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Effect of Passage History on Dengue-2 Virus Replication in Subpopulations of Human Leukocytes

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Three passage levels of dengue-2 virus strain PR-159, obtained during the course of deriving the attenuated S-1 vaccine, were tested for their ability to replicate in subpopulations of human peripheral blood leukocytes: (i) 6th primary African green monkey kidney (PGMK) cell passage (parent virus); (ii) 19th PGMK cell passage of a small-plaque-forming clone derived from the parent virus (S-1 PGMK virus); and (iii) virus derived by four additional passages of the S-1 PGMK virus in diploid fetal rhesus lung cells (S-1 vaccine virus). Replication of these PR-159 viruses and another strain of dengue-2 virus adapted to Raji cells (16681-Raji virus) was measured in adherent and nonadherent mononuclear cells. All viruses except the S-1 PGMK virus replicated in monocytes. Occasional replication of the S-1 PGMK virus was associated with reversion to parent virus. The addition to the monocyte cultures of low concentrations of homologous dengue-2 antibody or non-neutralizing heterologous antibody increased the yield of the parent virus as much as 400-fold. This phenomenon of immune enhancement usually enabled the S-1 PGMK virus to replicate slowly in monocytes, but the progeny virus produced large plaques similar to the parent virus. Replication of the S-1 vaccine virus in cultured monocytes did not result in the appearance of large plaques. We could not recover S-1 vaccine virus from monocytes harvested from infected volunteers in the same manner that monocytes from natural human infections yield wild virus. The three passage levels of PR-159 virus were tested for replication in lymphocytes in comparison with the 16681-Raji virus. Only the 16681-Raji virus replicated in human lymphocytes cultured with or without enhancing antibody.

Dengue viruses replicate in human and primate monocytes in vivo and in cell culture (6–8, 13, 14, 16). This replication, which can be enhanced by low concentrations of antibody in the culture medium, has been proposed as a major factor in the pathogenesis of dengue and dengue hemorrhagic fever (4–8). The present study was designed to investigate the interaction with macrophages of several dengue-2 viruses with different biological characteristics and passage histories. One of these viruses is a candidate live-virus vaccine (3; K. H. Eckels, V. R. Harrison, P. L. Summers, and P. K. Russell, Infect. Immun., in press). Replication of dengue viruses occurs also in stimulated lymphocytes (12, 16), so we examined virus replication in this leukocyte population as well.

Since heterologous antibody to other flaviviruses (i.e., to other dengue serotypes or to the yellow fever vaccine virus) could be present in the blood of recipients, we also tested the concept of immune enhancement of virus replication as described by Halstead and O'Rourke (6, 7). Very dilute homologous antibody or more concentrated heterologous antibody apparently complexes with the virus in a non-neutralizing manner, providing the virus with a molecular ride into the monocyte via the Fc receptor, and probably results in infection of more monocytes. In this study, both the replication of viruses with different passage histories and the concept of immune enhancement were tested in monocytes and lymphocytes.

MATERIALS AND METHODS

Virus strains. The passage history and characteristics of the dengue-2 viruses used in this study are presented in Table 1. The parent dengue-2 virus was isolated from a human serum (Puerto Rico patient 159) and passaged six times in certified primary African green monkey kidney (PGMK) cells (3). Cloning of isolated plaques and further passage in green monkey kidney cells resulted in a small-plaque clone at the 19th passage level (3), referred to herein as the S-1 PGMK virus. Subsequently, the S-1 PGMK virus underwent four additional passages in certified diploid fetal rhesus lung cells for preparation of an experimen-

TABLE 1. *Characteristics and passage history of dengue-2 viruses*[a]

Virus	Passage	Plaques	Ts	Monkey viremia	Antibody in monkeys
Parent[b]	PGMK 6	Large + small	−	+	+
S-1 PGMK[b]	PGMK 19	Small	+	−	+
S-1 vaccine[c]	PGMK 19/FRhL 4	Small	+	−	+
16681-Raji[d]	BS-C %/LLC-MK_2 3 Chronic Raji/acute Raji 2	Small	ND	+	+

[a] Abbreviations: Ts, temperature-sensitive replication (39°C); PGMK, primary African green monkey kidney cells; FRhL, fetal rhesus lung cells; BS-C, continuous green monkey kidney cells; LLC-MK_2, continuous rhesus monkey kidney cells; ND, not determined.

