirely different mechanism, by regulating the infection of cells (13). This phenomenon, immunological enhancement of dengue infection, has been observed in experimentally infected monkeys and in cultures of primate peripheral blood leukocytes (PBL). Monkeys monotypically immune to dengue viruses types 1, 3, or 4, when infected with dengue type 2 (D2V), circulated more virus than did nonimmune animals infected with the same virus strain administered by the same route and at the same dose (14). Similarly, PBL cultures prepared from dengue-immune monkeys or human beings readily supported dengue virus (D) replication while leukocyte cultures from non-immunes were not permissive (15-17). Recently, we have published preliminary evidence that non-neutralizing dengue antibody when added to PBL cultures from nonimmune primates results in productive infections similar to those occurring in cultures of immune PBL (18). The relevance of these observations are reinforced by virus recovery studies on dengue-infected human beings and monkeys which strongly suggest that leukocytes are the predominant or the only sites of D replication (19, 20).

This and the companion paper present further data on in vitro antibody-mediated enhancement of dengue infection in primate leukocytes (21). We report studies on the effect on viral replication of different methods of separating and culturing leukocytes, the kinetics of viral replication, the kinetics of internalization of virus, the immunoglobulin which mediates infection enhancement, the nature of the leukocyte antibody-binding site, and the attributes of virus-antibody-leukocyte interactions. Separately, we describe studies on the identification of blood and tissue leukocytes which support antibody-mediated dengue infection (21).

Materials and Methods

Virus Preparations and Assays. D types 1 (strain 16007), 2 (strain 16681), 3 (strain 16562), and 4 (strain 4328 S) were propagated either in continuous rhesus (LLC-MK2) or vervet (Vero) monkey kidney cells for 4-6 days in basal medium Eagle's (BME), with Earle's salt solution and 10% calf serum. Infected cells were disrupted by freeze-thawing and the suspension clarified by centrifugation at 2,000 g for 15 min. Virus in supernatant fluid was then concentrated by ultracentrifugation in a 10 × 10 angle head rotor either in a Spinco L-65 (Beckman Instruments, Inc., Spinco Div., Fullerton, Calif.) or MSE Superspeed 65 (MSE Scientific Equipment, Crawley, Sussex, England) at 100,000 g for 60 min. The pellet and the lowest 10% of the supernatant fluid were retained and resuspended by brief sonication. Fetal bovine serum (FBS), or agamma calf serum was added to a final concentration of 20-50% and the virus suspension stored at -70°C in multiple vials. Virus was quantitated by a plaque method in LLC-MK2 cells (22), or PS cells (23). For both systems, leukocyte suspensions were diluted 1:10 and the assay vol was 0.2 ml. For LLC-MK2 cells, inocula were added to each of three 1 ounce prescription bottles containing cell monolayers for 90 min at 37°C and then overlaid with Noble agar containing BME, 100% calf serum, and neutral red. The bottles were incubated in the dark for 7 days at 37°C, then for 7 days at room temperature and the plaques counted. In the PS cell assay, virus inoculum was added to cells to produce a total vol of 0.4 ml at a cell concentration of 1.5×10^6 cells/ml. After 4 h incubation of virus with cell suspension at 37°C, an overlay (0.4 ml) was added consisting of a final concentration of 1.5% carboxy methyl-cellulose (sodium salt; British Drug Houses, Ltd., London, England) supplemented with 3% FBS (GIBCO Bio-Cult, Ltd., Paisley, Scotland), L-glutamine, antibiotics, and with 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.). Assays were performed in at least three

replicate 16-mm diameter wells in plastic Disposo Trays (Model FB16-24TC; Linbro Chemical Co., New Haven, Conn.). After 96–108 h at 37°C the overlay medium was removed, cells rinsed in saline, and stained with a naphthalene black-acetic acid solution. PS cell concentrations required to form dense monolayers were found to vary with different cell lots between 1.5 and $3.0 \times 10^5/\text{ml}$. In some instances it proved necessary to increase the volume of L-15 medium and overlay to provide sufficient nutrients to maintain healthy cells throughout the 5-day incubation period.

Replicate plaque assays of individual samples are shown as mean values \pm one standard deviation; grouped data from several samples are shown as means \pm one standard error of the mean. Differences between mean values were tested by Student's *t* test for paired data using the square roots of plaque counts.

Antibody Production. Dengue antibodies were either obtained from naturally infected human beings or raised in rhesus monkeys or mice which were without neutralizing antibody to dengue types 1–4. Monkeys were infected by a single subcutaneous inoculation of approximately 1×10^4 to 1×10^5 plaque-forming units (PFU) of tissue culture passaged dengue viruses. Some animals were bled at intervals of 2–3 days for the first 2 wk after inoculation and then at 3 and 6 wk. For routine preparation of monkey anti-dengue, animals were bled at least 2 mo after a single inoculation. In mice, antibodies were raised by the immunization schedule of Brandt et al. (24) using saline suspensions of homogenized mouse brains infected with prototype mouse passaged dengue viruses. Antibodies were obtained in ascitic fluids induced by sarcoma 180 cells (25).

Antibody Measurement. Antibodies were measured by the hemagglutination-inhibition (HI) test and the plaque reduction neutralization test (PRNT). The HI test was performed in Microtitr-plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, Va.) using microvolumes and standard methods (26). All serum or plasma samples were treated with kaolin (Flow Laboratories, Inc., Rockville, Md.) to remove nonspecific hemagglutination inhibitors. Goose erythrocytes and eight antigen units were used in all tests. The 50% PRNT was performed in LLC-MK2 or PS cells using virus assay methods described above. Virus-serum mixtures were incubated for 60 min at 37°C before virus assay. Antibody titers were calculated on log probit paper by the method of Russell et al. (27).

