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Dengue Virus Replication Enhancement in Peripheral Blood Leukocytes from Immune Human Beings[1] (39160)

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Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are characterized by abnormal vascular permeability (1, 2). It has been suggested that this life-threatening capillary damage is mediated by complement components which are activated through rapid antigen–antibody complexing (3). If this were the case, the severity of a dengue infection might be directly related to the quantity of viral antigen produced. From experimental studies, we believe that dengue antigen production may be regulated paradoxically by an immune mechanism. In rhesus monkeys, viremia was enhanced when infections were established in partially immune compared with susceptible animals (4). There is an apparent *in vitro* correlate of this phenomenon. Nonmitogen-treated peripheral blood leukocyte (PBL) cultures prepared from dengue immune monkeys readily supported dengue replication while little growth was observed in PBL cultures from the same animals prior to dengue infection (5). In pathogenetic studies, lymphoid tissue and circulating blood leukocytes were found to be the most common sites of dengue virus recovery (4, 6). From this evidence it has been hypothesized that an expansible population of immune leukocytes may provide the cellular basis for the immunological regulation of dengue antigen production *in vivo*. This report relates experimental data developed in monkeys to man by providing the first evidence that immunologically enhanced viral replication occurs in human leukocytes *in vitro*.

Materials and methods. Leukocyte collection. Blood samples were obtained in Bangkok, Thailand from clinically normal children or pediatric patients with illnesses other than dengue hemorrhagic fever and in Suva, Fiji from young adults who were 6–15 weeks convalescent from dengue Type 2 infections (1972). Included were 22 Thai (T) children, ranging in age from 6 days to 13 yr, and from Fiji (F), five adult Indians or Fijians. PBL were separated from erythrocytes by gravity sedimentation in 2 vol of 2.4% dextran T250 (Pharmacia). Cells were washed three times in Hank's balanced salt solution to remove serum and resuspended to a concentration of 1.5×10^6 live mononuclear cells/ml in RPMI-1640 medium (Grand Island Biological Company) supplemented with 10% heat-inactivated fetal calf serum, glutamine, and antibiotics. The same lot of fetal calf serum was used throughout. In a few instances, PBL were not separated from the erythrocytes, but the whole blood was washed and cultured. A number of persons were bled more than once.

Virus. Dengue Type 2 (D2), strain 16681, isolated in BSC-1 cells from a patient with DHF was used to infect PBL cultures (7). Prior to use, this virus had been passed once in a susceptible rhesus monkey and twice in adult *Aedes albopictus* by intrathoracic inoculation. Seed virus was prepared in LLC-MK2 cells. A single lot of virus containing 1×10^6 PFU/ml was used in these experiments. D2 virus at multiplicities of infection of 0.01 to 0.15 was added to 1.5×10^6 live peripheral blood mononuclear cells/ml. Virus inoculum was not removed, as it had been determined from many experiments that extracellular Dengue 2 virus decays from 10^5 PFU/ml to less

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than 10 PFU/ml in 24–36 hr. PBL were placed in 1-dram vials and incubated at 37°C for 3–7 days. Single or duplicate cultures were frozen at −70°C until assayed for virus by the LLC-MK2 plaque method (9).

[^{3}H]Thymidine uptake. *In vitro* uptake of [^{3}H]thymidine of PBL following the addition of D2 virus was measured in serial daily samples as previously described (5). An uninfected LLC-MK2 cell suspension was inoculated into replicate leukocyte cultures as control antigen.

Antibody determinations. Plasma from each leukocyte donor was tested for hemagglutination-inhibition (HAI) antibody to D1, 2, 3, and 4, Zika, Japanese encephalitis, and chikungunya viruses using standard techniques (8). Dengue plaque reduction neutralization (PRNT) antibodies were measured in LLC-MK2 cells using the 50% plaque reduction endpoint (9). The immune status of each subject was established only after the completion of the virus replication experiments.

Results. Eleven Thai children (ages 6 days to 13 yr) and 5 Fijian adults had antibody patterns suggesting that they had prior infection with one or more dengue types or had passively acquired dengue antibody (Table I). Antibody in serums from the three infants with dengue antibody were subjected to ultracentrifugal partition. Antibody was of the IgG type. Two of the Fiji residents had antibody patterns indicative of more than one previous dengue infection.

Dengue virus multiplied to relatively high titer (285–4000 PFU/ml) in cultures sampled at daily intervals from two of three infants (TN, TK) with antibody of maternal origin, three of eight Thai children (TB, TG, TT) who had PRNT antibody indicating previous infection with dengue virus(es), and from two of five dengue immune adult Fijians (FD, FE). Virus titers of 25–75 PFU/ml were obtained in PBL cultures from two of the eight Thai children (TDD, TO) with endogenous dengue antibody, and three of the five adult Fijians (FB, FA, FC). PBL from one infant with maternal antibody (TII) and three Thai children (TEE, TU, TMM) with dengue antibody derived from previous infection failed to support dengue virus growth (Table I).

Dengue 2 virus added to PBL from immune subjects at doses described above did not stimulate the incorporation of greater amounts of [^{3}H]thymidine than by noninfected controls.

