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Phytohemagglutinin Enhancement of Dengue-2 Virus Replication in Nonimmune Rhesus Monkey Peripheral Blood Leukocytes

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Phytohemagglutinin treatment of peripheral blood leukocytes from dengue nonimmune monkeys enhanced dengue-2 virus replication. Enhancement was due primarily to an increase in the number of infected cells. Destruction of mononuclear phagocytes with silica did not significantly inhibit virus replication in phytohemagglutinin-treated cultures. Pokeweed mitogen, concanavalin A, and streptolysin O stimulated increased deoxyribonucleic acid synthesis in monkey leukocytes but did not enhance virus replication. None of the mitogens significantly affected virus replication in cultures of dengue-immune monkey peripheral blood leukocytes.

Dengue viruses replicate readily in cultures of peripheral blood leukocytes (PBL) prepared from dengue-immune primate donors but poorly or not at all in PBL from a nonimmune host (10, 11, 18-20). In preliminary studies, addition of dengue virus to dengue-immune rhesus monkey PBL resulted in lymphoblast transformation as evidenced by increased incorporation of tritiated thymidine (10). In parallel experiments, phytohemagglutinin (PHA)-treated nonimmune monkey PBL supported virus growth; untreated controls did not.

On the basis of these preliminary observations, we hypothesized that specifically committed lymphocytes transformed to lymphoblasts by dengue virus are inherently permissive to dengue infection (10). We now believe that dengue virus replication in immune PBL and in PHA-treated nonimmune PBL are separate and distinct phenomena. Subsequent experiments have shown that when mononuclear phagocytes were selectively destroyed by silica (E.J. O'Rourke et al., J. Immunol. Methods, in press), dengue virus replication was suppressed in dengue-immune human PBL cultures (unpublished data). This provides evidence that mononuclear phagocytes can support dengue virus replication in the absence of mitogen stimulation. However, since mitogens primarily affect lymphocytes, it is possible that dengue virus replication in PHA-treated PBL takes place in lymphocytes.

The present study presents further observations on the infection and replication of dengue-2 virus (D2V) in mitogen-treated monkey PBL.

MATERIALS AND METHODS

Virus. D2V (16681) (12) was prepared in LLC-MK2 cell cultures as previously described (17).

Rhesus monkey leukocyte donors. Juvenile and adult female rhesus monkeys (*Macaca mulatta*) from commercial sources were housed individually and fed commercial feed supplemented with fresh fruit and water ad libitum. They were without hemagglutination inhibition antibodies to flaviviruses (dengue-1, -2, -3, -4, Japanese encephalitis, and Zika) or neutralizing antibodies of dengue virus types 1, 2, 3, or 4 when selected for the study. These or similar monkeys were subsequently infected with dengue virus types 1, 2, 3, or 4 by subcutaneous inoculation (17) and were the source of dengue-immune leukocytes.

PBL. Mononuclear cells from heparinized (20 U/ml) peripheral blood of dengue-immune and nonimmune monkeys were routinely isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden; sodium diatrizoate, Winthrop, New York, N.Y.) gradient centrifugation (6). The cells were washed twice in Hanks balanced salt solution with 0.2% ethylenediaminetetraacetic acid (Sigma, St. Louis, Mo.) and once with Hanks balanced salt solution alone, suspended in complete RPMI 1640 medium (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) containing 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; Calbiochem, San Diego, Calif.), 2.0 g of $NaHCO_3$ per liter, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated (56°C, 30 min) fetal calf serum (GIBCO). A single lot of fetal calf serum low mitogenic activity was used throughout the study. Mononuclear cells were counted in a hemacytometer, the cell concentration was adjusted to 1.5×10^6 live mononuclear cells/ml as determined by the exclusion of trypan blue, and the suspension was dispensed in 1.0-ml

amounts into round-bottomed tissue culture tubes (15 by 100 mm), gassed with 5% CO_2 in air, tightly stoppered with rubber stoppers, and incubated at 36°C.