Separation and Chemical Modification of Antibodies. Anti-dengue serum was separated into different immunoglobulin types by two methods: (a) density gradient centrifugation. This was carried out in a discontinuous sucrose gradient consisting of successive layers of 1.0 ml of 40%, 1.5 ml of 25%, and 1.0 ml of 10% sucrose in a phosphate buffer, pH 7.4. After overnight incubation at 4°C, 0.4 ml of a 1:2 dilution of serum was layered on a 4.5 ml gradient and centrifuged in a 3 × 5 ml swing-out head (MSE Scientific Equipment) in a MSE Superspeed 65 ultracentrifuge at 35,000 rpm (100,000 g) for 18 h. Fractions of 0.5 ml were collected by piercing the bottom of the lusteroid tube. These were dialyzed against borate saline, pH 9.0. Fractions collected from the upper one half of the tube were treated with kaolin before measuring HI antibody. (b) DEAE-cellulose chromatography. Gamma globulins, precipitated twice by 33% saturated ammonium sulfate, were exhaustively dialyzed against 0.01 M phosphate buffer, pH 7.8, and then applied to a DEAE-cellulose column. The IgG fraction was obtained by pooling the first protein peak eluted by 0.01 M PO₄ buffer, pH 7.8, and concentrated to 10 mg/ml on an Amicon ultrafiltration unit (PM10 membrane; Amicon Corp., Lexington, Mass.). The IgG sample was further purified by Sephadex G-200 chromatography.

F(ab)₂ fragment was prepared from IgG by pepsin digestion (pepsin P 7012; Sigma Chemical Co., St. Louis, Mo.) at a 1:50 ratio of pepsin to sample by published methods (28) and separated by Sephadex G-200 gel filtration (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden). The protein content of purified IgG and F(ab)₂ was measured by the Lowry method (29). The dengue antibody activity of each immunoglobulin preparation was measured by the HI test using dengue 2 antigen and PRNT vs. D2V.

The specificity and purity of separated immunoglobulins was tested by immunoelectrophoresis and Ouchterlony using goat anti-human (anti- γ , μ , α , κ , λ) IgG and IgM (Nordic Diagnostics, Sera Service Limited, Maidenhead, Berkshire, England), and quantitated by radial immunoprecipitation using reagents prepared and standardized by the Immunology Division, Northwick Park-Clinical Research Centre, London. The purity of F(ab)₂ was determined by Ouchterlony using anti-human IgG, anti-F(ab), and anti-Fc serums (Nordic Diagnostics Sera Service Limited).

Leukocyte Separation and Culture. Blood was anti-coagulated either in heparin, 20 IU/ml final concentration (Heparin, B.P., without preservative; Paines and Byrne, Greenford, England);

citrate-phosphate-dextrose (CPD) solution, 63 ml in 450 ml blood, each 100 ml contained sodium citrate, 2.65 g; dextrose, 2.32 g; citric acid, 327 mg; and sodium acid phosphate, 251 mg (Fenwal Division, Travenol Laboratories, Ltd., Thetford, Norfolk, England), in dipotassium EDTA at a final concentration of 1.5 mg/ml (Stayne, London, England) or defibrinated using three glass beads in 20 ml blood. Leukocytes were recovered either by dextran sedimentation or by adding blood to 2 volumes of 2.4% dextran T-250 (Pharmacia Fine Chemicals Ltd.), incubating at 37°C for 30 min, and removing the leukocyte-rich plasma with a pipet, or by the density flotation method (30) in which anti-coagulated blood was diluted approximately 1:3 in phosphate-buffered saline without Ca⁺⁺ or Mg⁺⁺ (PBSA) and underlaid with a Ficoll-Triosil (Hypaque) solution prepared as described by Böyum (30) (Ficoll; Pharmacia Fine Chemicals Ltd., and Triosil 440; Nyegaard & Co., AS, Oslo). Mononuclear leukocytes were separated centrifugally at 400 g, and washed three times in PBSA, suspended in Hanks'-buffered salt solution (HBSS), counted, and resuspended at 1.5 or 1.0 × 10⁶ cells/ml in RPMI 1640 (GIBCO Bio-Cult, Ltd.) with 20 mM Hepes buffer and 0.02% sodium bicarbonate and 10% heat-inactivated FBS (complete RPMI). Leukocytes were incubated for up to 7 days without change of medium in stoppered or capped plastic or glass vials or tubes.

Infection of Leukocytes. Specific conditions for infection of PBL are described under Results. Three large lots of D2V were used. During the first 2 yr of the study, residual virus from experimental use was always assayed. When this experience showed that D2V viability in large seed lots was stable, in subsequent experiments, lots were titered at monthly intervals. Approximately one-half of the experiments described in this study utilized a single lot of monkey anti-dengue 4 serum (aD4), H-187, at a 1:200 final dilution.

Treatment of Leukocytes with Proteolytic Enzyme. Separated and washed human and monkey PBL were suspended at 1 × 10⁷ cells/ml in PBS, pH 7.8, containing 0.02% EDTA, following the methods of Kedar et al. (31). To each milliliter of PBL suspension was added an equal volume of serially 10-fold diluted trypsin (bovine pancreas type III, twice crystallized, 12,500 BAEE U/mg protein; Sigma Chemical Co.). Mixtures were placed in a 37°C water bath and shaken intermittently for 30 min. Cells were washed three times in HBSS, counted, resuspended to 1.5 × 10⁶/ml, and either D2V only or D2V plus aD4 added.

In other experiments, PBL were treated in similar fashion with twice crystallized papain, 17 uts/mg protein (Sigma Chemical Co.), and pronase, B-grade 45,000 PUK/g (Calbiochem, San Diego, Calif.).

