

# Dengue Virus, A Flavivirus, Propagates in Human Bone Marrow Progenitors and Hematopoietic Cell Lines

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Dengue and other arbovirus diseases are frequently associated with bone marrow failure. We show that dengue type 4 (DEN4) propagates in colonies derived from immature human bone marrow progenitors. DEN4 was propagated in BFU-E-derived colonies and replication was dependent on erythropoietin. DEN4 was not cytotoxic. In inoculated cultures, diffuse bursts with many clusters contained large amounts of DEN4 RNA. In contrast to dengue infection of macrophages, virus propagation in

semisolid culture was sustained and not enhanced by subneutralizing amounts of antibody. DEN4 also was efficiently propagated in human hematopoietic cell lines, especially those with erythroid properties. In K562 cells, DEN4 infection persisted for months; greatly slowed cell growth, again without cytotoxicity; and resulted in cytopathic changes in cell appearance. Flaviviruses can infect human hematopoietic cells and alter their proliferative capacity. © 1989 by Grune & Stratton, Inc.

**VIRUS INFECTION** of hematopoietic cells can cause bone marrow failure in animals and humans.<sup>1</sup> The mechanisms of virus-host interaction are diverse, including direct cytotoxicity (parvoviruses),<sup>2</sup> infection of supporting stromal cells (cytomegalovirus),<sup>3</sup> and incitement of a host cytotoxic immune response (Epstein-Barr virus).<sup>4</sup> Human arbovirus disease like dengue (Thai hematodepressive disease), Russian winter-spring hemorrhagic fever, Kyansur forest disease, and Semliki forest disease are often associated with significant hematologic abnormalities,<sup>1,5</sup> and experimental inoculation of volunteers has produced temporary marrow aplasia.<sup>6</sup> In dengue, neutropenia and thrombocytopenia are common, and the bone marrow may be markedly hypocellular with abnormal megakaryocytopoiesis.<sup>7</sup> Furthermore, the areas of the world in which arbovirus infections are endemic also have high rates of incidence of "idiopathic" aplastic anemia.<sup>8</sup> For these reasons, we investigated dengue virus infection of normal hematopoietic cells and hematopoietic cell lines in vitro.

## MATERIALS AND METHODS

**Infection, cultivation, and isolation of cells.** Bone marrow was aspirated from normal volunteers after informed consent under a protocol approved by the National Heart, Lung, and Blood Institute (NHLBI) institutional review board. Mononuclear cells were prepared by isopycnic centrifugation in Ficoll-Hypaque. Progenitor cultures were performed in Iscove's modification of Dulbecco's medium (IMDM) containing 30% fetal calf serum (FCS), 1% bovine serum albumin, 1.2% methylcellulose,  $5 \times 10^{-5}$  mol/L beta-mercaptoethanol, 30 U/mL recombinant erythropoietin (Genetics Institute, Cambridge, MA), and 1% C5MJ conditioned medium (a kind gift from Dr Makio Ogawa, Medical University of South Carolina, Charleston). Marrow cells were incubated in 100% humidity, 5% O<sub>2</sub>, 5% CO<sub>2</sub>, at 37°C. Hematopoietic cell lines were maintained in RPMI 1640-10% FCS (U937, HL60, K562, HEL) or IMDM-20% FCS (KG1). Cell lines were grown in 100% humidity, 5% CO<sub>2</sub> at 37°C.

In most experiments, normal bone marrow cells were infected with dengue type 4 (DEN4) (Caribbean strain 814669) produced in the C6/36 mosquito cell line; in some experiments, we tested dengue type 2 (DEN2) (New Guinea C strain, a kind gift from Dr Robert Putnak, Walter Reed Army Institute of Research, Washington, DC), cloned yellow fever virus derived from cDNA YF17D, and cloned Sindbis virus derived from cDNA Toto 1101 (the latter two kind gifts from Professor Charles Rice, Washington University, St Louis). For infection, bone marrow mononuclear cells or hematopoietic cell lines were incubated with virus at 1 plaque forming unit/cell for two hours at 37°C, excess virus was removed by

washing, and the cells were cultured for up to 20 days in methylcellulose at  $10^5$  marrow mononuclear cells/35 mm dish or for three days in suspension culture at  $10^5$  cells/mL. In some experiments, bone marrow mononuclear cells were cultured in suspension with growth factors but without methylcellulose for 15 days.