Treatment of leukocytes with mitogens. Freshly prepared cultures of mononuclear cells were treated with mitogens to achieve the following final concentrations (per milliliter per 1.5 × 10^6 cells): (i) 0.5 μl of purified PHA (Difco, Detroit, Mich.), (ii) 10 μg of pokeweed mitogen (PWM; GIBCO), (iii) 2.5 μg of concanavalin A (ConA; Calbiochem), and (iv) 100 μl of streptolysin O (SLO; GIBCO). Cell and mitogen concentrations for optimal cell transformation were determined by titration experiments. The area of the cell button at the bottom of the tube and culture conditions were essentially the same as those described by Moorhead et al. (18) as optimal for cell transformation.

The viability of macrophages in mitogen-treated PBL cultures was determined daily for up to 5 days. Samples from replicate mitogen-treated and control cultures were removed daily after incubation for 90 min with 0.81-μm latex beads (Difco) at a concentration of approximately 500 beads/cell, washed in saline, and mounted in a drop of 0.0002% acridine orange (Euchrysin 3R, G. T. Gurr, London). Phagocytic cells were counted with a Zeiss microscope with transmitted light-phase condenser and incident blue light from a 100-W halogen lamp passing through, a KP500 exciter, and LP 520 barrier filters. Live mononuclear phagocytes were defined as cells with ruffled cell membranes containing ingested latex beads and green-stained nuclei (11b).

Infection with D2V. D2V was diluted in RPMI complete medium to approximately 5.0 × 10^5 to 1.0 × 10^6 plaque-forming units/ml, and 0.1 ml was added to each cell culture at the same time as or 2, 24, 48, or 72 h after mitogen treatment. Untreated (control) cell cultures were infected in the same manner. Additional cultures of mitogen-treated and untreated cells received 0.1 ml of medium instead of virus and served as noninfected controls. The cultures were incubated at 36°C for up to 6 days after infection.

Selective destruction of mononuclear phagocytes. A suspension of particulate silica (Dorentrop quartz <5 μm in diameter) (2) containing 1.0 mg/ml in RPMI 1640 complete medium was dispersed by sonic treatment (O'Rourke et al., J. Immunol. Methods, in press), and 0.1 ml was added to PHA-treated and control cultures of PBL from nonimmune monkeys at the same time as D2V. On culture days 1 through 5, latex beads were added to replicate tubes as described, and samples were centrifuged onto slides in a cytospin (Shandon Southern Instruments, Sewickley, Pa.). The preparations were air dried, fixed in cold (−20°C) acetone for 10 min, and stained with a fluorescein-tagged specific monkey anti-D2V globulin purified by diethylaminoethyl-cellulose chromatography (9). The slides were examined with the Zeiss microscope described above fitted with a KP-490 exciter filter and an FT 510 beam splitter. Cells with specific cytoplasmic fluorescence were observed under phase contrast to determine the presence of phagocytized latex particles. The remainder of each culture was stored at −70°C for virus assay.

Virus assay. Infected cell cultures (in triplicate) were frozen at −70°C on days 1 through 6 after addition of D2V. A sample of the D2V inoculum in each experiment was similarly frozen at −70°C and stored until titrated. Virus was assayed by standard plaque assay in LLC-MK2 cells (12) or in porcine kidney (PS) cells (14). The minimum virus titer detectable was 1.0 $\log_{10}$ plaque-forming unit/ml of culture. Half the minimum value (0.5) was assigned for samples without plaques.

Assay for infected cells. The number of infected cells was determined by an infectious-center assay similar to that described by Bloom et al. (3) and Willems et al. (23) adapted to PS cells. Briefly, cultures of mitogen-treated or untreated cells were washed, incubated with D2V at 37°C for 90 min, and then washed again, and nonabsorbed virus was neutralized by incubation with specific anti-D2V serum for 30 min. The cells were then washed three times and suspended in 2.4 ml of L-15 medium (GIBCO) with 3% calf serum, and serial fivefold dilutions were made. The cells in each dilution were counted, and 0.4 ml was added to each of three 16-mm wells in tissue culture plates (Linbro, New Haven, Conn.); then 0.4 ml of PS cells (1.2 × 10^5 cells) was added to each well. Each experiment was done in triplicate, and the PS cell-leukocyte mixtures were incubated at 35°C for 4 h to allow the cells to settle and attach; 0.8 ml of carboxymethyl-cellulose (Sigma) overlay medium was added, and the cultures were incubated at 35°C. The cell sheets were stained, and plaques were counted on day 6. The results were expressed as plaque-forming units/10^6 live mononuclear cells actually plated.