Results

Effect of Methodological Variables on Infection

ANTI-COAGULANT. Heparin sodium, with or without preservative, was the anti-coagulant employed in initial experiments. In the whole series, 158 blood samples were anti-coagulated with heparin before attempts to study antibody-enhanced D2V replication in monkey or human PBL. Viral replication exceeded 50 plaque-forming units (PFU) per 10⁶ mononuclear PBL in 123 of these cultures. In an attempt to simplify blood collection, 11 human blood samples were defibrinated. D2V replication was observed in only three of these experiments. After this experience, the effect of anti-coagulant on D2V replication was compared in three experiments. Blood from human donors was defibrinated or anti-coagulated with heparin, CPD, or EDTA. Leukocytes were then separated by Ficoll-Hypaque, counted, suspended at 1 × 10⁶ mononuclear PBL/ml in RPMI-1640 with 10% heat-inactivated calf serum, and D2V added at a multiplicity of infection (MOI) of 0.01 together with a 1:200 final concentration of H-187 aD4. Cells were assayed on days 1–4. No significant difference in virus production was observed on any day in cultures prepared from blood anti-coagulated with heparin, CPD, or EDTA. No viral replication was observed in two of three defibrinated samples. In the third sample viral replication occurred at high titer in cells separated by each method.

LEUKOCYTE SEPARATION. The effect of leukocyte separation technique on

D2V replication was compared directly only two times. In approximately one-half of 158 experiments, PBL were separated from erythrocytes using dextran T-250, in the other half, PBL were separated on Ficoll-Hypaque. The average PFU per milliliter culture medium on days 1-4 did not differ with separation technique. In 10 experiments virus replicated well in whole blood anti-coagulated with heparin and diluted 1:5 in RPMI-1640 with 10% FBS or 10% autologous serum.

CULTURE MEDIA. Using RPMI-1640 medium in stoppered culture vessels, D2V yield was optimal when pH was regulated with 20 mM Hepes buffer together with 0.02% sodium bicarbonate. Gassing cultures with 5% CO₂ in air did not affect viral replication when compared with ungassed cultures. Satisfactory virus yields were also obtained in five experiments in which PBL were cultured in M-199 with 5% or 10% FBS plus 2% sodium bicarbonate. In four experiments, human PBL cultured in L-15 medium with 10% FBS did not support D2V synthesis, while virus replicated normally in control cells maintained in RPMI-1640.

Interactions between Leukocytes, Virus, and Antibody

KINETICS OF VIRUS REPLICATION. There was considerable variability in the amount of D2V replicated and the day of peak infection in primate PBL cultures. Even when PBL concentrations were standardized, D2V added at a narrow range of MOI (0.1-0.01) and one lot of aD4 used at a single final dilution, large differences in virus growth were observed. Six illustrative D2V growth curves in PBL cultures with and without antibody are shown in Fig. 1. In some cases (exp. I and II) viral replication occurred only in PBL cultures supplemented with antibody; in other instances, D2V growth in cultures with antibody showed only transient differences from control (exp. III) or no difference from control (exp. IV); in some experiments, the late production of D2V was observed in PBL cultures without antibody. Virus replicated was either significant, less than D2V grown in antibody-containing cultures (exp. V), or less often, differences were not significant (exp. VI).

In 112/145 paired experiments a significant difference in D2V replication between cultures with antibody compared to those without was observed on at least 1 day of culture. Because of the variation in day of peak virus production, virus assay results for 2 or more days were generally pooled. For monkeys this usually included days 3-5; for humans, days 2-4. This evaluative procedure resulted in large standard deviations and standard errors of the mean.

Similar variations in the replicative kinetics of D2V were observed in human PBL cultures. Paired data in Fig. 2 were obtained from PBL cultures prepared from the same donor over a period of 9 mo. A single batch of virus and aD4 were used.

ANTIBODY SPECIFICITY, IMMUNIZATION METHOD, AND SPECIES OF ANTIBODY DONOR. D2V infection enhancement in monkey or human PBL was produced by antisera raised to dengue types 1, 3, and 4 viruses using several species as antibody donors. We have tested one or more lots of anti-dengue raised in 10 monkeys, anti-D1 (2), anti-D3 (2), anti-D4 (6), 2 pools each of anti-D1, 3, and 4 mouse immune ascitic fluids, single rabbit antisera to each of these three viruses, and serums from five monotypic dengue-immune human beings. All of