Progeny cells from marrow cultures were isolated by several methods. Total cells from semisolid cultures were collected by washing in Hanks' balanced salt solution to remove methylcellulose. For some experiments, mature colonies were identified by their characteristic morphology in situ and physically removed from methylcellulose by aspiration through a tapered Pasteur pipette.<sup>10</sup> Cells were also fractionated by cell surface phenotype using a monoclonal antibody to glycophorin (10F7,<sup>11</sup> at 10 µg/mL final concentration) followed by reaction with a fluorescein-conjugated antimouse immunoglobulin (Becton-Dickinson) and separation on a Coulter EPICS V flow cytometer.

**Detection of viral RNA and protein and infectious virus.** After harvesting, cells were washed and RNA was prepared by extraction with phenol-chloroform<sup>12</sup> and hybridized<sup>13</sup> to a <sup>32</sup>P-labeled specific RNA probe corresponding to the DEN4 nucleotides 939-2307<sup>14</sup> in a slot blot apparatus (Schleicher and Schuell, Keene, NH). DEN4 RNA was measured by densitometric comparison of signals using known quantities of DEN4 RNA fragments of this region produced by in vitro transcription, and the total quantity of RNA expressed as picograms. For some experiments, colonies were applied directly to nitrocellulose without prior RNA extraction, immobilized by glutaraldehyde, and hybridized to the radioactive riboprobe.<sup>15</sup> Northern analysis was performed using 0.66 mol/L formaldehyde gels.<sup>13</sup>

Viral protein was determined by immunofluorescence after fixation of cytocentrifuge preparation of cells in 100% ethanol at -20°C followed by staining with mouse anti-DEN4 hyperimmune serum<sup>16</sup> (diluted 1:100) and fluorescein-conjugated antimouse IgG. For morphology, cells were stained using Wright's-Giemsa. Plaque formation was performed using LLC-MK2 monkey cells as described.<sup>17</sup>

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*Submitted April 13, 1989; accepted June 15, 1989.*