[b] From Eckels et al. and Harrison et al. (3, 9).

[c] From Eckels et al. (Infect. Immun., in press).

[d] From Sung et al. (15); personal communication, A. R. Diwan and S. B. Halstead and G. A. Eddy.

tal vaccine suitable for use in humans (Eckels et al., Infect. Immun., in press) and is referred to as the S-1 vaccine virus. For the following experiments, the parent and S-1 PGMK viruses were passaged once in continuous monkey kidney (LLC-MK_2) cells for preparation of seed stocks to conserve primary cells. The 16681 strain of dengue-2 was isolated from human serum in continuous monkey kidney cells. After several passages in monkey cells (both BS-C-1 and LLC-MK_2), a carrier culture of human lymphoblastoid cells was established at the University of Hawaii (15); it is referred to herein as the 16681-Raji virus and was kindly provided by A. R. Diwan and S. B. Halstead. The virus was passaged two additional times through Raji cells (acute infection; see Table 1). The four viruses were indistinguishable from one another by plaque reduction neutralization tests.

Isolation of adherent and nonadherent human leukocytes. The procedure was adopted from Böyum (1) and Theofilopoulos et al. (16). Blood (240 ml) was drawn from each donor into 60-ml syringes containing 1,000 U of preservative-free heparin. The heparinized blood was diluted 1:2.5 in Ca- and Mg-free Hanks balanced salt solution containing 0.0025 M ethylenediaminetetraacetic acid and 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (blood diluent) and then dispensed in 35-ml volumes into 50-ml conical disposable centrifuge tubes (Falcon Plastics, Oxnard, Calif.). Ficoll-Hypaque (15 ml; specific gravity, 1.077) was injected beneath the diluted blood with a spinal needle, and the tubes were centrifuged at 350 × *g* for 40 min at room temperature. Leukocyte layers were aspirated in 12-ml volumes with a 10-ml pipette, pooled, diluted 1:2 with blood diluent, and centrifuged at 350 × *g* for 7 min at 4°C. The leukocyte pellets were resuspended twice in 100 ml of diluent and centrifuged for 7 min to minimize platelet contamination. The yield from 17 donors ranged from 2.0×10^8 to 6.6×10^8 cells, and cells were at least 98% viable as determined by exclusion of trypan blue. Disposable petri dishes (50 cm^2; Falcon) were each seeded with 1.3×10^7 leukocytes in 20 ml of growth medium (RPMI 1640 containing 10% fetal bovine serum and 0.01 M HEPES buffer). After incubation for 3 h at 35°C in a humidified CO_2 incubator, the nonadherent cells were removed and incubated at 35°C for 3 days, the reported time required for nonadherent cells to become susceptible to infection with dengue virus (16). The dishes were washed with diluent, and the primary adherent cells (10 to 15% of the leukocyte suspension) were scraped up with a rubber policeman and reseeded at 5×10^6 cells per 5 ml of growth medium in 25-cm^2 culture flasks (Falcon). The secondary adherent cell population represented at most 10% of the reseeded cells and contained no lymphocytes. All of the secondary adherent cells phagocytized latex particles and were morphologically mononuclear phagocytes or monocytes. The adherent cells in representative areas were counted with the aid of a Whipple microscope eyepiece containing a grid. Dengue-2 virus (Raji) replicated in these cells whether they were infected within 24 h of drawing blood from the donor or up to 5 days later.

Immune enhancement of virus replication. The observation of increased yields of dengue virus from primate leukocytes cultured in medium containing very dilute antibody has been described by Halstead and O'Rourke (6, 7). Our human antisera were diluted in the culture medium beyond the dilution that was shown to neutralize the virus (Table 2). We defined enhancement titer as the dilution that results in 10-fold or greater yields of virus. The human anti-dengue-2 was used at 1:500 when included in immune enhancement experiments. The heterologous antisera (dengue-3 and West Nile) did not have dengue-2-neutralizing activity at 1:10 and were used at 1:20 in the culture medium without determining an immune enhancement endpoint. Normal serum was used at 1:20 when both homologous and heterologous antisera were used in the same experiment.