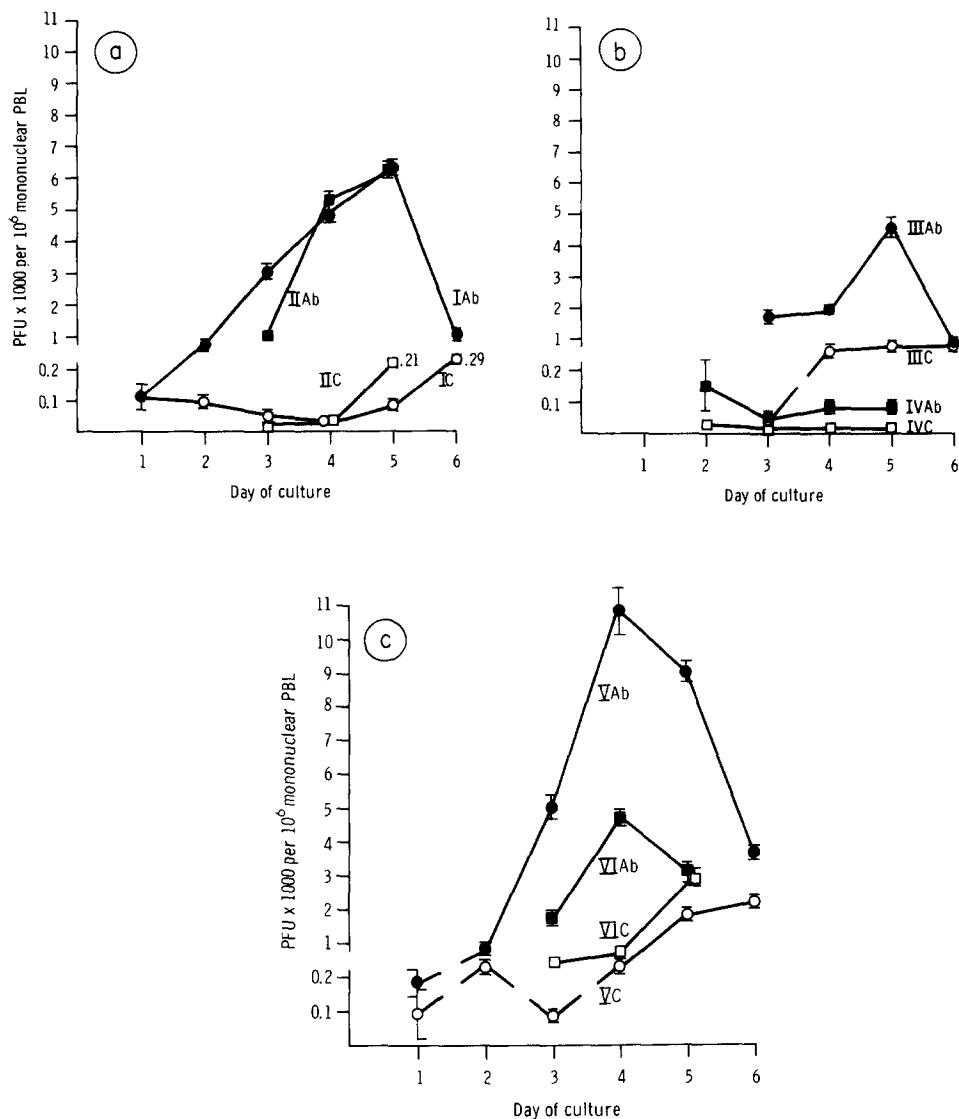


FIG. 1. (a, b, and c) Variability of antibody-enhanced D2V replication in six selected rhesus PBL cultures (exps. I-VI). PBL cultures were prepared at different times from monkey H-190 over a period of 5 mo. To each was added the same lot of D2V at a MOI of 0.1 and one half of each suspension received a 1:10 final concentration of rhesus H-30 aD4 (Ab). Cultures containing D2V only serve as control (C). After 120 min PBL were washed three times, counted, resuspended to 1.5×10^6 /ml, cultured days 1-6, and freeze-thawed suspensions assayed. Virus titers were standardized to 1×10^6 PBL.

these serums produced infection enhancement at or above 1:10 final dilutions. Representative results obtained from testing five serums simultaneously are shown in Fig. 3. Heterotypic anti-dengue serums had only low-titered 50% plaque reduction activity against D2V. From experimental results not shown the amplitude of enhancement and enhancing titers was shown to vary with individual sera and not with anti-dengue specificity.

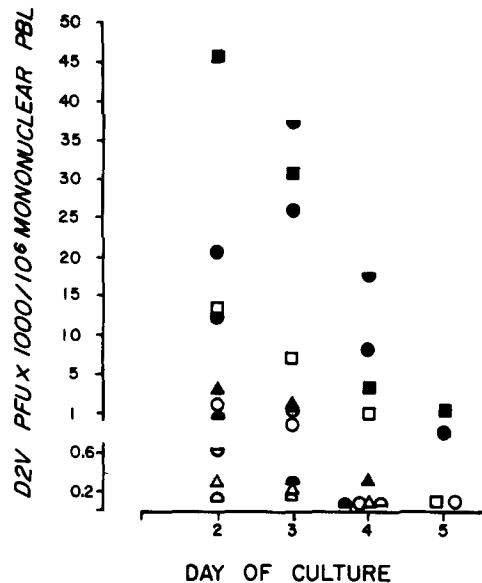


FIG. 2. Mean D2V titers on days 2-5 in five human PBL cultures incubated with and without H-187 aD4. Cultures prepared over a period of 9 mo from a single nonimmune donor. Paired results are shown in closed (with antibody) and open (no antibody) symbols.

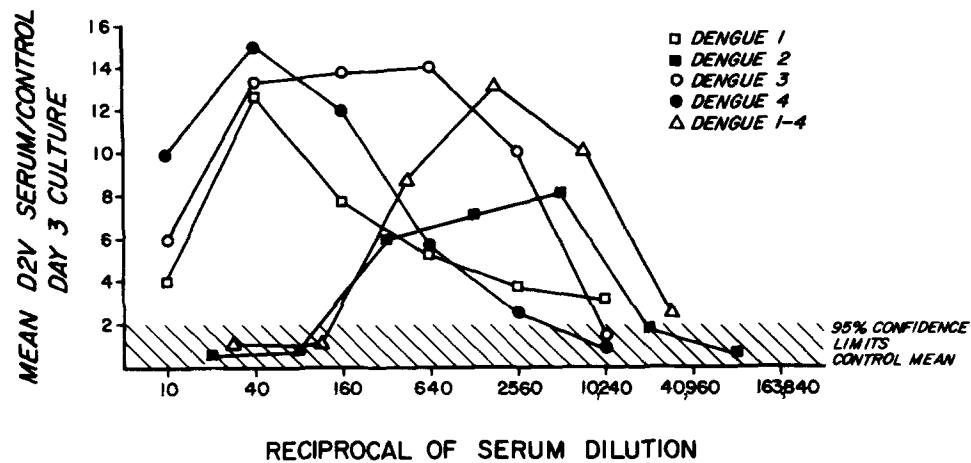


FIG. 3. Fold increase in D2V replication in human PBL cultures at varying dilutions of dengue antisera. Dengue 2 plaque reduction neutralization titers of tested sera: mouse anti-dengue 1, 1:15; rabbit anti-dengue 2, 1:320; rabbit anti-dengue 3, 1:40; monkey (H-187) anti-dengue 4, 1:20; human (SBH) anti-dengue 1-4, 1:717.