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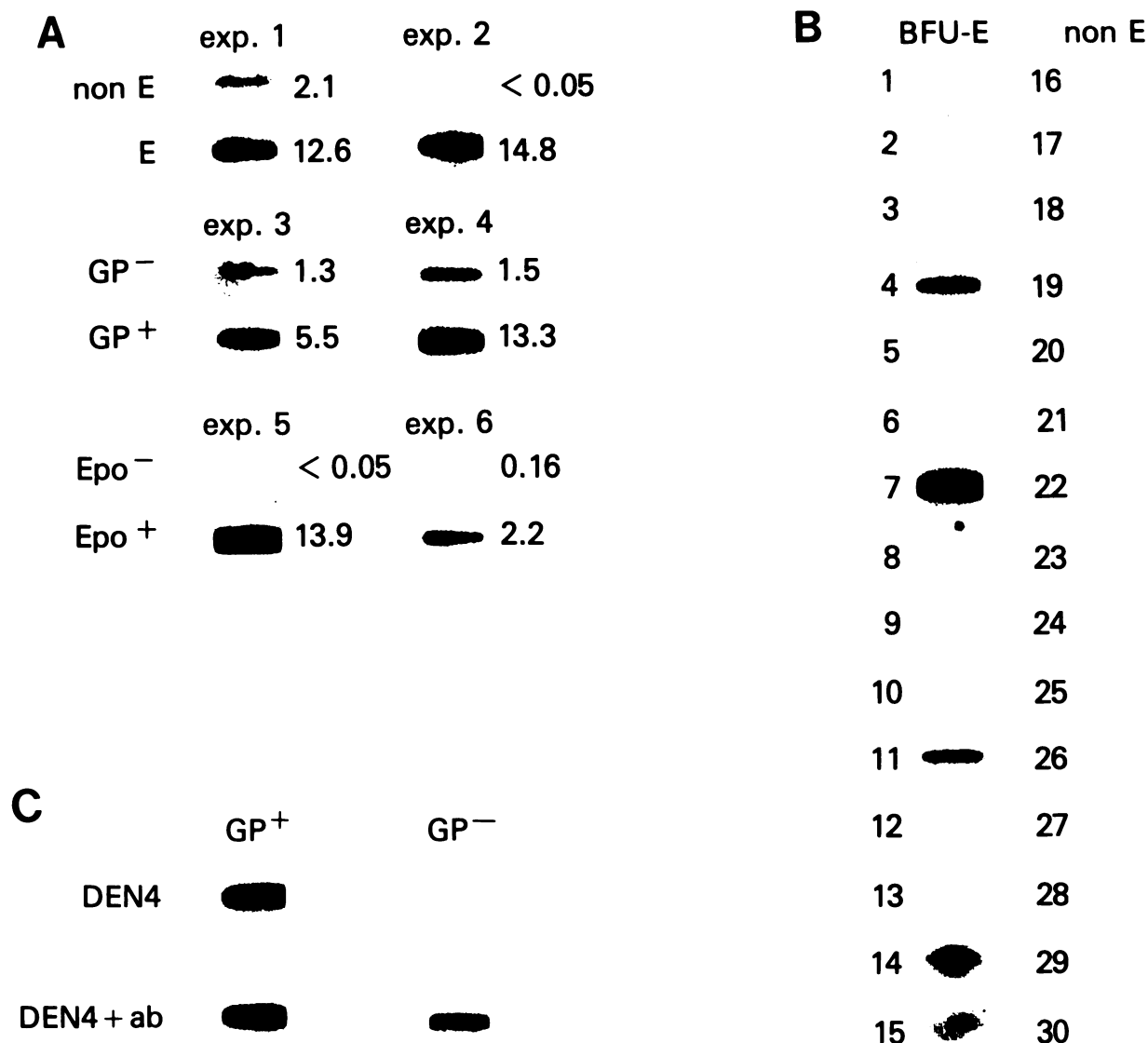
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## RESULTS AND DISCUSSION

**Infection of normal bone marrow by DEN4.** Normal bone marrow mononuclear cells were infected by exposure to fresh DEN4 virus followed by culture in semisolid medium for 14 days. We chose to focus our experiments on the analysis of virus infection of erythroid progenitors for two reasons: first, the ability of dengue to infect macrophages<sup>18,19</sup>

and a monocytic cell line<sup>20</sup> complicates interpretation of results with CFU-GM-derived colonies; and second, BFU-E undergo more extensive replication in vitro than myeloid cells. DEN4 propagation in hematopoietic cells was assessed by three methods. First, all individual colonies of different lineage were removed from cultures; under our culture conditions, nonerythroid colonies were more abundant than



**Fig 1.** DEN4 propagation in human hematopoietic progenitor cells and a cell line as analyzed by RNA hybridization. (A) Three types of experiments are shown: (1) DEN4 RNA in pooled bone marrow colonies (E, erythroid). (2) Separation of progeny based on binding to an antiglycophorin (GP) antibody. (3) Cultures performed in the presence (Epo<sup>+</sup>) or absence (Epo<sup>-</sup>) of erythropoietin. These cultures were grown in the absence of conditioned medium from the C5MJ cell line used as a source of hematopoietic growth factors. The number to the right of each radiograph indicates the amount of DEN4 RNA in pg. (B) Individual colonies derived from BFU-E and nonburst colonies applied to nitrocellulose membranes for direct hybridization. (C) Effect of subneutralizing quantities of anti-DEN4 serum on virus propagation in erythroid (GP<sup>+</sup>) and nonerythroid (GP<sup>-</sup>) progeny. DEN4 infection did not alter colony number. Colony number (mean/10<sup>5</sup> mononuclear cells  $\pm$  SEM of triplicate cultures) in the experiments described above under (A) (1) were as follows:

	exp 1		exp 2	
	control	DEN4	control	DEN4
BFU-E	65 $\pm$ 3	67 $\pm$ 4	43 $\pm$ 4	42 $\pm$ 2
CFU-GM	72 $\pm$ 2	75 $\pm$ 1	58 $\pm$ 2	61 $\pm$ 3
CFU-Eo				

erythroid colonies. Purified RNA from pooled erythroid BFU-E-derived colonies (about  $2 \times 10^5$  cells) and myeloid colonies (derived from the granulocyte-macrophage progenitor, CFU-GM, and eosinophil progenitor, CFU-Eo; about  $1 \times 10^5$  cells) was then assessed by hybridization with a DEN4-specific  $^{32}\text{P}$ -labeled riboprobe for the presence of DEN4 RNA. This method allowed detection of as little as 0.1 pg of DEN4 RNA, equivalent to a single plaque forming unit of virus. DEN4 RNA was present at higher concentrations in pools of erythroid compared to nonerythroid colonies (Fig 1A). Second, DEN4 RNA was more abundant in erythroid cells compared with nonerythroid cells, separated by flow cytometry after binding to an antibody to glycoporphin; glycoporphin-bearing ( $\text{GP}^+$ ) cells constituted about 40% to 50% of total cells. The average DEN4 RNA copy number was 50-150/ $\text{GP}^+$  cell. DEN4 RNA was increased when colonies were grown in the presence of erythropoietin (Fig 1A). When individual colonies derived from BFU-E, CFU-GM, and CFU-Eo were fixed on nitrocellulose and directly hybridized to the radioactive probe, signal was detectable only in erythroid colonies (5 of 15) and not in nonerythroid colonies (0 of 15) (Fig 1B). The glycoporphin-bearing progeny of DEN4-infected marrow cells produced full length dengue genomic RNA (Fig 2).

Several results suggested that infection of marrow hematopoietic cells differs in important respects from infection of

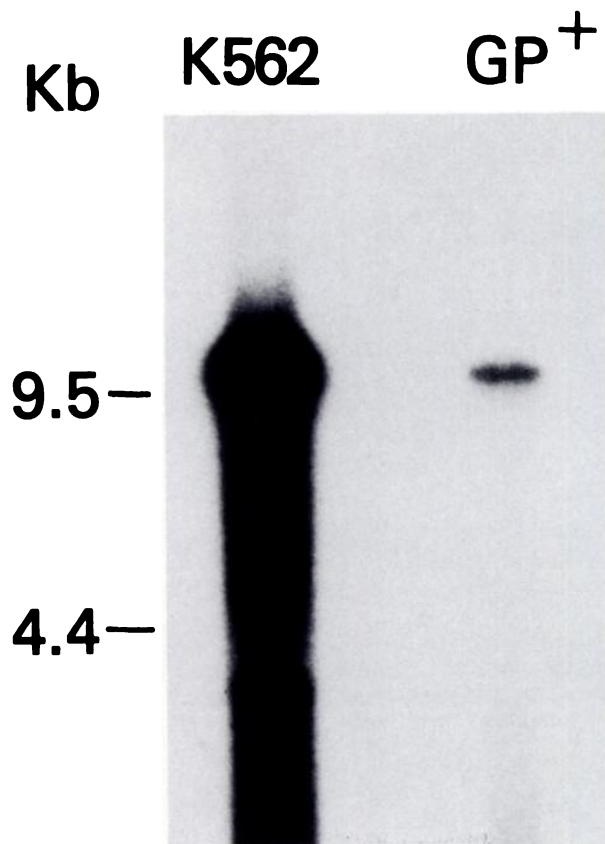


Fig 2. Northern analysis of RNA extracted from  $10^6$  DEN4-infected K562 cells and  $\text{GP}^+$  marrow cells.

