Dengue Virus Inhibits Human Hematopoietic Progenitor Growth In Vitro

Bernadette Murgue, Olivier Cassar, Martine Guigon, and Eliane Chungue

Dengue disease, whether it be classical dengue fever (DF), dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS), is frequently associated with hematologic disorders. The underlying cause of these abnormalities is unknown. To determine if an inhibitory effect on human hematopoietic progenitor growth can be observed, normal cord blood mononuclear cells were exposed to low-passaged clinical isolates from DF, DHF, and DSS patients and to the prototype strain of dengue-3 virus (H-87). In primary methylcellulose cultures, there was no inhibition of colony formation. After an initial 8-day liquid culture, inhibition was observed with the isolates, but strain H-87 had no effect. Furthermore, isolates from patients with DSS showed a more potent inhibitory effect. These data represent the first documented study of in vitro impaired progenitor cell growth by dengue virus and suggest that this inhibition could be dependent upon the isolate tested.

The four serotypes of dengue viruses (DEN-1, -2, -3, and -4) induce a large spectrum of clinical manifestations, which are often associated with hematologic disorders, such as leukopenia and thrombocytopenia [1]. In dengue fever (DF), a self-limited febrile disease, these manifestations are moderate, whereas in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are characterized by hemorrhagic manifestations and plasma leakage, these abnormalities are usually more pronounced, especially thrombocytopenia. Bone marrow examinations of persons with DF, DHF, or DSS have shown a hypocellular marrow with abnormal megakaryocytopenia [2, 3]. Marrow suppression observed during dengue infection may be due to a direct infection of hematopoietic progenitor cells or could be related to the release of cytokines down-regulating hematopoiesis (or both). Whether these mechanisms act alone or in combination to mediate the myelosuppression associated with dengue infection is still unknown.

In this study, we investigated the effects of dengue virus, using low-passaged clinical isolates of DEN-3, which is endemic in Tahiti, and prototype strain H-87 of DEN-3 on the in vitro growth of human hematopoietic progenitors.

Materials and Methods

Viruses and patients. Clinical isolates were obtained from vir-emic children presenting with primary dengue infection during the
epidemic of DEN-3 in French Polynesia. Severity of dengue disease was graded according to the criteria of the World Health Organization [4]. To determine the effects of DEN-3 on human hematopoietic progenitors, we used isolates 60-56DF, 136-116DHF, and 29-56DSS. Additional isolates (30-50DF, 30-56DF, and 276-43DSS) were used for some experiments.

Virus was isolated by inoculating the Aedes albopictus C6/36 cell line with acute-phase serum and passing 3 times using the Aedes pseudoscutellaris AP61 cell line. The infected culture supernatants were precipitated using 7% polyethylene glycol 8000 (Sigma, St. Louis). Virus titers ranged from 4 × 10⁴ to 2 × 10⁶ TCID₅₀/ml [5]. Prototype DEN-3 strain H-87 (obtained from passages on suckling mouse brain) and passages of AP61 cell culture fluid from a dengue-negative serum (virus-negative control stock) were prepared as above.

Cells. Normal cord blood was provided by Clinique Paofai (Papeete). Fresh cord blood mononuclear cells (CBMNC) were separated on ficoll-hypaque gradient (density 1.077 g/mL; GIBCO, Grand Island, NY). Plastic adherent cells were adsorbed by preincubation overnight in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO) with 10% fetal calf serum (D. Dutcher, Bru- math, France).

Inoculation of CBMNC with DEN-3 virus and liquid culture. CBMNC (4 × 10⁴ to 10 × 10⁴) were incubated with DEN-3 virus at MOIs ranging from 10⁻⁴ to 1 TCID₅₀/cell in IMDM for 2 h at 37°C (5% CO₂ in air). Thereafter, cells were washed 5 times in IMDM, counted, and seeded in liquid culture or methylcellulose culture. To control the efficiency of the washing procedure, we inoculated the supernatant from the last washing into C6/36 cells as previously described [6] and carried out viral RNA assay by reverse transcription–polymerase chain reaction (RT-PCR; see below). Negative controls were obtained by using either a virus-negative control stock or culture medium alone.

After infection, CBMNC (2 × 10⁶) were seeded in 24-well plates (Nunc, Roskilde, Denmark) in 1 mL of IMDM–10% fetal calf serum supplemented with 10 ng/ml interleukin (IL)-3, 10 ng/ml IL-6, and 50 ng/ml stem cell factor (SCF; Media Preparation Service, Terry Fox Laboratory, Vancouver, Canada). Cultures were incubated at 37°C for 8 days in a humidified atmosphere containing 5% CO₂ in air.

IFA. IFA was done on 50,000 cytocentrifuged cells from 8-day liquid cultures. After fixation with cold acetone, cells were incubated with anti-dengue mouse hyperimmune ascitic fluid (American Type Culture Collection, Rockville, MD). Presence of dengue antigens was revealed with fluorescein isothiocyanate–conjugated sheep anti-mouse immunoglobulin (Diagnostics Pasteur, Marnes-la-Coquette, France).