Homotypic anti-dengue 2 enhanced D2V replication at dilutions above neutralizing end points. A typical result is illustrated in Fig. 3; rabbit anti-dengue 2 with a 50% PRNT of 1:320 produced significant enhancement beginning at this dilution through 1:5,120. One anti-dengue 2 serum prepared in a rhesus monkey with a homologous PRNT₅₀ of 1:640 had an enhancement titer of 1:320,000.

ANTI-DENGUE CYTOPHILIA. Anti-dengue cytophilic for monkey and human PBL were studied in three experiments. Representative results from one experi-

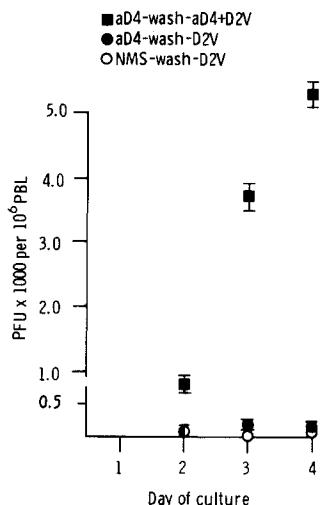


FIG. 4. Failure of aD4 to enhance D2V infection when preincubated with PBL. A 1:10 final concentration of heat-inactivated H-130 rhesus aD4 added to first and second PBL suspensions, and H-190 normal rhesus serum at 1:10 added to the third portion. Sera incubated for 120 min at 37°C and removed by washing. D2V (MOI, 0.1) added to all tubes. aD4 replaced in first tube. Means from six plaque assays \pm SE from two replicate experiments are plotted.

ment in monkey PBL are shown (Fig. 4). PBL were divided in three portions. Anti-dengue 4 was added to two samples, and FBS to the third. Cells and antibody were incubated at 37°C for 120 min and then washed three times. To the first portion, D2V plus aD4 were added, and to the second and third D2V only. D replication occurred only when aD4 was readded.

C DEPENDENCE. No difference in D2V replication was observed in two experiments in which late convalescent aD4 plus D2V was added to monkey PBL cultured in fresh or heat-inactivated FBS or autologous nonimmune rhesus serum.

ENHANCING ACTIVITY OF ANTI-DENGUE IgG AND IgM. Two nonimmune rhesus monkeys were infected with D2V and bled on days 7, 10, 14, 21, 28, and 45 after infection. Sera were tested for HI dengue 2 antibodies. The earliest serum containing antibody was fractionated on a sucrose density gradient (Table I). After dialysis and without further concentration, fractions were tested for HI and neutralizing antibodies and Ig content (Table II). For both animals, fractions 3 and 7 from day 14 postinfection sera contained uncontaminated IgM and IgG, respectively, as ascertained by immunoelectrophoresis against anti-Ig, anti-IgM, and anti-IgG. Immunoglobulins were quantitated by radial immunodiffusion. By comparing HI antibody titers, in serum and fractions, fraction 3 represented a 1:10 and 1:20 dilution of rhesus 111 and 112 sera, respectively. The concentration of IgG in fraction 7 of rhesus 111 and 112 was approximately 25% that of whole serum. D2V infection-enhancing activity was measured in whole serum and serum fractions using human PBL (Table III). Titers were expressed as dilution of serum antibody or IgG. Whole serum neutralized D2V at low dilutions and gave a weak enhancement at higher dilutions. Anti-dengue IgM did not enhance D2V replication at any (serum) dilution from 1:200 through 1:102,400. Fraction 7 from both animals had high-titered enhancing activity, but

TABLE I
*Temporal Development of HI and Neutralizing Antibody to D2V
 After Primary Infection of Two Rhesus Monkeys*

Rhesus 111			Rhesus 112		
Day of infection	Reciprocal titer		Day of infection	Reciprocal titer	
	HI	PRNT ₅₀		HI	PRNT ₅₀
0	<10	<10	0	<10	<10
7	<10		7	<10	
10	<10		10	10	
14	40	4,800	14	80	5,000
21	40		21	80	
28	40		28	80	
45	40		45	80	

TABLE II
*Immunoglobulin and Antibody Concentrations in Dialyzed Fractions Collected after
 Centrifugation of Day 14 Serums on a Sucrose Gradient at 100,000 g for 16 h*

Rhesus 111				Rhesus 112						
Fraction	IgM	IgG	Reciprocal titer		Fraction	IgM	IgG	Reciprocal titer		
	HI	PRNT ₅₀	mg/100 ml*	HI	PRNT ₅₀	mg/100 ml	HI	PRNT ₅₀		
<i>mg/100 ml*</i>										
1	0	0	<1			1	0	0	<1	
2	0	0	1			2	0	0	2	
3	<25 (+)	0	4	320		3	40	0	4	320
4	<25 (+)	0	2			4	+	0	1	
5	0	+	<1			5	0	+	<1	
6	0	+	<1			6	0	+	2	
7	0	305	<1	<10		7	0	481	2	<10
8	0	+	<1			8	0	+	<1	
Whole serum	68	1,250	40	4,800		Whole serum	172	1,960	80	5,000

* Fractions tested by Ouchterlony versus anti-IgM or anti-IgG. Fractions containing the highest concentration of IgM or IgG quantitated by radial immunoprecipitation.

TABLE III
*D2V Replication Enhancing Activity in Rhesus Monkey Serums and Sucrose Gradient
 Fractions Obtained 14 Days After Primary Infection*

Rhesus 111					Rhesus 112						
Serum		Fraction 3		Fraction 7	Serum		Fraction 3		Fraction 7		
Dilution	Enhancement*	Dilution‡	Enhancement	Dilution‡	Enhancement	Dilution	Enhancement	Dilution‡	Enhancement		
10	<1	200	<1	40	7.0	10	<1	400	<1	40	8.0
40	<1	800	<1	160	13.0	40	1.1	1,600	<1	160	12.0
160	2.9	3,200	<1	640	11.0	160	<1	6,400	<1	640	8.0
640	1.7	12,800	<1	2,560	4.4	640	1.3	25,600	<1	2,560	5.4
2,560	2.5	51,200	<1	10,240	2.1	2,560	3.2	102,400	<1	10,240	3.0
10,240	<1			40,960	<1	10,240	2.3			40,960	<1

* Day 3 PFU: test sample/control.