Cell transformation assay. On day 2 after culture, triplicate samples of all cultures received 1.2 μCi of [*methyl*-^{3}H]thymidine (specific activity, 2 μCi/mmol; New England Nuclear, Boston, Mass.). After 20 h of incubation at 36°C, the acid-insoluble residue of the cells was assayed for [*methyl*-^{3}H]thymidine uptake (4) in a Packard scintillation spectrometer. The results are expressed as counts per minute − background/milliliters of culture.

Analysis of results. Logarithmic or square-root transformation of the counts per minute and plaque-forming units as a measure of [*methyl*-^{3}H]thymidine incorporation and virus replication, respectively, was done to obtain normally distributed data. Student's t test of paired means, when appropriate, was then performed.

RESULTS

Effect of mitogens on D2V viability. D2V decays rapidly in cell-free medium at 36°C, reaching undetectable levels in 24 to 36 h (17). The presence in culture medium of PHA, PWM, ConA, or SLO at the concentrations used did not inactivate D2V or affect the rate of decay in any way. In all cases D2V was undetectable after 24 to 36 h of incubation in cell-free medium.

Effect of mitogens on viability of PBL in vitro. The viability and phagocytic activity of mononuclear phagocytes from three monkeys

was not significantly decreased by treatment with PWM or SLO for up to 5 days in culture. Clumping of PHA- and ConA-treated cells made accurate counting impossible, but the viability of the cells did not appear to differ from that of controls.

Replication of D2V in PHA-stimulated nonimmune monkey PBL. Cultures of nonimmune monkey PBL treated with PHA at the same time or 1, 2, or 3 days before D2V was added produced more virus than did untreated cultures (Table 1). Cultures of untreated PBL aged 1 to 3 days before infecting with D2V did not produce significantly more virus than cultures infected with D2V on day 0.

Treatment of PBL with PHA for 2 h significantly increased the number of permissive cells (Table 2). Cells in cultures treated with PHA for 24 h or longer before infection were too strongly agglutinated to disperse adequately, and the infectious-center assay did not accurately measure the number of infected cells.

Exposure of nonimmune monkey PBL to silica at the same time as infection with D2V and during incubation with or without PHA only slightly depressed deoxyribonucleic acid synthesis as measured by [*methyl*-^{3}H]thymidine uptake and did not significantly depress virus replication (Table 3).

Only an occasional cell with specific dengue antigen was seen by fluorescent microscopy in cultures of untreated nonimmune monkey PBL incubated for 2 to 5 days after addition of D2V. Treatment of replicate cultures with PHA increased the number of cells containing specific dengue antigen, but clumping of cells made accurate counts impossible. Within the limits of the technique, the presence of silica in amounts cytotoxic for mononuclear phagocytes did not appear to reduce the number of infected cells. Most of the infected cells had not phagocytosed latex beads.

Replication of D2V in PWM-, ConA-, and SLO-treated nonimmune monkey PBL. All three mitogens in the concentrations used stim-

TABLE 2. *Infectious-center assay of D2V-infected PBL from three nonimmune monkeys*

Monkey no.	No. of infected cells/10^6 cells (mean ± SD)[a]	
	RPMI	PHA
H-209	7.3 ± 2.0	28.5 ± 12.9
H-213	9.2 ± 1.7	29.9 ± 15.1
H-215	8.9 ± 10.5	38.2 ± 11.0
x̄ ± SE[b]	8.5 ± 0.6	32.2 ± 3.0[c]

[a] SD, Standard deviation.
[b] SE, Standard error of the mean.
[c] $P = 0.02$.

TABLE 3. *Failure of particulate silica toxic for mononuclear phagocytes to prevent D2V replication in PHA-treated PBL from nonimmune monkeys*

Donor monkey	PHA	PHA + silica
H-224	1.1[a]	0.8
H-226	1.4	0.8
H-234	2.2	2.1
H-235	1.8	1.2
H-236	1.2	1.4
x̄ ± SE[b]	1.5 ± 0.2	1.3 ± 0.2

[a] Mean $\log_{10}$ plaque-forming units per milliliter of triplicate 3- to 4-day cultures.
[b] SE, Standard error of the mean. The difference is not significant ($P = 0.15$).