Discussion. This study provides the first evidence of *in vitro* immunological enhancement of dengue virus replication in human beings. The relative amount of D2 virus recovered and the duration of virus production in immune PBL cultures were similar,

TABLE I. REPLICATION OF DENGUE 2 VIRUS IN PERIPHERAL LEUKOCYTE CULTURES FROM THAI CHILDREN AND FIJIAN ADULTS WITH DENGUE (D) ANTIBODY.[a]

Subject	Age (yr)	Sex	Reciprocal serum titer (50% PRNT)				Presumed immunological history	*In vitro* D2 replication (PFU/ml on indicated day of incubation)				
			D1	D2	D3	D4		3	4	5	6	7
TN	6/365	F	30	120	90	50	Maternal ab	100	200	1900	300	4000
TK	14/365	M	49	180	110	27	Maternal ab	0	650	25	0	580
TII	3/12	M	15	10	20	<10	Maternal ab			0	0	0
TDD	1.5	M	<10	1650	40	<10	D2 immune		75	50	0	0
TB	3	F	<10	235	40	<10	D2 immune	250	100	225	900	1375
TG	4	M	640	160	40	<10	D1 immune	175	225	575	400	25
TO	5	M	1280	64	20	<10	D1 immune		25	0	0	0
TEE	5	M	640	35	20	11	D1 immune		0	0	0	0
TU	11	M	48	510	80	320	Immune, types?			0	0	0
TMM	22	F	160	510	90	90	Immune, types?			0	0	0
TT	13	F	160	500	120	160	Immune, types?		50	50	350	800
FB	20	F	50	320	80	64	D2 immune	25	0	25	0	
FD	23	M	160	≥1280	40	20	D2 immune	285	150	120		
FA	28	M	≥5120	4000	2960	1400	D1,2 immune[b]	0	25	25	50	
FC	36	M	1280	640	1280	240	D1,2 immune[b]	0	30	0		
FE	38	F	1280	1280	128	320	D1,2 immune[b]	0	333	1400		

[a] D2 virus at multiplicities of infection of 0.01–0.15 was added to 1.5×10^6 live peripheral blood mononuclear cells/ml of RPMI 1640 medium. After incubation at 37°C for 3–7 days, single or duplicate cultures were frozen at −70°C and subsequently assayed for virus.

[b] Dengue 1 was transmitted on Fiji duing World War II and Dengue 2 in 1971–72.

TABLE II. REPLICATION OF DENGUE 2 VIRUS IN PERIPHERAL LEUKOCYTE CULTURES FROM THAI CHILDREN WITHOUT DENGUE 1–4 HAI OR NEUTRALIZING ANTIBODIES.

Subject	Age (yr)	Sex	PFU/ml on indicated day of incubation				
			3	4	5	6	7
TZ	710	F	0	0	0	0	0
TY	10/12	M		0	0	0	0
THH	1.5	M			0	0	
TJ	2	M	25	25	50	0	50
TJJ	2	M			0	0	0
TGG	2.5	F		0	0	0	0
TD	4	M	0	0	0	0	
TI	4	F	0	0	0	0	0
TQ	7	F			0	0	0
TX	8	F				0	
TW	11	M	0	0	25	0	

[a] 0 = less than 25 PFU/ml of leukocyte culture.

in 5 of 13 immune subjects, to results obtained when D2 virus was added to PBL cultures from recently infected dengue immune rhesus monkeys (5). When lower viral yields are included, virus replication was noted in PBL in 10 of 13 immune individuals, while growth occurred in PBL in only 2 of 11 nonimmune subjects. The failure of dengue virus to replicate in peripheral leukocytes from all dengue immune individuals is not understood. Sequential studies in immune monkeys have shown that permissiveness of PBL to dengue virus infection *in vitro* appears 2–3 weeks after experimental infection but may wane or disappear within several years (Marchette and Halstead, unpublished). It should be noted that each of the children with dengue antibody of endogenous origin whose leukocytes did not support virus growth *in vitro* were old enough to have had their previous dengue infection five or more years before our investigation. It is not possible to estimate when infections took place in these children as dengue viruses are transmitted endemically in Bangkok. In humans, as in the monkey, there may be a limit to the time after infection in the host after which PBL will no longer support viral replication.

In contrast to our study in rhesus monkeys, live D2 challenge of immune human PBL was not followed by significant uptake of [^{3}H]thymidine (5). We have recently observed that 2 to 3 months after primary dengue infection in the monkey, lymphoblast transformation induced by live virus is no longer observed, although the ability of PBL to support dengue replication was unchanged (Marchette and Halstead, unpublished). The observed inability to detect increased [^{3}H]thymidine uptake in dengue-stimulated immune human leukocytes similarly might result from a long interval between infection in the individual and the time of study.

Virus replication occurred to relatively high titer in leukocytes from two of three infants with dengue antibody, presumably of maternal origin (TN, TK), but did not occur in leukocytes from two infants who were without detectable antibody at the time of study. This could result from the passive acquisition by the infant of an as yet undescribed specific factor(s) that produces "permissive" infant leukocytes. Alternatively, leukocytes from infants normally may be receptive to dengue virus replication. Studies to investigate this phenomenon are now in progress.

Our findings establish biological similarities between simian and human PBL. Both monkeys and man possess a population of leukocytes permissive to dengue virus infection which are more abundant in immune than nonimmune hosts. The identification of cellular host (or hosts) dengue virus replication, the mechanism of virus penetration, and the biological significance of replication of dengue virus in immune leukocytes are now under active study in our laboratory.

Summary. Dengue 2 virus replication in peripheral blood leukocyte cultures from 10

of 13 Asian or Polynesian subjects with actively acquired dengue immunity and two of three infants with passively acquired dengue antibody. Only 2 of 11 cultures from nonimmune infants or children supported viral replication. This study establishes a parallel in the biological behavior of human and simian PBL with respect to the immunological dependence of dengue virus replication *in vitro*. Elucidation of the mechanism (or mechamisms) regulating growth of dengue virus in leukocytes from immune hosts may contribute to an understanding of the role that virus–leukocyte interactions play in the pathogenesis of human dengue illness.

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