† Serum equivalent.

TABLE IV

Immunoglobulin Concentrations, Anti-D2, and Replication Enhancement Titers in Serum, IgG, and F(ab)₂ Prepared from a D1-4 Immune Human

Specimen	Lowry	IgG	Reciprocal titer		D2V replication-enhancing titer	
			HI*	PRNT ₅₀ ‡	Exp. 1	Exp. 2
<i>mg/100 ml mg/100 ml</i>						
Serum (SBH)	4,750	1,440	88 ± 8	717	25,600	12,800
IgG (SBH)	170	114	8 ± 0	76	1,280	640
F(ab) ₂ (SBH)	130	§	5 ± 0.7	78	<1	<1

* Mean ± SD of 10 replicate titrations.

† Mean of two determinations.

§ Versus anti-human F(ab) = 80 mg/100 ml.

were without measureable HI or neutralizing activity at the lowest dilutions available for test (1:4 serum equivalent).

FC DEPENDENCE. D2V infection-enhancing, HI, and neutralizing activities of whole serum and F(ab)₂ preparations are shown in Table IV. The ratio between enhancing titers in serum and IgG was nearly the same as the ratios of HI and neutralizing titers between serum and IgG. Although anti-dengue 2 F(ab)₂ possessed both neutralizing and HI antibody to D2V, the preparation did not enhance D2V infection. Similar results were obtained in both monkey and human PBL.

PROTEOLYTIC ENZYMES. Monkey and human PBL were incubated with varying concentrations of several proteolytic enzymes for 30 min at 37°C. Enzyme was removed by washing, cells incubated with D2V plus aD4 or with D2V alone, and viral replication assayed. Heat-inactivated enzyme or no enzyme were used as controls. At some dilutions proteolytic enzyme treatment resulted in as much as an 18-fold increase in viral replication compared with non-enzyme-treated cells (Table V). Infection occurred only in the presence of aD4. Treatment of PBL with varying concentrations of enzymes did not increase the permissiveness of cells to infection by virus alone (data not shown). Papain and pronase were more active than trypsin under the conditions of these experiments.

PERMISSIVENESS OF PBL IN RELATION TO TIME IN CULTURE. Holding leukocytes in culture before infection was studied as a possible variable affecting infectibility of cells. Virus plus antibody or virus only was added to standard human PBL cultures shortly after Ficoll-Hypaque separation (time 0) and at 24-h intervals through 96 h. After infection, cultures were assayed on days 1–5. Average virus content of PBL cultures on days 2, 3, and 4 from eight experiments is shown in Table VI. Virus titer in cells infected after 24 h in culture was significantly greater than at other time periods. The 24-h titer differed from that at time 0 ($P < 0.001$). In cells infected at 48 h and later there was sharp decline in viral replication. Similar results were obtained in monkey leukocyte cultures.

KINETICS OF INTERNALIZATION OF D2V-aD4. D2V plus aD4 was added to replicate standardized monkey PBL suspensions and incubated at 37°C. At intervals tubes were removed and one half of cells were washed three times, counted, resuspended at $1 \times 10^6/\text{ml}$, incubated, and assayed on days 2–6. The other portion of cell suspension was washed once, anti-dengue 2 added at a final

TABLE V

D2V Replication in PBL Treated with Proteolytic Enzymes Before Incubation with D2V Plus aD4

Concentration of enzyme	Trypsin		Papain		Pronase	
	Mean PFU/ 10^6 PBL \pm SE*	Ratio of test:control†	Mean PFU/ 10^6 PBL \pm SE	Ratio of test:control	Mean PFU/ 10^6 PBL \pm SE	Ratio of test:control
<i>mg/ml</i>						
0.0001	476 \pm 50	1.0	1760 \pm 483	1.1	874 \pm 70	1.7
0.001	509 \pm 63	1.1	4566 \pm 320	2.8	2,792 \pm 130	5.5
0.01	641 \pm 84	1.4	22,224 \pm 3,513	13.6	3,497 \pm 210	6.9
0.1	1,589 \pm 133	3.4	14,865 \pm 2,366	9.1	5,671 \pm 269	11.2
1.0	1,640 \pm 179	3.5	14,732 \pm 2,167	9.0	9,091 \pm 74	17.9
10.0	190 \pm 27	0.4	96 \pm 19	0.04	2,454 \pm 147	4.8
None	470 \pm 45	—	1,633 \pm 320	—	508 \pm 60	—
Heat inactivated, 0.1 mg/ml	573 \pm 67	—	1,700 \pm 433	—	330 \pm 51	—
D2V only, 1.0 mg/ ml	26 \pm 4		23 \pm 5		13 \pm 2	

Results of single representative experiments are shown.

* Average of mean plaque counts on days 2-5 culture \pm standard error of mean.

† PFU enzyme-treated/nontreated PBL cultures.

TABLE VI
Replication of D2V in PBL Cultures from Eight Human Donors Incubated for Varying Periods Before Addition of D2V Plus D4

Days in culture before addition of D2V and aD4	Total leukocyte assays	Mean PFU/ 10^6 PBL \pm SE*	
		D2V and aD4	D2V only
0	21	4,487 \pm 850	125 \pm 6
1	24	11,025 \pm 3,425	76 \pm 4
2	24	6,493 \pm 2,717	111 \pm 5
3	20	1,838 \pm 978	176 \pm 7
4	24	1,243 \pm 424	185 \pm 8

* Mean plaque count on days 2-4 of culture after inoculation \pm one standard error of mean.

dilution of 1:10 for 30 min at 37°C, cells washed three times, counted, resuspended, incubated, and assayed. Control cells were not washed. Virus yields from PBL exposed to D2V for short periods were expressed as a ratio of control titers. Virus content of cells cultured for 3-5 days is shown in Table VII. After 10-15 min incubation, comparatively little virus could be removed from PBL by washing. A significant fraction of D2V was still accessible to neutralization by specific antibody through 30 min of incubation. D2V internalization was essentially complete at 60-90 min. The kinetics of internalization of D2V in human PBL are similar to those in monkey PBL.