Infection of adherent cells. Virus was diluted in growth medium containing either flavivirus immune serum at the dilutions stated above or normal human serum (normal medium) at the same dilutions. Due to the sparseness of the adherent cells, the number of cells and the titer of each inoculum (input) are given in each experiment rather than the multiplicity of infection. After adsorption for 1.5 to 2 h at 35°C, the inoculum was aspirated from the culture vessels and the monolayers were washed three times with blood diluent to remove residual inoculum. Six milliliters of growth medium with or without flavivirus antibody was added to the flasks, and 1 ml was removed immediately for a zero-time sample. The flasks were incubated at 35°C in 5% CO_2 for 5 days. One milliliter of medium was removed each day and replaced with

TABLE 2. *Characteristics of human flavivirus antiserum used in immune enhancement experiments*

Human antiserum	Dengue-2 neutralization titer[a]	Enhancing titer[b]	Final concn in medium
Dengue-2 (NG C)	1:80	1:1,000	1:500
Dengue-3 (H-87)	<1:10 (1:20)	—	1:20
West Nile	<1:10 (1:320)	—	1:20

[a] 50% plaque reduction neutralization test (parentheses contain homologous titer); the dengue-3 antiserum was drawn 9.5 years post-disease.

[b] Dilution which increased the yield of virus over the control medium by at least 10-fold. —, Not done.

an equal volume of fresh medium. The samples were stored at −70°C and assayed for infectious virus by plaque formation in LLC-MK_2 cells (3).

Infection of nonadherent cells. Infection was accomplished by suspending 2×10^7 nonadherent cells in 1 ml of virus inoculum to obtain multiplicities of 0.002 to 0.002. After incubation for 1.5 to 2 h at 35°C under CO_2 with occasional agitation, the cells were centrifuged from the viral inoculum at $350 \times g$ for 7 min at 4°C. Residual virus was removed from the cells by suspending them twice in 12 ml of blood diluent followed by centrifugation. A third suspension was carried out in 12 ml of growth medium (with or without flavivirus antibody) which was dispensed in 2-ml portions in small petri dishes (35 by 10 mm; Falcon). The remainder was frozen as a zero-time sample. Daily samples were obtained by freezing the contents of a petri dish.

Vaccine virus isolation attempts from monocytes of volunteers. Dengue-2 live virus vaccine trials were carried out in six human volunteers who had received the 17D live yellow fever vaccine within the past 10 years (W. H. Bancroft, F. H. Top, Jr., K. H. Eckels, J. M. McCowan, J. H. Anderson, Jr., and P. K. Russell, manuscript in preparation). Primary adherent and nonadherent cells were obtained from 10 ml of blood and cultured for 5 days. This was done each day for 21 days post-inoculation with the vaccine virus. In an attempt to determine if vaccine virus could be recovered from the mononuclear leukocytes infected in vivo as wild dengue virus is isolated from dengue patients (14), the culture supernatants were sampled for the presence of virus by plaque assay. In addition, an infectious center assay was carried out with primary adherent cells. They were overlaid with LLC-MK_2 cells and the following day overlaid with nutrient agar as described for a plaque assay (3; R. M. Scott, A. Nisalak, V. Cheamudon, S. Seridhoranakul, and S. Nimmannitya, J. Infect. Dis., in press). We tested 1.8×10^5 to 5.4×10^5 adherent cells by each assay from each donor per day and at least 10^6 nonadherent cells each day from four of the donors. This number of cells was similar to or greater than the number from which wild dengue virus was isolated during studies of dengue fever patients (Scott et al., J. Infect. Dis., in press).

RESULTS

Replication of parent and S-1 PGMK viruses in human monocytes. Adherent cells from two donors were tested simultaneously; one had no history of exposure to the serologically related flaviviruses, and the other had been immunized with the live yellow fever vaccine. The parent dengue-2 virus replicated to similar levels in cells from both donors, whereas little if any of the S-1 PGMK virus was found during the 5-day culture period (Fig. 1).