TABLE 1. *D2V replication in PHA-treated PBL from nonimmune monkeys*

Expt[a]	D2V added on day:							
	0[b]		1		2		3	
	Control	PHA	Control	PHA	Control	PHA	Control	PHA
1	0.5[c]	3.3	0.5	1.2	0.5	1.3	0.5	2.6
2	0.5	1.2	1.4	2.4	0.6	3.3	0.5	1.1
3	0.5	0.7	0.5	0.8	1.4	2.4	1.7	3.1
4	0.6	2.0					0.5	0.7
5	0.5	2.0						
6	0.6	2.4						
x̄ ± SD[d]	0.6 ± 0.1	1.8 ± 0.7	0.8 ± 0.5	1.5 ± 0.8	0.8 ± 0.5	2.3 ± 1.0	0.9 ± 0.7	1.9 ± 1.2
P	0.01		<0.1 > 0.05		<0.2 > 0.1		<0.1 > 0.05	

[a] A different PBL donor monkey was used in each experiment.
[b] Days in culture with PHA or RPMI (control) before D2V added.
[c] Mean $\log_{10}$ plaque-forming units per milliliter in replicate cultures assayed on days 3 to 5 after infection.
[d] SD, Standard deviation.

ulated lymphoblast transformation of monkey PBL. Cultures treated with PWM 0, 1, 2, or 3 days before D2V infection did not produce significantly more virus than untreated cultures (Table 4). Treatment with ConA or SLO at the same time as D2V infection did not enhance virus production in nonimmune PBL (Table 5).

Replication of D2V in mitogen-treated PBL from immune monkeys. Treatment of PBL from dengue-immune monkeys with PHA, PWM, or SLO did not significantly enhance or inhibit virus production. Cells treated with ConA produced less virus, but the results were not significant at the 5% level (Table 6).

DISCUSSION

The present study confirms our original observations (10) that PHA treatment of nonimmune monkey PBL renders them permissive to D2V. The number and function of mononuclear phagocytes were not altered by PHA treatment, but it cannot be concluded that PHA had no effect on them. There is no evidence that PHA is a mitogen for these cells, but it may induce less dramatic metabolic changes (13). However, silica, which is cytotoxic for mononuclear phagocytes (1) but does not affect lymphocytes (O'Rourke et al., J. Immunol. Methods, in press), did not significantly reduce D2V replication in PHA-treated cultures. Therefore, PHA treatment presumably increased permissiveness by action on some cell other than the mononuclear phagocyte. This observation is consistent with the hypothesis previously advanced that dengue antigen transforms specifically sensitized lymphocytes, and these serve as the permissive host cells.

Other data, however, suggest that this mechanism is not operating in immune monkey PBL or in nonimmune PBL in the presence of nonneutralizing anti-dengue serum (11a). In these cases, silica treatment completely suppresses D2V replication. In untreated PBL, mononuclear phagocytes are the dengue virus-permissive cells. Thus, there appear to be two distinct and separate phenomena: (i) antibody-enhanced infection of mononuclear phagocytes and (ii) PHA-enhanced infection of lymphocytes.

Similar PHA enhancement of virus replication in lymphocytes has been reported in vesicular stomatitis virus-infected mouse spleen cells (19) and measles virus-infected human PBL (20). Yellow fever virus, which resembles dengue virus in many ways, also replicates in untreated human monocytes; treatment of PBL cultures with PHA resulted in virus replication in lymphocytes but no enhancement of replication in monocytes (22). In PHA-treated human PBL,

TABLE 5. *Failure of D2V to replicate in nonimmune monkey PBL treated with ConA or SLO*

Expt[a]	Control	ConA	Control	SLO
1	1.3[b]	1.4	0.7	1.6
2	0.6	0.8	0.9	0.5
3	0.8	0.8	0.6	0.5
4			0.7	0.5
5			0.6	0.6
$\bar{x} \pm$ SD[c]	0.9 ± 0.4	1.0 ± 0.4	0.7 ± 0.1	0.7 ± 0.5
P	0.4		0.9	

[a] A different donor monkey was used in each experiment.
[b] Log_{10} plaque-forming units per milliliter of triplicate cultures assayed on days 3 to 5 after D2V addition.
[c] SD, Standard deviation.