MOI. The effect of virus dose on the kinetics of replication and the threshold of infectivity of PBL was measured in human and monkey PBL. Serial 1-fold dilutions of stock virus was added to PBL in the presence of a constant concen-

TABLE VII
Kinetics of Internalization of D2V Plus aD4 in Rhesus PBL

Method	Exp. no.	Time (min)	PFU days 3-5 washed/nonwashed PBL						
			5	10	15	30	60	90	120
Wash \times 3	1		ND	ND	ND	ND	1.0	ND	ND
	2		0.4	0.8	ND	1.0	1.0	ND	ND
	3		ND	ND	0.8	1.0	1.0	ND	1.0
Wash \times 1	1		0.0	0.1	ND	0.1	0.3	ND	1.0
Anti-D2, 37°C \times 30 min	2		0.1	0.1	0.2	0.4	0.7	1.0	ND
Wash \times 3	3		ND	ND	0.3	0.5	0.8	ND	1.0

Virus-antibody added to PBL for progressively longer times, then leukocytes are washed or incubated with anti-dengue 2 before culture.

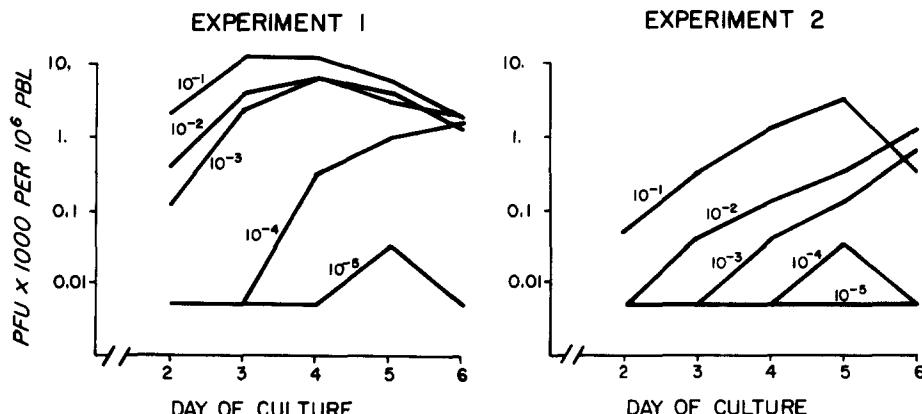


FIG. 5. Kinetics of dengue 2 replication in relation to infecting virus dose in two experiments. Mean PFU per 10^6 mononuclear PBL are shown for each dose on days 2-6 of culture. aD4 concentration constant in each culture vessel.

tration of antibody. Virus replication measured in two separate experiments with monkey PBL is shown in Fig. 5. These results are illustrative of the variation in D2V replication observed in different PBL donors. In exp. 1 comparatively little difference was observed in virus yield or in the day of peak virus production over a ratio of virus:PBL of 10^{-1} - 10^{-3} . In exp. 2, dose-related differences in virus yield were somewhat larger. In both experiments, a virus: PBL ratio of 10^{-5} (an inoculated dose of approximately 100 PFU) was at the threshold of infection. With decreasing virus inocula there was retardation in the day of peak virus titer.

Discussion

In a large series of experiments performed under relatively basal conditions, D2V complexed with antibody entered and replicated in mononuclear leukocytes with high relative efficiency but in the absence of antibody did so at lower efficiency. Anti-dengue serums prepared in several mammalian species were equally efficient in mediating D2V infection. An Fc terminus was a requirement for infection and the Fc receptor of permissive leukocytes was resistant to

treatment with several proteases. In the absence of C, infection was mediated by IgG but not IgM. The kinetics of internalization of virus-antibody complexes was temporally similar to the kinetics of phagocytosis by mononuclear phagocytes (32). These observations are compatible with the data described in the companion paper which identify the antibody-dependent dengue-permissive primate leukocyte as a mononuclear phagocyte (21).

We have shown that D2V infection of primate mononuclear phagocytes can be mediated by both homotypic and heterotypic anti-dengue IgG. Little is known of the molecular and cellular events which result in infection of a mononuclear phagocyte rather than destruction of virus. Before this study, a reasonable speculation would have been that strong interactions between virus and antibody might be lethal, while weak interactions might be capable of promoting immune phagocytosis, but the high rate of disassociation of the complex results in infection of the cell. We report for the first time the existence of an antibody subpopulation which appears to have as its sole measureable function the enhancement of viral infection in mononuclear phagocytes. Two D2V-infected monkeys produced large quantities of infection-enhancing IgG in serums obtained 14 days postinfection. These fractions had no detectable HI or neutralizing activity against D2V. The IgG subtype of this enhancing IgG has not yet been determined. It will be important to learn whether separate populations of IgG are active in the neutralization and enhancement phenomena.

The remarkable ability of very low concentrations of anti-dengue IgG to promote dengue infection of mononuclear phagocytes is reminiscent of a similar sensitivity of leukocytes to the antibody-dependent cell-mediated cytotoxicity phenomenon (33). In most of our experiments the D to leukocyte ratio was less than 1. In experiments in which anti-dengue was diluted to 500-fold above the neutralization end point, it seems reasonable to conclude that a single virus-antibody complex may suffice to initiate infection.