Replication of parent and S-1 PGMK viruses in monocytes cultured in medium containing dilute antibody. We next tested whether the presence of very dilute antibody in the inoculum and the culture medium would enhance the yields of these viruses in adherent cells as previously described for leukocyte suspension cultures (6, 7). The parent virus did not replicate very well in cells from this particular donor (Fig. 2). However, the addition of heterologous dengue-3 antibody or dilute homologous dengue-2 antibody to the culture medium resulted in up to a 400-fold increase in the yield of only the parent virus (Fig. 2). This type of experiment was carried out with monocytes from other donors and provided the following results: (i) the immune enhancement effect of dilute antibody on the yield of parent virus decreased as monocytes produced greater quantities of virus in antibody-free medium; and (ii) the S-1 PGMK small-plaque virus generally replicated in monocytes when dilute antibody was included in the culture medium, but the progeny virus produced large plaques similar to those of the parent virus. For example, when monocytes replicated the parent virus to 10^4 plaque-forming units (PFU), the addition of antibody to the inoculum and to the culture medium increased the yield no more than 10-fold (Fig. 3); however, the total amount of virus represented by this increase was greater than that represented by the 400-fold increase in Fig. 2. In monocytes from another donor, the parent virus replicated to well over 10^6 PFU, and the presence of dilute antibody did not increase the yield any further (Fig. 4). The S-1 PGMK virus did not replicate at all unless antibody was present, and then it gradually reached 10^3 to 10^4 PFU by the 5th day of culture (Fig. 3 and 4). The progeny virus produced large plaques in addition to the small plaques, a characteristic of the parent virus. Occasionally, the S-1 PGMK virus replicated when normal medium was used, but virus did not appear until the 4th day postinfection; again, replication was associated with reversion (appearance of large plaques). Thus, the S-1 PGMK

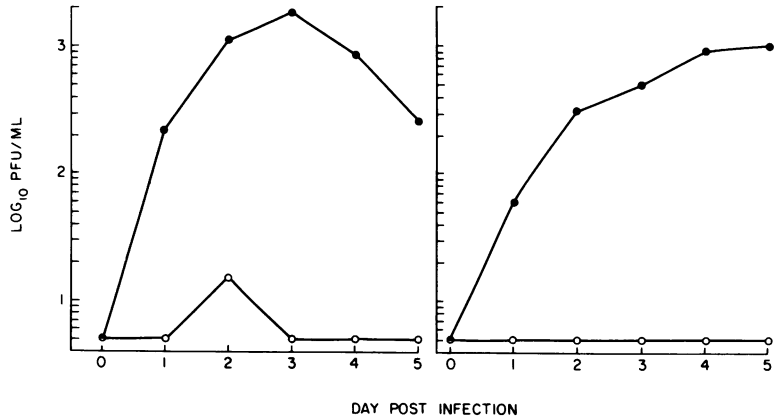

FIG. 1. *Parent and S-1 PGMK virus replication in monocytes from a normal donor (A, left panel, 1.1 × 10^5 cells) and a yellow fever-immunized donor (B, right panel, 1.2 × 10^5 cells). Parent virus (●) input = 1.6 × 10^4 PFU; S-1 PGMK virus (○) input = 3.2 × 10^4 PFU.*

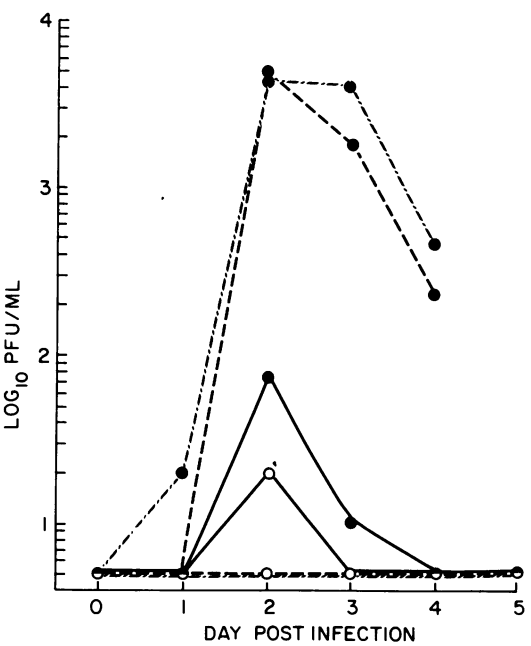

FIG. 2. *Parent and S-1 PGMK virus replication in 1.6 × 10^4 monocytes from a yellow fever-immunized donor (C). The adherent cells were infected and cultured either in normal medium (——) or in medium containing dengue-2 antibody (- - -) or dengue-3 antibody (-·-·-). Parent virus (●) input = 2.0 × 10^4 PFU; S-1 PGMK virus (○) input = 2.4 × 10^4 PFU.*