macrophages. DEN4 infection of macrophages is enhanced by antibody; viral propagation in these cells is rapid but transient, and accompanied by release of significant amounts of extracellular virus.<sup>18,19</sup> In contrast, DEN4 RNA appeared in marrow cells five days after inoculation, and sustained high levels were present for at least 2 weeks of methylcellulose culture; there was little DEN4 propagation in cultures lacking hematopoietic growth factors (Fig 3A). DEN4 was released at a low level into the extracellular medium, as determined by plaque assay ( $<30$  plaque forming units/ $10^5$  DEN4-infected cells). In contrast to semisolid cultures, suspension cultures of DEN4-infected marrow cells showed much lower levels of intracellular virus and virus was released transiently into supernatant, consistent with viral replication in macrophages<sup>18,19</sup> or B lymphocytes<sup>21</sup> (Fig 3). Mixing of DEN4 with subneutralizing amounts of antibody to DEN4 did not enhance virus propagation in glycoporphin-bearing progeny, although it did increase DEN4 replication in cells that lacked glycoporphin (Fig 1C). These data suggest that DEN4 enters hematopoietic cells via specific receptors rather than as an immune complex attached to immunoglobulin receptors.

The plating efficiency of hematopoietic progenitors was not changed following DEN4 inoculation (see table, Fig 1 legend). However, some erythroid colonies in infected cultures appeared abnormally diffuse with increased numbers of small clusters of normoblasts (Fig 4A, B). When individual colonies were analyzed by immunofluorescence, about one third stained with antiserum against DEN4 protein; within positive colonies, 10% to 60% of cells showed bright specific staining (varying from experiment to experiment), and fluorescence roughly correlated with the morphologic appearance of the bursts (Fig 4C). Colonies with an abnormal appearance showed the highest quantities of DEN4 RNA when individually applied to nitrocellulose and hybridized with specific probe (ie, colony #7 in Fig 1B). Normoblasts from infected colonies appeared normal on Wright's-Giemsa staining, and the concentration of hemoglobin determined spectrophotometrically was also normal (data not shown).

**Propagation of DEN4 in hematopoietic cell lines.** DEN4 was efficiently propagated in several human hematopoietic cell lines. Cells with erythroid properties were most permissive (K562 [ $10^4$ ] > HEL [ $10^3$ ] > U937 [ $10$ ] > KG1 [ $3$ ] > HL60 [ $1$ ]; for comparison, bracketed numbers refer to the ratio of viral RNA/cell relative to HL60). Full-length genomic viral RNA was present in infected K562 cells as determined by Northern analysis (Fig 2). As with marrow cells, DEN4 infection did not result in killing of K562 cells, as determined by dye exclusion, but it did inhibit cell proliferation. Infected cultures of K562 could be maintained for several months after DEN4 inoculation with continued bright specific immunofluorescence in almost all cells (Fig 4D). The morphologic appearance of infected K562 cells was abnormal, with multiple prominent nucleoli, scanty cytoplasm, and a deeply azurophilic nucleus (Fig 4D and E).

**Propagation of other arboviruses.** The ability of other arboviruses to infect hematopoietic cells was investigated. DEN2 propagated primarily in nonerythroid cells and less