Enhancing antibody does not attach to mononuclear phagocytes before the formation of immune complexes. This experiment thus did not provide insight into the nature of the D receptor on the surface of dengue-permissive leukocytes obtained from immune donors. It has been speculated that this receptor is a cytophilic antibody (15). This speculation receives support from unpublished observations showing that silica treatment of dengue-immune PBL ablates in vitro dengue infection. This identifies the permissive cell as a mononuclear phagocyte, a cell frequently associated with cytophilic antibody. It will be of interest to determine if the dengue cell-associated receptor is an antibody and if so to identify the immunoglobulin type.

The biological implications of in vitro antibody-mediated dengue infection of mononuclear phagocytes require comment. Severe dengue disease occurs in infants, a majority of whom are 6- to 10-mo-old. There is evidence that these infants develop DSS during a primary dengue infection (6). In an age-stratified serological study in Bangkok it was shown that over 90% of women of child-bearing age are immune to one or more D types (8). Thus, most Bangkok-born infants acquire dengue antibody at birth. By 6-10 mo, it is likely that maternal anti-dengue IgG has waned to below the protective threshold. But, as demonstrated in this study, antibodies at nonprotective concentrations may be infection enhancing. The age-related responses to dengue infection seen in infants

during the 1st yr of life resembles the "prozone" in the in vitro antibody infection enhancement system. When homotypic antibody was used, neutralization of virus occurred with high concentrations of antibody (protection of the young infant) and enhancement of virus infection at low concentrations (infection enhancement in the older infant).

When studies were done using PBL from different individuals or from the same individual bled repeatedly, the variation in the amount of virus replicated was notable. Separately, we have shown that virus production in vitro is related directly to the number of cells infected (21). Since in each of our experiments the concentrations of infecting virus and antibody were held constant, the most plausible explanation for variation in virus production is a variation in the number of cells available to support dengue infection. The number of circulating permissive cells might vary between different persons and in individuals serially studied. The identity of the small fraction of mononuclear phagocytes which are permissive to dengue infection receives further comment elsewhere (21). Individual variation in the size of a leukocyte population which can be rendered permissive by antibody might be related to the observation that only a small minority of persons undergoing secondary dengue infections develop DSS (11). Regulation of the size of mononuclear phagocyte populations is incompletely understood, but genetic, hormonal, and nutritional factors and preceding or accompanying infections may be important. The hypothesis that the permissive monocyte population varies according to the severity of dengue disease could be tested directly in man. We propose that the concentration of dengue-permissive PBL in patients with secondary dengue infections be compared in a matched series of children with severe and mild disease.

Studies should also be initiated on the role of biologically active molecules in regulating the penetration, synthesis, and release of dengue viruses from infected mononuclear phagocytes. Epidemiological and autopsy studies have demonstrated that secondary dengue infections are more severe in girls than in boys (6, 34). This anomalous response to infectious disease might be explained by our results which relate infection of mononuclear phagocytes to the phenomenon of immune phagocytosis plus the observation of Vernon-Roberts who showed that some steroid hormones, including progesterone, increase phagocytosis by mononuclear phagocytes, *in vivo* (35).

The identification of the mononuclear phagocyte as a host for dengue replication in vitro, and the demonstration of a mechanism by which antibody can regulate dengue infection of these cells serves to focus attention on the disease-producing consequences of infection in this cell system. Recently it has been shown that various stimuli to mononuclear phagocytes results in the release of lysosomal enzymes (36). Recently it has been shown that C3a and C3b can release lysosomal hydrolases from guinea pig macrophages (36). Macrophage as well as granulocyte lysosomal enzymes can cleave C3 to C3b (37, 38). Activated C3b with factor B functions as a proteinase, cleaving further C3 to C3b (39). Thus, through a positive feedback mechanism macrophages are potentially powerful activators of C via the alternative pathway. It is not yet established that D infection of human mononuclear phagocytes can result in release of lysosomal enzymes or other C-activating factors. Viral infections in other cell systems do result in the release of factors which activate C3 and C5 (40, 41). If

dengue infection of mononuclear phagocytes results in C activation, the degree of activation should correlate with the number of mononuclear phagocytes infected and render it unnecessary to invoke the production of virus-antibody complexes to explain C consumption. Such an hypothesis would be more in accord with conditions under which DSS occurs in primarily infected infants (very low levels of residual maternal anti-dengue IgG) or with the shock and C activation recently described in primarily infected children older than 1 yr (42). Indeed, vasoactive or hemorrhagic compounds whose existence is as yet unsuspected, may be released either as a result of virus infection or when infected mononuclear phagocytes are under immunological attack. We are actively studying these interesting possibilities.

Summary

Cultured mononuclear peripheral blood leukocytes (PBL) from nonimmune human beings and monkeys are nonpermissive to dengue 2 virus (D2V) infection at multiplicities of infection of 0.001–0.1, but become permissive when non-neutralizing dengue antibody is added to medium. D2V infection occurred in PBL prepared from anti-coagulated but not from defibrinated plasma. Infection enhancement was produced by multiple lots of heterotypic anti-dengue raised in several mammalian species. Homotypic anti-dengue neutralized D2V at high concentrations but enhanced at low concentrations; enhancement end point in one serum was 1:320,000. The infection-enhancing factor was a noncytophilic antibody of the IgG class. D2V infection occurred in the absence of heat-labile complement components but did not occur when complexes were prepared with anti-dengue F(ab)₂. Treatment of PBL with several proteases increased permissiveness to D2V infection by immune complexes but not by virus alone. Two rhesus monkey serums collected 14 days after D2V infection contained an IgG antibody with high-titered enhancing activity but with no hemagglutination-inhibition or neutralizing activity. Virus-antibody complexes are irreversibly attached to PBL within 15 min and completely internalized in 60 min. There was considerable variation in cellular infection in different experiments, however, maximum virus yields usually exceeded 1,000 plaque-forming units per 1×10^6 PBL occurring between 2 and 4 days in culture. In vitro antibody-dependent infection of PBL provides a possible model for study of pathogenetic mechanisms in infants with dengue shock syndrome who passively acquire maternal anti-dengue IgG.

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