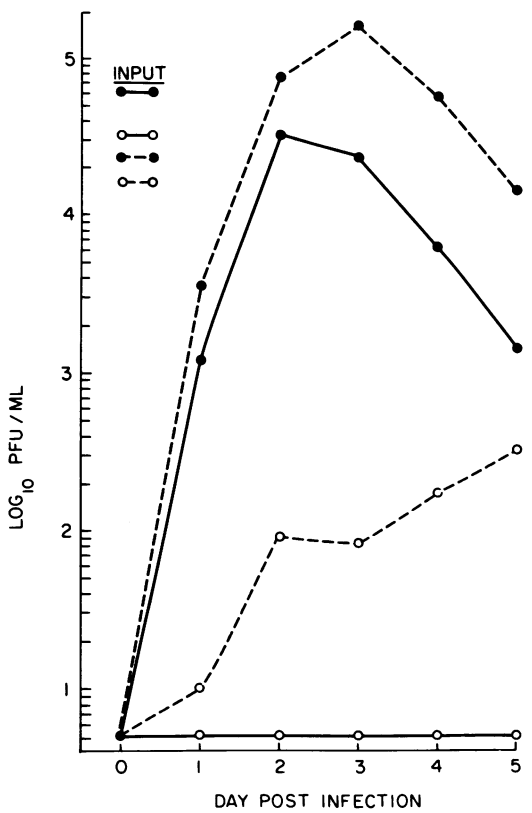

FIG. 3. *Parent (●) and S-1 PGMK (○) virus replication in 9.0 × 10^4 monocytes from a West Nile-immune donor (D). Adherent cells were infected and cultured in either normal medium (——) or medium containing West Nile antibody (- - -). Input titers of the viruses in each medium varied slightly and are represented at the top left of the figure.*

virus could be distinguished from the parent virus by the inability of the S-1 PGMK virus to replicate in monocytes unless it reverted.

Replication of vaccine virus in human monocytes. S-1 vaccine virus was prepared by passage of the S-1 PGMK virus in fetal rhesus

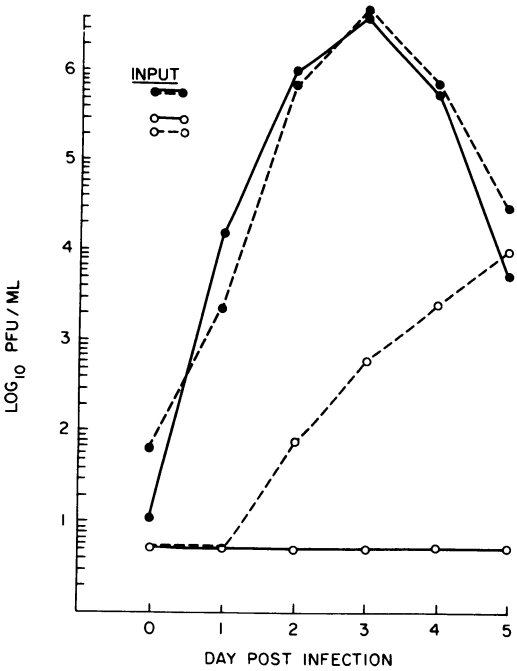

FIG. 4. *Parent (●) and S-1 PGMK (○) virus replication in 5.4 × 10⁵ monocytes from a yellow fever immunized donor (E). Adherent cells were infected and cultured in either normal medium (——) or medium containing dengue-2 antibody (- - -).*

lung cells. The vaccine virus unexpectedly gained the ability to replicate in human monocytes cultured in normal medium to almost the same titer as the parent virus, and the vaccine virus progeny retained its small-plaque characteristics (Fig. 5). We confirmed this change by examining the replication of the S-1 PGMK and S-1 vaccine viruses in the same experiment; even when a slightly greater inoculum of the S-1 PGMK virus was used, only the vaccine virus replicated in monocytes cultured in normal medium (Fig. 6).