TABLE 4. *Failure of D2V to replicate in PWM-treated PBL from nonimmune monkeys*

Expt[a]	D2V added on day: 0[b]		1		2		3	
	Control	PWM	Control	PWM	Control	PWM	Control	PWM
1	0.5[c]	0.9	0.5	0.5	0.5	0.5	0.5	0.5
2	0.8	0.9	0.5	0.5	0.5	0.5	0.6	0.5
3	0.5	0.5	0.6	0.7	0.5	0.5	0.5	0.5
4	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5
5	0.7	0.8			0.8	0.5		
6					0.5	0.9		
$\bar{x} \pm$ SD[d]	0.6 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.5
P	0.3		0.4		0.9		0.4	

[a] A different donor monkey was used in each experiment.
[b] Days in culture with PWM or RPMI (control) before D2V added.
[c] Log_{10} plaque-forming units per milliliter of triplicate cultures assayed on days 3 to 6 after D2V addition.
[d] SD, Standard deviation.

TABLE 6. *Replication of D2V in mitogen-treated PBL from dengue-immune monkeys*

Expt[a]	Control	PHA	PWM	SLO	Control[b]	ConA
1	2.7[c]	2.4	ND[d]	2.8	2.7	1.9
2	1.8	2.4	ND	ND	1.0	0.8
3	1.9	0.5	1.0	1.9	2.8	1.4
4	1.8	1.9	2.3	1.6	2.4	2.1
$\bar{x} \pm$ SD[e]	2.0 ± 0.4	1.8 ± 0.9	1.6	2.1 ± 0.6	2.2 ± 0.8	1.6 ± 0.6
P		0.6	0.8	0.8		<0.1 > 0.05

[a] A different donor monkey immune to dengue-1, -2, -3, or -4 was used for each experiment.
[b] A different group of monkeys was used in the ConA experiments.
[c] Log_{10} plaque-forming units per milliliter of triplicate cultures assayed on days 3 to 5 of culture.
[d] ND, Not done.
[e] SD, Standard deviation.

poliovirus initially infects monocytes and then spreads to lymphocytes (23).

The monkey lymphocyte that supports D2V replication after PHA treatment has not been identified. The specific lymphocyte subpopulation responding to PHA stimulation appears to differ from species to species, and no data are available for rhesus monkeys. In the human system, for example, PWM rather than PHA is reported to enhance D2V permissiveness of PBL (21). Preliminary evidence suggests that different cells or different mechanisms are operating in the monkey and human PBL-mitogen-dengue system.

The failure of mitogens other than PHA to enhance or induce permissiveness to D2V in nonimmune monkey PBL suggests that transformation per se may not be the event that confers permissiveness. Specific alteration of the lymphocyte membrane by PHA may allow the virus to adsorb to and enter the cell. This membrane change must be stable since cells treated with PHA for 3 days before adding virus are essentially as permissive as those treated for only 2 h before D2V infection. The initial effect of PHA on the cell is to modulate membrane phospholipids (10, 14). Thus, a reasonable hypothesis is that PHA uncovers or induces the formation of membrane receptors capable of interacting with D2V, thereby allowing infection to occur. In other systems, enhanced virus replication in PHA-treated PBL also is due, at least partly, to an increase in the number of cells that become infected (5, 23), presumably for similar reasons.

We have no explanation for the inability of PWM, ConA, and SLO to enhance D2V replication in monkey PBL. The initial action of these mitogens is also on the cell membrane, but different receptors are involved, and the subsequent alteration of membrane structure or physiology probably differs. There is no evidence that any of these mitogens except possibly ConA interferes with virus replication in permissive dengue-immune PBL (Table 6), ruling out a toxic effect or the production of virus-inhibitory substances by the mitogen-stimulated cells.

PHA treatment resulted in a more than 10-fold increase in D2V yield 3 to 6 days postinfection, but the effect of PHA on internalization of virus (as measured by the infectious-center assay) resulted in only a three- to fourfold increase in the number of infected cells. These data suggest not only that PHA increases the number of cells capable of internalizing the virus, but also that replication may depend upon the cytoplasmic changes accompanying cell transformation. The increased virus yield in PHA-treated cultures may also depend to some extent upon secondary or tertiary spread of virus to cells transformed during the culture period.

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