Assay for clonogenic progenitors. The number of clonogenic hematopoietic progenitors was determined by assay in methylcellulose cultures as described previously [7]. In brief, 2 h after inoculation (primary methylcellulose culture) and on day 8 of liquid culture, cells were counted in trypan blue, and 2 × 10⁴ to 5 × 10⁴ cells were plated in triplicate in 35-mm dishes in 1 mL of methylcellulose supplemented with 10% agar-leukocyte–conditioned medium (vol/vol) and 3 U/ml erythropoietin (Media Preparation Service, Terry Fox Laboratory). Cultures were incubated at 37°C (5% CO₂ in air). Colony-forming unit–granulocyte-macrophages (cfu-GM), containing at least 50 cells, and burst-forming unit–erythroid (bfu-E), consisting of at least three bursts, were scored by use of an inverted microscope after 14–18 days of culture. In some experiments, colonies were plucked separately, and pools of 20–30 bfu-E or cfu-GM were analyzed by RT-PCR.

Detection of viral RNA. RT was done for 1 h at 42°C with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and anti-genomic sense primer. cDNA was amplified by 35 cycles of PCR, using Taq polymerase (Promega) and internal genomic sense and anti-genomic sense primers. Semi-nested PCR was subsequently done on the first-round amplified products [8].

Statistical analysis. Results are expressed as the mean ± SE. The numbers of colonies generated by infected and noninfected CBMNC in primary methylcellulose cultures were compared by use of a t test. At a given MOI, the percentages of inhibition of colony formation observed with the different isolates after an 8-day liquid culture were compared by analysis of variance (LSD post-hoc test). P < .05 was considered statistically significant.

Results

Effects of DEN-3 virus on the growth of progenitor cells from CBMNC in primary methylcellulose cultures. There was no change with any of the strains tested at an MOI of 1 or 10⁻¹ in the number of bfu-E and cfu-GM colonies generated by infected CBMNC (data not shown). Viral RNA was detected in cfu-GM colonies (30 pooled colonies) but not in bfu-E colonies from CBMNC infected at an MOI of 1 with 29-56DSS (n = 3) or 136-116DHF (n = 1).

Effects of DEN-3 virus on hematopoietic progenitor growth after an 8-day liquid culture. At an MOI of 1, the number of viable cells was reduced with the 3 clinical isolates and the prototype strain H-87 (table 1). The number of colonies was also decreased. We expressed the results at this time point as the total number of colonies because the number of bfu-E colonies in control cultures was too low to be taken into account separately. The inhibition of colony formation was not different between the 3 isolates tested (P > .05); however, prototype strain H-87 induced a weak inhibition of 18% ± 9%.

When the same experiments were done at MOIs ranging from 10⁻⁴ to 10⁻², inhibition of colony formation was dose dependent (figure 1A) with both clinical isolates. The number of viable cells was also reduced in a dose-dependent manner (data not shown). The level of colony formation inhibition between DSS and DF isolates or DSS and DHF isolates was significantly different (P < .05) at MOIs <10⁻¹. By contrast, no difference was found in the level of inhibition induced by DF or DHF isolates (P > .05).

In addition, we tested the effects of 2 DF isolates (30-50DF and 30-56DF) and one fatal grade IV DSS isolate (276-43DSS) at an MOI of 10⁻² and found that the differences in colony formation inhibition were significantly different between isolates 60-56DF and 29-56DSS (P < .01). The results of three separate experiments with each isolate, showed an inhibition of 28% ± 13%, 29% ± 15%, and 72% ± 3% for 30-50DF, 30-56DF, and 276-43DSS, respectively (P < .05, between 30-50DF or 30-56DF and 276-43DSS). No difference was
Table 1. Reduction of the number of viable cells and inhibition of the number of progenitors after an 8-day liquid culture of CBMNC infected by DEN-3 virus at MOI of 1 TCID₅₀/cell.

<table>
<thead>
<tr>
<th>DEN-3 strains, experiment no.</th>
<th>Viable cells/well (x10⁶)</th>
<th>No. of progenitors/well*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Infected</td>
</tr>
<tr>
<td>60-56DF</td>
<td>1</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.68</td>
</tr>
<tr>
<td>136-116DHF</td>
<td>1</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.05</td>
</tr>
<tr>
<td>29-56DSS</td>
<td>1</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.03</td>
</tr>
<tr>
<td>H-87</td>
<td>1</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.87</td>
</tr>
</tbody>
</table>

NOTE. Infected (with clinical isolates of dengue-3 virus [DEN-3] or H-87 strain) or noninfected cord blood mononuclear cells (CBMNC) were seeded in 24-well plates (2 x 10⁶ cells/well) in 8-day liquid culture in presence of interleukin-3 and -6 and stem cell factor. Thereafter, cells were counted in trypan blue and plated in triplicate at 20,000 cells/mL in 35-mm dishes in methylcellulose containing agar-leukocyte-conditioned medium and erythropoietin. After 14 days, total no. of colonies (erythropoietic /granulocyte-macrophage) was determined.