Attempted isolation of vaccine virus from mononuclear leukocytes from volunteers. The replication of vaccine virus in vitro described above prompted us to determine if monocytes (as well as lymphocytes) were infected in vivo after inoculation of human volunteers with the vaccine virus. Even though five of the six volunteers had low-level viremia (Bancroft et al., manuscript in preparation), no virus was recovered from monocyte or lymphocyte culture supernatants or cells.

Replication of dengue viruses in human lymphocytes cultured in medium with and without enhancing antibody. Replication of dengue viruses in lymphocytes (nonadherent cells) was not pursued to the extent that it was in monocytes since lymphocytes are not susceptible to infection during the first 2 days in culture

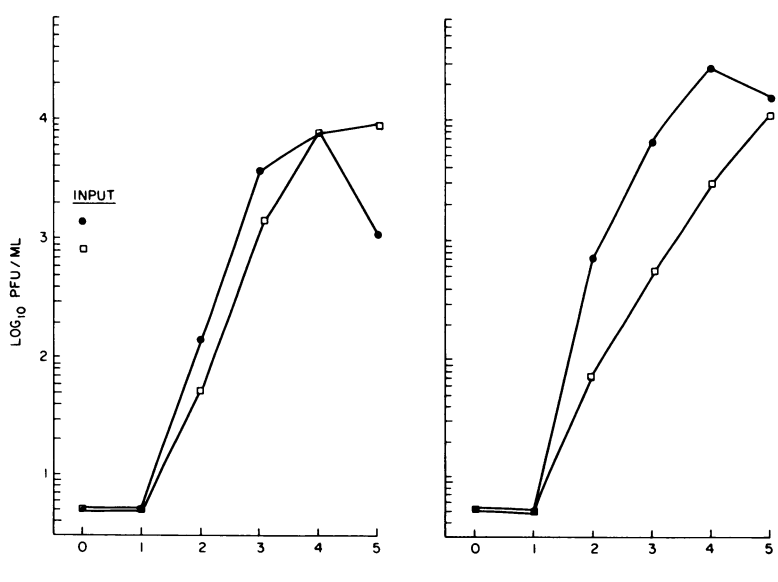

FIG. 5. *Replication of parent (●) and S-1 vaccine (□) viruses in monocytes from yellow fever-immunized donors. (F, left panel), 5.4 × 10⁴ cells; (G, right panel), 4.0 × 10⁴ cells.*

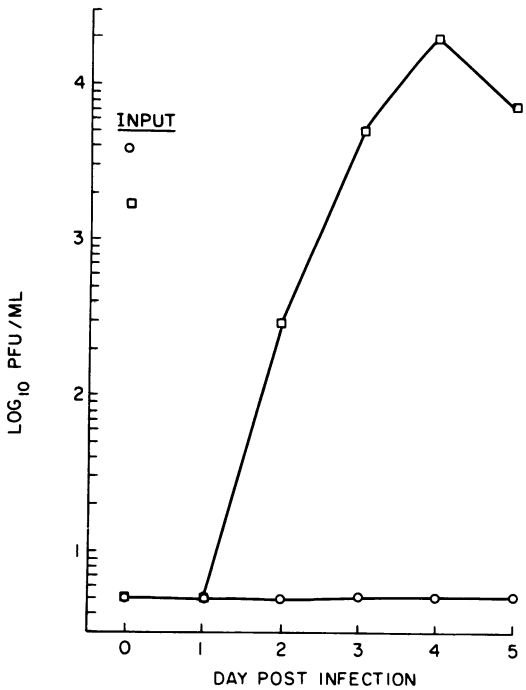

FIG. 6. *Replication of S-1 vaccine* (□) *and S-1 PGMK* (○) *viruses in 1.1 × 10⁵ monocytes from a yellow fever-immunized donor (H).*