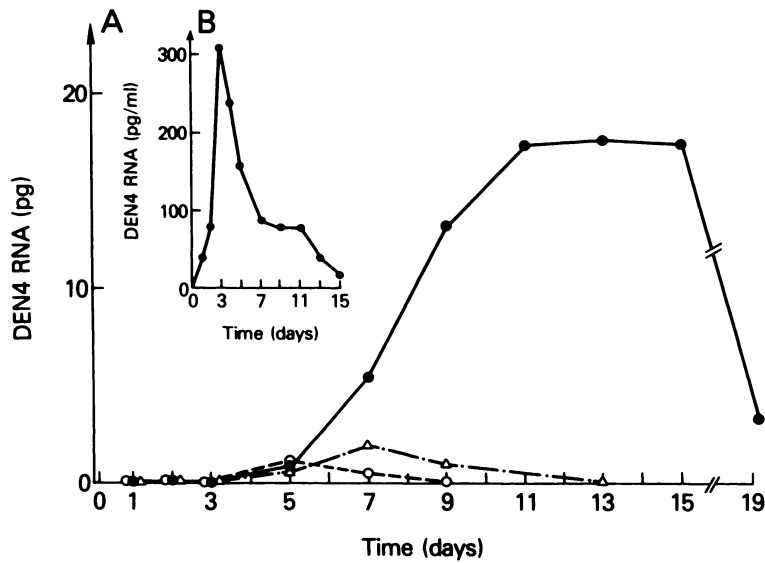


Fig 3. Time course of DEN4 propagation in bone marrow cells. (A) Marrow mononuclear cells in methylcellulose with (●—●) or without (○—○) Epo plus C5MJ medium or in suspension culture in the presence of these erythropoietic growth factors (Δ—Δ). Data are expressed as picograms of DEN4 RNA in total cultures. (B) DEN4 RNA in supernatant of suspension culture. Data are expressed as picograms DEN4 RNA/mL.