* Total no. of progenitors per well was calculated as (no. of progenitors/dish x no. of cells/well)/no. of cells/dish. Results are mean ± SE of triplicate experiments.

² % reduction of viable cells was calculated as 100 x [1 - (no. of viable cells in infected cultures/no. of viable cells in noninfected cultures)].

³ % inhibition of no. of progenitors was calculated as 100 x [1 - (no. of colonies in infected cultures/no. of colonies in uninfected cultures)].

Replication of dengue virus in CBMNC. After 2 h of incubation with dengue virus, viral RNA was detected in CBMNC. Neither viral RNA nor infectious particles were detected in the supernatant from the last wash.

After 8 days of liquid culture at an MOI of 1, viral antigens were intermittently detected by IFA in cells infected by either virus, and no difference in the number of positive cells was observed between the viruses tested (two experiments; data not shown). We also inoculated cells and supernatants from 8-day liquid culture of cell line C6/36. The results of experiments showed that no matter the virus tested, cultures were not constantly positive (at least 2 experiments, data not shown).

Viral replication was also tested in three experiments using both viruses at an MOI of 10⁻², a level at which inhibition between DSS and other isolates was significantly different. Results showed that cultures of cell line C6/36 inoculated with cells and supernatants were intermittently positive. However, RT-PCR detected cellular DEN RNA at a similar level for the viruses tested, as shown for 1 representative experiment of 2 experiments in figure 1B.

Discussion

The in vitro effects of dengue virus on human hematopoietic cell growth have not been extensively studied. The only documented report is that of Nakao et al. [9], who demonstrated that dengue virus propagated in human bone marrow progenitors but did not induce a decrease in the number of progenitors in primary methylcellulose cultures. Our data are in agreement with this work since we did not observe any colony formation inhibition in primary methylcellulose cultures, although viral RNA was detected in cfu-GM colonies.

By contrast, when cells infected with either clinical isolate at an MOI of 1 were initially cultured for 8 days in the presence of growth factors, we observed an inhibition of progenitor growth. However, viral antigens and viral particles were intermittently detected for both viruses tested, suggesting a low level of replication in cord blood mononuclear cells as previously shown in peripheral blood leukocytes by Waterman et al. [10]. The absence of a significant inhibitory effect with
the prototype strain H-87 suggests that this strain may have undergone a shift in cell tropism as a result of extended propagation on various growth systems.

A dose-dependent inhibition of colony formation was observed with the 3 isolates, but isolate 29-56DSS induced a more potent inhibitory effect than did 60-56DF and 136-116DHF. Furthermore, at an MOI of $10^{-2}$, viral replication was very low, but there was no evidence of differences in the level of DEN RNA in cells infected by either virus. These data suggest that the level of colony formation inhibition is not related to the level of viral replication.

The differences in the level of colony formation inhibition were confirmed when 3 additional isolates (2 from DF and 1 from fatal grade IV DSS cases) were used. However, the number of isolates studied was small, and from these data it is difficult to definitely conclude that the differences in inhibition are related to virulence differences between isolates. However, this in vitro culture system can provide a basis by which to compare dengue isolates.

The pathogenesis of DHF and DSS is not fully understood. Multiple factors, including host, epidemiologic, and virus factors, may contribute to the severity of the disease [11]. Many studies suggest a role for the so-called antibody-dependent enhancement phenomenon [12] during secondary dengue infection. However, the pathogenesis of primary DHF and DSS is still unresolved. During the 1989–1990 DEN-3 epidemic in Tahiti, among 178 children hospitalized for laboratory-confirmed dengue fever and for whom the type of antibody response was determined, 20 children >1 year old had a primary infection, and 4 of them presented with DSS [13]. Viral factors may also influence the severity of dengue infection [14, 15]. Our results are in agreement with this hypothesis since the inhibition of colony formation appeared to be dependent upon the isolate tested. However, viral virulence is not the only explanation for severe disease.

Whether the mechanism of hematopoietic progenitor growth inhibition induced by dengue virus is related directly to viral replication or to factors released by cells of mononuclear phagocyte lineage or progenitors themselves must be further investigated.

Acknowledgments

We are grateful to the nurses and Dr. Thibault (obstetric department, Clinique Paofai, Papeete) for providing cord blood samples.
and to Paul M. V. Martin for his continual support and Lam N’Guyen for statistical analysis. We thank Claudine Roche, Claude Baillou, and Marie-Laure Bonnet for technical assistance, and we acknowledge Luc Nicolas and F. Lemoine for helpful discussions.

References