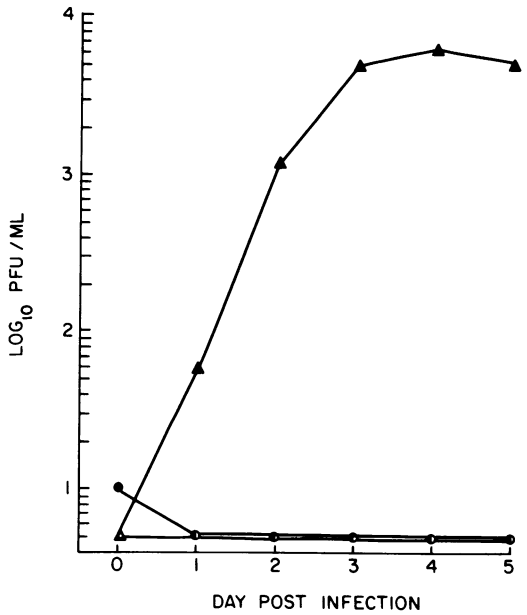

FIG. 7. *Replication of parent* (●), *S-1 PGMK* (○), *and 16681-Raji* (▲) *viruses in lymphocytes (nonadherent cells) from a yellow fever-immunized donor (B). Multiplicity of infection = 0.003, 0.004, and 0.003, respectively.*

(16). It was shown in that study that a Raji cell-adapted strain of dengue-2 virus would replicate only in lymphocytes that were cultured for several days. Therefore, we evaluated the ability of the parent and S-1 PGMK viruses, as well as the Raji-adapted dengue-2 virus, to replicate in cultured lymphocytes. A representative experiment (Fig. 7) indicated that neither the parent nor the S-1 PGMK virus replicated in lymphocytes, whereas the Raji-adapted virus replicated relatively well. We determined next if the parent, S-1 PGMK, and S-1 vaccine viruses would replicate in lymphocytes when antibody was included in the inoculum and the culture medium. These viruses still did not replicate.

The Raji-adapted dengue-2 virus, which was the only dengue-2 virus in this study that replicated in lymphocytes, was inhibited by the same dengue-2 antibody that enhanced virus production in monocytes (Fig. 8). Almost identical results were obtained with lymphocytes from separate donors studied 1 month apart but plotted together in Fig. 8. The virus yield from lymphocytes cultured in normal medium was over 10^4 PFU by the 5th day postinfection, whereas the yield from lymphocytes cultured in medium containing enhancing antibody was well over 100-fold less by the same day.

DISCUSSION

In the present study, an uncloned dengue-2 virus (parent) passaged in monkey kidney cells usually replicated in adherent human monocytes. A small-plaque clone derived from the parent virus by additional passage of isolated plaques in monkey kidney cells (S-1 PGMK virus) did not replicate in monocytes. The defect may have been at the attachment phase, since small-plaque and large-plaque viruses of the same strain have been shown to have differing absorption characteristics (10, 11). However, further passage of the S-1 PGMK virus in fetal rhesus lung cells (S-1 vaccine virus) enabled it to replicate in human monocytes even though the vaccine virus maintained the small plaque size and other markers (Table 1) characteristic of the S-1 PGMK virus. Since flaviviruses emerging from an infected cell contain lipid moieties from the intracellular membranes of that cell (D. W. Trent, *in* T. Monath, ed., *St. Louis Encephalitis,* in press), a virus propagated in different cells probably has different surface properties, which could affect attachment to the monocyte.

The S-1 PGMK virus replicated occasionally in monocytes, but replication in these instances was associated with reversion as judged by the

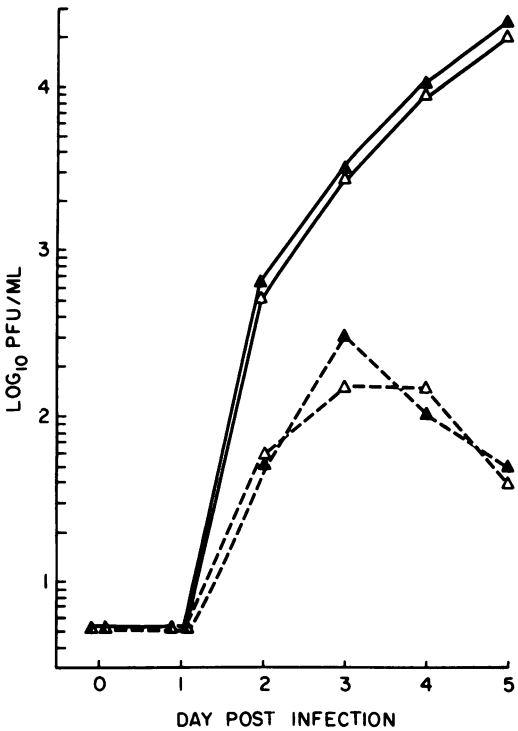

FIG. 8. *Replication of the 16681-Raji virus in lymphocytes from two separate donors. Donor C (▲), multiplicity of infection (MOI) in normal medium (——) = 0.002, MOI in medium containing dengue-2 antibody (- - -) = 0.0008. Donor J (△), MOI in normal medium (——) = 0.0003, MOI in medium containing dengue-2 antibody (- - -) = 0.0002.*