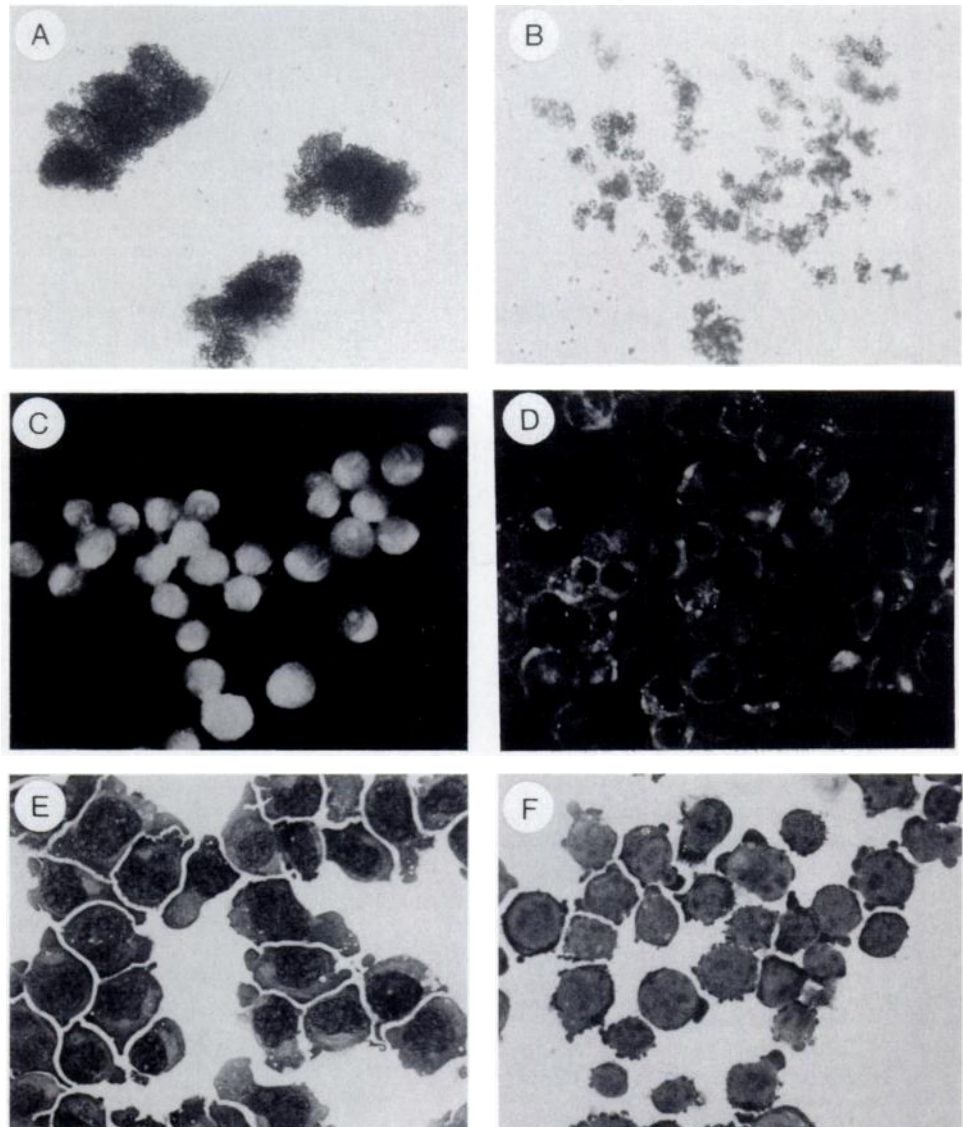


Fig 4. Morphology of DEN4-infected hematopoietic cells. (A) Uninfected mature BFU-E-derived colony and (B) DEN4-infected burst (original magnification  $\times 80$ ). (C) Immunofluorescence of cytocentrifuge preparations of a DEN4-infected burst and (D) DEN4-infected K562 cells (original magnification  $\times 500$ ) after staining with murine hyperimmune serum and FITC-conjugated goat antimouse IgG. (E) Wright's Giemsa staining of cytocentrifuge preparations of uninfected K562 cells and (F) DEN4-infected K562 cells (original magnification  $\times 500$ ).



efficiently than DEN4. We could not detect propagation in human bone marrow of the togavirus Sindbis or the flavivirus yellow fever (data now shown). However, all these arboviruses were efficiently propagated in K562 cells (yellow fever was cytotoxic).

**Conclusions.** Our results show that members of the *Flaviviridae* family can propagate in human bone marrow cells and leukemic cell lines. This is also the first demonstration of persistent viral infection in human hematopoietic cells. Although DEN4 infection was more readily detected in erythroid progeny, this is unlikely to represent lineage specificity, as DEN4 RNA was also present in pooled nonerythroid colonies and the virus propagated in nonerythroid cell lines. We have also found that DEN2, a closely related strain, propagated better in nonerythroid than in erythroid colonies (data not shown). That DEN4 did not propagate as well in cells cultured in suspension as in methylcellulose may reflect the dominance of macrophages and granulocytes in the absence of semisolid medium or the lack of intimate cell-cell contact.

DEN4 infection did not kill cells but affected their proliferation in vitro, altering burst morphology and slowing reproduction of K562 cells. Oldstone has proposed that viral pathogenicity may occur in the absence of cytotoxicity due to viral effects on cellular "luxury" functions; for example,

lymphocytic choriomeningitis virus is trophic to cells of the anterior pituitary gland, and infection inhibits growth hormone synthesis without cell necrosis or inflammation.<sup>22</sup> As proliferation in response to growth factors is a special quality of marrow cells, DEN4 inoculation may be viewed as resulting in virus inhibition of this hematopoietic cellular "luxury" function. Practically, the proliferative and cytopathic effects of DEN4 infection in K562 cells make this culture system a potential model of persistent flavivirus infection in human hematopoietic cells.

Clinically, the early course of dengue is dominated by viremia-associated marrow failure, with poorly characterized immune responses probably mediating the severe hemorrhagic shock syndrome. Our results suggest that the bone marrow may be a major site of flavivirus replication during clinical infection. Hepatitis C virus, the etiologic agent of most cases of non-A non-B hepatitis, recently has been shown to have the properties of a flavivirus.<sup>23</sup> In addition, dengue virus antigens incite in vitro an immune response, T lymphocyte activation and  $\gamma$ -interferon production,<sup>24</sup> similar to abnormalities present in patients with aplastic anemia.<sup>25</sup> Hepatitis-associated aplastic anemia may be the result of an intrinsic proliferative defect due to virus infection in combination with an inhibitory immune response directed against viral antigens on the host cell surface.

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