appearance of large-plaque-forming virus. The use of dilute antibody with S-1 PGMK virus inoculum increased the frequency of appearance of large-plaque virus. Reversion of the S-1 PGMK virus was shown previously to be enhanced by increasing the multiplicity of infection (3). In the present experiments, small aggregates of virus may have been produced by the antibody which effected a higher multiplicity on some cells, or some undetectable large-plaque virus in the input inoculum was assured of infecting monocytes when complexed with antibody.

The addition of dilute antibody to infected monocyte cultures usually increased the yield of parent virus unless it had already replicated to some maximum level (>10^5 PFU/ml) in monocytes cultured in normal medium. The yield of virus was often not predictable even from cells harvested at different times from the same donor with the same media. Halstead and O'Rourke (7) encountered the same variation and felt that it was due to variation in the number of permissive cells, which varied between individuals and in individuals serially studied. Even though less than 1% of the mononuclear phagocytes exhibited dengue-specific fluorescence, there was a correlation between the number of fluorescent cells and the virus yield (8). We monitored the flavivirus-immune status of the donors (yellow fever immunization, previous natural infection, or normal), and this status did not appear to have a bearing on the virus yield. It was shown previously that enhancing antibody is not cytophilic since it could be washed from the cells and had to be re-added to effect immune enhancement (7). In our studies, antibodies from the donor would have been removed during the cell separation procedures, which involved extensive cell washings.

The three viruses passaged in monkey kidney or lung cells (parent, S-1 PGMK, and S-1 vaccine viruses) did not replicate in cultured lymphocytes. The addition of enhancing antibody to the culture system did not alter this result. Thus, the lack of replication of a dengue virus with wild-type virulence characteristics indicates that lymphocytes are not one of the target cell populations in natural disease. As described previously (16), we were able to demonstrate replication in lymphocyte cultures of a strain of dengue-2 virus passaged through a human lymphoblastoid cell line (16681-Raji virus). However, the same dilution of antibody that enhanced viral replication in monocytes depressed replication in lymphocytes. Thus, enhancing antibody in one cell system was suppressive in another. The differing susceptibilities of each cell population to the variously passaged viruses and the differing effects of dilute antibody on the replication that does occur indicate that separation of the cell populations by adherence, after Ficoll-Hypaque centrifugation, was sufficient for these experiments.

Monocytes and lymphocytes obtained in this manner from infected volunteers inoculated with the vaccine virus did not yield virus. On the other hand, wild virus is recovered readily from monocytes obtained from dengue fever patients (14); up to twice as many isolates were obtained from adherent cells as from the plasma (14; Scott et al., J. Infect. Dis., in press). Even though the volunteers converted serologically and exhibited a very low level of viremia (Bancroft et al., manuscript in preparation), it appears that circulating monocytes were not infected as they are in actual cases of dengue fever. This may reflect a fundamental difference between the S-1 vaccine and wild strains of dengue.

The apparent dichotomy between virus replication in monocytes infected with the vaccine

virus in vitro and no recovery of virus from monocytes taken from viremic volunteers infected with the vaccine virus may be related to the physiological condition of cultured adherent monocytes. They are in a stimulated state (2) and therefore may have been in a more active form, facilitating infection. This may explain why dengue virus replication usually can be demonstrated in adherent monocytes from normal donors. Unattached monocytes in peripheral blood leukocyte suspensions from normal donors do not usually replicate dengue virus unless dilute antibody is present in the culture system (4, 5, 13). Thus, passage history of the virus and physiological condition of the target cell under study are at least two reasons why virus replication in a certain cell as an in vitro correlate of virulence, or as a stable genetic marker, should be interpreted with caution.

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